

Aus dem Institut für Pflanzenbau und Pflanzenzüchtung
der Christian-Albrechts-Universität zu Kiel

**Investigations on flowering time genes from oilseed rape (*Brassica
napus* L.)**

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List of Abbreviations

%	Per cent
°C	Degree Celsius
<i>A. thaliana</i> , <i>At</i>	<i>Arabidopsis thaliana</i>
AFLP	Amplified fragment length polymorphism
<i>AGL</i>	<i>AGAMOUS LIKE</i>
ANOVA	Analysis of variance
<i>API</i>	<i>APETALA1</i>
<i>AP2</i>	<i>APETALA2</i>
<i>AP3</i>	<i>APETALA3</i>
<i>B. napus</i> , <i>Bna</i>	<i>Brassica napus</i>
<i>B. oleracea</i> , <i>Bo</i>	<i>Brassica oleracea</i>
<i>B. rapa</i> , <i>Br</i>	<i>Brassica rapa</i>
BLAST	Basic local alignment search tool
bp	Base pairs
<i>CAL</i>	<i>CAULIFLOWER</i>
Cas	CRISPR-associated
<i>CCA1</i>	<i>CIRCADIAN CLOCK ASSOCIATED 1</i>
<i>CDF1</i>	<i>CYCLING DOF FACTOR 1</i>
cDNA	Complementary DNA
CDS	Coding sequence
CI	Confidence interval
CK2	Casein kinase II
cm	centimeter
<i>CO</i>	<i>CONSTANS</i>
CRISPR	Clustered regularly interspaced short palindromic repeats

List of abbreviations

<i>CSTF66</i>	<i>CLEAVAGE STIMULATING FACTOR 66</i>
<i>CSTF77</i>	<i>CLEAVAGE STIMULATING FACTOR 77</i>
CTAB	Cetyltrimethyl ammonium bromide
DEGs	Differentially expressed genes
DH	Doubled haploid
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ECJ	court of justice of the European Union
EDTA	Ethylene diaminetetraacetic acid
EMS	Ethyl methanesulfonate
FAO	Food and Agriculture Organization of the United Nations
<i>FCA</i>	<i>FLOWERING LOCUS CA</i>
FDR	False discovery rate
<i>FIO1</i>	<i>FIONA1</i>
<i>FK</i>	<i>FLOWERING LOCUS KH DOMAIN</i>
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLD</i>	<i>FLOWERING LOCUS D</i>
<i>FPA</i>	<i>FLOWERING LOCUS PA</i>
<i>FRI</i>	<i>FRIGIDA</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FUL</i>	<i>FRUITFULL</i>
<i>GA2OX6</i>	<i>GIBBERELLIN 2-OXIDASE 6</i>
Gb	Giga base pairs
<i>GI</i>	<i>GIGANTEA</i>
gm	gram

List of abbreviations

GM(O)	Genetically modified (organism)
GO	Gene ontology
GWAS	Genome wide association study
h	hour
<i>HTA11</i>	<i>HISTONE H2A 11</i>
<i>HUA2</i>	<i>ENHANCER OF AG- 4 2</i>
<i>IND</i>	<i>INDEHISCENT</i>
indel	Insertion/ deletion
IPA	Ideal plant architecture
kb	Kilo base pairs
kg	kilogram
LD	Linkage disequilibrium
<i>LFY</i>	<i>LEAFY</i>
<i>LNK2</i>	<i>NIGHT LIGHT INDUCIBLE AND CLOCK REGULATED- 2</i>
m ²	Square meter
Mb	Mega base pairs
min	minute
miRNA	Micro RNA
MITE	Miniature Inverted-repeat Transposable Element
mRNA	Messenger ribonucleic acid
MSL	Male sterile line
MYA	Million years ago
NCBI	National Center for Biotechnology Information
nm	Nanometer
NPZ	Norddeutsche Pflanzenzucht Hans-Georg Lembke KG
NV	Non-vernalized

List of abbreviations

PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHD	Plant homeodomain
<i>PhyB</i>	<i>PHYTOCHROME B</i>
<i>PI</i>	<i>PISTILLATA</i>
<i>PIFs</i>	<i>PHYTOCHROME INTERACTING FACTORS</i>
<i>PP2A</i>	<i>PROTEIN PHOSPHATASE 2A</i>
PRC2	Polycomb repressive complex
<i>PRR</i>	<i>PSEUDO RESPONSE REGULATOR</i>
QTL	Quantitative trait loci
<i>REF6</i>	<i>RELATIVE OF EARLY FLOWERING 6</i>
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT-qPCR	Real-time quantitative PCR
s	second
SAM	Shoot apical meristem
SD	Standard deviation
SEM	Scanning electron microscopy
<i>SEP</i>	<i>SEPALLATA</i>
<i>SFT</i>	<i>SINGLE FLOWER TRUSS</i>
SNP	Single nucleotide polymorphism
<i>SOC1</i>	<i>SUPPRESSOR OF OVEREXPRESSION OF CO1</i>
<i>SP</i>	<i>SELF PRUNING</i>

List of abbreviations

<i>SPL</i>	<i>SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE</i>
<i>SUF4</i>	<i>SUPPRESSOR OF FRIGIDA 4</i>
TAIR	The Arabidopsis Information Resource
TE	Transposable element
<i>TFL1</i>	<i>TERMINAL FLOWER 1</i>
TILLING	Targeting induced local lesions in genomes
TN	Tapidor X Ningyou7
UTR	Untranslated region
V	Vernalized
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
<i>VRN5</i>	<i>VERNALIZATION5</i>
χ^2	Chi square

1 General introduction

1.1 Oilseed rape- its importance and genetic architecture

Rapeseed (*Brassica napus* L.) is one of the most important sources of vegetable oils and protein-rich meals worldwide. Apart from conventional uses, rapeseed oil is also used in the production of biodiesel in Europe. According to the Food and Agriculture Organization of the United Nations (FAO), 68.8 million tons of rapeseed were harvested from a worldwide area of 33.7 million hectares in 2016. The availability of high yielding cultivars and advances in harvesting techniques facilitate rapeseed cultivation. However, farmers have experienced a decline in overall yield in recent years due to unfavorable environmental conditions. Based on the requirement of vernalization, rapeseed is grown across the world as three different types. In Europe, rapeseed is grown as a winter crop, which is usually sown in autumn and can only flower after vernalization. In contrast, spring type rapeseed, which is mainly grown in Canada and Australia, can flower rapidly in the same season without vernalization. The third type of rapeseed, semi-winter type, is widely grown in China and has a moderate vernalization requirement. These three ecotypes of rapeseed have substantial genetic diversity in spite of the short domestication history of rapeseed (Wang et al. 2014).

Brassica napus (AACC, $2n = 38$) is an allopolyploid species and comprises two subgenomes A and C, originating from a natural hybridization of *Brassica rapa* (AA, $2n = 20$) and *Brassica oleracea* (CC, $2n = 18$). It is presumed that this spontaneous hybridization occurred 7,500 to 12,500 years ago (Chalhoub et al. 2014). The reference genome of rapeseed (winter rapeseed line Darmor-*bzh*) contains 314.2 Mb of the A subgenome and 525.8 Mb of the C subgenome, which accounts for a total of 101,040 annotated gene models (Chalhoub et al. 2014). *B. napus* and its progenitors, *B. rapa* and *B. oleracea*, are believed to share a common ancestor with *Arabidopsis thaliana*, which also belongs to the *Brassicaceae* family. Although the two species are closely related, the transfer of knowledge from *A. thaliana* to *B. napus* is hindered by the complexity of *B. napus* genome. Genome triplication took place during the evolution process of *Brassicaceae* (Mandáková and Lysak 2018). As *B. napus* originated from the hybridization between *B. rapa* and *B. oleracea*, it has approximately six copies for each gene in *Arabidopsis*. Nevertheless, the number of paralogs for each gene varies due to deletion and duplication events (Chalhoub et al. 2014). This variation in copy number creates a major obstacle generating simultaneous mutagenesis in multiple homoeologous sites. The fate of duplicated copies of a gene is not well understood. They might be eventually lost or mutated (Cheng et al. 2018). Studies show that many copies of genes are retained in the genome via sub- or neo-functionalization. These functional changes can be helpful in increasing adaptability to a specific environment (Cheng et al. 2018). In rapeseed, there have been several reports in the past few years showing evidence of functional divergence from *A. thaliana* (Dun et al. 2014; Lloyd et al. 2018; Xia et al. 2016).

1.2 Morphology of rapeseed

Rapeseed belongs to the *Brassicaceae* family, a medium-sized and economically important family of flowering plants which contains about 341 genera and 3,973 species distributed worldwide (Koch et al. 2018). The floral structure in the *Brassicaceae* family is highly conserved. The *B. napus* flower has four distinct yellow color petals, six stamens, and one pistil. During the growth of rapeseed, a rosette of 5-6 leaves is formed by the seedling. In rosette, the younger and smaller leaves stay at the top, and the older leaves are at the base. Followed by the rosette stage, the plant undergoes bolting, stem extension, branching and flowering (Gulden et al. 2008).

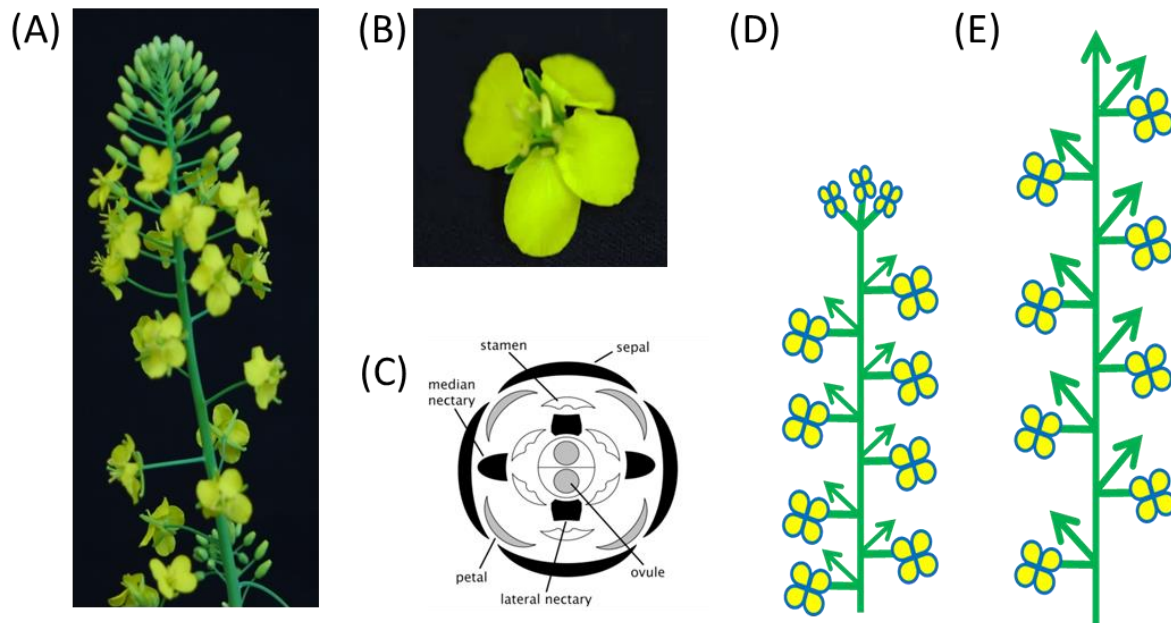


Figure 1: Inflorescence, flower and plant architecture of rapeseed. (A) Rapeseed inflorescence, (B) Rapeseed flower, (C) Floral diagram of *Brassica* species, (D) Determinate growth habit, and (E) Indeterminate growth habit.

The inflorescences of *B. napus* are quite similar to *Arabidopsis*. They are classified as raceme (unbranched, indeterminate) type inflorescences with flowers produced in acropetal succession (Figure 1(A)). In *Brassica* species, the pedicels are elongated and produce well spread out flowers in the inflorescence (Figure 1(B)). This results in a canopy cover that decreases light penetration to the photosynthetic leaves (Dumoncaux et al. 2009). Rapeseed has an indeterminate growth habit, meaning that the plant keeps generating a continuous main axis that laterally produces floral meristems and eventually sets more seeds until environmental cues terminate these processes (Figure 1(E)). Depending on the sowing density and row spacing, the number of siliques per plant varies between 100 and 300 (Kuai et al. 2015). In contrast to this, the plants having a determinate growth habit, lack the main axis. Moreover, the main shoot terminates in a flower, while growth continues through lateral axes produced below the terminal flower (Figure 1(D)). Following this, the lateral axes also form terminate flowers. A determinate growth habit results in synchronized fruit maturation, which is a desirable trait for mechanical harvesting. There also exists a variation in determinate growth, which is termed as ‘sympodial growth,’ a growth type observed in tomato (Zhang et al. 2018). Plants having a sympodial growth type generate a new axis of growth from an axillary meristem, which also produces leaves before forming a terminal flower. This process continues generating a plant with a continuous growing axis, having an alternate production of leaves and lateral inflorescences (Chomicki et al. 2017).

Rapeseed is reproduced by seeds and is predominantly self-fertile. Being a majorly self-pollinating crop, the majority of rapeseed cultivars are pure lines and obtained from breeding programs designed for self-pollinating crops. However, rapeseed also tends to outcross (~30%) and receive pollen from other plants (Soengas et al. 2013). Apart from these conventional methods, the doubled haploid (DH) technique is also widely applied in rapeseed breeding programs for the generation of completely homozygous lines (Gilles et al. 2017). DH lines are beneficial as it allows rapid development of fixed genotypes, which can be propagated indefinitely by sexual means. These fixed genotypes facilitate the estimation of genetic and environmental components of phenotypic variation. For example, the Tapidor × Ningyou7 DH population (TN-DH), generated by crossing the winter rapeseed Tapidor and semi-winter type Ningyou7, is used to map QTLs for various traits (Luo et al. 2017).

1.3 Significance of flowering time in rapeseed breeding and cultivation

The transition from the vegetative to the reproductive phase is a crucial developmental stage for the environmental adaptation of the plant. Environmental adaptation mainly includes adaptation to prevailing climatic conditions (for example season, day length, and temperature), as well as biotic and abiotic stresses. This environmental adaptation of rapeseed is important to maximize the yield of the plant (Schiessl et al. 2015). In the *Brassicaceae* family, there is a considerable variation in flowering time and includes species with both an annual and a biennial growth habit. This variation in growth habit and flowering time has been thought to have evolved as a mechanism for plants to tolerate cold and warm temperatures (Shea et al. 2018). Floral induction is a key developmental switch in Brassica species for the timely production of seeds. This is especially important for Northern Hemisphere countries, in which short growing seasons and early winter can reduce the amount and quality of the harvest significantly. For example, in annual plants such as cauliflower (*B. oleracea*), an early spring and other adverse environmental conditions can lead to early flowering and cause yield loss. Late flowering in crops such as rapeseed can also be a problem in the Northern Hemisphere because the yield can be limited by restricted spring growth due to cold temperatures between late fall and early spring (Jung and Muller 2009). However, early flowering and maturity can be important traits for growing spring rapeseed under long day conditions. Traditional plant breeding methods mainly aim at the directed re-assortment of the flowering time allelic variants to produce allele combinations that have an optimum flowering time and are assumed to be the best in specific environmental conditions. Different molecular markers are also widely used to map flowering time genes and play an important role in the breeding and selection of rapeseed. There have been several QTL analyses using a varying number of molecular markers to find loci responsible for flowering time (Javed et al. 2016; Liu et al. 2016; Luo et al. 2014). As a recent approach, a high-density marker set, like the Brassica Infinium 60K SNP Chip, is also used to perform genetic mapping as well as allele-trait association studies and to identify flowering time regulators in rapeseed (Luo et al. 2017; Schiessl et al. 2015). The molecular markers for favorable flowering time alleles can be used to perform screening of the rapeseed diversity pool, as well as the elite varieties, and ultimately to select the best crossing parent. Additionally, targeted mutagenesis or other genetic modification techniques can be used to create a material with varying flowering time characteristics, which can be suitable for changing environmental conditions. For instance, it was shown that overexpression of the MADS gene caused early flowering in summer rapeseed and also altered the vernalization requirement in winter rapeseed lines (Chandler et al. 2005). It was also shown that allelic variations in *Bna.FLC.A10* are responsible for most of the flowering time variation between spring- and winter-type rapeseed and these variations can be used by breeders as functional markers (Hou et al. 2012). Schiessl et al. (2014) performed targeted deep sequencing of 29 regulatory flowering-time genes in four diverse rapeseed accessions and found a broad range of variation for the target genes in the accessions. These genes and their variations can be used by breeders as markers to select rapeseed genotypes suitable for certain environments.

1.4 Flowering time regulation in Arabidopsis

In the life cycle of flowering plants, the transition from vegetative growth to reproductive development (producing flowers) is a major and one of the most complex processes, which is mainly regulated by several environmental and endogenous cues (Cho et al. 2017). There has been remarkable progress in unveiling the genetic and molecular mechanisms of flowering time in Arabidopsis and crops after the work of Koornneef et al. (1991) who characterized numerous flowering time mutants in Arabidopsis. Various regulatory pathways have been defined that are different in their response to distinct internal and external cues (Figure 2). The external cues mainly include low as well as high temperatures and day length (Cho et al. 2017), while the internal cues include hormone signaling (Gibberellins and Cytokinins) general and autonomous pathway, circadian

clock pathway, aging pathway (*miRNA156/miRNA172* and *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKEs* (*SPLs*)) and sugar budget pathways (Lee et al. 2018).

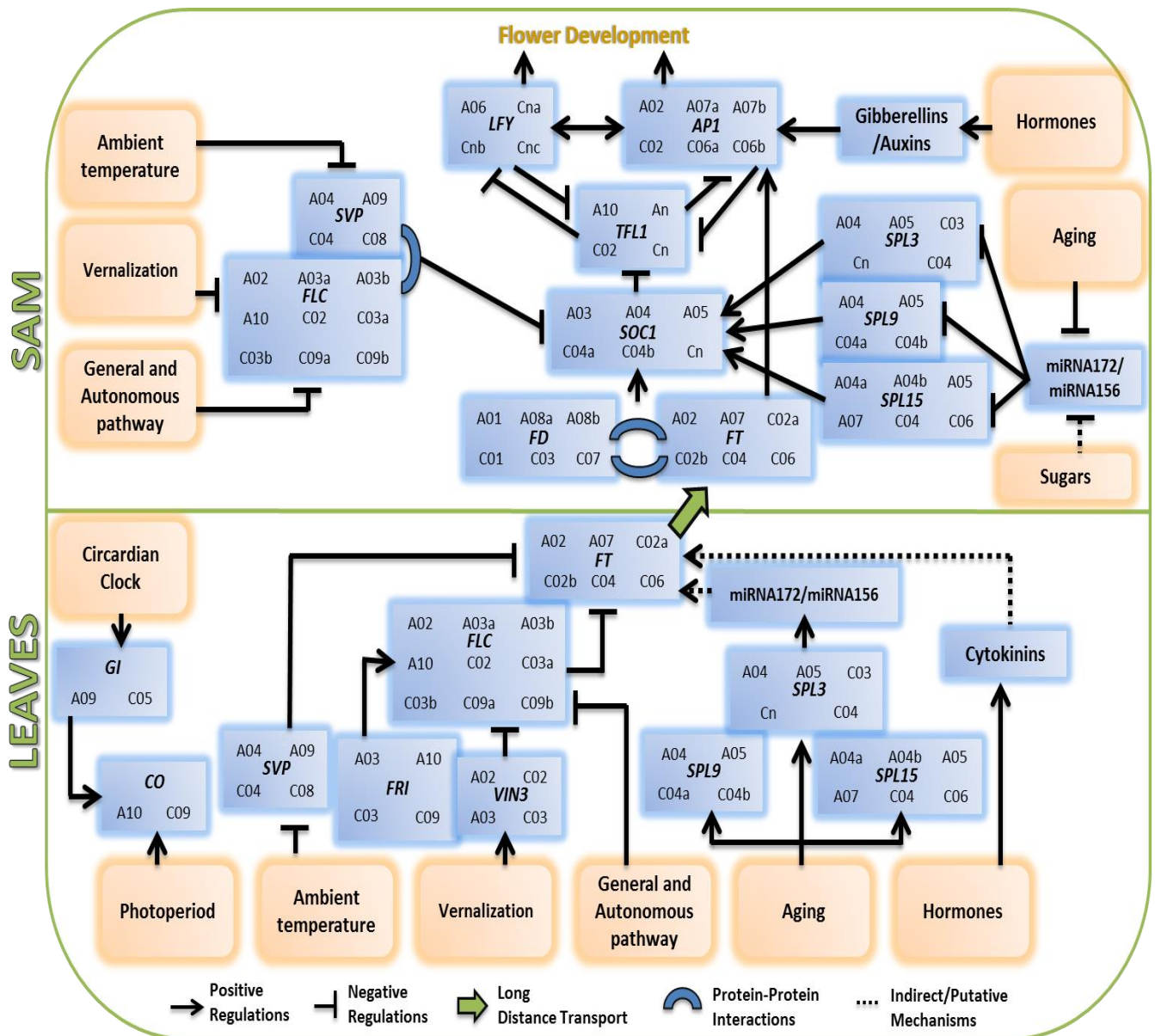


Figure 2: Gene network of the major flowering time regulators in Arabidopsis with their respective orthologs in rapeseed. Arabidopsis gene names are mentioned in the middle of the box. In each box, rapeseed orthologs for each gene are mentioned by chromosome number they are located on. Blue boxes represent major flowering regulators in Arabidopsis, while orange boxes represent environmental as well as endogenous cues regulating flowering time.

Many flowering plant species exhibit variation in flowering time. This varying flowering time in Arabidopsis accessions can be attributed to the requirement of vernalization (Duncan et al. 2015). There are three distinct phases involved in vernalization in most of the *A. thaliana* accessions: (1) Establishment of a vernalization requirement by activation of *FLOWERING LOCUS C* (*FLC*) (2) cold-induced *FLC* inactivation and (3) maintenance of *FLC* in a silent stage through epigenetic modifications (Song et al. 2013). Active *FLC* in plants leads to a need for vernalization during winter before flowering. Low *FLC* leads to rapid-cycling, which allows multiple generations a year. *FRIGIDA* (*FRI*) is a coiled-coil domain protein and activates the expression of *FLC* through a co-transcription regulatory mechanism and chromatin modifications (Amasino 2018). During winter, a series of cold-

induced processes converts active *FLC* into a silenced state. As soon as the *FLC* transcripts are reduced, the *FLC* gene loop is disrupted and expression of the *FLC* antisense transcript, *COOLAIR* is upregulated (Csorba et al. 2014). Upon contentious exposure to cold, Polycomb-mediated histone modification, H3K27me3 is accumulated at the *FLC* site. Moreover, the cold-induced form of polycomb repressive complex (PRC2) also accumulates at the *FLC* site, which causes a higher accumulation of H3K27me3. Prolonged cold also induces the interaction of PRC2 with PHD-containing proteins such as VERNALIZATION5 (VRN5/VIL1) and VERNALIZATION INSENSITIVE 3 (VIN3) (Kim and Sung 2013). The quantitative increase in H3K27me3 is proportional to the length of the cold exposure and is important for mitotically stable silencing of *FLC* during rest of the development (Kim and Sung 2013).

As the plants use the photoperiod information to precisely align the time of flowering, it is necessary to understand how the plants integrated photoperiodic signals to control flowering. In Arabidopsis, flowering is accelerated under long day condition through the function of FLOWERING LOCUS T (FT) protein (Andrés and Coupland 2012; Pose et al. 2012). FT acts as florigen, which is synthesized in the leaf vasculature, it moves through the phloem to the shoot apical meristem (Tsuji 2017). Different factors like temperature, photoperiod, plant age, and gibberellic acid control the expression of the *FT* gene. Moreover, its expression is strongly induced by *CONSTANS* (*CO*) (Andrés and Coupland 2012; Pin and Nilsson 2012). Upon activation, the FT protein is transported to the shoot apex, where it forms a heterodimer with the FD protein. Subsequently, this FD/FT complex activates the expression of *SUPPRESSOR OF CONSTANS1* (*SOC1*) and the meristem identity genes (*APETALA1* (*API*) and *LEAFY* (*LFY*)). Activation of meristem identity genes in the shoot apex leads to floral initiation. In Arabidopsis, expression of *API* and *LFY* is suppressed by *TERMINAL FLOWER 1* (*TFL1*), and through this suppression, *TFL1* maintains the indeterminate growth of shoot apical meristem (SAM). Although *FT* and *TFL1* share high sequence similarity, *TFL1* acts antagonistically and delays flowering (Ho and Weigel 2014).

Genes of the autonomous pathway regulate *FLC* expression through chromatin modification and RNA-based post-transcriptional regulation. In total, nine different proteins mediate RNA regulatory processes (Cheng et al. 2017). Out of nine proteins, FLOWERING LOCUS CA (FCA) and FLOWERING LOCUS PA (FPA) mediate alternative polyadenylation of antisense RNAs and plays an important role in 3'-end formation at the *FLC* (Hornyik et al. 2010; Liu and Mara 2010). Moreover, *FPA* and *FCA* genes also interact with *FLOWERING LOCUS D* (*FLD*), which links RNA processing to chromatin changes in the autonomous pathway (Liu et al. 2007). In contrast to these RNA processing genes, *FLD*, *FLOWERING LOCUS KH DOMAIN* (*FK*), *RELATIVE OF EARLY FLOWERING 6* (*REF6*) and *LUMINIDEPENDENS* (*LD*) can also regulate *FLC* expression through chromatin modification (Cheng et al. 2017). Additionally, post-translational modification of proteins has been shown to play an important role in *FLC* regulation. *Casein kinase II* (*CK2*) and *Protein phosphatase 2A* (*PP2A*) repress *FLC* to promote flowering through phosphorylation and dephosphorylation (Mulekar and Huq 2015). Apart from the above-mentioned pathways, the GA (gibberellic acid) biosynthesis pathway also plays an important role in bolting and flower formation. In previous studies, it was shown that under long day conditions, a mutation in GA receptors causes a delay in flowering even when the *CO* pathway is active. GA promotes the degradation of the DELLA proteins, and subsequently, it promotes plant growth (Galvão et al. 2012). It has been proposed that the DELLA proteins function as transcriptional repressors. They block the activity of PHYTOCHROME INTERACTING FACTORS (PIFs) and suppress their ability to promote growth (Li et al. 2016a). Gibberellins also promote the expression of *SOC1* and *LFY* by DELLA mediated pathways. GAs are most influential for flowering under short-day conditions when the major photoperiod-inductive pathway via *CO* is absent (Li et al. 2016b). Apart from hormones and sugar

assimilates, the age of the plant also plays an important role in the flowering of Arabidopsis. Two miRNAs, *miRNA156* and *miRNA172*, along with *SPLs*, act as major regulators of the age pathway (Teotia and Tang 2015). As the plants get older, the expression of *miRNA156* decreases and the expression of *miRNA172* as well as *SPLs* increase and promote flowering (Luo et al. 2013). Despite detailed knowledge about flowering time regulators in Arabidopsis, it may not always represent the best tool to characterize novel genes in other crops. Due to evolutionary diversification, new flowering time regulators have been found in crops that are not characterized in Arabidopsis (Blümel et al. 2015). Therefore, crop specific studies to unveil the flowering time network are important to increase the genetic variation in crops.

1.5 Flowering time regulation in rapeseed

Recently several efforts have been made in mapping genomic locations related with flowering time in *B. napus* (Figure 3) (Baojun et al. 2018; Rahman et al. 2017; Shen et al. 2018a). Moreover, a number of studies have been performed in *B. napus*, *B. rapa* and *B. oleracea* to functionally characterize flowering time orthologs (Table 1). Despite Arabidopsis and rapeseed being close relatives, transfer of knowledge from Arabidopsis to rapeseed is hindered due to the presence of multiple copies of genes in rapeseed (Figure 2). For example, one of the important vernalization pathway genes, *FLC* has nine copies in rapeseed (Zou et al. 2012). To functionally characterize *FLC* in *B. napus*, one of the pioneer works was done by Tadege et al. (2001), who isolated five *FLC*-related sequences from *B. napus* (*BnFLC1–5*). They showed that expression of these five *BnFLC* sequences in Arabidopsis delayed flowering from three weeks to more than seven months. Moreover, they could also show that vernalization response as well as flowering time is correlated with *BnFLC* expression. In another study, Zou et al. (2012) identified nine paralogs of *BnFLC* in rapeseed and they also showed that all nine *BnFLCs* were relatively conserved but the intronic and promoter regions were more divergent. Moreover, based on QTL analysis conducted in the same study, four paralogs out of nine were within flowering time QTLs in two different mapping populations. One of these four paralogs, *BnFLC.A3b* showed differential expression between winter and semi-winter type rapeseed, making it a putative candidate for ecotype differentiation in rapeseed. In another study, *BnFLC.A10* was mapped within flowering time QTL in a doubled haploid (DH) mapping population derived from a cross between Tapidor (winter-type) and Ningyou7 (semi-winter). In the same study, they identified 12 polymorphic sites between *BnFLC.A10* parental alleles of the TNDH population. Moreover, they could also identify the association of one of the polymorphic sites to the vernalization requirement of rapeseed. This polymorphic site was a *Tourist*-like miniature inverted-repeat transposable element (MITE) insertion/deletion in the upstream region of *BnFLC.A10*, which was absent in the spring type. Apart from this, *BnFLC* paralogs have been mapped to flowering time QTLs in different studies conducted using different mapping populations in varying environmental conditions (Fletcher et al. 2014; Nelson et al. 2014; Raman et al. 2013).

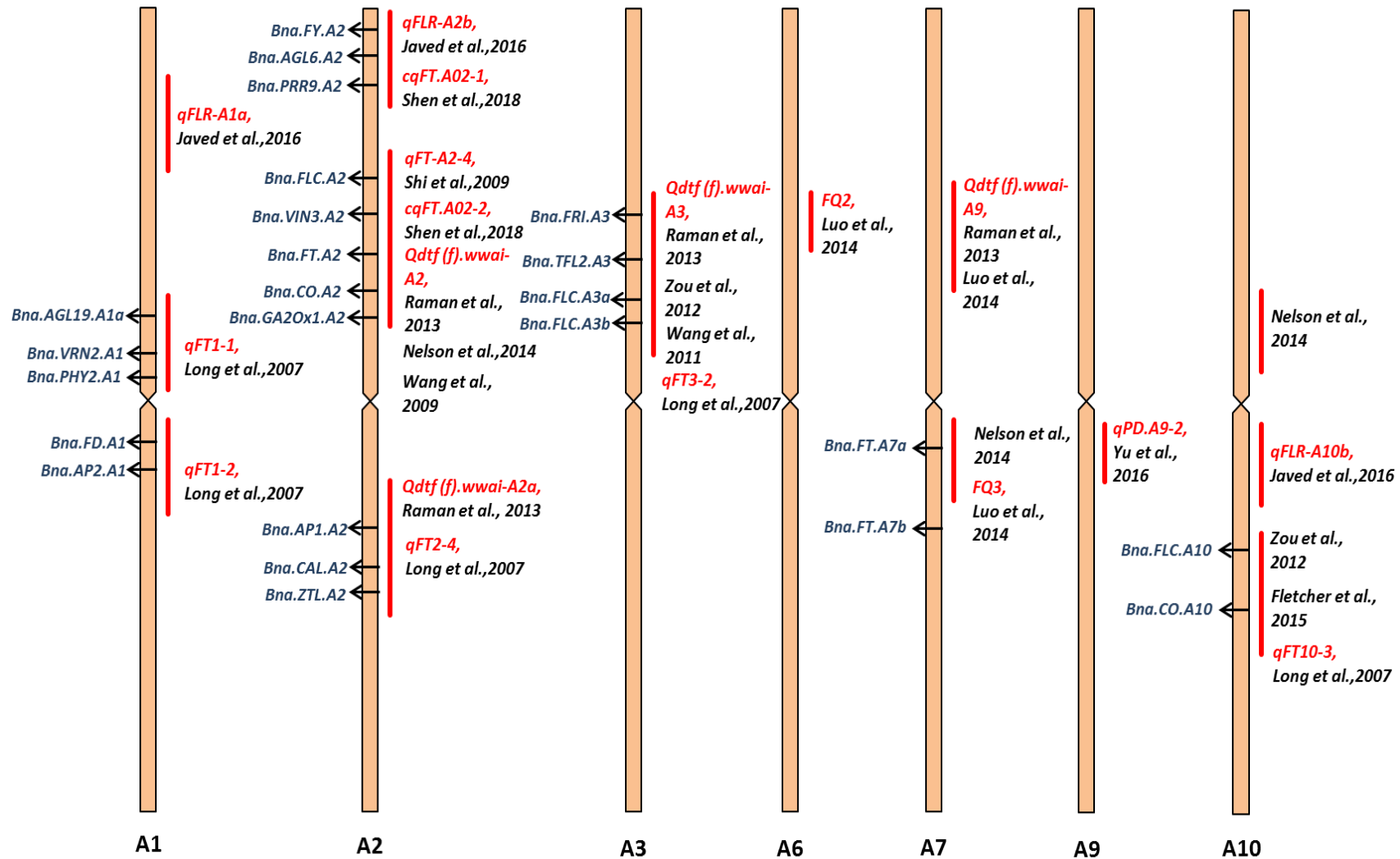
For another vernalization pathway gene *FRIGIDA*, four paralogs (*BnaA.FRI.a*, *BnaX.FRI.b*, *BnaX.FRI.c*, and *BnaX.FRI.d*) were identified in rapeseed by BAC libraries screening and PCR-based cloning (Wang et al. 2011a). In a recent study (Yi et al. 2018), these paralogs were renamed as *BnaA3.FRI*, *BnaA10.FRI*, *BnaC3.FRI* and *BnaC9.FRI*, according to their locations in the ‘Darmor-*bzh*’ reference genome (Chalhoub et al. 2014). Wang et al. (2011a) showed that out of these four paralogs, two paralogs (*BnaA3.FRI* and *BnaC9.FRI*) were differentially expressed in aerial plant organs in three different rapeseed accessions. Moreover, *BnaA3.FRI* was mapped within a flowering time QTL on chromosome A03 in TN DH population phenotyped in multiple environments (Wang et al. 2011a). The association analysis conducted in the same study revealed that six SNPs, including at least one at a putative functional site, are associated with flowering time variation in 248 accessions. The results of the association analysis were further confirmed in another study including 174 rapeseed accessions

analyzed in two successive years (2013-2014) in North China. In a recent study (Yi et al. 2018), the function of *BnaA3.FRI* was further verified by overexpression of different *BnaA3.FRI* alleles in *Arabidopsis col-0* lines. The overexpression of *BnaA3.FRI* showed that variation in the coding sequence of *BnaA3.FRI* led to weak alleles and this caused variation in flowering time in *Arabidopsis* (Yi et al. 2018).

FT, one of the major floral inducer in *Arabidopsis*, has six paralogs in rapeseed (Wang et al. 2009), and all of them are homologous to a common ancestral block (E) of *Arabidopsis* chromosome 1. Wang et al. (2009) reported that four of the six paralogs were present within inverted duplicated regions of chromosomes A07 and C06. It was also observed that all of these six *Bna.FT* paralogs shared high sequence similarity among themselves (92%-99%) and also with *Arabidopsis* (85%-87%) (Wang et al. 2009). In the same study, it was observed that an FLC protein binding site, CARG box was missing in two of the paralogs present on chromosome A02 and C02. Moreover, three paralogs (*BnA2.FT*, *BnC6.FT.a*, and *BnC6.FT.b*) were found within flowering time QTL in the TNDH population. The role of *BnC6.FT.b* was further confirmed by Guo et al. (2014) who performed a phenotypic analysis of TILLING mutants under greenhouse conditions. It was shown that plants carrying splice site mutation in *BnC6.FT.b* copy flowered 29 days later than the control. However, a missense mutation in another paralog, *BnC6.FT.a*, did not show any effect on flowering time. In another study, a set of three conserved blocks A, B, and C which are essential for FT activation in *Arabidopsis* were found to be conserved in rapeseed (Wang et al. 2012). Nevertheless, it was found that the paralog present on chromosome C02 had an insertion of a DNA transposable element (TE) and a retroelement in *FT* upstream blocks A and B, which contributed to silencing of *BnC2.FT* paralog.

TFL1, which acts as a floral repressor in *Arabidopsis* has four copies in rapeseed. Out of these four, it was possible to extract sequence information from three copies (*BnTFL1-1*, *BnTFL1-2*, and *BnTFL1-3*) (Mimida et al. 1999). The sequence comparison among three paralogs showed that *BnTFL1-1* and *BnTFL1-3* share higher sequence similarity and are inherited from *B. oleracea*, whereas *BnTFL1-2* carries an insertion and is inherited from *B. rapa* (Mimida et al. 1999). Expression analysis of these paralogs conducted in the same study showed predominant expression in flowers. In a transcriptome analysis conducted on SAM of rapeseed, *BnTFL1-2* and *BnTFL1-3* displayed similar expression pattern to *Arabidopsis* as both showed increased expression during the floral transition (Jones et al. 2018). Phenotypic analysis TILLING mutant carrying missense mutation in *BnTFL1-2* paralog showed no effect on flowering time, while it showed an effect on seed yield components (Guo et al. 2014).

General introduction



General introduction

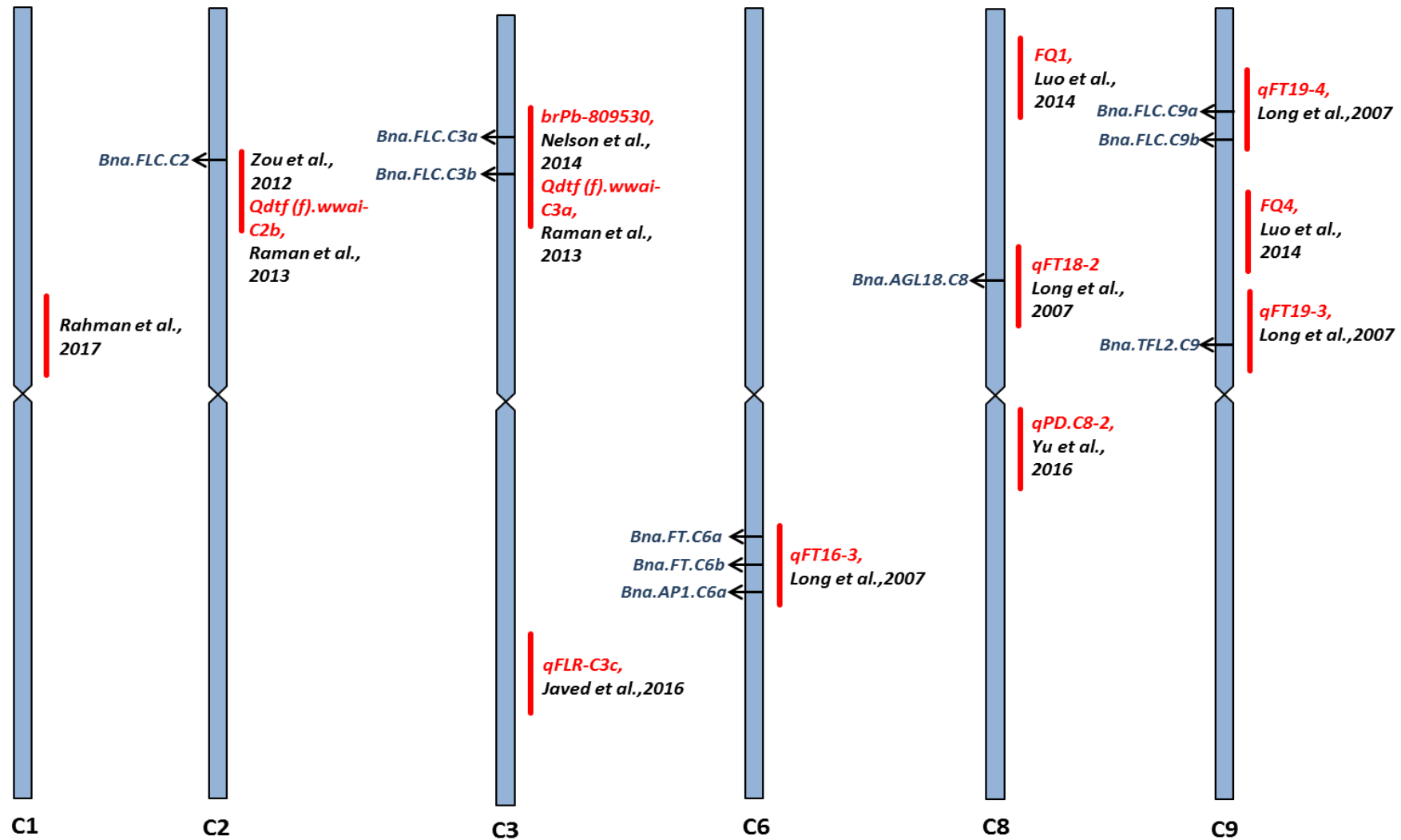


Figure 3: Overview of the flowering time QTL studies in rapeseed. The red bar represents a previously identified flowering time QTL, while the gene name on the left represents rapeseed flowering time gene within a specific QTL. Name of the QTL and its reference is mentioned on the right side of the red bar.

Table 1: Overview of the functionally characterized flowering time orthologs in *B. napus*, *B. oleracea* and *B. rapa* in previous studies.

<i>A. thaliana</i> gene name	<i>B. napus</i> gene name	Copy number			Functional analysis			References
		<i>B. napus</i>	<i>B. oleracea</i>	<i>B. rapa</i>	<i>B. napus</i>	<i>B. oleracea</i>	<i>B. rapa</i>	
<i>FT</i>	<i>Bna.FT</i>	6	2	2	<i>Bna.FT.C6b</i> mutation delays flowering	-	An insertion in <i>BrFT</i> delays flowering	(Guo et al. 2014; Zhang et al. 2015)
<i>TFL1</i>	<i>Bna.TFL1</i>	4	2	2	<i>Bna.TFL1.A10</i> mutation affects seed yield and flowering time	-	-	(Guo et al. 2014)
<i>FLC</i>	<i>Bna.FLC</i>	9	3	3	- Overexpression of five <i>Bna.FLC</i> copies delays flowering in Arabidopsis - Presence of a <i>Tourist</i> -like MITE insertion in <i>Bna.FLC.A10</i> of winter type, absent in spring type	-	- <i>BrFLC3</i> overexpression delays flowering in Arabidopsis - <i>BrFLC2</i> overexpression delays flowering in Arabidopsis	(Hou et al. 2012; Huang et al. 2018; Kim et al. 2007; Tadege et al. 2001)
<i>CO</i>	<i>Bna.CO</i>	2	1	1	<i>Bna.COa1</i> allele complements <i>co-2</i> mutation in Arabidopsis	-	-	(Robert et al. 1998)
<i>FRI</i>	<i>Bna.FRI</i>	4	2	2	<i>Bna.FRI.A3</i> overexpression delays flowering in Arabidopsis	<i>BoFRIa</i> causes late flowering in <i>FRI</i> mutant in Arabidopsis	Naturally occurring splicing mutation in the <i>BrFLC1</i> alters flowering time	(Irwin et al. 2012; Yi et al. 2018; Yuan et al. 2009)
<i>SOC1</i>	<i>Bna.SOC1</i>	6	3	3	-	-	<i>BrSOC1</i> overexpression in	(Hong et al. 2013)

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							rapeseed causes early flowering	
<i>API</i>	<i>Bna.API</i>	6	3	3	-	Stop codon mutation in <i>BoAPI-B</i> causes cauliflower phenotype	-	(Lowman and D Purugganan 1999)
<i>LFY</i>	<i>Bna.LFY</i>	4	2	2	-	-	<i>BrLFY</i> overexpression causes early flowering in Arabidopsis	(Qi et al. 2015)
<i>CCA1</i>	<i>Bna.CCA1</i>	2	1	1	-	-	Naturally occurring allelic variation in the <i>BrCCA1</i> alters flowering time	(Yi et al. 2016)
<i>GA2Ox6</i>	<i>Bna.GA2Ox6</i>	3	1	2	<i>Bna.GA2Ox6</i> overexpression delays flowering in Arabidopsis	-	-	(Yan et al. 2017)
<i>GI</i>	<i>Bna.GI</i>	2	1	1	-	Expression of antisense <i>BoGI</i> caused delayed flowering in <i>B. oleracea</i>	- <i>BrGI</i> could rescue Arabidopsis GI mutant phenotype. - <i>BrGI</i> TILLING mutants flower later	(Thiruvengadam et al. 2015; Xie et al. 2015)
<i>CDF1</i>	<i>Bna.CDF1</i>	3	2	1	<i>Bna.CDF1</i> overexpression delays flowering in Arabidopsis	-	-	(Xu and Dai 2016)
<i>PhyB</i>	<i>Bna.PhyB</i>	3	2	0	-	-	<i>BrPhyB</i> overexpression delays flowering in Arabidopsis	(Song et al. 2015)

1.6 Pleiotropic effect of *FT* orthologs in crops

Flowering time genes showed various pleiotropic effects in different crops. In tomato (*Solanum lycopersicon*) for instance, overexpression of the gene *SFT* (*SINGLE FLOWER TRUSS*, *FT* ortholog) caused early flowering along with a reduction in leaf complexity, shorter internodes, thinner stems, and arrested apices (Lifschitz et al. 2006). In another study, it was reported that heterozygosity for a missense mutation in *SFT* increased the tomato yield by 60% (Krieger et al. 2010). It was shown that this pleiotropic effect of *SFT* was due to a suppression of growth termination mediated by *SELF PRUNING* (*SP*) - an ortholog of Arabidopsis *TFL1*. In another study (Jiang et al. 2013), it was observed that reducing the transcript level of *SFT* in *SP* mutant plants by artificial microRNAs causes modification of shoot and inflorescence development. Similar to tomato, there have been several studies in rice providing evidence for the pleiotropy of flowering genes in controlling yield traits (Endo-Higashi and Izawa 2011; Xu et al. 2014; Yan et al. 2013; Zhang et al. 2012). Besides this, an ortholog of Arabidopsis *FT* gene, *Hd3a* was found within a heterosis related QTL in rice (Huang et al. 2016). They observed a two-base substitution in the mutated *hd3a* allele, which was associated with heterosis in rice. Consistent with the findings in tomato, heterozygosity of the mutated *hd3a* allele resulted in an increase of 7.4% seed setting rate and 9.9% grain yield per plant (Huang et al. 2016).

1.7 Plant architecture and its role in optimizing yield

Plant architecture is defined as the three-dimensional organization of the above ground and includes plant size, shape, and orientation of shoot components (Kaggwa-Asiimwe et al. 2013). These plant architecture components affect crop resistance, environmental adaptability, and yield of the plant (Maddonni et al. 2001). Therefore, breeding crops with optimum plant architecture has gained increasing importance over the years. For instance, plant architecture and plant height were significantly altered in wheat and rice which ultimately caused increased seed yield (Evenson and Gollin 2003; Peng et al. 1999). Studies conducted in maize have also shown that modification in the plant architecture leads to increase in yield (Duvick 2005; Ku et al. 2010; Tian et al. 2011). Ideal plant architecture (IPA) in rice has been proposed which contains less unproductive tillers which lead to a plant with sturdier stems and also more grains per panicle (Jiao et al. 2010). Nearly all plant architecture related traits are quantitative traits and are influenced by various environmental conditions like temperature, light, nutrition and plant density (Cai et al. 2016). Over the years, there have been several studies to identify genes controlling plant architecture through various reverse genetics approaches. Reports have shown that most of the genes responsible for plant architecture are mainly involved in hormone biosynthesis (Cai et al. 2016). For instance, it was confirmed that the use of a brassinosteroid biosynthesis inhibitor combined with a gibberellin biosynthesis inhibitor might be useful in controlling rice plant height (Matusmoto et al. 2016). Recently, there have been many studies showing genes involved in cytokinin regulation, gibberellin, and brassinosteroid biosynthesis as well as strigolactone metabolism controlling plant architecture related traits in various crops (Butt et al. 2018; Matusmoto et al. 2016). Apart from hormone metabolism genes, several transcription factors and cell cycle genes are also known to affect plant architecture (Dai et al. 2018; Tian et al. 2017; Yang et al. 2018; Zhou et al. 2018).

Rapeseed plant architecture is determined by plant height and branch number, as well as inflorescence morphology, which indirectly influences the yield of rapeseed by affecting a number of siliques per plant (Chen et al. 2014). Therefore, several studies aimed to find genes controlling rapeseed plant architecture and to study their pleiotropic effect on rapeseed yield (Cai et al. 2016; Li et al. 2014; Li et al. 2017b). In one of such studies, 24 plant architecture related and 23 plant yield related QTL were

found in a rapeseed DH population (Cai et al. 2016). Out of all the identified QTL in this study, 52% QTL had pleiotropic effect, simultaneously controlling plant architecture and plant yield. Dissection of plant architecture related QTL showed the involvement of the transcription factor, auxin/IAA, and gibberellin biosynthesis genes in the regulation of plant architecture related traits in rapeseed (Cai et al. 2016). In another study, Schiessl et al. (2015) performed GWAS on 158 winter rapeseed lines using 60 K-SNP Illumina® Infinium consortium array and found 68 cross-trait regions associated with flowering time, seed yield and plant height. Among the genes within these cross-trait regions were orthologs of, *CIRCADIAN CLOCK ASSISTED 1* (*Bna.CCA1*), *FT* (*Bna.FT*) and *FRUITFUL* (*FUL*) (*Bna.FUL*). These genes with pleiotropic effects can be better targets in breeding to optimize multiple traits via targeting a single gene.

1.8 Scientific hypotheses and Objectives

I expected that flowering time regulators in rapeseed are differentially expressed among different developmental stages. Therefore, I aimed to identify flowering time genes in rapeseed by RNA-seq and verify them by genetic mapping and association mapping. I further expected that mutation in *Bna.API* alters plant architecture and yield related traits. For that reason, I aimed to phenotypically characterize *Bna.API* EMS mutants in the greenhouse. Furthermore, I expected that plants carrying mutations in two of the major flowering time regulators (*Bna.FT.C6b* and *Bna.TFL1.A10*) suffer from a significant delay in the flowering time. To verify this, I aimed to perform phenotypic analysis of *Bna.FT.C6b/Bna.TFL1.A10* double mutant plants in the greenhouse. Moreover, I also expected that F₁ plants heterozygous for *Bna.FT* and *Bna.TFL1* missense mutations display better yield performance than the parents. Therefore, I aimed to evaluate F₁ hybrid plants under field conditions in two successive years.

The current study was conducted with following hypotheses: (1) Flowering time regulatory network is conserved between Arabidopsis and rapeseed; (2) Plant architecture and yield of rapeseed are affected by a mutation in *Bna.API*; (3) In the case of EMS mutant plants, background mutations have a significant effect on flowering time and yield related traits; (4) Due to heterosis effect, F₁ plants heterozygous for *Bna.FT* and *Bna.TFL1* missense mutations have better yield performance than the individual parents and (5) Flowering can be severely delayed in the plants carrying mutations in both *Bna.TFL1.A10* and *Bna.FT.C6b*.

2 Whole transcriptome analysis reveals genetic factors underlying flowering time regulation in rapeseed (*B. napus* L.)

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2.1 Abstract

Rapeseed (*Brassica napus* L.), one of the most important sources of vegetable oil and protein-rich meals worldwide, is adapted to different geographical regions by modification of flowering time. Rapeseed cultivars have different day length and vernalization requirements, which categorize them into winter, spring and semi-winter ecotypes. To gain a deeper insight into genetic factors controlling floral transition in *B. napus*, we performed RNA-seq in the semi-winter doubled haploid line, Ningyou7, at different developmental stages and temperature regimes. The expression profiles of more than 54,000 gene models were compared between different treatments and developmental stages and the differentially expressed genes were considered as targets for association analysis and genetic mapping to confirm their role in floral transition. Consequently, 36 genes with association to flowering time, seed yield or both were identified. We found novel indications for neofunctionalization in homologs of known flowering time regulators like *VIN3* and *FUL*. Our study proved the potential of RNA-seq along with association analysis and genetic mapping to identify candidate genes for floral transition in rapeseed. The candidate genes identified in this study could be subjected to genetic modification or targeted mutagenesis and genotype building to breed rapeseed adapted to certain environments.

2.2 Introduction

Oilseed rape (*Brassica napus* L.) is the second most important source of plant oil worldwide. Moreover, the protein-rich seed cake is used as animal feed. Rapeseed is grown in Europe, East Asia, Australia, and North America, where intensive selection and adaptation to local environments gave rise to different ecotypes. Spring type rapeseed which can flower without prolonged exposure to cold temperature, a process known as vernalization, is mainly grown in Canada. Winter rapeseed which is typically grown in Central and Northern Europe needs a period of prolonged cold to initiate flowering. In comparison to winter types, semi-winter rapeseed has a lower vernalization requirement. They are grown in regions with moderate winter temperatures like central China. *B. napus* is an allotetraploid species (~1,130Mb, 2n=4x=38, AACCC), which originated from a spontaneous hybridization between *B. rapa* (AA) and *B. oleracea* (CC) about 7,500 years ago (Chalhoub et al., 2014). Both parental species underwent genome triplication events after the split of Brassica from the Arabidopsis clade (~14.5 MYA) (Cheng et al., 2017, Cheng et al., 2013). Because of these evolutionary events, a six-fold redundancy of Arabidopsis orthologs can be expected in rapeseed (Schranz, Lysak & Mitchell-Olds, 2006). The European winter oilseed cultivar 'Darmor-bzh,' which serves as a reference genome contains 101,040 gene models out of 91,167, showed similarity to *B. rapa* and/or *B. oleracea* predicted proteomes (Chalhoub et al., 2014).

The genetic diversity in the rapeseed gene pool is relatively small (Hasan et al., 2006). Thus, gene introgression from different ecotypes or related species is considered as a mean to increase genetic variation (Zou et al., 2010). Moreover, hybrids between different ecotypes display high heterosis (Qian et al., 2007). However, the use of non-adapted ecotypes in breeding programs is limited due to large differences in their phenological development (Girke, Schierholt & Becker, 2012). The time of flowering is a major factor for the local adaptation of rapeseed and a key determinant of seed yield

(Raman et al., 2013). Therefore, understanding the genetic network underlying this character is of great importance in rapeseed breeding programs.

Rapeseed is closely related to *Arabidopsis*, and therefore, it is relatively easy to transfer information about gene functions between both species (Schiessl, Huettel, Kuehn, Reinhardt & Snowdon, 2017). However, despite of massive knowledge about flowering time regulation in *Arabidopsis* (Blümel, Dally & Jung, 2015), assigning the information to *B. napus* is hampered by its genome complexity. Different approaches have been followed to identify flowering time regulators in rapeseed. One strategy is based on biparental populations where QTL for flowering time and other agronomic traits have been mapped (Javed et al., 2016, Liu et al., 2016, Luo et al., 2014). Wang et al. (2016) identified 20 flowering time QTLs and 224 overlapping flowering time genes using 448 inbred lines genotyped with 20,342 single nucleotide polymorphism (SNP) markers of the Brassica 60K Illumina Infinium array. Raman et al. (2013) reported that flowering time is controlled by at least 20 different loci, localized on 10 different chromosomes in a doubled haploid (DH) population derived from the vernalization responsive rapeseed cultivars Skipton and Ag-Spectrum. As an alternative strategy, Schiessl, Iniguez-Luy, Qian & Snowdon (2015) genotyped 158 winter type accessions with 60 K-SNP array. They found 181 orthologs of flowering related genes to be associated with the onset of flowering in their diversity panel. In another study, Raman et al. (2016) conducted a genome wide association study (GWAS) using 69 flowering time associated SNP markers and by combining the data with expression QTL and RT-qPCR analyses, they could confirm roles of several loci controlling flowering time through vernalization. Several studies took advantage of sequence conservation between *Arabidopsis* and Brassica species, including *B. rapa* and *B. oleracea*, to dissect the genetic factors underlying flowering time in rapeseed. As a result, one *FRIGIDA (FRI)* homologue (*BnaA.FRI.a*), two *FLOWERING LOCUS C (FLC)* homologs and three *FLOWERING LOCUS T (FT)* homologs (*BnA2.FT*, *BnC6.FT.a* and *BnC6.FT.b*) have been shown to be associated with flowering time regulation in rapeseed (Wang et al., 2009, Wang et al., 2011, Zou et al., 2012). *FLC* orthologs *BrFLC.A2* and *BrFLC.A10* in *B. rapa* (Yuan et al., 2009, Zhao et al., 2010) and *BoFLC.C2* in *B. oleracea* (Okazaki et al., 2007) showed association with flowering time variation. In rapeseed, one *FLC* paralog was mapped to a major flowering time QTL on chromosome A10 in Tapidor × Ningyou7 DH (TNDH) population (Long et al., 2007). Other paralogs (*BnFLC.A3a* and *BnFLC.C2*) were found to co-localize with a QTL cluster for flowering time on chromosome A03 and C02, respectively in a Skipton/Ag-Spectrum population (Raman et al., 2013; Zou et al., 2012). Interestingly, a paralog of *FRI* which controls the *FLC* expression in *Arabidopsis* was mapped to the same position (Zou et al., 2012). Furthermore, the pleiotropic effect of a *TERMINAL FLOWER 1 (TFL1)* paralog in oilseed rape was reported by Guo, Harloff, Jung & Molina (2014), who observed that mutations within the *Bna.TFL1.A10* paralog had an impact on yield components in rapeseed. This finding is in line with studies in tomato, where a *TFL1* ortholog has a great impact on fruit yield heterosis (Krieger, Lippman & Zamir, 2010).

The RNA-seq technology enables a new perspective for transcriptome sequence analysis by providing a complete coverage of transcripts. It can be used as an alternative to other transcript quantification approaches with the benefit of higher sensitivity and the potential to distinguish between very similar paralogs of a gene differing only in a few nucleotides. In one of such studies, RNA-seq was performed on shoot apical meristem of two semi-winter rapeseed lines (Ye et al., 2017) that resulted in the identification of 9,135 differentially expressed genes (DEGs) between both lines, out of which 647 DEGs were within QTLs for pod number. In another study, Yu et al. (2016) performed transcriptome analysis on apetalous and an early flowering rapeseed line and developed an early flowering gene network comprised of mainly vernalization and photoperiod pathway genes.

Here, we present a comprehensive survey of gene expression in rapeseed at different developmental stages using RNA-seq. We studied the transcriptomes from leaves of the semi-winter type cultivar Ningyou7 at different developmental stages with and without cold treatment. We hypothesize that the regulatory network underlying flowering time regulation in rapeseed is similar to Arabidopsis. We expect that vernalization-regulated flowering time genes are differentially expressed between vernalized and non-vernalized plants and between different stages of development before (BBCH20) and after vernalization (BBCH30). We also hypothesize that genes directing flower development are differentially expressed between early (BBCH20) and late (BBCH50) stages of development irrespective of vernalization. To verify the function of the DEGs, we applied genetic mapping and association analysis using a biparental population and a diversity panel which had been genotyped with the 60K SNP array. Ultimately, we could provide strong indications for the role of 36 orthologs of known Arabidopsis flowering time genes in regulation of flowering time or yield related traits in rapeseed. The complexity of flowering time control in rapeseed in comparison to Arabidopsis was further demonstrated by the high number of paralogs and their different expression pattern.

2.3 Materials and methods

2.3.1 Plant material and growth conditions

Six rapeseed accessions representing the three main growth types were grown in two experiments with and without vernalization to select a genotype for the main RNA-seq experiment (Table S1). In the first experiment, ten plants per accession were grown in 3 x 3 cm 35-multi-well palettes (Hermann Meyer KG, Germany) for four weeks under constant 22°C and 16h light (900 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands). Then, the plants were transferred to a climate chamber at 4°C (vernalization) under long day conditions (16h light, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Osram Lumilux T8 L 58W/840, Osram AG, München, Germany) for a period of six weeks. Vernalized plants were then returned to the greenhouse and further cultivated in 9 x 9 cm soil pots (Hermann Meyer KG, Germany). In the second experiment, plants were grown until maturity in 9 x 9 cm pots under constant temperature (22°C) and long day conditions (16h light, 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands). We recorded BBCH stages two times per week to track the development of each plant (Meier, 2001).

For the transcriptome experiment, Ningyou7 was chosen because it displayed the highest differences in phenological development with and without vernalization. Plants were grown in 9 x 9 cm soil pots (Hermann Meyer KG, Germany) either with (V) (experiment 1, winter conditions) or without vernalization (NV) (experiment 2, spring conditions) under the same conditions as described above. We performed three greenhouse trials in three different seasons. For each trial, we collected leaf samples at noon from four different plants at three different developmental stages (Figure 4). To synchronize their growth, plants exposed to cold were sown six weeks earlier.

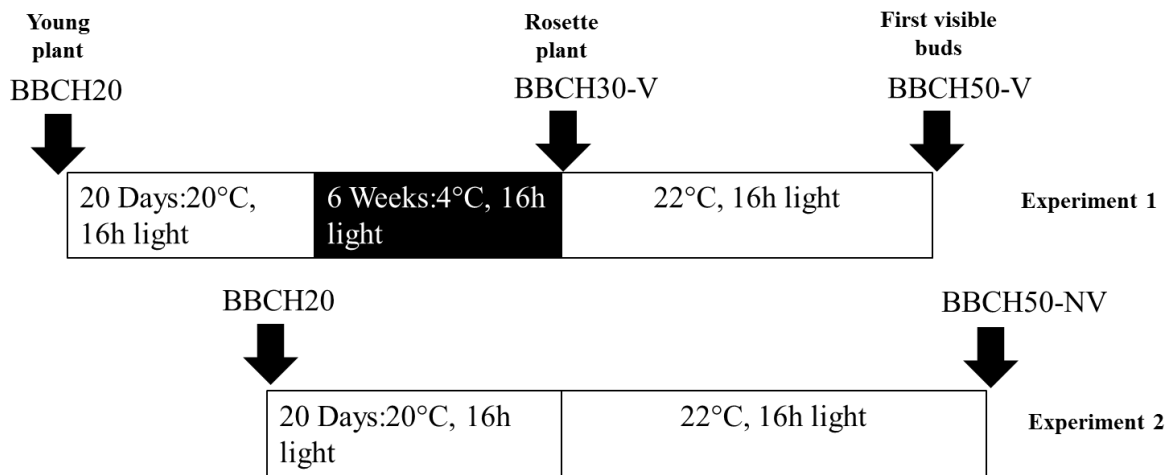


Figure 4: Experimental setup for two experiments with Ningyou7 differing by a vernalization period. For RNA-sequencing, young leaves (4-8 leaves per sampling point) were taken at stages BBCH20, BBCH30 and BBCH50. Non-vernalized plants: all plants were grown till mature stage in the greenhouse at constant temperature (22°C) and LD (16h light); Vernalized plants: all plants were grown in the greenhouse at constant temperature (22°C) and LD (16h light) after vernalization (4°C, 16h light, 6 weeks). The black arrows show the sampling points for RNA-seq library preparation.

2.3.2 RNA isolation, library preparation, and RNA-sequencing

We isolated total RNA using the PeqGold plant RNA extraction and PeqLab DNase kits (Peqlab, Germany) according to the manufacturer's protocol. RNA concentrations were checked with a Nanodrop spectrometer (Thermo scientific, Germany). RNA quality for each library was estimated via Agilent Bioanalyzer System (Agilent, Germany) through the calculation of the RNA Integrity Number (RIN) values for each sample. According to previous studies, only RNA integrity numbers above 7.0 were accepted for further treatment of the extracted RNAs (Brisco & Morley, 2012). We removed ribosomal RNA from 10 µg of the isolated RNA using the RiboMinus™ Plant Kit for RNA-Seq (Invitrogen, Germany) according to the manufacturer's protocol and stored the mRNA at -80°C in 1x TE-buffer until sequencing. The depleted RNA quality was re-tested with the Agilent Bioanalyzer 2100. RNA free of ribosomal RNA was set to a final concentration of 150 ng/µl by adding RNase-free water to the stock solutions. For RNA-seq libraries production, 2.25 µg RNA per library were processed at the Institute of Clinical Molecular Biology (IKMB, Kiel) for Illumina HiSeq2000 paired-end sequencing. For high-throughput sequencing at high coverage (>100x), we used five out of eight lanes from an Illumina flow-cell, by sequencing three paired-end RNA-seq libraries per lane.

2.3.3 Data analysis and bioinformatics workflow

A primary quality trimming and quality filtering of the raw paired-end reads was carried out using sickle with parameters $-l\ 50$ and $-q\ 30$ (Joshi & Fass, 2011). Afterwards, we mapped the quality filtered paired-end reads by STAR (v2.5.2a) (Dobin et al., 2013) to the rapeseed reference genome (Chalhoub et al., 2014) using the gene annotation file. We counted the non-chimeric uniquely and multiple mapped read-pairs using featureCounts (v1.5.0) (Liao, Smyth & Shi, 2014) with the annotation file from Chalhoub et al. (2014). A gene model was considered as 'expressed' when it had more than five average raw counts for at least one condition. Finally, we performed the differential expression analysis for the set of expressed genes using Bioconductor package edgeR (v3.14) with TMM normalization and applying the *exactTest* function (McCarthy, Chen & Smyth, 2012). We declared a gene as 'differentially expressed' (DEG) if the false discovery rate (FDR) was below 0.05 and the log₂ fold change was bigger than 1 (or smaller than -1). We validated the bioinformatics

pipeline using quantitative real-time RT-PCR (RT-qPCR) values from 63 paralogs of 21 Arabidopsis flowering time orthologs along with 14 uncharacterized genes.

We filtered out the candidate genes by comparing the expression patterns among different developmental stages and growth conditions. First, we analyzed genes differentially expressed between BBCH20 and BBCH50 (in both vernalized and non-vernalized conditions). As a second criterion, we looked for the DEGs between BBCH20 and BBCH30 in vernalized plants. For gene ontology (GO) functional categories analysis, we used the online tool PANTHER (Thomas et al., 2003). As input, we provided the corresponding Arabidopsis gene IDs of the DEGs in *B. napus*. For creating heat maps, we provided for each condition the average of the log₂ TMM normalized counts of the replicates.

2.3.4 Association mapping and linkage mapping

Linkage disequilibrium (LD) decay in the A- and C-genomes is in a range of 0.25 – 0.30 Mb and 2.00-2.50 Mb, respectively (Qian, Qian & Snowdon, 2014). We used two alternative approaches to find the region which is in LD with a candidate gene. As a first preferred approach, we considered the region after which the coefficient of determination (r^2) decayed to less than 0.25 as the region of interest around the candidate gene and all SNP markers within this region were considered as ‘linked markers’. As an alternative approach for the candidate genes in regions with highly varying r^2 value, we considered 200 kb up- and 200 kb downstream of the candidate genes to be in LD with that gene, which is a widely used approach in rapeseed (Raman et al., 2016). We used the data from a recent association analysis with 158 winter-type accessions using 21,623 unique SNP markers from the Brassica Infinium 60K SNP Chip. The accessions had been grown in 11 different environments in Germany, China and Chile (Schiessl et al., 2015). For our study, we used marker trait association data for flowering time and seed yield obtained from Schiessl et al. (2015). Based on the strategies mentioned above, we identified SNP markers in LD with candidate genes depending on their position in the reference genome (Chalhoub et al., 2014). Then, all these SNP markers were checked for marker-trait association. For each marker in LD, we analyzed $-\log_{10}P$ value for all 11 environments. The SNP markers with $-\log_{10}P > 2.0$ for at least one out of 11 environments were considered as associated with trait variation.

In a second approach, we analyzed the QTL data from TNDH population (Luo et al., 2017). The population consisting of 202 DH lines had been phenotyped under seven different environments in China and genotyped using the Brassica Infinium 60K SNP Chip (Luo et al., 2017). The genetic map was constructed with 13,296 SNP markers. Based on the positions of linked SNP markers, we identified the candidate genes which were within the confidence intervals (CI) of QTL for flowering time and/or yield related traits (pod number, seed number, seed weight and seed yield).

2.4 Results

2.4.1 A semi-winter type rapeseed was selected for transcriptome analysis

We conducted an experiment to choose a suitable rapeseed genotype for the subsequent RNA-seq experiments. We analyzed a small diversity set of six rapeseed accessions under constant temperature (22°C) and long day conditions (Table S1) with and without vernalization. The semi-winter accession, Ningyou7, flowered with and without vernalization, although it showed significant differences in phenological development between vernalized and non-vernalized conditions (Figure S1). The present results suggested that there are differentially expressed set of genes between equivalent BBCH stages in both conditions in Ningyou7 plants. Accordingly, vernalization-responsive as well as vernalization-independent flowering time regulators are expected to be present within mRNA libraries assessed

through RNA-seq. Analyzing vernalization-dependent and independent genes in simultaneous experiments was only possible with a semi-winter type and not with winter or spring type rapeseed. Since due to the limitation and the size of the experiment, we could only consider one genotype for transcriptome analysis, Ningyou7 was selected for further analysis of winter- and spring-regulated flowering time genes using RNA-seq.

2.4.2 Transcriptome sequencing

For the main RNA-seq experiments, Ningyou7 was grown under long day conditions in the greenhouse with (experiment 1) and without cold treatment (experiment 2). Vernalized plants showed a faster transition between BBCH50 and BBCH60 (12 to 19 days) than plants grown under non-vernalized experiment (25 to 30 Days) (Figure S1).

We sequenced 14 RNA libraries from leaves picked at three developmental stages (BBCH20, BBCH30, and BBCH50). We used rRNA depleted RNA for sequencing (Table S2). We obtained ca. 1.4×10^9 paired-end reads (2×100 bp) (Table S3). With this setup, we generated ~78.2 million paired end reads per library with an average read length of 75 bp amounting to a total of 11.73 Gb per library. Given the number of gene models in the rapeseed genome (101,040; (Chalhoub et al., 2014), we estimated an average size of the transcriptomes between 100 and 120 Mb. Therefore, by dividing the total generated sequence information (11 Gb) by the transcriptome size (~0.1 Gb), we calculated a 100x coverage per library.

We conducted iterative cycles of RT-qPCR (Method S1) and bioinformatics quantification. In this way, the bioinformatics pipelines were optimized using the RT-qPCR values to choose the most accurate parameters. RT-qPCR values from 63 paralogs of 21 Arabidopsis flowering time orthologs along with 14 uncharacterized genes with single melting curve peaks and relative low variation among biological replicas were used for the validation of bioinformatics pipeline, consisting of sickle (quality filtering), STAR (read mapping), FeatureCounts (read quantification), and edgeR (differential expression analysis and normalization). The scatter plots including the Pearson correlation coefficients (Figure S2) between log₂ fold changes of RNA-seq and log₂ fold changes of RT-qPCR show good the agreement of both techniques.

In total, we found 54,777 gene models as expressed in the Ningyou7 leaf transcriptome after applying the 'expressed genes' filter to RNA-seq raw counts, (Figure 5(A)). Therefore, 54% of the gene models proposed for the rapeseed genome (Chalhoub et al., 2014) were expressed in Ningyou7 leaves under two environmental regimes at three developmental stages.

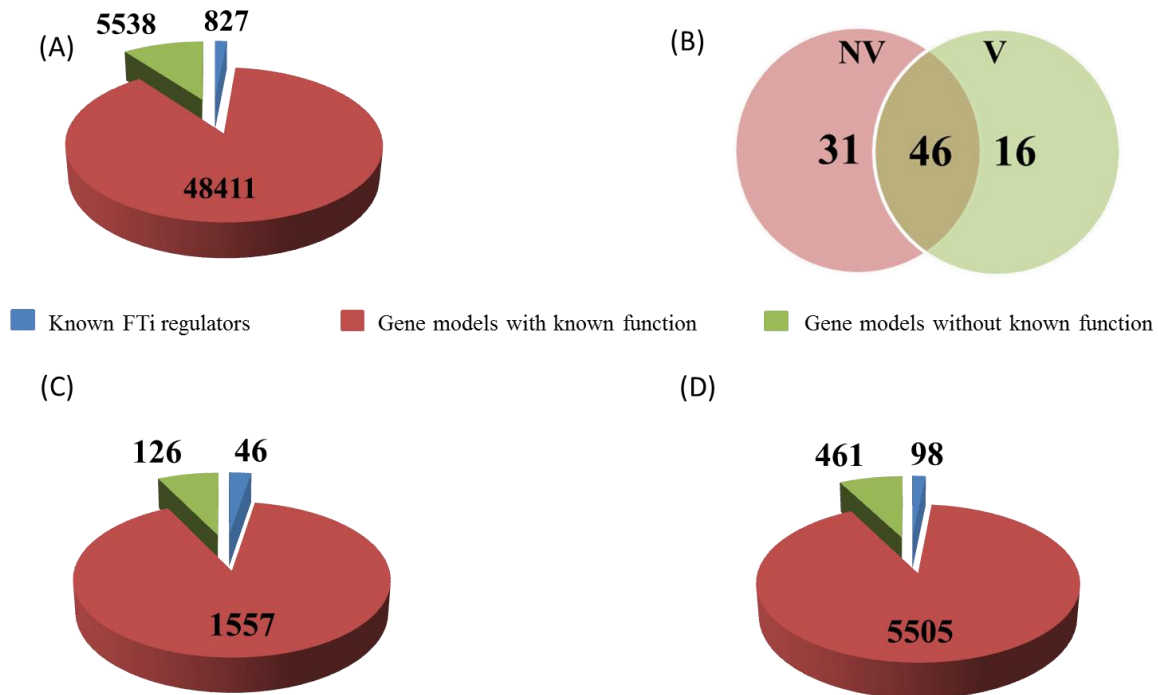


Figure 5: Graphical representation of a total number of expressed and differentially expressed genes. (A) Total number of gene models which have more than 5 reads mapped for at least 2 out of 14 RNA-seq libraries (B) Venn diagram showing number of differentially expressed genes which are common between BBCH20 vs BBCH50-V and BBCH20 vs BBCH50-NV (C) Differentially expressed gene models between BBCH20 and BBCH50-V as well as BBCH20 and BBCH50-NV ($FDR \leq 0.05$; $\text{Log}_2\text{foldchange} \geq 1$ or ≤ -1) (D) Differentially expressed gene models between BBCH20 and BBCH30-V ($FDR \leq 0.05$; $\text{Log}_2\text{foldchange} \geq 1$ or ≤ -1); Gene models in blue color are orthologs of Arabidopsis flowering time regulators; Orthologs of the gene models in red have known function in Arabidopsis other than flowering time regulation; Orthologs of the gene models in green have no known function in Arabidopsis. FTi: flowering time; V: Vernalized experiment; NV: Non-vernalized experiment

2.4.3 Identifying genes in response to vernalization and phenological development

We hypothesized that some flowering time regulators respond to cold because the phenological development was strikingly different between plants grown under different vernalization regimes. Moreover, we expected that their transcriptional activities vary over time of development. Therefore, we considered two criteria for DEG identification.

As the first criterion, we analyzed genes differentially expressed between early and late stages of development, both under vernalized and non-vernalized conditions (BBCH20 vs. BBCH50-V/BBCH50-NV). As plants were grown under two different temperature regimes, we expected key genes of the vernalization pathway to be differentially regulated between vernalized and non-vernalized plants. Under non-vernalized condition, we observed that 2,107 genes were up-regulated and 1,543 genes were down-regulated between BBCH20 and BBCH50. These numbers reduced in vernalized condition to 1,130 up-regulated and 1,090 down-regulated genes (Figure 6).

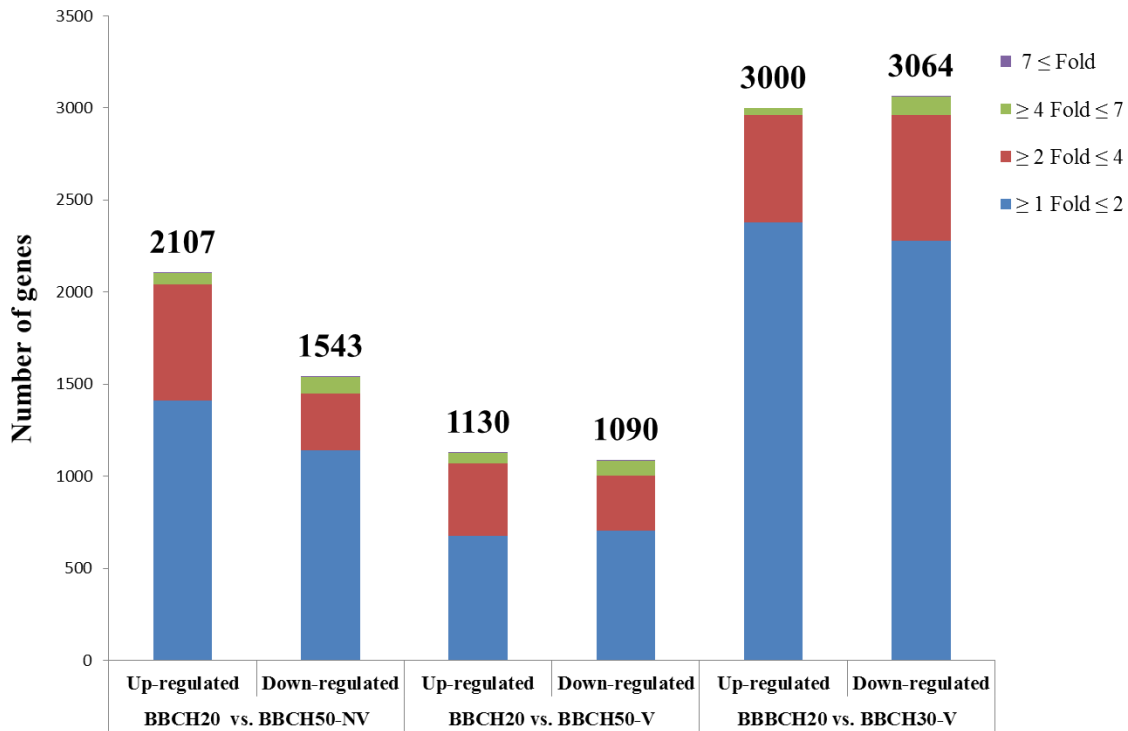


Figure 6: Up and down-regulated genes and the respected fold changes between different developmental stages. The total number of gene models is given on top of each bar; V: Vernalized experiment; NV: Non-vernalized experiment

In total, 49% of the differentially expressed Arabidopsis flowering time homologs between BBCH20 and BBCH50 displayed the same transcriptional activities under vernalized and non-vernalized conditions (Figure 5(B)). We reason that these flowering time regulators are acting independent from vernalization. Out of 1,729 differentially regulated genes common in both experiments, the vast majority of genes had a different (1,557) or unknown (126) gene function in Arabidopsis (Figure 5(C)). Based on gene ontology classification, the majority of DEGs were grouped into biological processes like metabolic processes (39%) and cellular processes (29%) (Figure 7(A)).

Among homologs of flowering time regulators in Arabidopsis, an ortholog of *FRUITFUL (FUL)* had seven differentially expressed paralogs. Moreover, we could identify three orthologs of each, *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)* and *SQUAMOSA PROMOTER BINDING PROTEIN LIKE 15 (SPL15)* among the DEGs. We also found that 6 orthologs of 4 different *AGAMOUS-LIKE* genes were differentially expressed. All these paralogs showed significantly higher expression at BBCH50 compared to BBCH20 in both vernalized and non-vernalized conditions (data not shown).

As the second criterion, we looked for genes which were up- or downregulated after the cold period in experiment 1. We anticipated that the vernalization pathway in rapeseed is similar to Arabidopsis and that the orthologous genes respond to cold in the same way. Therefore, we compared the expression of the genes before (BBCH20) and after vernalization (BBCH30-V). In total, 6,064 genes were differentially regulated, out of which 98 gene models were annotated as putative orthologs of Arabidopsis flowering time regulators; 5,505 gene models had not been annotated as flowering time regulators and 461 gene models had no Arabidopsis annotation at all (Figure 5(D)). Based on gene ontology, DEGs were classified into different subgroups of biological processes. Most DEGs encode for metabolic processes (35%) and cellular processes (31%) (Figure 7(B)). Out of 6,064 DEGs, 3,000

genes were up-regulated and 3,064 genes were down-regulated after vernalization (Figure 6), from which 10 genes displayed high homology to two cold-responsive genes from the vernalization pathway in Arabidopsis. The most prominent gene family among the DEGs displayed homology to *FLC*. Except *Bna.FLC.C3b*, all paralogs of *Bna.FLC* were differentially expressed. The eight differentially expressed *Bna.FLC* paralogs showed lower expression after vernalization. Later on, the *Bna.FLC* activities increased from BBCH 30 to BBCH50 in vernalized and non-vernalized plants. Along with *Bna.FLC*, two paralogs of an upstream regulator of *FLC* in Arabidopsis, *VERNALIZATION INSENSITIVE 3 (VIN3)*, also showed differential expression between BBCH20 and BBCH30 under vernalized condition. We observed that the expression of two out of four *Bna.VIN3* was higher after vernalization (Figure 8). Subsequently, we looked for genes which are not likely members of the vernalization pathway. *SOC1* homologs (*Bna.SOC1.A3* and *Bna.SOC1.A5*) were up-regulated after vernalization (Figure 8). Along with *Bna.SOC1*, orthologs of other *AGAMOUS- LIKE* genes (*AGL19*, *AGL6*, *AGL16* and *AGL24*) were also differentially expressed. Moreover, we observed differential expression of two orthologs of Arabidopsis *PHYTOCHROME INTERACTING FACTOR 4 (PIF4)*, which is known as a temperature dependent flowering time regulator (Kumar et al., 2012). Among DEGs, we found genes whose Arabidopsis orthologs are part of circadian clock like *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *FIONA 1 (FIO1)* and *PSEUDO RESPONSE REGULATOR 9 (PRR9)*. Additionally, we also observed differential expression of orthologs of Arabidopsis autonomous pathway genes like *ENHANCER OF AG- 4 2 (HUA2)* and *HISTONE H2A 11 (HTA11)*.

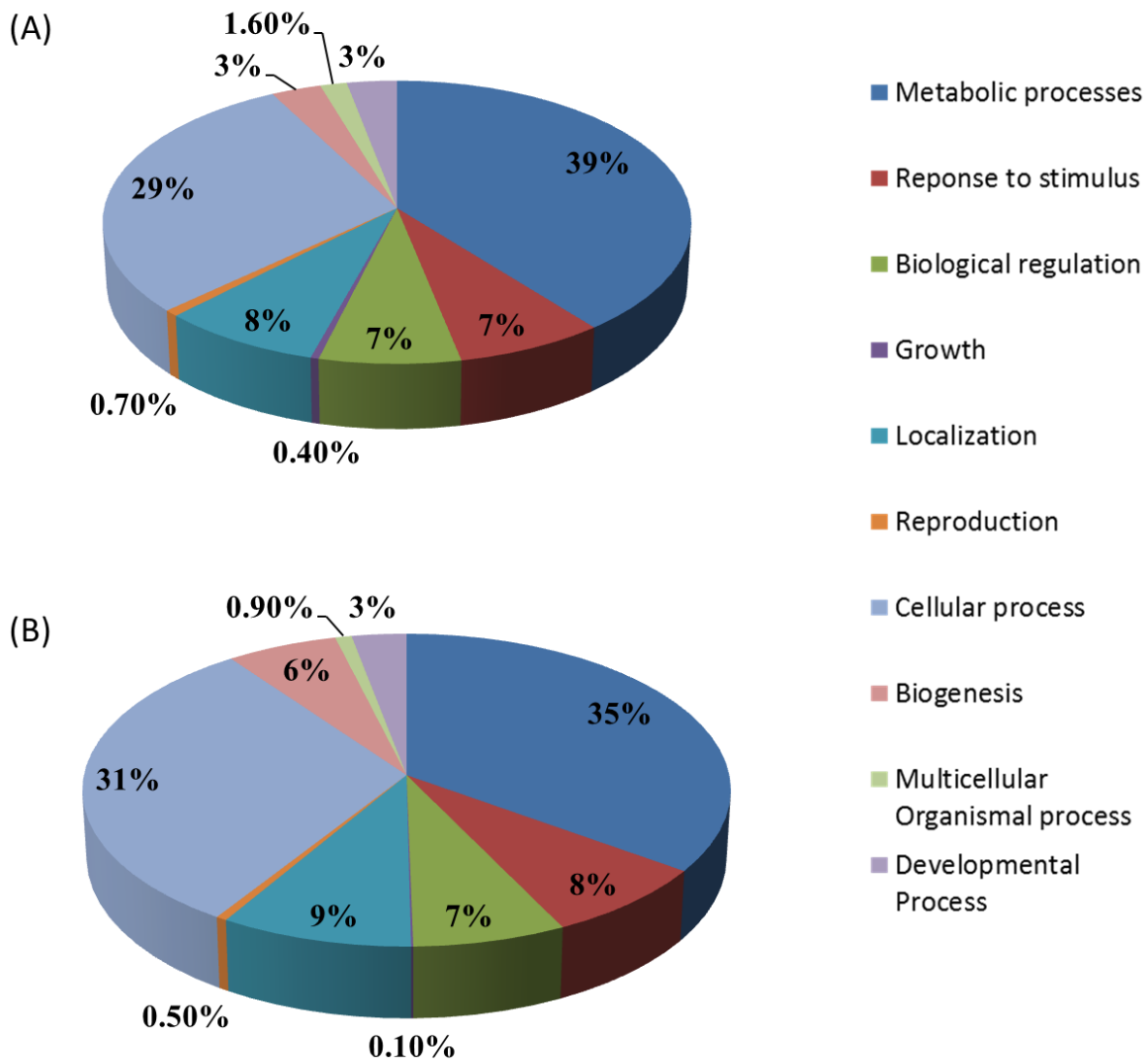


Figure 7: Functional categories of differentially expressed genes. (A) Differentially expressed genes which are common between BBCH20 vs BBCH50-V and BBCH20 vs BBCH50-NV ($FDR \leq 0.05$; $\text{Log}_2\text{foldchange} \geq 1$ or ≤ -1) (B) Differentially expressed gene models between BBCH20 and BBCH30-V ($FDR \leq 0.05$; $\text{Log}_2\text{foldchange} \geq 1$ or ≤ -1); The classification was performed according to gene ontology (GO) biological process. The percentage of genes was shown for each category.

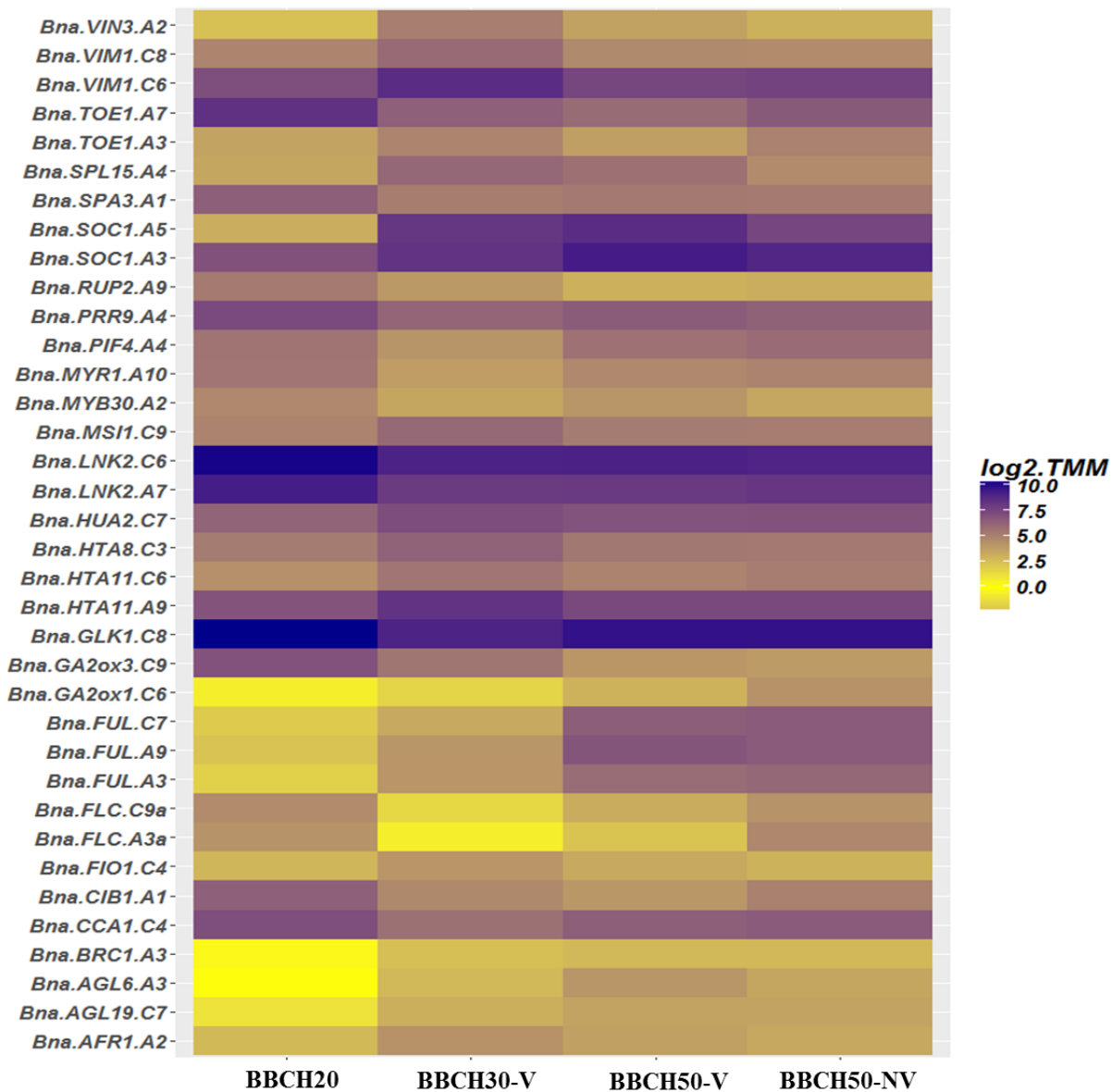


Figure 8: Heatmap showing the gene expression of 36 candidate genes from the current study. A DEG was only considered as a candidate gene, if its association with flowering time or yield was confirmed through genetic mapping, GWAS or both. Heatmap was constructed using R package ggplot2. Intensity of the color for each gene model corresponds to the average of log2 TMM normalized counts for that developmental stage (1 = 1 log2 TMM; 10 = 10 log2 TMM).

2.4.4 Differentially regulated genes map to genome regions which control flowering time and yield characters

We aimed to map DEGs, which are orthologs of Arabidopsis flowering time genes either by association analysis or linkage mapping. We assumed a function for the candidate genes, if they were in close linkage to a QTL for flowering time or yield characters. Therefore, a DEG was only considered as a candidate gene, if its association with flowering time or yield was confirmed through genetic mapping, GWAS or both. First, we used a large data set from a genome-wide association study (GWAS) (Schiessl et al., 2015). We considered the SNP markers which were in LD with our candidate genes and mapped them in a DH population (TNDH) (Figure S3) grown in diverse environments (Luo et al., 2017). Second, we assessed association of the SNP markers in LD to flowering time or seed

yield (Figure S4). Here, we considered the candidate genes to be involved in the regulation of flowering time or seed yield, if they were located within the CI of the QTLs.

We analyzed the genome positions of 46 homologs of Arabidopsis flowering time genes, which were differentially expressed between BBCH20 and BBCH50 (vernalized and non-vernalized, Table 2). An ortholog of an Arabidopsis floral activator *SPL15* (*Bna.SPL15.A4*) was associated with flowering time. Likewise, a homolog of the floral meristem identity gene *FUL* (*Bna.FUL.A9*) was associated with flowering time and seed yield. The same paralog was localized within QTLs related to seed yield ($R^2=5.37\%$) and seed number ($R^2=5.93\%$) on chromosome A09. In addition, another ortholog of *FUL*, *Bna.FUL.C7* was associated with flowering time, while *Bna.FUL.A3* was associated with seed yield. We also found the association of an ortholog of Arabidopsis *SOCI* (*Bna.SOCI.A3*) with flowering time and seed yield related QTL on chromosome A03 ($R^2=3.86\%$). Moreover, a *SOCI* ortholog (*Bna.SOCI.A5*) was associated with seed yield and was also localized within a QTL for seed weight ($R^2=9.4\%$). An ortholog of circadian clock pathway gene, *NIGHT LIGHT INDUCIBLE AND CLOCK REGULATED-2* (*LNK2*, *Bna.LNK2.C6*) was associated with flowering time and yield, while the other (*Bna.LNK2.A7*) was associated with seed yield.

Among the differentially expressed flowering time orthologs between BBCH20 and BBCH30- V (98 genes), *Bna.VIN3.A2*, was in LD with flowering time and seed yield (Fig. S4) and it was located within the CI of flowering and yield related QTLs ($R^2=3.1\%$ and 11.4% , respectively) at the same positions on chromosome A02 (Table 2). As *Bna.VIN3.A2* was the only candidate gene from our study showing positive results from genetic and association mapping for flowering time, we considered it as our most promising candidate gene. Therefore, we analyzed the sequence level variation for *Bna.VIN3.A2* between winter, spring and semi-winter accessions of ERANET-ASSYST panel (Method S2). We observed one silent mutation and one missense mutation, which caused a single amino acid change (Leucine to Valine) at position 177 in PHD zinc finger domain between winter and spring ecotypes as well as between winter and semi-winter accessions (Figure S5).

Out of eight differentially expressed paralogs of *Bna.FLC*, one paralog (*Bna.FLC.A3a*) was associated with flowering time, while another paralog (*Bna.FLC.C9a*) was associated with seed yield. Likewise, we found positive associations for *AGAMOUS-LIKE* (*AGL*) orthologs (*Bna.AGL19.C7* and *Bna.AGL6.A3*) with flowering time. Furthermore, an ortholog of *PIF4* (*Bna.PIF4.A04*) was associated with seed yield. Overall, we identified 36 homologs of flowering time genes as candidate genes using association analysis and genetic mapping.

Table 2: Flowering time genes and their position next to flowering time and yield QTL. Genes were selected based on two criteria. 1) They were differentially expressed (between BBCH20 and BBCH50 in vernalized and non-vernalized or between BBCH20 and BBCH30-V) ($FDR \leq 0.05$ and \log_2 fold change ≥ 1 or ≤ -1) 2) They showed positive results for genetic mapping (TNDH population) or association analysis (Schiessl et al.,2015). Genome position of the SNP markers from 60K Chip is given for the markers which showed association with flowering time or yield in GWAS study or localized within QTL confidence interval. For genetic mapping, the name and the position of the linked marker which was within the confidence interval of the QTL are given.

		LD mapping (SNP marker/genome position)		Genetic mapping (SNP marker/genome position)	
<i>B. napus</i> gene ID	<i>A. thaliana</i> homolog	Flowering time	Yield/yield related traits	Flowering time	Yield/yield related traits
DEGs between BBCH20 and BBCH50- Vernalized and non-vernalized					
<i>Bna.FULA3</i>	<i>FUL</i>	-	Bn-A03-p20983398/ 19,750,133	-	-
<i>Bna.FULA9*</i>	<i>FUL</i>	Bn-A01-p9004629/2,580,835	Bn-A01-p9004629/ 2,580,835	-	Bn-A01-p9080573 (SY (5.37%) & SN (5.93%))/ 2507154
<i>Bna.FUL.C7*</i>	<i>FUL</i>	Bn-scaff_15762_1-p717223/ 2,201,425	-	-	-
<i>Bna.CIB1.A1*</i>	<i>CIB1</i>	-	Bn-A01-p1770291/ 1,310,265	-	-
<i>Bna.SPA3.A1*</i>	<i>SPA3</i>	-	-	-	Bn-A01-p23842865 (SW (3.4%))/ 19,908,475
<i>Bna.AGL6.A3*</i>	<i>AGL6</i>	Bn-A03-p1050893/ 883,287	-	-	Bn-A03-p1297873(SY (3.86%))/ 929,858
<i>Bna.AGL19.C7*</i>	<i>AGL19</i>	Bn-scaff_16069_1-p2897181/ 39,369,786	-	-	-

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<i>Bna.SOC1.A3*</i>	<i>SOC1</i>	Bn-A03-p1050893/ 883,287	-	-	Bn-A03-p1297873(SY (3.86%))/ 929,858
<i>Bna.SOC1.A5*</i>	<i>SOC1</i>	-	-	A05-p2497466/ 2,601,479	A05-p2497466 (SW (9.4%))/ 2,601,479
<i>Bna.SPL15.A4*</i>	<i>SPL15</i>	Bn-A04-p261340/ 194,583	-	-	-
<i>Bna.TOE1.A7*</i>	<i>TOE1</i>	Bn-A07-p11152131/ 12,442,850	-	-	-
<i>Bna.LNK2.A7*</i>	<i>LNK2</i>	-	Bn-A07-p11813549/ 13,966,389	-	-
<i>Bna.LNK2.C6*</i>	<i>LNK2</i>	Bn-scaff_15818_2- p1567500/17,932,787	Bn-scaff_15818_2-p1699411/ 18,048,792	-	-
<i>Bna.RUP2A9*</i>	<i>RUP2</i>	-	Bn-A01-p9030262/ 2,563,530	-	-
<i>Bna.GA2ox1.C6*</i>	<i>GA2ox1</i>	Bn-scaff_17799_1-p300944/ 36,342,566	-	-	-
<i>Bna.BRC1.A3*</i>	<i>BRC1</i>	-	-	-	Bn-A03-p18025753 (PN & SY)/ 17,031,377
DEGs between BBCH20 and BBCH30- V					
<i>Bna.VIN3.A2</i>	<i>VIN3</i>	Bn-A02-p7012860/4,047,981	Bn-A02-p6564861/3,784,981	Bn-A02-p6473898 (3.1%)/3,687,810	Bn-A02-p6473898 (SY (11.4%))/3,687,810
<i>Bna.AFR1.A2</i>	<i>AFR1</i>	Bn-A02-p13782884/ 10,402,649	-	-	Bn-A02-p13782884(SW (5.25%))& SN (10.3%)) /10,402,649
<i>Bna.MYB30.A2</i>	<i>MYB30</i>	Bn-A03-p1070680/ 21,520,725	-	-	-
<i>Bna.FLC.A3a</i>	<i>FLC</i>	Bn-A03-p6994294/ 6,278,471	-	-	-

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<i>Bna.FLC.C9b</i>	<i>FLC</i>	-	Bn-scaff_17750_1-p1905208/ 46,574,560	-	-
<i>Bna.TOE1.A3</i>	<i>TOE1</i>	-	-	-	Bn-scaff_22466_1-p980721(SW (4.7%))/ 10,475,804
<i>Bna.PIF4.A4</i>	<i>PIF4</i>	-	Bn-A04-p17976746/ 18,056,886	-	-
<i>Bna.HTA11.A9</i>	<i>HTA11</i>	-	-	-	Bn-scaff_22728_1-p576821 (SY (5.13%))/ 24,330,467
<i>Bna.HTA11.C6</i>	<i>HTA11</i>	Bn-scaff_15818_2- p1567500/17,932,787	Bn-scaff_15818_2-p1698069/ 18,046,506	-	-
<i>Bna.MYR1.A10</i>	<i>MYR1</i>	-	Bn-A10-p11230418/ 12,543,348	-	Bn-A10-p11272184 (SW (3.92%))/ 12,567,405
<i>Bna.HTA8.C3</i>	<i>HTA8</i>	-	Bn-scaff_23954_1-p72433/ 11,751,481	-	-
<i>Bna.CCA1.C4</i>	<i>CCA1</i>	Bn-scaff_16534_1-p2222544/ 4,794,748	-	-	-
<i>Bna.VIM1.C6</i>	<i>VIM1</i>	Bn-scaff_16116_1-p50186/ 27,985,350	Bn-scaff_16116_1-p50186/ 27,985,350	-	-
<i>Bna.VIM1.C8</i>	<i>VIM1</i>	-	Bn-scaff_16197_1-p1008308/ 33,079,933	-	-
<i>Bna.GLK1.C8</i>	<i>GLK1</i>	Bn-scaff_16197_1-p438035/ 33,580,092	-	-	-
<i>Bna.MSII.C9</i>	<i>MSII</i>	Bn-scaff_15808_1-p31258/ 36,873,261	-	-	-

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<i>Bna.GA20ox3.C9</i>	<i>GA20ox3</i>	-	Bn-scaff_17750_1-p1810873/ 46,663,402	-	-
<i>Bna.PRR9.A4</i>	<i>PRR9</i>	Bn-A04-p18913495/18,961,269	-	-	-
<i>Bna.FIO1.C4</i>	<i>FIO1</i>	Bn-scaff_19208_1-p442021/ 35,248,108	Bn-scaff_19208_1-p442021/ 35,248,108	-	-
<i>Bna.HUA2.C7</i>	<i>HUA2</i>	Bn-scaff_15762_1-p717223/ 2,201,425	-	-	-

2.5 Discussion

We aimed to understand the regulation of flowering time in rapeseed through transcriptome analysis. We assumed that the regulatory pathways between Arabidopsis and rapeseed are highly conserved. The main findings of this study can be summarized as follows: (1) we provided the first report on the paralog specific expression dynamics of the flowering time regulating genes in leaves under vernalized and non-vernalized conditions along the flowering transition in a semi-winter rapeseed cultivar, (2) we identified 36 orthologs of known flowering time regulators in Arabidopsis as putative candidate genes, analyzing the paralog specific expression of more than 54,000 gene models along with genetic mapping and association analysis, (3) we provided novel evidence for neofunctionalization of flowering time regulators in rapeseed. Combining all these data, we found compelling evidence for orthologs of *VIN3* and *FUL* not only as flowering time genes, but also as seed yield regulators in rapeseed.

Transcriptome analysis provides valuable information about gene expression patterns, new genes discovery and post-transcriptional regulation of different genotypes under different environmental conditions. The candidate genes identified based on whole transcriptome studies need to be confirmed through additional investigations. Genetic mapping can be applied as a second tool to verify the effect of the candidate genes derived from the whole transcriptome analysis. Yu et al. (2016) compared the transcriptomes of an apetalous line with a petalled line and found 36 putative regulators of the apetalous character in rapeseed, from which six candidate genes were located within QTLs regulating petal organization. Apart from QTL analysis, GWAS in combination with transcriptome analysis also offers a powerful platform to dissect the genetic architecture of complex traits. Lu et al. (2016) identified 33 candidate genes responsible for harvest index in rapeseed using the combination of transcriptome analysis and GWAS. In the current study, we applied both QTL analysis and GWAS to confirm the RNA-seq-derived-candidate genes associated with flowering time and yield related traits in rapeseed. Our study is the first that combines three approaches for candidate gene identification. However, for deeper understanding of the role of the candidate genes identified in this study and their interaction with other flowering time regulators, further functional analysis is inevitable.

2.5.1 Vernalization-responsive candidate genes for flowering time in rapeseed

In the current study, we identified orthologs of vernalization pathway genes which regulate flowering time in rapeseed by further characterization of genes differentially expressed after vernalization through genetic mapping or GWAS.

VIN3, *FLC*, *REDUCED VERNALIZATION RESPONSE (VRN)* complex genes, *SUPPRESSOR OF FRIGIDA 4 (SUF4)* and *CLEAVAGE STIMULATING FACTOR 77 & 66 (CSTF77 & CSTF 66)* are major vernalization pathway genes in Arabidopsis. *VIN3* in Arabidopsis controls the transcript level of *FLC* by H3 dimethylation and promotes flowering. *VIN3* expression increases after vernalization and becomes undetectable 3 days after return to higher temperatures (22°C) (Sung & Amasino, 2004). In our study, we observed similar expression patterns for two *Bna.VIN3* paralogs, which was in accordance with observations in *Brassica rapa* ssp. *chinensis* (Sun et al., 2015) and *Brassica oleracea* (Ridge, Brown, Hecht, Driessen & Weller, 2014). *Bna.VIN3.A2* was the only gene that fulfilled all our criteria for candidate gene identification. It was mapped to a flowering time QTL on chromosome A02 and showed a positive association with flowering time based on our association analysis. This result was in line with Nelson et al. (2014), who mapped *Bna.VIN3* to a flowering time QTL on chromosome A02 in a rapeseed population made by a cross between a European and an Australian spring-type rapeseed line. In another study, Raman et al. (2016) found SNP marker in LD with *Bna.VIN3* to be

associated with flowering time. Apart from flowering time, positive association of *Bna.VIN3.A2* with seed yield and localization within seed yield related QTL in our study provided hints for its pleiotropic role in semi-winter rapeseed. Zhao, Hu, Guo, Qian & Xue (2010) observed that a VIN3- like protein, leaf inclination2 (lc2), regulates inclination of leaves in rice which is important for plant architecture and grain yield. Similarly, *Bna.VIN3.A2* might affect yield through regulation of plant architecture. In Arabidopsis, a single amino acid change in the PHD zinc finger domain completely abolishes the binding ability of the VIN3 protein and results in altered vernalization response (Kim & Sung, 2017). We performed sequence analysis in different rapeseed ecotypes for *Bna.VIN3.A2*. We expected differences in the coding sequences of *Bna.VIN3.A2* among rapeseed accessions with varying growth habits. The difference of single amino acid in PHD zinc finger domain observed in the current study (Figure S5(C)) can be a possible reason for variation in vernalization requirements of different rapeseed ecotypes. Nevertheless, further sequence analysis with more diverse accessions as well as between early and late flowering accessions, for which the sequence data is publically available can shed more light on the role of *Bna.VIN3.A2* in rapeseed. The function of *Bna.VIN3.A2* needs to be further confirmed through mutant analysis for which we will use the rapeseed EMS population that has been previously constructed in our institute (Harloff et al., 2012).

Since *FLC* is another major vernalization pathway gene in Arabidopsis, we investigated the expression pattern of its orthologs in our study. The downregulation of eight out of nine *Bna.FLC* paralogs after vernalization in the current study was in line with previous studies in rapeseed (Zou et al., 2012), *B. rapa* (Zhao et al., 2010) and *B. oleraceae* (Lin et al., 2005). Based on association mapping, we found that only one paralog (*Bna.FLC.A3a*) was associated with flowering time, which was in accordance with the results obtained by Zou et al. (2012) and Raman et al. (2016). In a recent study, it was observed that homoeologous exchange and genomic introgression in *Bna.FLC.A3a* causes genetic differences in controlling vernalization in semi-winter types (Sun et al., 2017). Therefore, from current and past studies, it is evident that *Bna.FLC.A3a* plays an important role in flowering time and vernalization control in rapeseed. Additionally, Hou et al. (2012) mapped *Bna.FLC.A10* on a flowering time QTL, while we did not find any evidence for involvement of this paralog in flowering time control. This contradictory result can be due to a varying number of markers, number of individuals and phenotyping strategy between different QTL studies. *FLC* in Arabidopsis is known to have more than 500 binding sites indicating alternative regulatory roles to the repression of flowering. Majority of the target sites of *FLC* in Arabidopsis are on the promoters of the genes, which are involved in development pathway and reproductive development (Deng et al., 2011). Similarly, *Bna.FLC* paralogs might have functions other than flowering time regulation, as indicated by the association of *Bna.FLC.C9a* with seed yield in our study.

Except *Bna.FLC* and *Bna.VIN3*, we did not find any other vernalization pathway genes to be differentially expressed before and after vernalization. Orthologs of Arabidopsis vernalization pathway genes, *FRI* and *VRN* complexes showed constitutive expression pattern before and after vernalization. This is in accordance with the expression pattern in Arabidopsis, where *FRI* mRNA level was not affected by vernalization (Michaels, Bezerra & Amasino, 2004). Similarly, expression patterns of *VRN* complex genes (*VRN1* and *VRN2*) were also constant before and after vernalization in Arabidopsis (Gendall, Levy, Wilson & Dean, 2001, Levy, Mesnage, Mylne, Gendall & Dean, 2002).

2.5.2 Vernalization-independent candidate genes for flowering time in rapeseed

We considered the common DEGs between BBCH20 and BBCH50 in both temperature conditions to find flowering time regulators irrespective of vernalization treatment. Two orthologs of *FUL* showed positive association with flowering time and were upregulated at BBCH50 compared to BBCH30. In contrast, the Arabidopsis *FUL* gene is strongly upregulated only in the shoot apical meristem after

floral transition (Hempel et al., 1997). Nevertheless, previous studies showing expression of orthologs of *API* and *FUL* in barley leaves and their involvement in tomato leaf development (Burko et al., 2013) reveal that meristem identity genes can be also expressed in leaves aiding vegetative growth. Notably, we observed association of *Bna.FULA9* with seed yield based on QTL analysis and GWAS, which makes *Bna.FULA9* one of our most promising candidate genes for controlling yield related traits and hints towards neofunctionalization of a *FUL* homolog in rapeseed.

Our study suggests a role of two *Bna.SOC1* paralogs in controlling yield related traits in rapeseed (Table 2), which has not been reported before. Remarkably, two *SOC1* homologs in barley showed higher expression in later seed development stage suggesting their possible role in seed development (Papaefthimiou, Kapazoglou & Tsaftaris, 2012). Therefore, from the current study and the previous reports, it is evident that *SOC1* orthologs have functions beyond floral transition. In addition to yield related traits, we confirmed the role of two *Bna.SOC1* paralogs in flowering time regulation through QTL mapping and association analysis. These results were in accordance with the observations in Arabidopsis, that *SOC1* is a floral inducer which integrates signals from the photoperiod, vernalization and autonomous pathways (Lee & Park, 2015). The up-regulation of *Bna.SOC1* paralogs after vernalization was in accordance with the observation made in Arabidopsis (Sheldon, Jean Finnegan, Dennis & James Peacock, 2006).

The Arabidopsis SBP- box transcription factor *SPL15* plays an important role in the juvenile-to-adult phase transition (Schwarz, Grande, Bujdoso, Saedler & Huijser, 2008). In our study, we observed differential expression of one (*Bna.SPL15.A4*) of four expressed *Bna.SPL15* paralogs. This paralog showed positive association with flowering time, which was never reported before in rapeseed. In Arabidopsis, *SPL15* is predominantly expressed only in shoot apical meristem and young flowers. We found that all four *Bna.SPL15* paralogs were expressed in leaves, which is in accordance with Cheng et al. (2016). *SPL*-homologs and their upstream micro-RNA regulators miR156/157/529 came into focus because their role in controlling plant growth and development in different crops like Arabidopsis, maize and rice is conserved (Poethig, 2009, Wang & Zhang, 2017). The positive association of *Bna.SPL15* in the current study provides indications of rapeseed being another plant having conserved role of *SPL* homologs in the transition from juvenile to adult development. In our experiments, orthologs of some major flowering time genes like *FT* and *TFL* were not differentially expressed. From the studies conducted in Arabidopsis, it is evident that *FT* is diurnally regulated and it shows the highest expression during the evening and early in the night (Krzymuski et al., 2015). In our experiment, leaves were sampled at noon, which can be the reason for the absence of *Bna.FT* mRNAs. Hence, the choice of plant material and the experimental design can strongly affect the identification of candidate genes regulating major processes by transcriptome analysis. Multiple sampling of plant material throughout the day is necessary to unravel the transcriptional activity of diurnally regulated flowering time genes. *TFL1* as an inflorescence meristem identity gene is only expressed in cells just below the apical dome of inflorescence and co-flourescence meristems (Bradley, Ratcliffe, Vincent, Carpenter & Coen, 1997). Since we analyzed leaf material for transcriptome analysis, we observed very low expression for *Bna.TFL* paralogs. For the future studies we suggest assessing the expression of genes in different tissues (e.g. shoot apical meristem) using additional developmental stages to provide a more comprehensive model for flowering time regulation in rapeseed. Moreover, analyzing the expression between winter and spring type rapeseed could uncover ecotype-specific gene regulation. However, we expect a number of candidate genes identified based on our criteria in the current study to regulate the flowering time in winter and spring rapeseed.

In conclusion, we demonstrated the power of transcriptome analysis in combination with QTL and association mapping to identify candidate genes regulating traits of agronomic importance in rapeseed. Based on our data, we conclude that the expression patterns of major vernalization pathway genes of

Arabidopsis are conserved in rapeseed. Moreover, based on differential expression of paralogs, association mapping and genetic mapping studies, we showed evidence of sub- or neofunctionalization for certain flowering time genes which showed positive association with yield related traits (Table 2 and Figure 8). There is increasing evidence, that despite of its quantitative inheritance, single genes play an important role in the regulation of yield related traits (Wang & Zhang, 2017). In support of this hypothesis, our data suggest that orthologs of *VIN3* and *FUL* play an important role as flowering time and yield regulators in rapeseed.

The result of the current study can be useful for selection of rapeseed varieties adapted to different environments. Using the sequences from flowering time genes, the Brassica gene pool can be screened for new alleles of the candidate genes, which can later be combined to create genotypes better adapted to certain environmental conditions. For example, *Bna.VIN3* (*Bna.VIN3.A2*) and *Bna.FLC* (*Bna.FLC.A3*), which showed a strong association with flowering time, can be useful for breeding varieties, which can better adapt to certain growth conditions. Genetic variation can also be improved by stable introgression of spring rapeseed alleles into winter ecotypes, as spring cultivars have a substantially higher genetic variation compared to winter cultivars. Recently, targeted genetic modification by CRISPR-CAS technology has been established and demonstrated to be highly efficient in rapeseed (Braatz et al., 2017). All paralogs of a given Arabidopsis ortholog in rapeseed could be mutagenized at a time. This offers interesting perspectives for further functional characterization and modification of flowering time genes to produce new plant prototypes with superior agronomic performance.

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2.7 Supplementary Data

Table S1: The *B. napus* mini diversity panel for the initial growth experiment in the climate chamber. For each accession, 20 plants were split into two groups of vernalized and non-vernalized plants. Seeds were provided by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) and Dr. Habibur Rahman (University of Alberta, Canada); DH: doubled haploid line.

Table S2: Total number of RNA probes (rRNA-depleted) for sequencing of 14 Ningyou7 RNA-seq libraries through the Illumina HiSeq2000 platform; RIN: RNA integrity number

Table S3: Quality filtering and raw read statistics for Ningyou7 transcriptome libraries. Three biological replicates grown under two different conditions (with and without cold treatment) were sequenced from three developmental stages; V: Vernalized plants, NV: Non-vernalized plants.

Figure S1: Phenological development of Ningyou7 under greenhouse conditions. Plants were grown in two different experiments 1) Vernalized and 2) Non-vernalized. The number of days to flowering is given as days from sowing to first flower. Each column represents number of days to flowering for each biological replicate which is the mean of four plants; BR: Biological replicate; Exp.1: Experiment 1 (Vernalized conditions); Exp.2: Experiment 2 (Non-vernalized conditions).

Figure S2: Scatter plot showing correlation between expression data from RNA-seq and RT-qPCR. RT-qPCR values from 63 paralogs of 21 *Arabidopsis* flowering time orthologs and 14 uncharacterized gene models were used to optimize bioinformatics pipeline. A) log₂fold change data between BBCH20 and BBCH50-V; B) log₂fold change data between BBCH20 and BBCH50-NV; C) log₂fold change data between BBCH20 and BBCH30-V.

Figure S3: Genetic positions of QTLs related to 22 phenological and yield related traits along with the positions of the candidate genes from current study which mapped to the QTLs. The map was constructed with 202 DH lines of the TN population. Phenotypic traits were measured under 19 environments (Luo et al., 2017). The position of candidate genes from the current transcriptome study is marked with a black arrow. Vertical lines on the left side of the linkage groups indicate the confidence interval for QTLs; Marker positions mentioned on the right side of the chromosome are in cM.

Figure S4: Manhattan plots representing marker-trait associations of *Bna.VIN3.A2* as an example for the candidate genes with absolute values for flowering time (A) and seed yield (B) data from Schiessl et al. (2015). Each color key represents one environment out of 11 different environments from Germany, China and Chile as mentioned by Schiessl et al. (2015). The solid black line represents the position of closest SNP marker to *Bna.VIN3.A02* and the dotted lines on each sides represent the region within LD of the gene. The SNP markers within LD and having $-\log_{10}P > 2.0$ for at least one out of 11 environments were considered as associated with trait.

Figure S5: Sequence variation in *Bna.VIN3.A2* among rapeseed accessions with different growth habits. A) Sequence comparison between two winter type and two spring type rapeseed cultivars. B) Sequence comparison between two winter type and two semi-winter type rapeseed cultivars. C) Protein sequence comparison for *Bna.VIN3.A2* PHD zinc finger domain among winter type, semi-winter type, spring type and 'Darmor-bzh'; black arrow indicates sequence level variation. Sequence analysis was performed using CLC Main Workbench.

Method S1: Quantitative real-time RT-PCR

Method S2: Identification of sequence variations in the candidate gene

2.8 References

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3 Stop codon mutation in an *APETALA1* homoeolog affects plant architecture and seed yield component in oilseed rape (*Brassica napus* L.)

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3.1 Abstract

3.1.1 Background

Increasing the productivity of rapeseed as one of the widely cultivated oil crops in the world is of utmost importance. As flowering time and plant architecture play a key role in the regulation of rapeseed yield, understanding the genetic mechanism underlying these traits can boost the rapeseed breeding. Meristem identity genes are known to have pleiotropic effects on plant architecture and seed yield in various crops. To understand the function of one of the meristem identity genes, *APETALA1* (*API*) in rapeseed, we performed a phenotypic analysis of TILLING mutants under greenhouse conditions. Three stop codon mutant families carrying a mutation in *Bna.API.A02* paralog were analyzed for different plant architecture and seed yield related traits.

3.1.2 Results

It was evident that stop codon mutation in the K domain of *Bna.API.A02* paralog caused significant changes in flower morphology as well as plant architecture related traits like plant height, branch height and branch number. Furthermore, yield related traits like seed yield per plant and number of seeds per plants were also significantly altered in the same mutant family. Apart from phenotypic changes, stop codon mutation in K domain of *Bna.API.A02* paralog also altered the expression of putative downstream target genes like *Bna.TFL1* and *Bna.FUL* in shoot apical meristem (SAM) of rapeseed. Mutant plants carrying stop codon mutations in the COOH domain of *Bna.API.A02* paralog did not have a significant effect on plant architecture, yield related traits or the expression of the downstream targets.

3.1.3 Conclusions

We found that *Bna.API.A02* paralog has pleiotropic effect on plant architecture and yield related traits in rapeseed. The allele we found in the current study with a beneficial effect on seed yield can be incorporated into rapeseed breeding pool to develop new varieties.

3.2 Introduction

Rapeseed (*Brassica napus* L., AACC, $2n = 38$) is one of the most important oil crops in the world for the production of vegetable oil and animal feed. The productivity of rapeseed has been significantly increased in the last ten years, mainly due to the high yielding cultivars, mechanical harvesting and better agronomic practices. Nevertheless, in order to meet the increasing demand for edible oil worldwide, it is important to understand the genetic mechanism underlying rapeseed productivity (Cai et al. 2016). To increase the productivity, optimization of flowering time and plant architecture is fundamental.

Flowering time plays a very crucial role in the environmental adaptation of the plant. Environmental adaptation mainly includes the adaptation to prevailing climatic conditions (for example season, day length, and temperature), as well as biotic and abiotic stresses. The adaptation to the environment is

important for the overall yield of the plant. It is known from the model plant *Arabidopsis* that environmental factors like cold temperature, photoperiod and ambient temperature, as well as, genetic and epigenetic factors, influence floral transition. Under long day conditions, the floral inducers *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) are activated and trigger the expression of meristem identity genes like *LEAFY* (*LFY*), *APETALA1* (*API*), *SEPALLATA3* (*SEP3*) and *FRUITFULL* (*FUL*). Subsequently, meristem identity genes transform the *Arabidopsis* shoot apical meristem into a floral meristem (Blümel et al. 2015). In contrast to *FT*, *TERMINAL FLOWER-1* (*TFL1*), which shares high sequence similarity (71%) with *FT*, represses downstream meristem identity genes such as *API* and *LFY* in the central zone of the meristem. Considering the close phylogenetic relationship between rapeseed and *Arabidopsis*, knowledge of flowering time control in *Arabidopsis* provides the basis to understand the regulatory network in rapeseed. However, knowledge transfer from *Arabidopsis* to rapeseed is hindered by the complexity of the rapeseed genome. Different approaches have been applied to unveil the flowering time mechanism in rapeseed. Application of biparental populations for mapping quantitative trait loci (QTL) for flowering time and other agronomic traits is one of the widely used approaches (Javed et al. 2016; Liu et al. 2016; Luo et al. 2014). In one of such studies, Raman et al. (2013) mapped 20 different flowering time loci to 10 different chromosomes using a doubled haploid (DH) population, derived from a cross between two vernalization responsive rapeseed cultivars. They reported that three paralogs of *Bna.API* coincided with flowering time QTL on chromosomes A02, A07, and A08. Nevertheless, up to now no studies in rapeseed have demonstrated the phenotypic effect of *Bna.API* overexpression or mutation on flowering time.

In addition to flowering time, selecting plants with ideal architecture is also crucial for crop domestication and improvement. (Jiao et al. 2010). Therefore, over the years, there have been several studies in major crops to understand the mechanisms, which control plant architecture. For example, in rice, the ideal plant architecture (IPA) plant was reported to have thicker and more robust stems with more grains per panicle (Jiao et al. 2010). Variation in the leaf angle also showed a significant effect on maize grain yield, underpinning the importance of plant architecture in optimizing crop yield (Ku et al. 2010; Tian et al. 2011). Plant height, branch length, branch angle, length of main inflorescences, leaf angle and branch number per plant define plant architecture in *B. napus*, which affect seed yield components like silique number per plant and also number of seeds per plant (Chen et al. 2014; Qiu et al. 2006). Cai et al. (2016) mapped 163 QTL related to plant architecture and yield related traits in a DH population comprising 254 individuals. In another study, Shen et al. (2018a) could identify 19 QTL related to plant height, branch initiation height, stem diameter and flowering time in a DH population with 208 individuals. Using the same population, 17 QTL for branch angle were found, of which, three major QTL were steadily expressed, each explaining more than 10% of the phenotypic variation (Shen et al. 2018b). Besides genetic mapping, association mapping has also been used to find candidate genes for the trait of interest. In one of such GWAS studies (Schiessl et al. 2015), 158 winter rapeseed accessions were phenotyped for flowering time, plant height and seed yield in 11 different environments across Germany, China and Chile. These accessions were genotyped using the Brassica 60 K-SNP Illumina® Infinium consortium array. They found 68 regions across the rapeseed genome, which showed multi-trait associations. Using the same SNP array, Li et al. (2017b) genotyped 472 diverse rapeseed accessions to find regions associated with rapeseed branch angle. They found 21 loci across the genome with significant associations with branch angle. In another GWAS study, Zheng et al. (2017) genotyped and phenotyped 333 rapeseed accessions across 4 years and found seven loci for plant height, four for branch initiation height, and five for branch number.

During plant development, the shoot apical meristem (SAM) transforms into an inflorescence meristem and finally into a floral meristem. Subsequently, sepals, petals, stamen, and carpel of the

flower are developed from the floral meristem. Several meristem identity genes play an important role in the development of floral organs. Moreover, there are reports in crops suggesting the role of meristem identity genes in controlling plant architecture (Burko et al. 2013; Lu et al. 2012; Rao et al. 2008). Meristem identity and determinacy are controlled by the overlapping expression of meristem identity genes of the ABCE model (Hugouvieux et al. 2018). In Arabidopsis, A-functional genes include *APETALA1* (*API*) and *APETALA2* (*AP2*), B- functional genes include *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), C- functional genes include *AGAMOUS* (*AG*), and E- functional genes include *SEPALLATA* orthologs (*SEPI–SEP4*) (Liu and Mara 2010). *API* plays a crucial role in floral meristem identity and also in sepal and petal development in Arabidopsis (Alejandra Mandel et al. 1992). In Arabidopsis, a mutation in *API* causes the conversion of sepals into bracts as well as the development of floral buds in the axil of transformed sepals. Moreover, the flowers of the mutant plants also lack petals (Irish and Sussex 1990). Previous studies demonstrated the ability of *API* orthologs from various plant species (*Jatropha curcas* (Tang et al. 2016), Orange (Sun et al. 2014), Pea (Berbel et al. 2001) and Lily (Chen et al. 2008)) to complement Arabidopsis *ap1* phenotype, which indicates the conservation of the role of *API* between different species. However, orthologs of *API* have not been yet functionally characterized in rapeseed.

In the current study, we aimed to characterize the function of an *API* ortholog in rapeseed using TILLING (Targeting Induced Local Lesions in Genomes). Based on our phenotypic evaluation, we report that a stop codon mutation in *Bna.API.A02* alters plant architecture in rapeseed and increases the number of seeds per plant. Moreover, we found that a stop codon mutation in *Bna.API.A02* also leads to modifications in floral architecture similar to an Arabidopsis *API* mutant phenotype. Our data suggest that EMS-generated alleles can be useful to develop high yielding varieties by conventional breeding and can also be valuable to increase genetic diversity for rapeseed breeding.

3.3 Materials and Methods

3.3.1 Mutation screening

We screened 3,840 M₂ plants of the EMS Express617 winter rapeseed mutant population (Harloff et al. 2012) by TILLING. For TILLING, we used normalized DNA (5ng/μl) from M₂ plants which was arranged into ten 96-well microtiter plates using two-dimensional (2D) eight-fold (8x) pooling strategy (Harloff et al. 2012). We designed paralog-specific primers for *Bna.API.A02* and *Bna.API.C02* (Table S4) using the published reference genome sequence (Chalhoub et al. 2014). Subsequently, we performed *CelI* digestion of heteroduplexes, sample purification and polyacrylamide gel electrophoresis (PAGE) on a LI-COR 4300 DNA analyzer (LI-COR Biosciences, www.licor.com) according to Harloff et al. (2012). We used GelBuddy Software (Zerr and Henikoff 2005) to identify mutations.

3.3.2 Plant material and growth conditions

M₄ seeds were produced by selfing of M₃ plants of three *Bna.API.A02* stop codon mutant families. From each family, 20 M₄ seeds per genotype (mutant and wildtype) together with Express617 were sown in the greenhouse for phenotyping. All plants were grown in the greenhouse under constant temperature (22°C) and long day conditions (16h light, 900 μmol m⁻² s⁻¹, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands). After three weeks of pre-culture, we transferred the plants to a cold chamber at 4°C (vernalization) under long day conditions (16h light, 200 μmol m⁻² s⁻¹, Osram Lumilux T8 L 58W/840, Osram AG, München, Germany) for eight weeks. After vernalization, we transferred the plants to the initial greenhouse conditions and transplanted them into 11x11 cm pots. We randomized the plants two times a week.

3.3.3 Plant phenotyping and statistical analysis

We measured the total number of seeds per plant, seed yield per plant (g) and a total number of healthy and filled siliques per plant. Moreover, we measured plant height (length of the plant from the base of stem to the top of the main inflorescence at maturity), branch height (distance from the base of the stem to the first branch (Cai et al. 2016)) and branch number (the total number of primary and secondary branches at maturity). The means of 15 plants were compared among different genotypes using the Least Significant Difference (LSD) test ($\alpha \leq 0.05$). For LSD test, we used the R package 'Agricolae' version 1.2-8.

3.3.4 Gene expression analysis

We collected SAM tissue from three M_4 vernalized plants (BBCH 30) (Meier 2001) at Zeitgeber time (ZT) 8. We performed RNA isolation from three biological replicates with the peqGold Plant RNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined by agarose gel electrophoresis and photometric quantification with a NanoDrop spectrophotometer (Thermo Scientific). We treated total RNA with DNase I (Fermentas Inc., Maryland, USA) to remove genomic DNA. Subsequently, we synthesized the first-strand cDNA from 1 μ g of DNA-depleted RNA using Oligo(dT)18 primers and the M-MuLV Reverse Transcriptase (ThermoFisher Scientific, Waltham, United States). For quality check, a standard PCR of the synthesized cDNAs (1:10 dilution) was performed with the housekeeping gene *Bna.Actin* (rapeseed actin gene, GenBank Accession No. AF111812). Prior to expression analysis, we developed primers for *Bna.TFL*, *Bna.LFY*, *Bna.SEP4*, *Bna.FUL* and *Bna.AP1* (Table S4). Moreover, we designed paralog-specific primers for *Bna.TFL* paralogs (*Bna.TFL1.A10*, *Bna.TFL1.C3* and *Bna.TFL1.Ann*) and the amplicons were Sanger sequenced.

We performed quantitative real-time RT-PCR (RT-qPCR) with SYBR qPCR Super mix w/ROX (Invitrogen Corporation, Carlsbad, USA) using a CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). For each reaction, we used a total volume of 20 μ l containing 100 nM of each primer and 2 μ l of diluted cDNA templates with the following cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by 95°C for 10 min. Primer efficiencies for the different targets were determined using four 2-fold serial dilutions of cDNA and were included in the calculation of relative expression levels in Bio-Rad CFX Manager 3.1. We analyzed the amplification curves and used the average Ct values of three technical replicates to calculate relative expression in comparison to the reference gene (*Bna.Actin*) using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001).

3.4 Results

3.4.1 Identification of *Bna.AP1* stop codon mutants by TILLING

We performed amino acid sequence alignments between *A. thaliana* (AtAP1) and six rapeseed (*Bna.AP1*) AP1 proteins to identify the conserved regions. Based on protein alignments, amino acid sequences are highly conserved between Arabidopsis and rapeseed proteins (Figure 9). We concluded that all four functional domains present in AtAP1 (MADS domain, I domain, K domain, and COOH domain) are also present in five out of six predicted *Bna.AP1* proteins. Only, *Bna.AP1.C02* lacks a MADS-box domain based on the published rapeseed genome sequence (Chalhoub et al. 2014). We selected two paralogs, *Bna.AP1.A02* and *Bna.AP1.C02* for mutation screening, to study the function of *AP1* orthologs in rapeseed. These two paralogs were selected based on leaf transcriptome analysis data of semi-winter rapeseed cultivar "Ningyou7" (Shah et al. 2018), expressed sequence tags (EST) data

available for winter rapeseed cultivar ‘Darmor-bzh’ in the genome database (Chalhoub et al. 2014) and the study of genetic variation in *Bna.API* paralogs between different *B. napus* morphotypes (Schiessl et al. 2014). We designed paralog-specific primers and the *Bna.API.A02* and *Bna.API.C02* amplicons covered 88.7% and 55.9% of the coding sequences, respectively. We screened 3,488 M₂ plants for *Bna.API.A02* and 2,720 M₂ plants for *Bna.API.C02* paralog to find mutations. After confirmation by sequencing, we found 164 mutations in the *Bna.API.A02* paralog and 32 mutations in the *Bna.API.C02* paralog (Table S5). For *Bna.API.A02*, we identified six premature stop codon mutations (nonsense) in exon 4, exon 7 and exon 8. Moreover, we also found one splice site mutation in *Bna.API.C02* at the 5’ end of intron 4 (Figure 10). We considered three premature stop codon mutants of *Bna.API.A02* (annotated as *ap1_1*, *ap1_2*, and *ap1_3*) for further phenotyping in the greenhouse (Table S6). We selected these three mutants, because the premature stop codon for each of the selected mutant families was on a different exon, resulting in truncated proteins of different lengths. The splice site mutant found in *Bna.API.C02* copy was not considered for phenotyping along with three stop codon mutant families, because in an earlier greenhouse experiment, no phenotypic difference was observed between *Bna.API.C02* splice site mutant plants and the controls (data not shown).

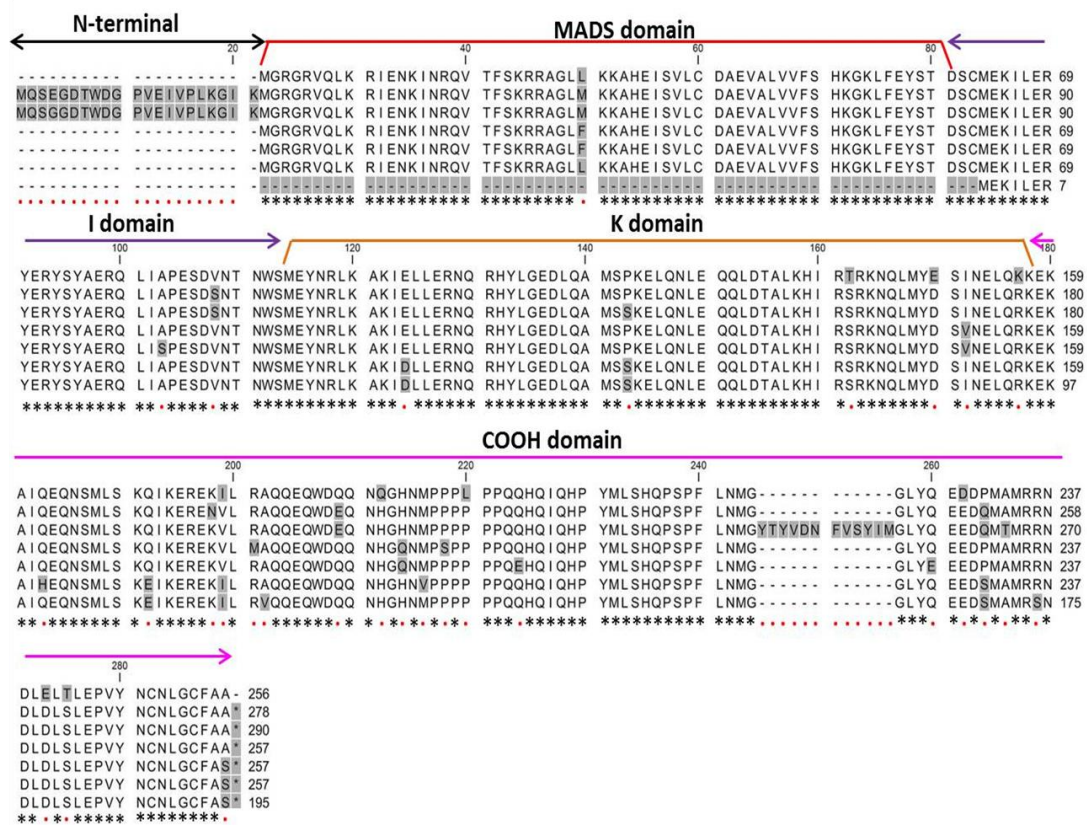


Figure 9: Amino acid alignment between *Bna.API* proteins and *AtAPI* protein. Conserved amino acids between proteins are indicated by asterisks (*), and red color dots (.) indicate non-conserved amino acids. Changes in the amino acids are shaded in grey.

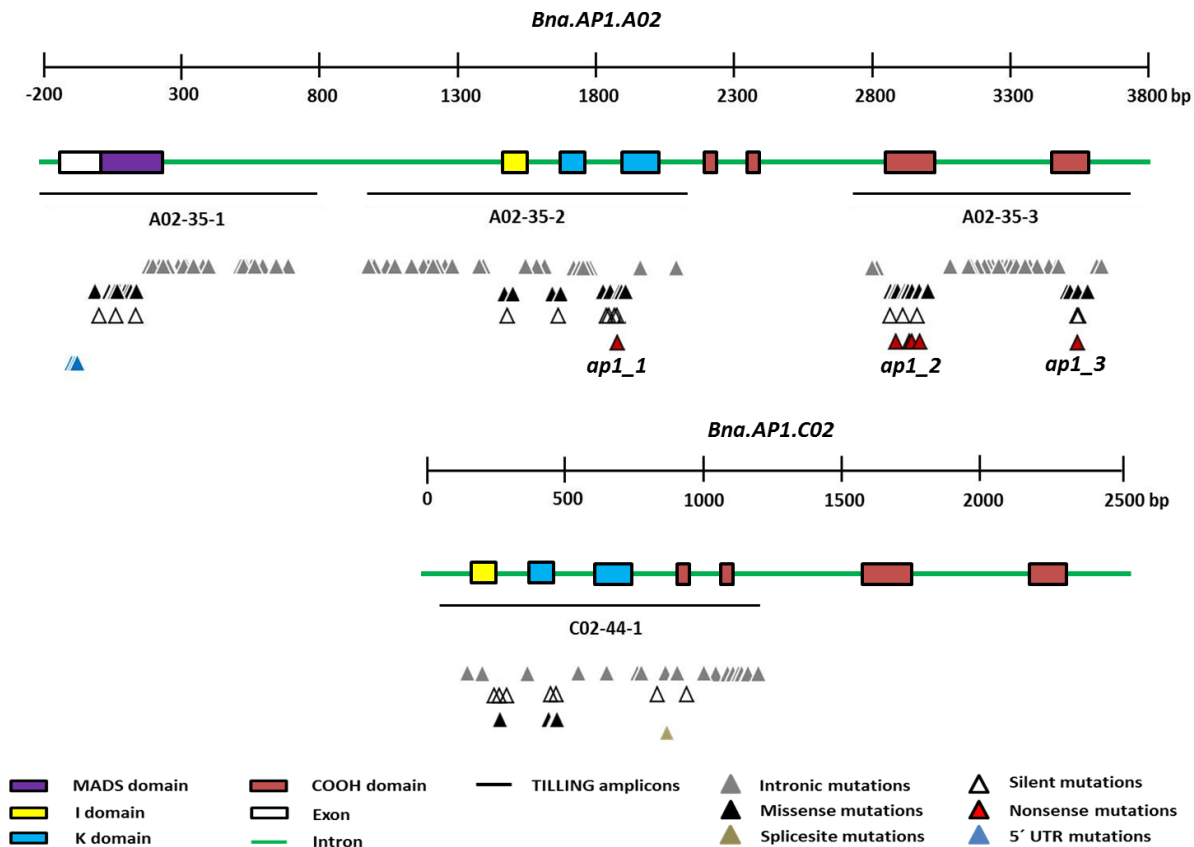


Figure 10: Graphical presentation of EMS induced mutations in *Bna.AP1.A02* and *Bna.AP1.C02* paralog in Express617. All mutations verified by sequencing are shown.

3.4.2 Effect of *Bna.AP1.A02* on flowering time, plant architecture and seed yield components

We recorded flowering time, plant height, branch height, number of seeds/plant, seed yield/plant, siliques/plant and branch number/plant of 15 M₄ plants per genotype for three stop codon mutant families, to investigate the effect of the mutations in *Bna.AP1.A02*. For ease of understanding, the mutant allele was termed 'a' and the wildtype allele was termed as 'A'. All three families consisted of mutant (*aa*) and wildtype (*AA*) genotypes, which were obtained by selfing of the M₃ plants (Table S6). In this context, a wildtype (*AA*) genotype was generated from the same mutant family and hence, it is expected to have all background mutations as they are in the mutant genotype (*aa*), except at the loci of interest (*Bna.AP1.A02*). Therefore, within each mutant family, we compared the mutant (*aa*) with the wildtype genotype (*AA*). We used Express617 as a non-mutated control. We observed that there was no significant difference in the flowering time between mutant and wildtype plants for all three families (data not shown).

The homozygous *ap1_1* mutant plants (*aa*) displayed secondary flower buds instead of sepals, which later developed into siliques (Figure 11). The same mutant family also displayed modified plant architecture-related traits like plant height, branch height, and branch number compared to the wildtype plants (*AA*) of the same family (Figure 12). We observed that plant height and branch height was increased in the mutant plants (116.9 ± 5 cm and 43.0 ± 10.66 cm, respectively) compared to the wildtype plants (109.3 ± 6.3 cm and 31.0 ± 6.8 cm, respectively). Moreover, *ap1_1* mutants demonstrated significant differences in plant yield related traits like seed yield per plant, seed number

per plant and siliques per plant compared to the wildtype plants (Figure 12). *apl_1* mutant plants had an average seed yield per plant of 1.75 ± 1.1 g compared to 0.84 ± 0.7 g for the wildtype plants.

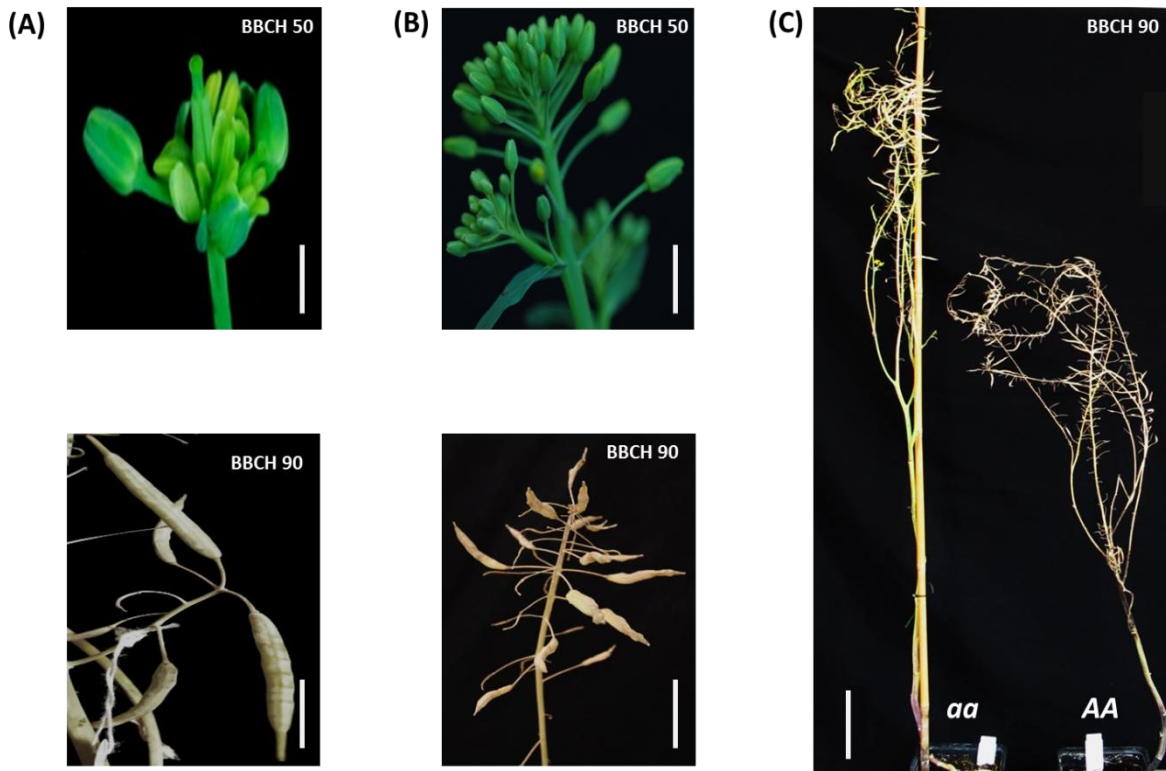
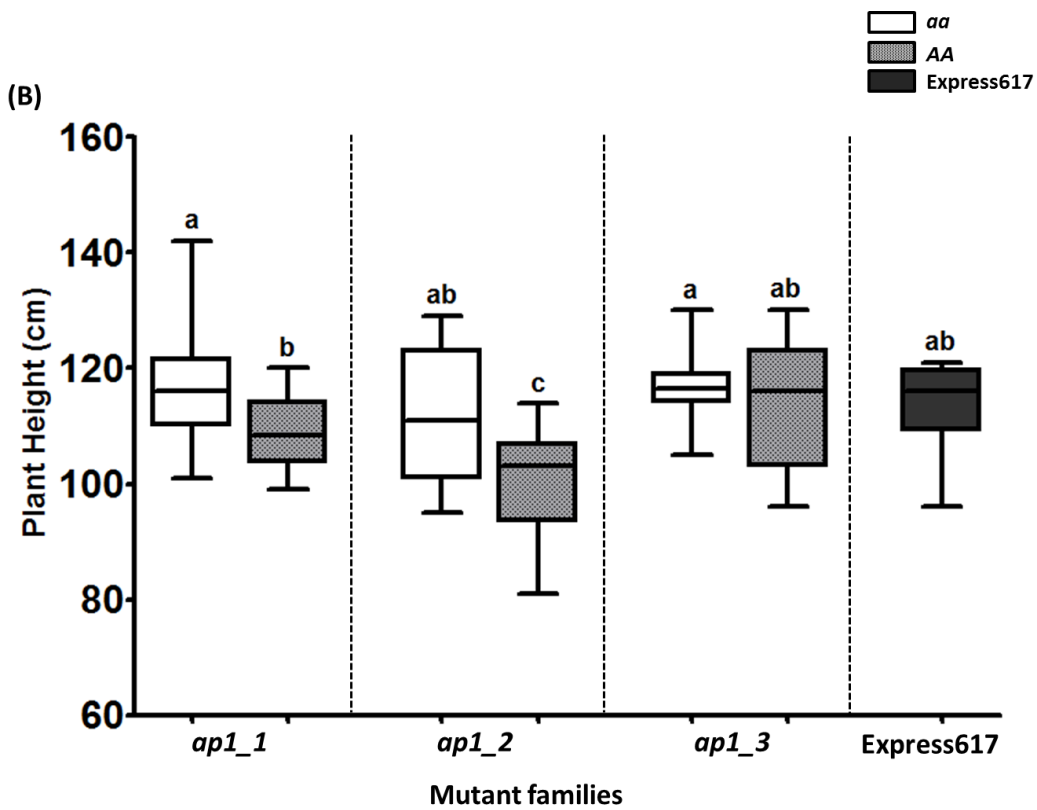
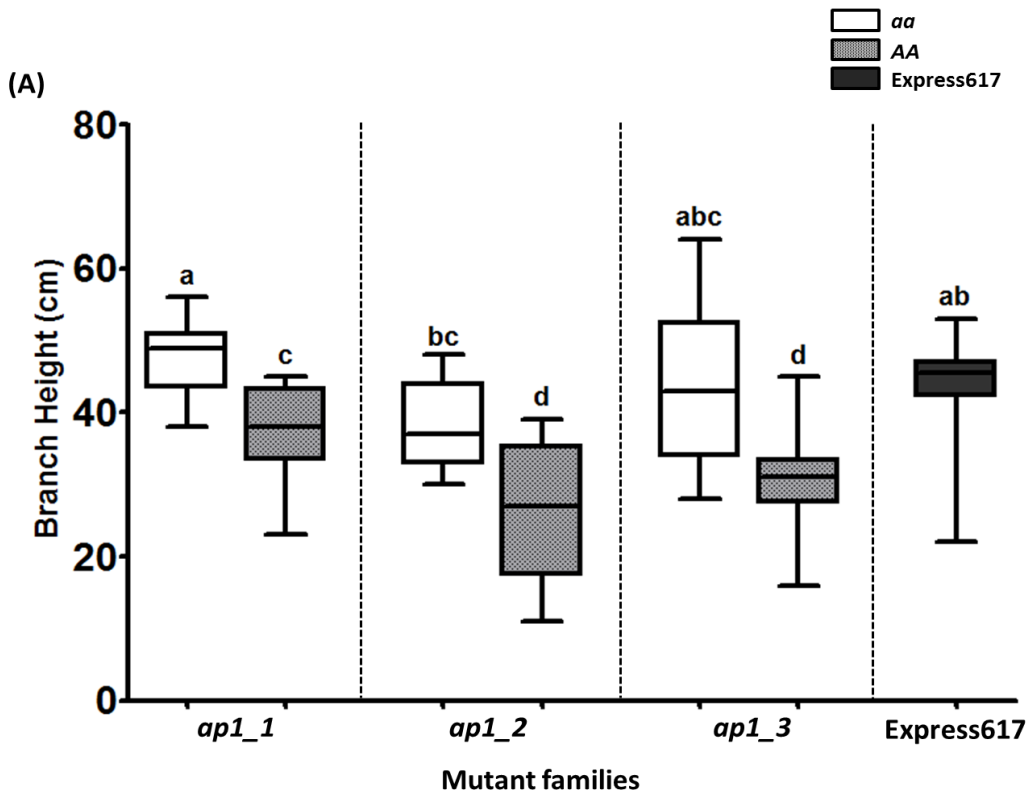
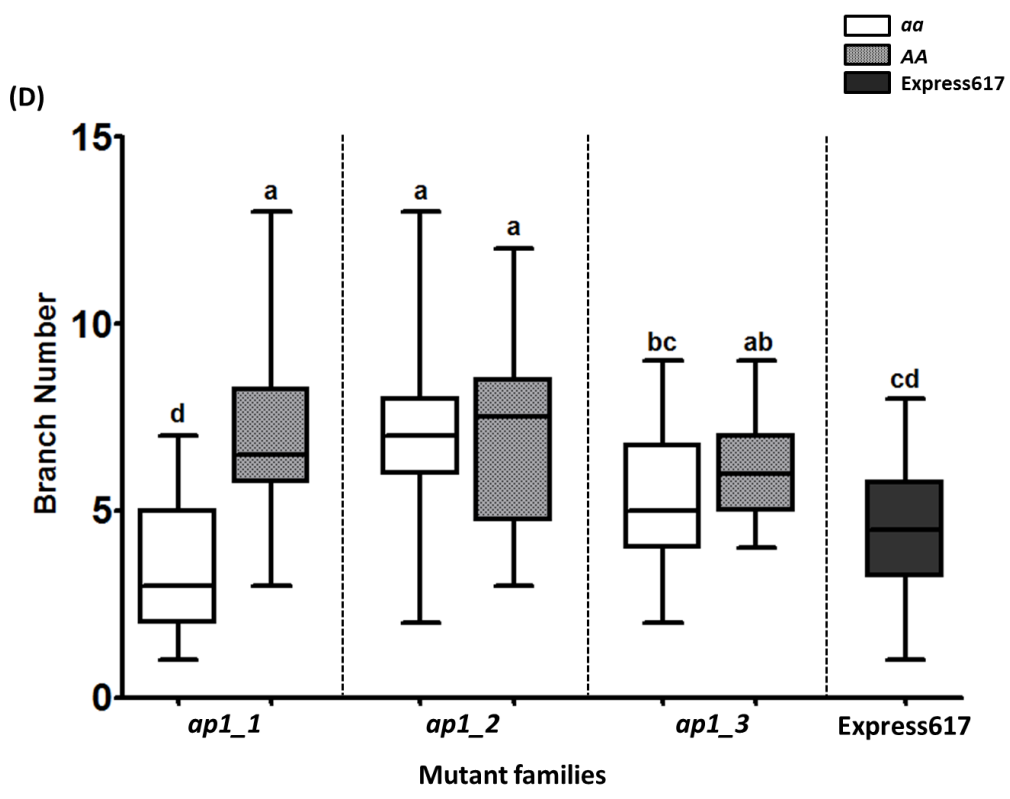
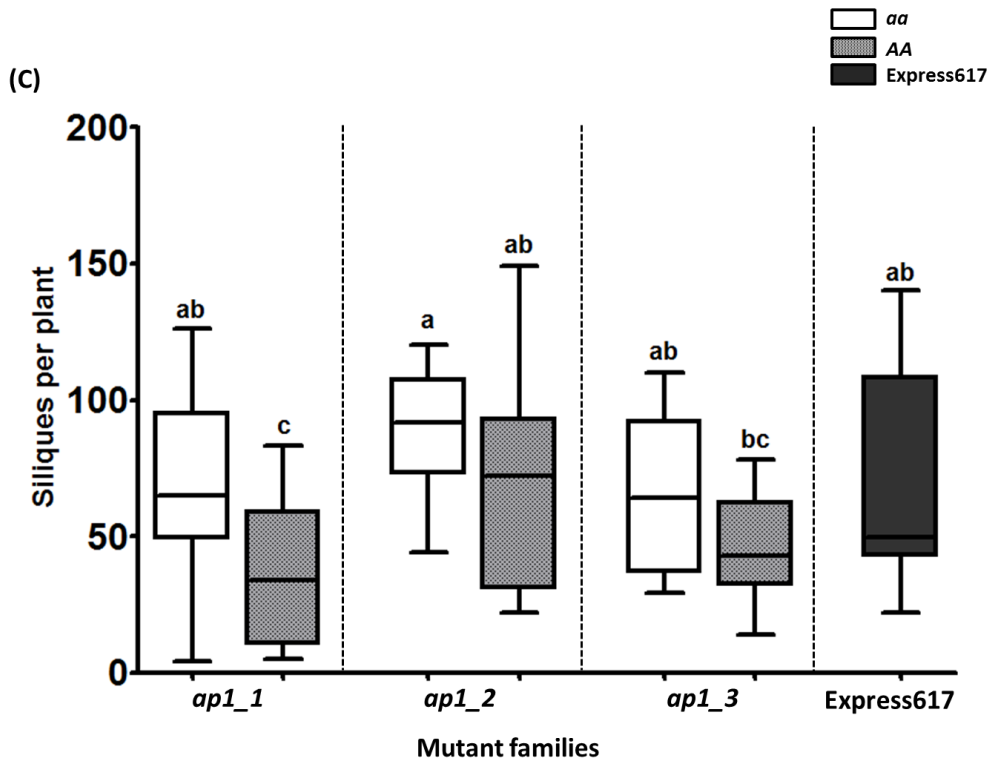


Figure 11: Morphological features of *Bna.API.A02* stop codon mutant lines (*aa*) and wildtype controls (*AA*). Floral buds and silique development in plants homozygous for the *Bna.API.A02* stop codon mutation (*aa*) are shown on the left (Fig. A; scale bar: 1 cm); Floral buds and silique development in homozygous wildtype plants (*AA*) in the center (Fig. B; scale bar: 1 cm for BBCH50 and 10 cm for BBCH90) and whole plant pictures with both genotypes on the right (Fig. C; scale bar: 10 cm). Plants were grown in the greenhouse at constant temperature (22°C), under long day conditions (16 h light) after vernalization (4°C, 16 h light, 8 weeks).

We observed that *apl_2* and *apl_3* mutant plants did not display any variation in floral bud development compared to their respective wildtype genotypes. Nevertheless, *apl_2* mutant plants exhibited significant differences in plant and branch height, while *apl_3* mutant plants had significantly different branch height and branch numbers (Figure 12). We did not observe any significant difference in yield related traits for *apl_2* and *apl_3* mutant plants.





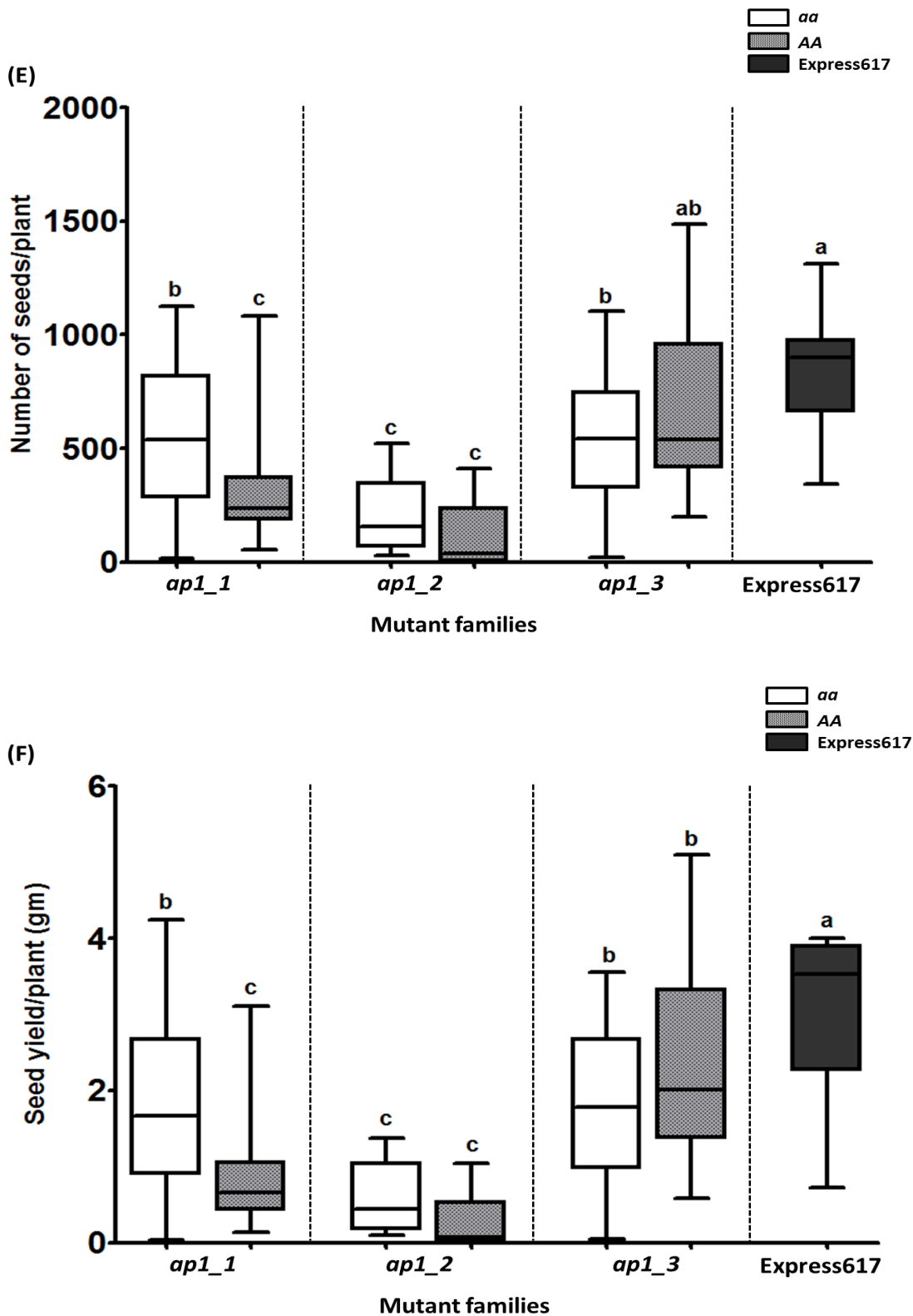
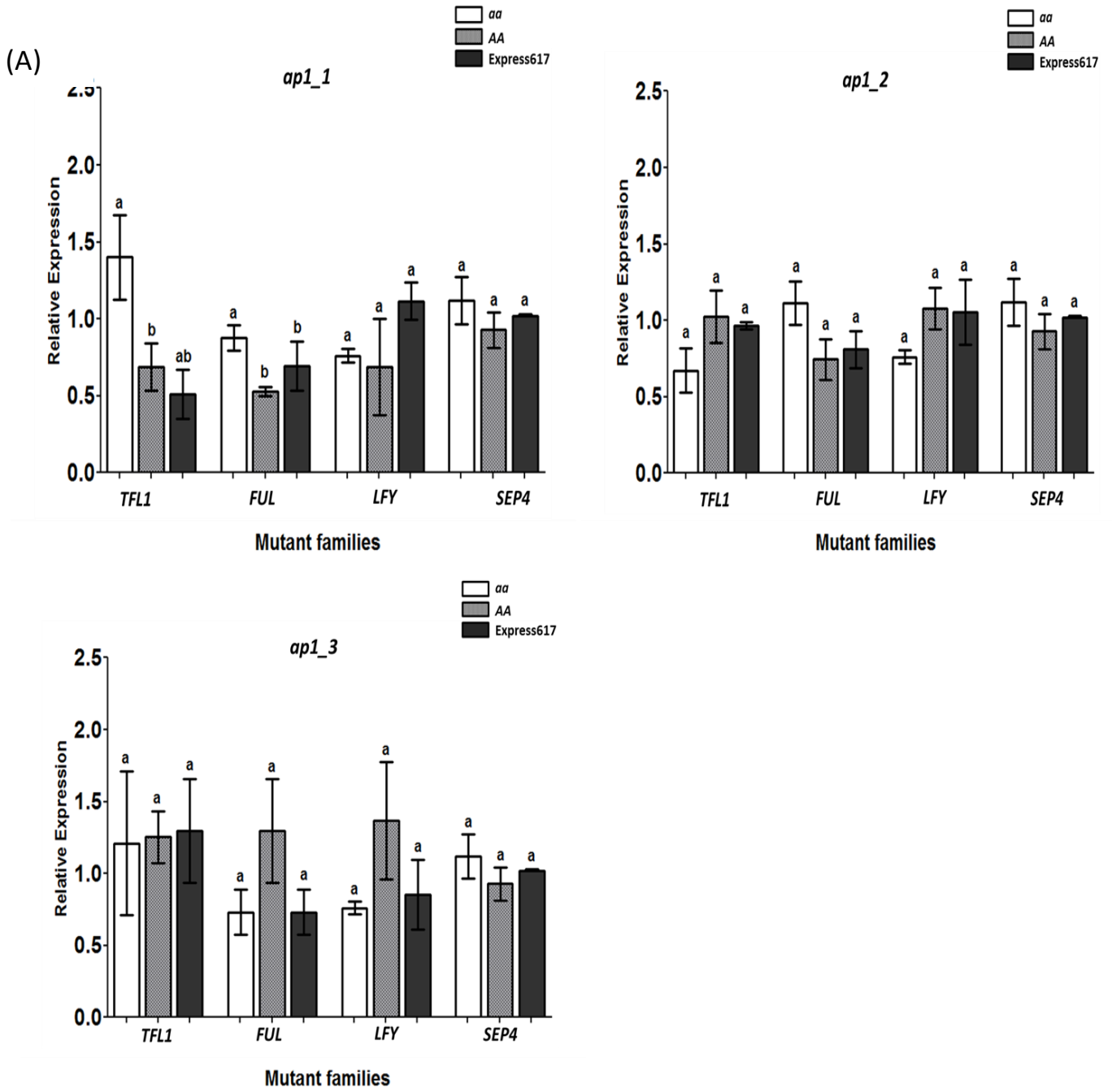


Figure 12: Box plots showing phenotypic analysis of *Bna.API.A02* stop codon mutant lines under the greenhouse conditions. (A) Branch height, (B) Plant height, (C) Siliques per plant, (D) Branch number, (E) Number of seeds/plant and (F) Seed yield/plant. 15 plants per genotypes were used for phenotyping. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Error bars: standard error of the mean for 15 plants. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using the LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

3.4.3 A stop codon mutation in *Bna.AP1.A02* alters the expression of *Bna.TFL1* and *Bna.FUL* in the SAM

We expected that a stop codon mutation in *Bna.AP1.A02* paralog impacts the expression of its downstream target genes. Therefore, we measured the expression of putative downstream target genes in the SAM of *Bna.AP1.A02* stop codon mutant families. We took SAM samples from the greenhouse-grown plants between zeitgeber time 8 and 9. Based on the knowledge from Arabidopsis, we selected *Bna.TFL1*, *Bna.FUL*, *Bna.LFY* and *Bna.SEP4* as putative downstream targets of *Bna.AP1*. We observed 2-fold higher joined expression of all *Bna.TFL1* paralogs in *ap1_1* mutants compared to wildtype plants (Figure 13(A)). When we measured the expression of *Bna.TFL1* paralogs separately, we observed a similar expression pattern for three out of four paralogs in *ap1_1* mutants (Figure 13(B)). Due to high sequence similarity, we could not design paralog-specific primers for *Bna.TFL.Cnn*. We did not observe any significant difference in the expression of *Bna.TFL1* paralogs in *ap1_2* and *ap1_3* mutant plants. To further study the interaction between *Bna.AP1* and *Bna.TFL1*, we reasoned that the expression of *Bna.AP1* also decreases in *Bna.TFL1* mutants, as it is the case for Arabidopsis. For this purpose, we investigated the combined as well as paralog-specific (*Bna.AP1.A02*) expression of *BnAP1* in a *Bna.TFL1.A10* missense mutant family identified in a previous study (2014). We observed that after two generations of backcrossing, the plants carrying a missense mutation in *Bna.TFL1.A10* showed lower combined and paralog-specific (*Bna.AP1.A02*) expression compared to the wildtype plants (Figure S6).

Besides *Bna.TFL1*, we detected significantly lower expression of *Bna.FUL* in *ap1_1* mutants compared to wildtype, but we did not observe any significant difference in the expression of *Bna.SEP4* and *Bna.LFY* between the mutants and controls. Moreover, we also did not detect any significant difference in the expression of any of the putative downstream target genes in *ap1_2* and *ap1_3* mutant plants (Figure 13). In order to investigate the nonsense-mediated mRNA decay phenomenon, we measured the expression of *Bna.AP1.A02* paralog in *ap1_1*, *ap1_2* and *ap1_3* mutant families. We observed that there was no significant difference in the *Bna.AP1* combined and paralog-specific (*Bna.AP1.A02*) expression between the mutant and wildtype genotypes for *ap1_1* and *ap1_2* mutant families (Figure S7). Nevertheless, there was a significant difference in combined and paralog-specific (*Bna.AP1.A02*) expression between the mutant and wildtype genotypes for *ap1_3* mutant family.



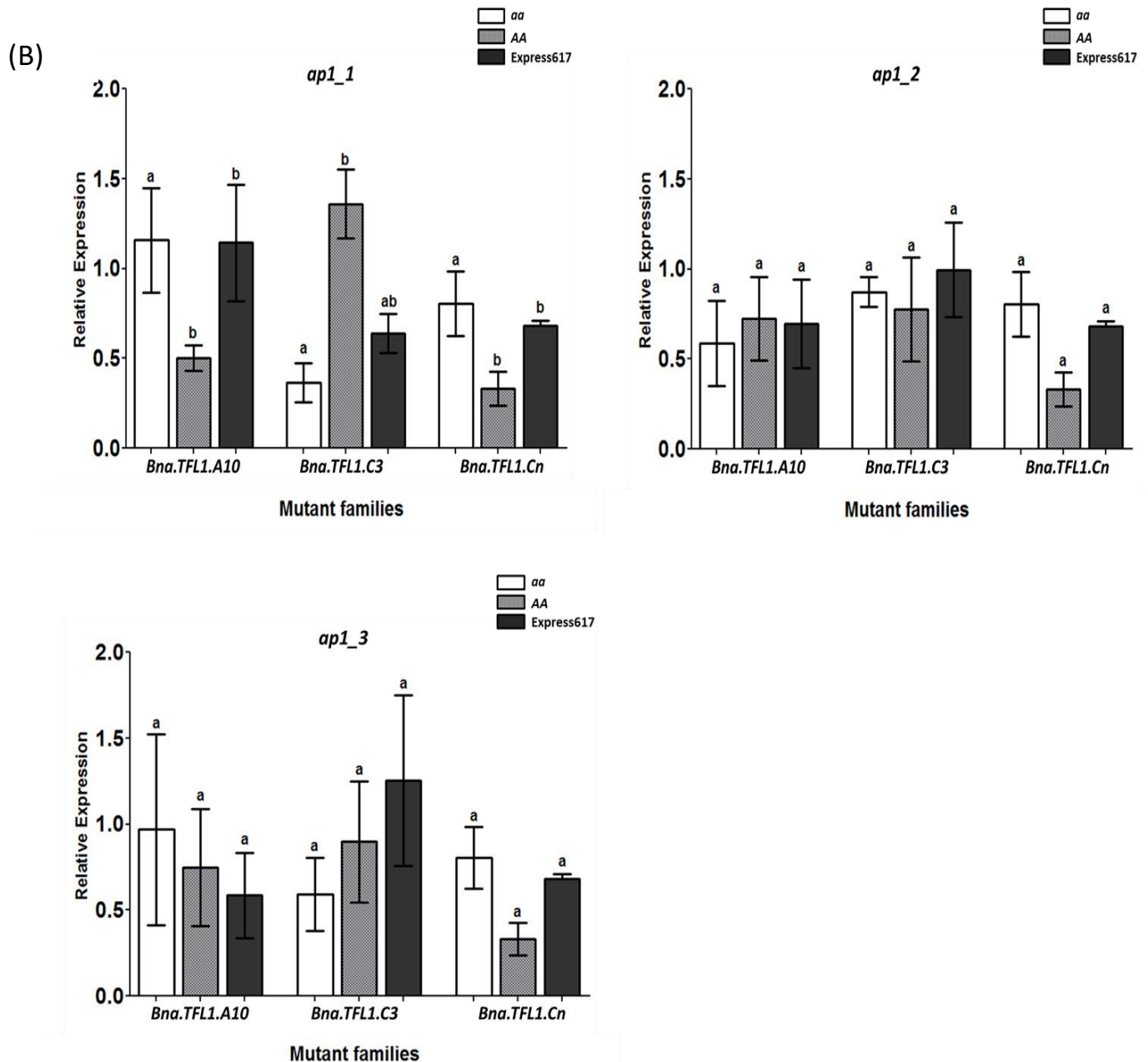


Figure 13: Expression analysis of *Bna.API.A02* stop codon mutant lines. (A) Relative expression of *Bna.API* putative downstream target genes (B) Relative expression of *Bna.TFL1* paralogs. *aa*: Genotype carrying *Bna.API.A02* mutant allele (M_4 generation); *AA*: Genotype carrying *Bna.API.A02* wildtype allele (M_4 generation); Express617: Control. Expression levels of target genes were normalized against *Bna.Actin* total expression. For all genotypes tissue (SAM) sampling was done at BBCH30 between zeitgeber 8 h and 9 h. Three biological replicates and three technical replicates were used for each genotype. Error bars: standard error of the mean for biological replicates. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using the LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

3.5 Discussion

The aim of this study was to characterize the role of *API* orthologs in rapeseed. We hypothesized that the function of *API* is conserved between rapeseed and Arabidopsis. The main findings of this study can be summarized as follows: (1) a stop codon mutation in *Bna.API.A02* strongly affects plant

architecture and seed yield related traits, (2) the same mutation alters the plant architecture and increases the number of seeds per plant. (3) Moreover, the stop codon mutation in the K domain of *Bna.API.A02* leads to altered expression of the putative downstream target genes *Bna.TFL1* and *Bna.FUL*. Combining all these data, we found compelling evidence that the stop codon mutation in the K domain of *Bna.API.A02* leads to architectural changes in rapeseed, with an impact on seed yield components.

TILLING offers a non-transgenic, rapid and cost-efficient method for detection of point mutations, which can be used to target each homologue of a multigene family independently. After phenotypic evaluation of TILLING mutants, favorable alleles can be combined into a single line by crossing single mutant parents. The frequency of EMS mutations depends on several factors like plant species, target tissue, the developmental stage of the target tissue, mutagen and also the concentration of mutagen (Harloff et al. 2012). Based on the previous studies, mutation frequencies vary in Brassicaceae family. For example, it was reported to be 1/345 kb in Arabidopsis (Lai et al. 2012), which was lower than mutation frequency in rapeseed (1/41.5 kb, (Wang et al. 2008). Moreover, a higher mutation frequency of 1/56 kb was observed in *B. rapa* (Stephenson et al. 2010) compared to *B. oleracea*; (1/447 kb, (Himelblau et al. 2009). Apart from Brassicaceae family, Chen et al. (2012a; 2012b) reported 1/47 kb mutation frequency in hexaploid wheat, while Till et al. (2007) reported 1/300 kb mutation frequency in rice. Based on these studies, it was evident that the mutation frequency in diploid species is expected to be lower than in polyploid species. In our study, we calculated the mutation frequencies of 1/17.4 kb and 1/26 kb for *Bna.API.A02* and *Bna.API.C02*, respectively. Mutation frequency observed in this study is similar to mutation frequencies reported by Harloff et al. (2012) for sinapine biosynthesis genes; (1/12 kb to 1/22 kb) but higher than reported mutation frequencies by Guo et al. (2014) for flowering time genes *Bna.TFL1* and *Bna.FT*; (1/24 kb to 1/72 kb), using the same EMS population.

3.5.1 *Bna.API* has functions beyond conferring meristem identity in rapeseed

In the current study, our aim was to evaluate the function of an *API* homolog in rapeseed. In Arabidopsis, *API* confers meristem identity with an important role in sepal and petal development (Irish and Sussex 1990). Moreover, an *API* mutation in Arabidopsis resulted in delayed flowering (Piñeiro and Coupland 1998). Considering the close phylogenetic relationships between Arabidopsis and rapeseed, we expected that rapeseed plants carrying missense or nonsense mutations in *API* paralogs show the same phenotype as Arabidopsis *API* mutants. Unlike Arabidopsis, we did not observe any significant difference in flowering time between mutant and wildtype plants, which indicates that *Bna.API.A02* does not affect flowering time in rapeseed. However, in a previous study, Schiessl et al. (2014) found SNPs in *Bna.API* between early and late flowering winter rapeseed lines, suggesting a potential role of *Bna.API* in controlling flowering time in rapeseed. One possible explanation for the identical flowering time phenotype of mutant and wildtype plants can be the presence of five non-mutated *Bna.API* paralogs in *ap1_1* mutant plants.

We also evaluated floral and plant architecture in *ap1_1* mutant and wildtype plants. We observed the development of floral buds in the axil of transformed sepals in *ap1_1* mutant plants, which confirmed the conserved role of *API* as meristem identity gene in rapeseed. However, we did not observe this phenotype in all flowers of the mutant plants. The presence of flowers with normally developed sepals and petals on *ap1_1* mutant plants can be due to the compensation by the other non-mutated paralogs of *Bna.API*. A similar phenomenon has been reported for the *INDEHISCENT* (*Bna.IND*) gene, where a mutation in a single paralog of *Bna.IND* gene does not affect the shatter resistance in rapeseed due to the presence of other functional copies of the gene (Braatz et al. 2018a). However, the authors observed a significant increase in shatter resistance, when both paralogs of *Bna.IND* were mutated.

Therefore, inducing mutation in all functional copies of *Bna.API* might result in a stronger *apl_1* phenotype.

In polyploid plants like rapeseed, duplicated genes may undergo varying fate like sub-/neo-functionalization (Force et al. 1999) or gene loss (Blanc and Wolfe 2004). We wanted to analyze whether *Bna.API.A02* paralog has other functions beyond conferring floral meristem identity in rapeseed. For this purpose, we analyzed the effect of the mutation on plant architecture, yield related traits as well as the transcriptional activities of its putative downstream target genes. We observed that apart from floral architecture, a stop codon mutation in the K domain of *Bna.API.A02* also altered plant architecture-related traits like branch height, plant height and branch number, which is in accordance with studies in other crop plants. In rice (*Oryza sativa* L), plant architecture was altered after overexpression of *OsMADS15*, an ortholog of Arabidopsis *API* (Lu et al. 2012). Burko et al. (2013) showed a role of tomato *API/FUL* in tomato leaf development. Besides plant architecture, in previous studies, it has been shown that meristem identity genes also affect seed yield related traits. For instance, in a previous study, it was reported that meristem identity gene, *APETALA2* (*AP2*) controls seed yield and seed mass in Arabidopsis (Jofuku et al. 2005). Furthermore, a homolog of meristem identity gene *FUL* also showed indication of neo-functionalization in rapeseed (Shah et al. 2018). Nevertheless, there is no previous report on the role of *API* in controlling seed yield related traits in Arabidopsis. Hence, the results from the current study, indicating the involvement of *Bna.API.A02* in controlling plant architecture and seed yield related traits hints towards neo-functionalization of the meristem identity gene *API* in rapeseed.

3.5.2 *Bna.API.A02* is an upstream regulator of *Bna.TFL1* and *Bna.FUL* in rapeseed meristem

Because *Bna.API.A02* stop codon mutation had a strong effect on plant architecture and yield related traits; we expected an altered expression of genes which are transcriptionally regulated by *Bna.API*. The increased expression of *Bna.TFL1* paralogs in the *apl-1* mutant in rapeseed is in accordance with the relationship between *TFL1* and *API* in Arabidopsis, where constitutive expression of *API* downregulated the activity of *TFL1* (Liljegren et al. 1999). A higher expression of *Bna.TFL1* might be the reason for the increased number of branches and plant height in *apl_1* mutant plants compared to wildtype plants, since previous studies in Arabidopsis and other species have shown that the overexpression of *TFL1* and its homologs causes highly branched inflorescences and thus, altered plant architecture (Carmona et al. 2007; Danilevskaya et al. 2010; Jensen et al. 2001; Nakagawa et al. 2002). However, the decreased expression of *Bna.API* in *Bna.TFL1* missense mutant plants hints towards a different transcriptional regulation between *Bna.API* and *Bna.TFL1* in rapeseed. Moreover, the lower expression of *Bna.FUL* in *apl_1* mutant in the current study was also in contrast with the expression profile in Arabidopsis, where *FUL* was ectopically expressed in the floral meristem of an *API* mutant (Ferrandiz et al. 2000). Nevertheless, in another study, it was observed that overexpression of *Jatropha API* ortholog (*JcAPI*) in Arabidopsis caused higher expression of *FUL* (Tang et al. 2016). Hence, the data from current and previous studies suggest that despite functional conservation, meristem identity genes might display varying transcriptional regulation in other crops compared to Arabidopsis. Based on transcriptional data of *Bna.API*, *Bna.FUL* and *Bna.TFL1* from the current study, we propose a model of gene interaction: (1) *Bna.API.A02* suppresses the expression of three *Bna.TFL1* paralogs (2) *Bna.TFL1.A10* induces or maintains the expression of *Bna.API.A02* and (3) *Bna.API.A02* is necessary for maintaining the transcript level of *Bna.FUL*. We propose that analyzing the expression of *Bna.TFL1*, *Bna.FUL* and *Bna.API* in CRISPR-Cas mutants of meristem identity genes can depict the transcriptional regulation between these genes in a much clearer way because CRISPR-Cas technology can generate site-specific mutants without any background mutations. The expression and phenotypic data of *Bna.API.A02* mutant plants from the current study provide strong evidence for the involvement

of this homolog of *API* in controlling processes beyond its function as a meristem identity gene in rapeseed.

Based on phenotypic and expression data, *apl_1* showed a stronger effect on floral architecture, plant architecture and yield related traits compared to *apl_2* and *apl_3*. This was in accordance with our expectations because *apl_1* carried a mutation in exon four, which results in the shortest protein among all mutants, lacking a K- and COOH-domain. The other two mutants, *apl_2* and *apl_3*, carried mutations in exon 7 and exon 8, respectively. Hence, proteins encoded by these mutants are only lacking the COOH domain. There is a considerable amount of evidence in Arabidopsis, emphasizing the significant role of different AP1 domains in the protein function and its interaction with other proteins for the functional specificity. In a previous study (Alvarez-Buylla et al. 2006), chimeric constructs between *AtAPI* and *CAULIFLOWER (AtCAL)* were used to demonstrate that K domain and COOH domain of *API* are important for conferring floral meristem identity. In another study (Lowman and D Purugganan 1999), a 9bp insertion was found in the exon 4 of *BoAPI-B* gene in *B. oleracea ssp. botrytis* (domesticated cauliflower) and in *B. oleracea ssp. oleracea* (wild cabbage) that lead to a premature stop codon. As a result of these mutations, in both cases, *BoAPI-B* gene was coding for a truncated protein lacking a part of K domain and the entire COOH domain, leading to the cauliflower phenotype.

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3.7 Supplementary Data

Table S4: Primers used in this study for screening mutations in Express617 EMS population and for expression analysis by RT-qPCR.

Table S5: Details of EMS mutations in *Bna.API.A02* and *Bna.API.C02* paralogs detected by TILLING of Express617.

Table S6: Nucleotide position and amino acid changes in different splice site, non-sense and UTR mutants from *Bna.API.A02* and *Bna.API.C02* paralogs. The phenotyping was performed with plants from the M4 generation.

Figure S6: Relative expression of *Bna.API* in *Bna.TFL1.A10* missense mutant (Guo et al., 2014) plants. (A) Joint expression of *Bna.API* (B) Paralog-specific (*Bna.API.A02*) expression. M4: Genotype carrying *Bna.TFL1.A10* missense mutation (M4 generation); *aa-*: Genotype carrying homozygous mutant allele of *Bna.TFL1.A10* in BC2F3 generation; *AA*: Genotype carrying homozygous wildtype allele of *Bna.TFL1.A10* in BC2F3 generation; Express617: Control. Expression levels of target genes were normalized against *Bna.Actin* total expression. For all genotypes tissue (SAM) sampling was done between zeitgeber 8 h and 9 h. Three biological replicates and three technical replicates were used for each genotype. Error bars: standard error of the mean for biological replicates. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

Figure S7: Relative paralogue-specific (*Bna.API.A02*) (Fig. A) and joint expression of *Bna.API* (Fig. B) in *Bna.API.A02* stop codon mutant lines. *aa*: Genotype carrying homozygous mutant allele of *Bna.API.A02* (M4 generation); *AA*: Genotype carrying homozygous wildtype allele of *Bna.API.A02* (M4 generation); Express617: Control. Expression levels of target genes were normalized against *Bna.Actin* total expression. For all genotypes tissue (SAM) sampling was done between zeitgeber 8 h and 9 h. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Three biological replicates and three technical replicates were used for each genotype. Error bars: standard error of the mean for biological replicates. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

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4 Phenotypic evaluation of Brassica *TFL1/FT* mutants and F₁ hybrids

4.1 Introduction

Rapeseed (*Brassica napus*), is one of the important sources of edible oil, animal fodder, vegetables, condiments, and biodiesel. It plays a significant role in securing edible oil production worldwide accounting for more than 20 million tons of extracted oil worldwide (<http://www.worldoil.com/>). Rapeseed is grown in different environments over the world and therefore, adaptation to the environmental condition is of utmost importance to maximize yield. As environmental adaptation heavily relies on the flowering time of the variety, understanding its genetic mechanism can be useful to produce elite varieties with optimum flowering time and high yield. Rapeseed is an allopolyploid species which diverged from the Arabidopsis lineage approximately 14.5 to 20.4 MYA (Chalhoub et al. 2014). Though having high level of sequence collinearity, the rapeseed genome is more complex due to whole genome triplication and reshuffling of the diploid ancestors of rapeseed. As a consequence of this, single Arabidopsis orthologs are represented by one to twelve rapeseed paralogs (Schiessl et al. 2014).

Orthologs of *FT* and *TFL1* play an important role in controlling flowering time and a number of other phenotypic characters like seed yield and plant architecture in various crops (Guo et al. 2014). In Arabidopsis, plants carrying a nonsense mutation in *TFL1* flowers earlier compared to the non-mutated line, which is similar to the plants overexpressing *FT*. This concludes that in Arabidopsis, *TFL1* represses the transcription of genes which are activated by *FT* (Hanano and Goto 2011). Apart from Arabidopsis, orthologs of *FT* and *TFL1* have been also analyzed in various other crops like sugar beet (Pin et al. 2010), potato (Navarro et al. 2011), wheat (Yan et al. 2006), rice (Kojima et al. 2002) and barley (Faure et al. 2007). Along with flowering time, the pleiotropic effect of *FT/TFL1* orthologs was shown in tomato, where *SFT* (*FT* ortholog) and *SP* (*TFL1* ortholog) genes have a significant impact on heterosis (Krieger et al. 2010). In another study in tomato, heterozygous *sft* mutants (*sft/+*) showed a weak semi-dominant delay in flowering and when it was combined with *sp* mutant plants, the productivity was increased drastically (Jiang et al. 2013). This demonstrates that exploiting dosage sensitivity of florigen and its family members provides a way to enhance productivity in other crops, but species-specific tuning is required. In rapeseed, out of the six *Bna.FT* paralogs (Wang et al. 2012), one paralog on chromosome C02 is silent due to the insertion of a miniature inverted-repeat transposable element (MITE) in its promoter region. The other five paralogs are detectable in rapeseed as well as in both ancestors, *B. rapa* and *B. oleracea* (Wang et al. 2012). Three out of six *Bna.FT* paralogs (*Bna.FT.A2*, *Bna.FT.C6a*, and *Bna.FT.C6b*) were localized within flowering time QTL (Baojun et al. 2018; Wang et al. 2009). A missense mutation in *Bna.FT.C6b* showed a significant delay in flowering, while a *Bna.FT.C6a* missense mutation did not have any effect on flowering time (Guo et al. 2014). Moreover, it was also shown that a missense mutation in one of the four *Bna.TFL1* paralogs also delayed flowering (Guo et al. 2014).

The mutants for *Bna.FT* and *Bna.TFL1* analyzed by Guo et al. (2014) were identified via TILLING, which is based on screening EMS-treated population for desired point mutations. This method provides a valuable advantage for pyramiding beneficial mutant alleles with varying functions (Till et al. 2018). Chemical mutagenesis provides an opportunity to construct a large mutant population to find mutations in a large number of gene loci because mutations are induced randomly across the genome. TILLING offers a non-transgenic approach to identify mutations in genes of interest, and therefore, it has been applied for over 25 plant species so far (Till et al. 2018). Polyploids like rapeseed are better suited for TILLING compared to the diploids, as they can withstand high mutation densities. One of the drawbacks of chemical mutagenesis method is thousands of random background mutations across the whole genome. These mutations in addition to the mutation in the gene of interest can affect plant

phenotype and thus, complicate the analysis of the possible effects of the gene of interest. Additionally, random mutagenesis does not offer any possibility to mutate the desired number of gene copies in polyploids like rapeseed. This also makes it difficult to functionally characterize any gene, as there might always be non-mutated copies of the gene present in the genome to complement the function of the mutated copy. The Background mutation load of TILLING mutants can be reduced via repeated backcrossing with an elite line. For winter crops such as rapeseed which needs a period of vernalization before flowering, backcrossing till complete exclusion of background mutations can be time-consuming. Therefore, acceleration of the vegetation period of the plants can help to reduce the backcrossing time. In a recent study, speed breeding method was used to achieve up to 6 generations per year for spring wheat (*Triticum aestivum*), durum wheat (*T. durum*), spring rapeseed (*B. napus*) barley (*Hordeum vulgare*), chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) (Watson et al. 2018). Apart from these species, speed breeding methods have also been successfully applied to accelerate breeding objectives for amaranth (*Amaranthus* spp.) (Stetter et al. 2016), and peanut (*Arachis hypogaea*) (O'Connor et al. 2013). As an alternative time-saving approach, marker-assisted background selection can also be applied, where numerous markers are used to select BC₁ with a higher share of the recipient genome (Jung 2010).

Phenotypic analysis of *Bna.TFL1* TILLING mutants carried out by Guo et al. (2014) showed that missense a mutation in *Bna.TFL1* affects seed yield components. The authors observed increased seed yield in F₁ plants carrying a mutated *Bna.TFL1* allele on the Express617 background. When different plant varieties are intercrossed, the resulting hybrid shows better performance than the mean performance of the parents for the trait of interest; a phenomenon termed as heterosis (Schnable and Springer 2013). The superior performance of hybrids can appear as biomass, yield, abiotic and biotic stress tolerance, which makes heterosis an agronomically important phenomenon (Fujimoto et al. 2018). Since the 1990s, there have been mainly three hypotheses put forward to understand the genetic basis of heterosis. The most widely accepted hypothesis is the dominance hypothesis. According to the dominance hypothesis, heterosis results from suppression/complementation of harmful or deleterious recessive alleles from one parent by dominant alleles from the other parent. Another hypothesis, the overdominance hypothesis, states that heterosis can be attributed to heterozygosity at individual loci (Fujimoto et al. 2018). Both hypotheses are based on single-locus as well as quantitative models. The third hypothesis is based on epistasis in which interaction between two or more genes from the parental lines leads to heterosis. For crops like maize and rice, different studies have demonstrated that all three hypotheses play an important role in heterosis (Frascaroli et al. 2007; Li et al. 2017a; Li et al. 2001). A study conducted in tomato gave the first evidence of single gene overdominance in plants where heterozygosity of *SINGLE FLOWER TRUSS* (*SFT*) contributes to fruit yield heterosis (Krieger et al. 2010). Nevertheless, the extent of heterosis depends on various factors like plant species, parental combination and also the trait of interest. Therefore, heterosis cannot be explained by any single hypothesis (Fujimoto et al. 2018).

The present study was carried out to test the following hypotheses: (1) Variation in the vernalization period, and the light regime affects the vegetation period of rapeseed; (2) Reduction in the background mutation load of flowering time mutants can clearly depict the role of certain paralogs in flowering time; (3) Due to the heterosis effect, *Bna.TFL1/Bna.FT*- F₁ hybrids have higher yield performance than the individual mutants, and the controls and (4) Flowering can be severely delayed by mutations in both *Bna.TFL1.A10* and *Bna.FT.C6b*. I could shorten the generation time of rapeseed by five weeks. Analysis of four backcrossed mutants clearly showed the effect of background mutations on flowering time phenotype. My data indicates that *Bna.FT*- F₁ hybrids do not have any effect on yield performance compared to the controls (Express617 × MSL1 and Express617 × MSL2). I observed that plants carrying mutations in both *Bna.TFL1.A10* and *Bna.FT.C6b* showed a severe delay in flowering.

4.2 Materials and Methods

4.2.1 Plant material and genotyping

To shorten the vegetation period of rapeseed in the greenhouse, I conducted an experiment with varying growth conditions. For this experiment, winter type cultivar Express617 was used as plant material. To see the effect of background mutations on flowering time, I selected four mutant families (*ft_1*, *ft_2*, *ft_3*, and *tfl_1*) for background reduction by crossing with the donor line Express617 (Table 3). To perform phenotypic analysis of *Bna.FT/Bna.TFL1* double mutants, I sowed 120 F₂ seeds of *Bna.FT.C6b.G2009A* Splice (mutant code: *ft_1*) × *Bna.TFL1.A10.G750A* (mutant code: *tfl_2*) double mutants. Out of 120, 86 seeds germinated. As there were not enough F₂ plants for all the genotypes, I selfed the F₂ plants to produce F₃ seeds. For phenotypic analysis, I sowed 40 F₃ seeds of double heterozygous genotype (*AaBb*), 10 F₃ seeds of double homozygous mutant genotype (*aabb*), and 20 F₃ seeds for the rest of the genotypes (*AAbb*, *aaBB*, and *AABB*) in another greenhouse experiment. To select the desired genotype for backcrossing and double mutant analysis, I isolated genomic DNA from leaf samples of all plants using CTAB (Saghai-Marroof et al. 1984) method. Followed by DNA isolation, I amplified genomic DNA using gene-specific primers (Table S4) and performed sequencing.

To check the effect of *Bna.FT* and *Bna.TFL1* mutations on seed yield heterosis, a field trial was conducted at Norddeutsche Planzenzucht Hans-Georg Lembke KG (NPZ) in Groß Lüsewitz in the year 2015/16. For this field trial, I used F₁ seeds, along with the crossing parents (homozygous M₄ mutants and Express617/Express617 MSL) for five mutant families. During the season 2015/16, F₁ hybrid seeds were also produced in the field located at Hohenlieth, Germany (NPZ). Due to the poor performance of the pollinator (mutant lines), I could not get F₁ hybrid seeds for *Bna.TFL1.A10* mutants. The possible reasons for poor performance of the pollinator lines are thousands of background mutations present in the mutant plants, winter losses and also dry weather condition, which was there for 4 weeks in Hohenlieth, Germany (NPZ). For this reason, a field experiment in 2016/17 was conducted only with hybrids generated from four different *Bna.FT* mutant lines. For the year 2016/17, I conducted the field trial in two different locations within Germany: 1) NPZ in Groß Lüsewitz and 2) Hohenschulen (Achterwehr). For both locations, I used M₄ seeds from four mutant lines along with their respective F₁ hybrids produced from a cross between the mutant line and two male sterile lines (MSL1 and MSL2). MSL1 and MSL2 are male sterile lines which are presently being used by NPZ to produce rapeseed hybrids. Apart from this, I also used the seeds produced from a cross between Express617 and both male sterile lines (MSL1 and MSL2) as controls. Additionally, cultivars Express617, Penn and Avatar were also considered as controls in both locations (Table 4).

Table 3: Timeline of the backcrossing program for four mutant families. For phenotyping of the backcrossed mutant families, 30 seeds of BC₂F₃ homozygous and wildtype genotypes were sown under greenhouse conditions. The number in the bracket next to the harvest date indicates seed code.

Gene name	Mutation	Mutation type	Mutant code	F ₁	BC ₁	BC ₂	BC ₂ F ₂	BC ₂ F ₃	
								Homozygous	Wildtype
<i>Bna.FT.C6b_</i>	G2009A	Splice	<i>ft_1</i>	Available* (122136)	07.2015 (150446)	02.2016 (160196)	08.2016 (162133)	02.2017 (170078)	02.2017(170079)
<i>Bna.FT.C6b</i>	G2122A	Missense	<i>ft_2</i>	Available* (122160)	07.2015 (150448)	02.2016 (160223)	08.2016 (162155)	02.2017(170076)	02.2017(170077)
<i>Bna.TFL1.A1 0</i>	G52A	Missense	<i>tfl_1</i>	Available* (122088)	07.2015 (150458)	02.2016 (160216)	08.2016 (162147)	02.2017(170072)	02.2017(170073)
<i>Bna.FT.C6b</i>	G2009A	Splice	<i>ft_3</i>	Available* (122145)	07.2015 (150451)	02.2016 (160207)	08.2016 (162138)	02.2017(170074)	02.2017(170075)

Table 4: Plant material used for the field experiment conducted at Groß Lüsewitz and Hohenschulen (Achterwehr) in 2016/17.

Gene	Mutation	Mutation type	Mutant code	Generation	Seed codes
<i>Bna.FT.C6b</i>	G2009A	Splice site	<i>ft_1</i>	M ₄	162161
				F ₁ (M ₄ × MSL1)	162159
				F ₁ (M ₄ × MSL2)	162160
<i>Bna.FT.C6b</i>	G2122A	Missense	<i>ft_2</i>	M ₄	162165
				F ₁ (M ₄ × MSL1)	162163
				F ₁ (M ₄ × MSL2)	162164
<i>Bna.FT.C6a</i>	G163A	Missense	<i>ft_4</i>	M ₄	162169
				F ₁ (M ₄ × MSL1)	162167
				F ₁ (M ₄ × MSL2)	162168
<i>Bna.FT.C6a</i>	G37A	Missense	<i>ft_5</i>	M ₄	162173
				F ₁ (M ₄ × MSL1)	162171
				F ₁ (M ₄ × MSL2)	162172
Express617				Control	161537
Penn				Control	162175
Atora				Control	162176
Express617 × MSL031				Control	162157
Express617 × MSL211				Control	162158

4.2.2 Growth conditions and phenotyping

In order to develop a rapid cycle protocol for rapeseed, I designed four experiments (A, B, C, and D) with varying pre-culture time (time between sowing and start of vernalization) and vernalization period to see which treatment accelerates the rapeseed vegetation period the most (Table 5). All four experiments consisted of two sets of plants (10 plants per set), one set was treated with 16 hours of light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands) at constant temperature (22°C) after vernalization and the other with 22 hours light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands) at constant temperature of 22°C . In experiment A, I put the plants under vernalization for 8 weeks at 4°C (16h light, $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, Osram Lumilux T8 L 58W/840, Osram AG, München, Germany) after 2 weeks of pre-culture, while in experiment B, I put them under vernalization after 1 week of pre-culture. In experiment C and experiment D, I put the plants under 6 weeks of vernalization after 2 and 1 weeks of pre-culture, respectively. I counted the days between sowing and flowering to select the best growth conditions for rapid rapeseed growth. For both experiments, I used 7 cm pots.

Table 5: Experimental design for shortening the rapeseed generation time. For each greenhouse experiment winter rapeseed cultivar, Express617 was used.

Experiment	Pre-culture time	Vernalization period	Light regime after vernalization
A	2 weeks	8 weeks	10 plants under 22 hours and 10 plants under 16 hours light
B	1 week	8 weeks	10 plants under 22 hours and 10 plants under 16 hours light
C	2 weeks	6 weeks	10 plants under 22 hours and 10 plants under 16 hours light
D	1 week	6 weeks	10 plants under 22 hours and 10 plants under 16 hours light

To perform backcrossing of the mutant families, I sowed five F_1 seeds for each of the four mutant families in 3 x 3 cm 35-multi-well palettes in the greenhouse. Small seedlings were grown for four weeks under constant 22°C and long day conditions (LD, 16h light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands) / 8 hours dark). After that, I transferred the plants to the cold chamber for vernalization at 4°C under LD conditions (16h light, $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, Osram Lumilux T8 L 58W/840, Osram AG, München, Germany) for 8 weeks. After the cold treatment, I transferred the plants with desired genotypes to the greenhouse and transplanted them into 9 x 9 cm soil pots. They were grown under long day conditions until maturity (16h light) and constant temperature (22°C). I performed the hand crossing (for two subsequent generations until BC_2F_3 generation) between the heterozygous plants and Express617. I performed a phenotypic analysis of four mutant families in the greenhouse. For each family, I sowed 30 seeds for each, mutant lines (M_4 generation without any backcrossing), homozygous mutant lines (aa) (BC_2F_3) and homozygous wildtype line (AA) (BC_2F_3) in 3 x 3 cm 35-multi-well palettes. I also sowed 20 seeds of Express617 as a control. I kept the same greenhouse conditions as mentioned above for the phenotypic analysis of *Bna.FT/Bna.TFL1* backcross as well as double mutant families. For phenotypic analysis in both experiments, I measured traits like flowering time (the time when the first flower opens), number of seeds per plant and seed yield per plant (g).

For the field experiment conducted in 2015/16, I experimented with 5.25m² observation plots at NPZ. While for the field experiment conducted in 2016/17, I conducted the experiment in 2.7m x 12m = 32.4m² yield plots with plot-in-plot design and row distance of 20 cm at Groß Lüsewitz. The seeds were sown by seed drilling machine (Carrier: GTP100, Parcel technology: Hege 80) on 24th August 2016 at a seeding density of 50 seeds/m² at Groß Lüsewitz. As the middle five rows were harvested, harvestable plot size at Groß Lüsewitz was 1.5m x 9.5m = 14.25m². The plot size at Hohenschulen was 4.5m x 3m = 12.5 m² with row distance of 28 cm. At Hohenschulen (Achterwehr), harvestable plot size was 3m x 3m = 9m². At Hohenschulen (Achterwehr), the sowing was done also done by seed

drilling machine on 27th August 2016 at seeding densities of 55 seeds/m² for controls and 110 seeds/m² for M₄ and F₁. For both the locations, yield per plot was measured as kilograms of seeds harvested from middle five rows of each plot using combine harvester. Besides yield per plot, I also calculated plant height and seeds per siliques. For measuring plant height and seeds per siliques, I collected plants from 1 m² area from each plot and measured the traits off the field.

4.2.3 Statistical analysis

Based on the genotyping results, I calculated the genotype frequency in the segregating mutant families and also performed the chi-square test if the population segregates according to the expected 1:2:1 ratio. For the phenotypic analysis of *Bna.FT/Bna.TFL1* backcross as well as double mutant families, I measured the traits on 15 and 10 plants, respectively. For all the measured traits, I performed the analysis of variance for the investigated traits using ANOVA test (P value=0.0001), while I performed the LSD test ($\alpha \leq 0.05$) to compare the mean of the genotypes. I performed both these statistical tests using the R package 'Agricolae' version 1.2-8.

For field experiment conducted in Hohenschulen (Achterwehr) in 2016, I calculated the phenotypic traits from two replications, while for the experiment conducted in Groß Lüsewitz, I took the average of three replications. I performed the analysis of variance for the investigated traits using ANOVA test (P value=0.0001), while I performed the LSD test ($\alpha \leq 0.05$) to compare the mean of the genotypes. I performed both these statistical tests using the R package 'Agricolae' version 1.2-8.

4.3 Results

4.3.1 Rapid cycle protocol development for rapeseed

To produce more than one backcrossing generations per year, I needed to develop a rapid cycle protocol. For this purpose, I sowed 20 Express617 seeds under different conditions for optimization (Table 5). I expected that plants grown under longer light period after vernalization would flower faster. I observed that plants grown under 22 hours light after vernalization flowered up to 20 days earlier compared to the plants grown under 16 hours light (Figure 14). Apart from this, I also observed that plants which were vernalized for six weeks flowered later than the plants which underwent 8 weeks of vernalization. Moreover, plants which were put under vernalization after two weeks of pre-culture flowered earlier than the ones which were transferred to the cold chamber after 1 week pre-culture.

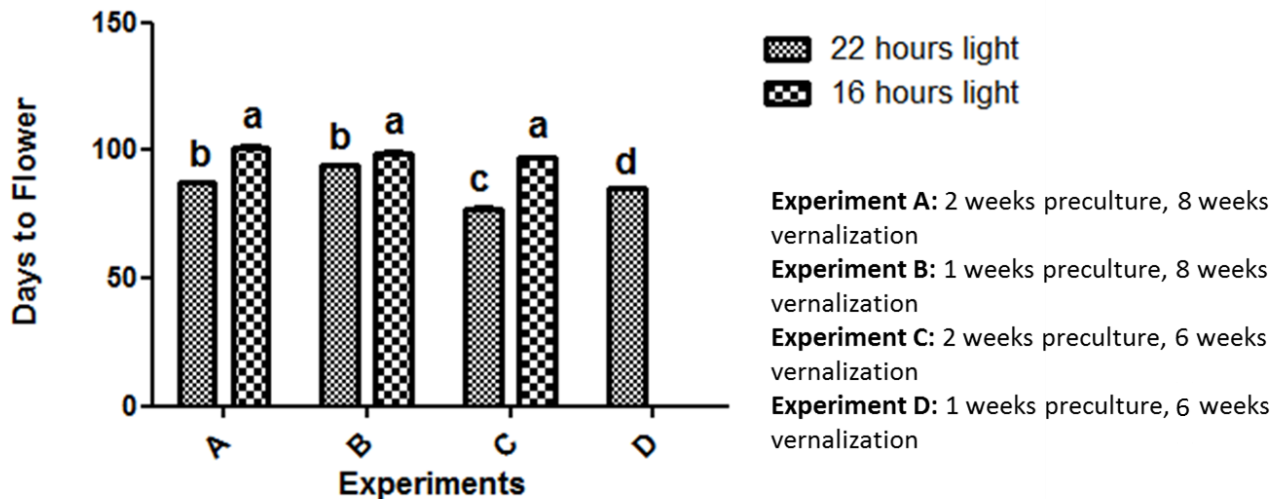


Figure 14: Days to flower for Express617 plants grown under varying growth conditions; number of days to flowering was measured as the average of 10 Express617 plants for each experiment; Error bars: standard error of the mean for biological replicates. Before and after vernalization (4°C, 16 h light, 8 weeks), plants were grown in the greenhouse at a constant temperature (22°C). The mean comparison was made using ANOVA test (P value=0.0001), while the statistical grouping was done using the LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

In conclusion, I propose the following protocol for rapid generation time in winter type rapeseed: Two weeks of pre-culture (under constant 22°C and long day conditions (LD, 16h light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands)), 6 weeks of vernalization under LD conditions (16h light, $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, Osram Lumilux T8 L 58W/840, Osram AG, München, Germany) and plants under 22 hours light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$, Son-T Agro 400 W, Koninklijke Philips Electronics N.V., Eindhoven, Netherlands) at constant 22°C after vernalization. Using above mentioned growth conditions, the time from sowing to harvest is expected in range between 130 and 150 days. This can accelerate the growth cycle of rapeseed by five weeks and therefore, the total generation time sums up to five months/generation. In this way, the rapeseed growth cycle can be fastened and the cultivation of two generations per year will be possible.

4.3.2 Phenotypic evaluation of *Bna.FT* / *Bna.TFL1.A10* backcross families in greenhouse

As the mutant lines investigated by Yuan Guo during her Ph.D. work were in the M_3 generation, a large number of background mutations can be expected, which might hinder the phenotypic evaluation of the interested genes. To eliminate the background mutations, I initiated backcrossing for four mutant families (Table 3). For backcrossing, I used the rapid cycle protocol described under 4.3.1 that shortened the generation time. For phenotypic characterization, I produced BC_2F_3 seeds by selfing BC_2F_2 plants (Figure 15). I performed the Chi-square test for the BC_2F_3 progenies and found out that the segregation for all three mutant families is in accordance with expected 1:2:1 ratio (Table S7). I performed the phenotypic analysis of four backcrossed mutant families (*ft_1*, *ft_2*, *ft_3*, and *tfl_1*) (Table 3) under the greenhouse conditions to check the effect of background mutations on flowering time, number of seeds per plant and seed yield per plant. For this purpose, I grew 15 plants per genotype under greenhouse conditions as mentioned under the section 4.2.2.

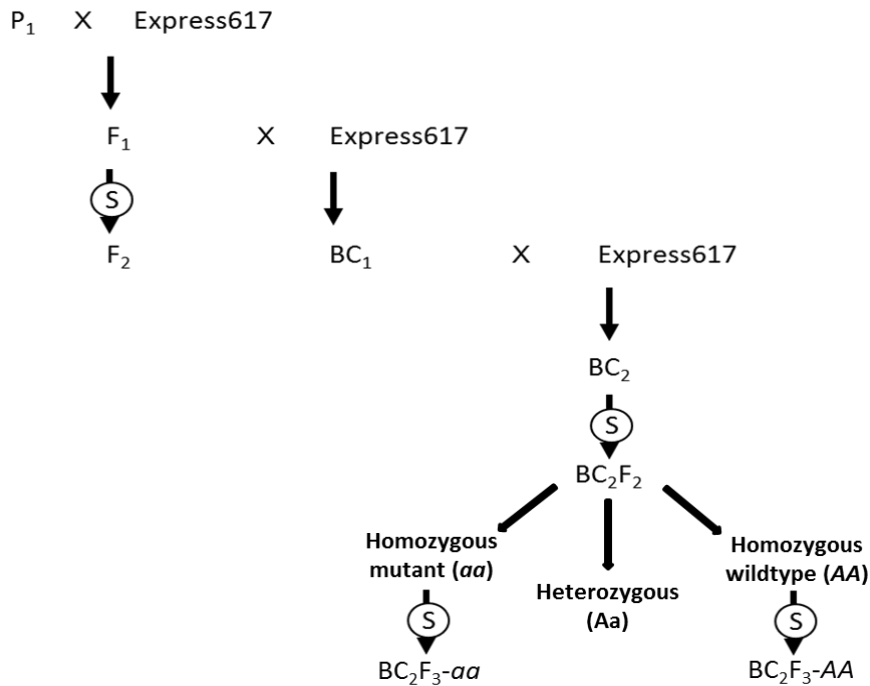
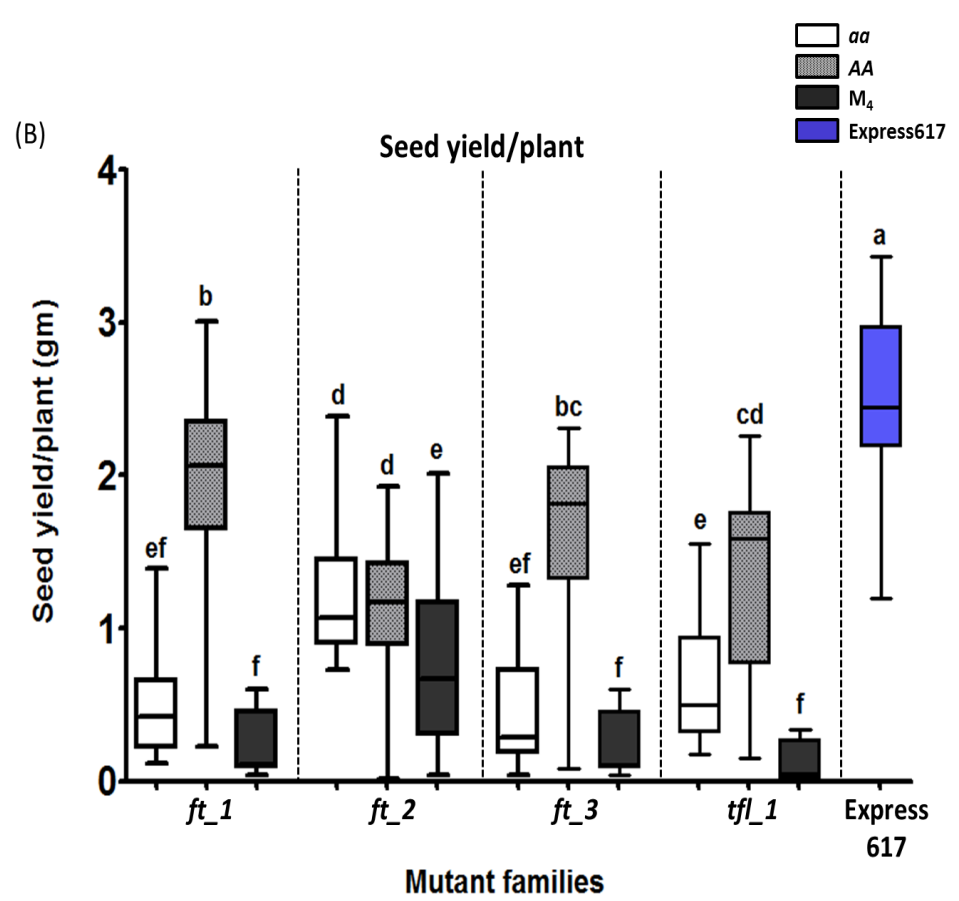
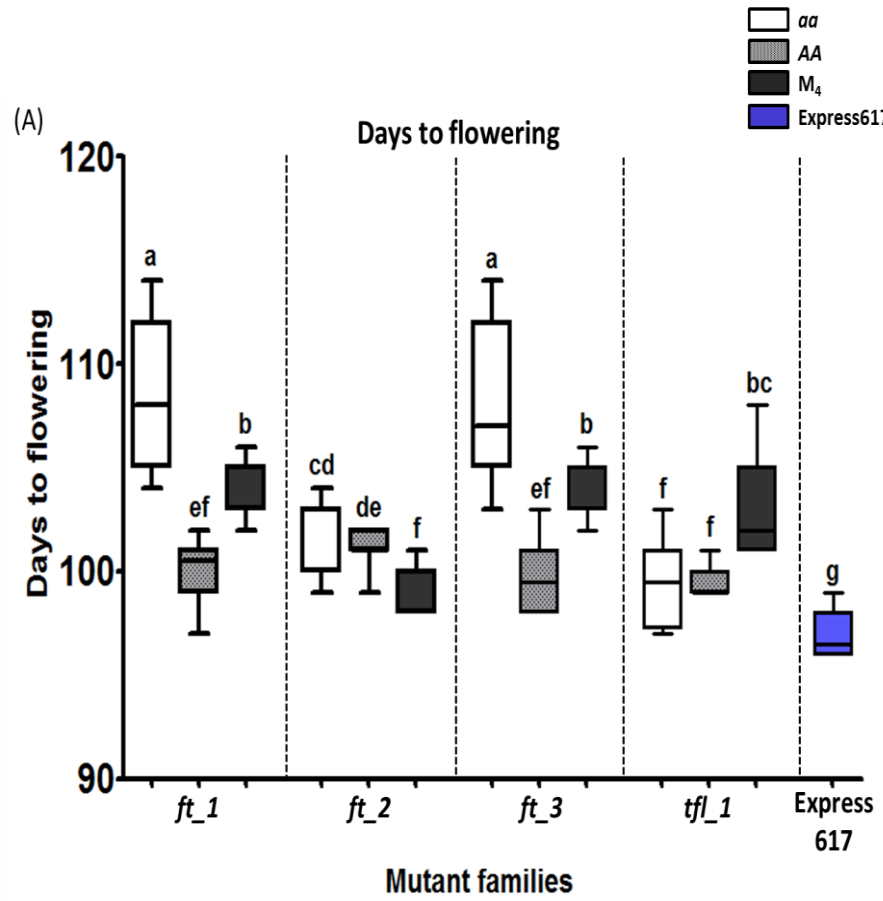


Figure 15: Backcrossing scheme used to reduce the background mutations in four rapeseed mutant families in the current study. For phenotypic analysis, 15 seeds of each, BC_2F_3-aa and BC_2F_3-AA were sown for four mutant families in the greenhouse. Express617 was used as non-mutated control.

I measured the germination rate for all four mutant families to check the effect of background mutation on germination. All the mutant families showed a better germination rate after backcrossing (BC_2F_3) compared to M_4 generation (Table S8). Besides germination, I also recorded days to flowering for all mutant families and control plants. To study the effect of background mutations, I compared backcrossed mutant plants (aa) with M_4 plants and also the wildtypes (AA). After backcrossing, $aa-ft_1$ flowered eight days later as compared to $AA-ft_1$, while four days and twelve days later as compared to M_4-ft_1 and Express617, respectively (Figure 16(A)). For ft_2 mutant family, I did not observe a difference in flowering time between $aa-ft_2$ and $AA-ft_2$. Nevertheless, $aa-ft_2$ flowering was significantly delayed as compared to M_4-ft_2 mutant plants and Express617 showing the effect of background mutations (Figure 16(A)). Similarly, for $aa-tfl_1$ mutant plants, I did not observe any significant difference in the flowering time compared to $AA-tfl_1$ and Express617. But, I observed a significant difference in flowering time between $aa-tfl_1$ and M_4-tfl_1 (Figure 16(A)). Apart from flowering time, I also investigated the effect of background mutation on seed yield related traits (number of seeds per plant and seed yield per plant). I observed that for the mutant families ft_1 , ft_3 and tfl_1 , there was no effect of background mutations on total number of seeds per plant or seed yield per plant (Figure 16(B and C)). In contrast, $aa-ft_2$ had ~60.5% higher number of seeds per plant as well as seed yield per plant as compared to M_4-ft_2 which shows the effect of background mutations on seed yield related traits.



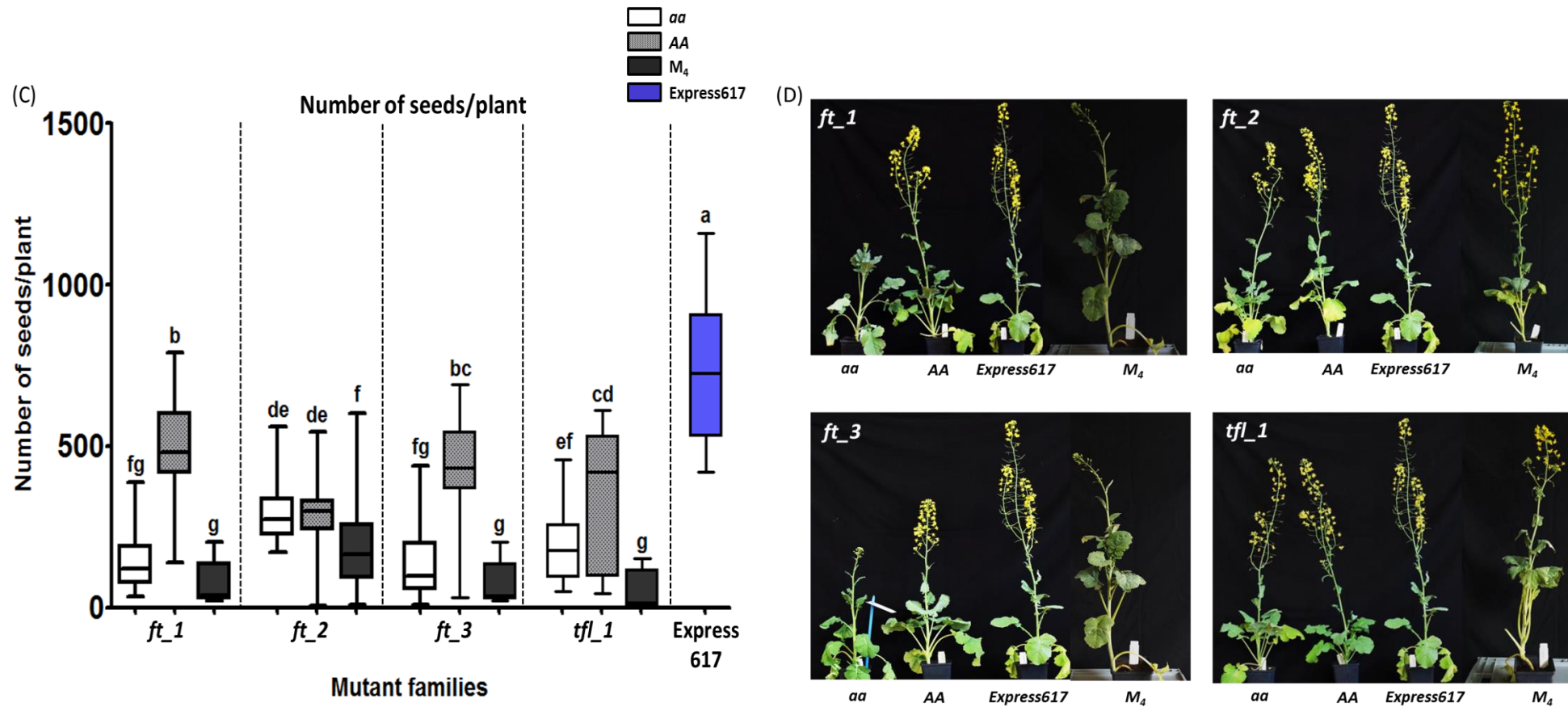


Figure 16: Phenotypic characterization of four backcross mutant families grown in the greenhouse. (A) Days to flowering (B) Seed yield per plant (g) (C) Number of seeds per plant (D) Plant pictures taken 101 days after germination. *aa*: Genotype carrying mutant allele after two generations of backcrossing (BC_2F_3); *AA*: Genotype carrying wildtype allele after two generations of backcrossing (BC_2F_3); *M₄*: Genotype carrying mutant allele without backcrossing; Express617: Control. For all phenotypic traits average was taken from 15 plants per genotypes. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Error bars: standard error of the mean for 15 plants. The mean comparison was made using the ANOVA test (P value=0.0001), while the statistical grouping was done using the LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

4.3.3 Field performance of *Bna.FT* F₁ hybrids

For the field experiment conducted at Groß Lüsewitz in 2015, I sowed *Bna.FT/Bna.TFL1.A10* F₁ hybrids together with mutant plants and the control Express617. I counted emerged plants per plot in relation to the number of sown seeds to assess the field emergence before winter. Based on the number of sown seeds, I expected to have 275 plants per plot considering the plot size of 5 m² and a seeding density of 55 seeds/m². Field emergence for mutant plants varied from 44% to 56%, while for F₁ hybrids, it was significantly higher, varying from 64% to 78%. The field experiment at Groß Lüsewitz in 2015 was severely affected by harsh winter conditions of -20°C without snow cover and this reduced the number of plants after winter (Figure 17). I observed that only 1 - 5% of the M₄ plants survived after winter, while for F₁ hybrids, 8 to 19% of plants survived after winter. Furthermore, for Express617 and MSL00B (MSL line used by NPZ to produce F₁ seeds in the year 2015/16), 16% and 19% plants survived after winter, respectively.

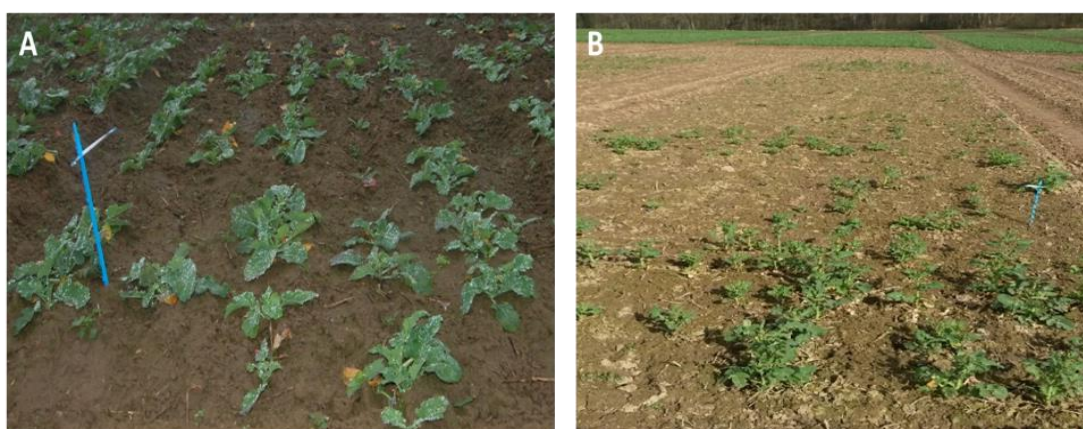


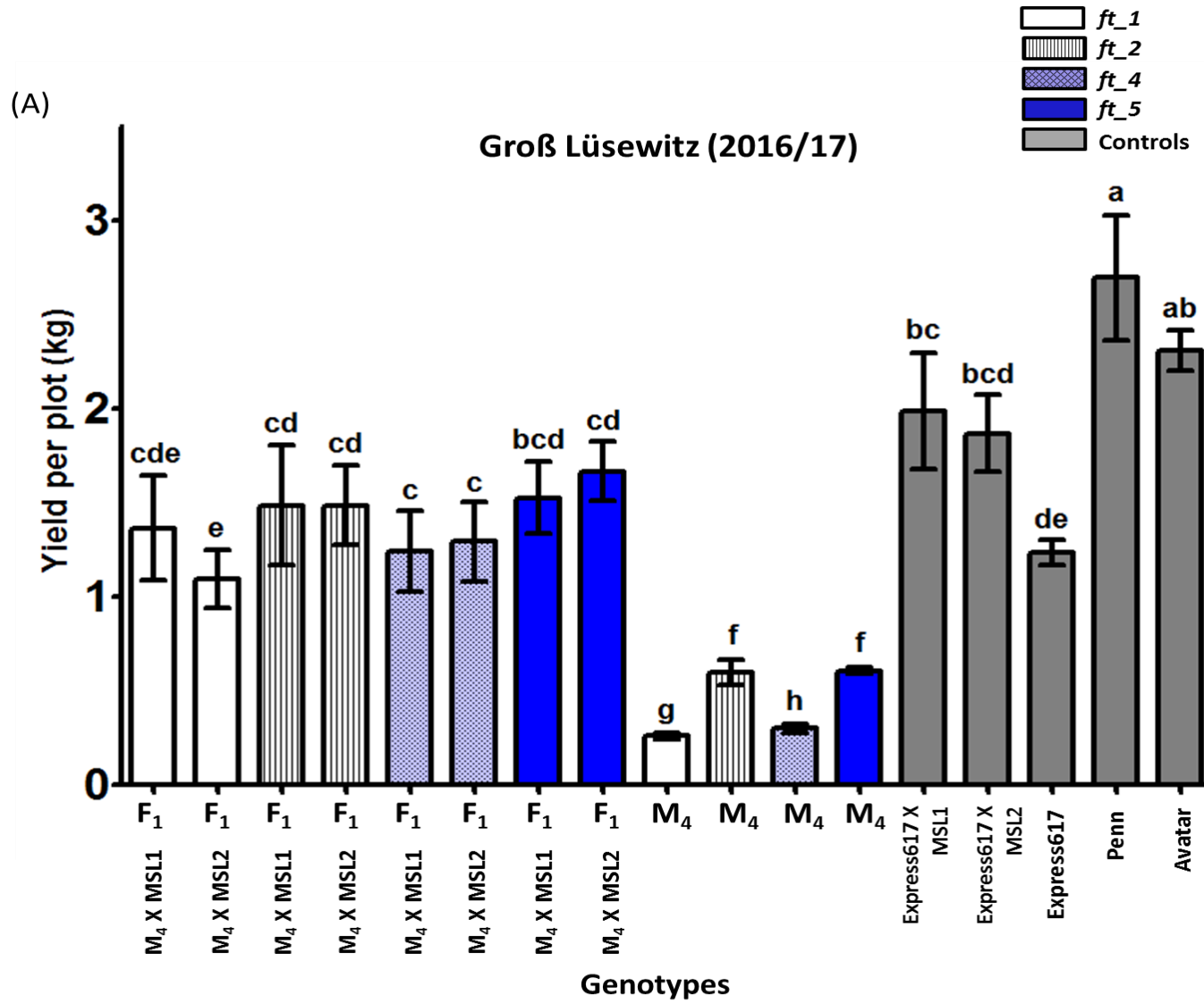
Figure 17: F₁ hybrid plants of *ft_1* mutant family during the field experiment at Groß Lüsewitz in 2015/16 (A) Before winter (B) After winter. Plants were severely affected by harsh winter conditions of -20°C without snow cover.

For the year 2016, I repeated the field experiment with *Bna.FT* F₁ hybrids at two locations (Groß Lüsewitz and Hohenschulen (Achterwehr)). The number of plants before and after winter was counted at Hohenschulen (Achterwehr). The field emergence for mutant plants before winter was varying between 63% and 79%, while for F₁ hybrids the field emergence was between 74% - 100%. Field emergence of controls was varying between 70% and 100%. For mutant genotypes, 13% to 31% plants survived after winter, which was much lower compared to F₁ hybrids and controls. For F₁ hybrids, 55% to 100% and for controls, 87% to 100% plants survived after winter (Table 6). As the field experiment at Groß Lüsewitz was conducted in 2.7m × 12m = 32.4m² yield plots, counting of single plants was not possible.

Yield per plot for each genotype was measured as kilograms of seeds harvested from middle five rows of each plot using combine harvester. I observed that yield per plot for F₁ hybrids of all four mutant families were significantly lower compared to the controls (Express617 × MSL1 and Express617 × MSL2) and commercially available cultivars (Penn and Avatar) (Table 6 and Figure 18). However, F₁ hybrids of all four mutant families had higher yield per plot compared to the M₄ mutant plants. To measure seeds per silique, plants were collected from 1m² area of each plot. From the collected plants, I counted the total number of seeds and also total number of healthy siliques to obtain seeds per silique data for each genotype. Based on the data, the F₁ hybrids produced from a cross between *ft_4* missense mutants and MSL1 had higher seeds per siliques in both locations compared to the control plants (Table 6 and Figure S8).

Table 6: Phenotypic data from the field experiment conducted at Groß Lüsewitz and Hohenschulen in 2016/17. Values are means of three plots for Groß Lüsewitz and two plots Hohenschulen. The field experiments at both the locations were conducted in a completely randomized block design. Plot size at Groß Lüsewitz was 14.25 m², while at Hohenschulen it was 9 m².

Gene	Mutation	Mutation type	Mutant code	Generation	Seed codes	Winter survival (%) - Hohenschulen	Field emergence (%) - Hohenschulen	Yield per plot (kg)		Seeds per silique	
								Groß Lüsewitz	Hohenschulen	Groß Lüsewitz	Hohenschulen
<i>Bna.FT.C6b</i>	G2009A	Splice site	<i>ft_1</i>	M ₄	162161	59.30	13.26	0.26±0.02	-	6.47±2.83	-
				F ₁ (M ₄ × MSL1)	162159	68	100	136±0.48	4.4±0.15	14.52±2.18	21.64±2.22
				F ₁ (M ₄ × MSL2)	162160	71.60	100	1.09±0.26	4.32±0.32	16.51±4.85	21.24±4.34
<i>Bna.FT.C6b</i>	G2122A	Missense	<i>ft_2</i>	M ₄	162165	75.90	27.94	0.59±0.11	-	9.36±0.64	-
				F ₁ (M ₄ × MSL1)	162163	75	99.59	1.48±0.54	4.03±0.16	13.82±4.57	15.87±0.43
				F ₁ (M ₄ × MSL2)	162164	83.93	95.30	1.48±0.36	3.89±0.19	15.4±1.54	22.54±2.66
<i>Bna.FT.C6a</i>	G163A	Missense	<i>ft_4</i>	M ₄	162169	63	15.62	0.30±0.03	-	10.89±3.28	-
				F ₁ (M ₄ × MSL1)	162167	74.60	100	1.24±0.36	4.50±0.07	18.84±0.81	22.75±3.06
				F ₁ (M ₄ × MSL2)	162168	68.78	100	1.29±0.36	4.34±0.10	19.29±1.57	22.49±3.28
<i>Bna.FT.C6a</i>	G37A	Missense	<i>ft_5</i>	M ₄	162173	78.93	31.28	0.60±0.02	-	9.09±2.86	-
				F ₁ (M ₄ × MSL1)	162171	85.60	100	1.52±0.33	4.53±0.02	14.27±2.89	17.05±3.11
				F ₁ (M ₄ × MSL2)	162172	99.24	55.38	1.66±0.27	4.32±0.00	17±7.85	17.79±1.37
Express617				Control	161537	70.30	98.70	1.23±0.11	4.2±0.01	14.9±2.75	21.69±3.48
Penn				Control	162175	96.66	100	2.69±0.57	4.59±0.09	17.25±1.05	23.74±2.95
Avatar				Control	162176	81.27	100	2.31±0.15	4.58±0.07	16.3±1.91	20.65±3.50
Express617 × MSL1				Control	162157	99.60	95.49	1.98±0.53	4.57±0.09	12.2±3.20	17.95±0.94
Express617 × MSL2				Control	162158	93.93	86.77	1.86±0.35	4.43±0.23	16.56±3.03	19.05±4.53



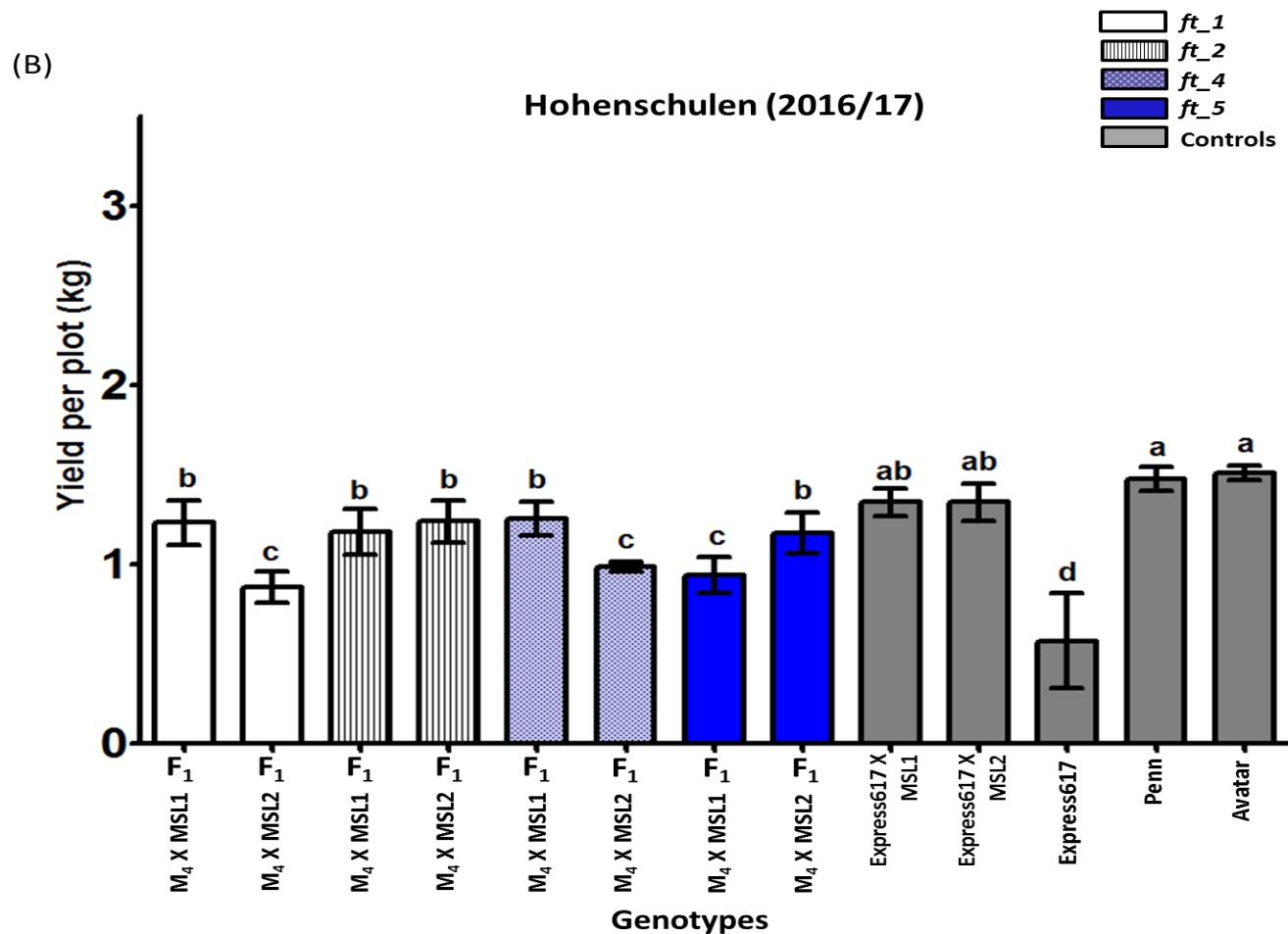


Figure 18: Yield per plot data of the field experiment conducted at Groß Lüsewitz (Fig. A) and Hohenschulen (Fig. B) in 2016/17. Yield per plot was calculated as kilograms of seeds harvested from the middle five rows of each plot. Values are means of three plots for Groß Lüsewitz and two plots Hohenschulen. The field experiments at both the locations were conducted in a completely randomized block design. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using the LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

4.3.4 Phenotypic evaluation of *Bna.FT.C6b* - *Bna.TFL1.A10* double mutants in the greenhouse

It has been reported that under greenhouse conditions, the *ft_1* mutant plants flowered up to 30 days later than Express617, while the *tfl_2* mutant plants showed 12 days flowering delay compared to Express617 (Guo et al., 2014). Therefore, it was expected that the plant carrying homozygous mutations for both, *Bna.FT.C6b* and *Bna.TFL1.A10* would flower the latest. To verify this hypothesis, Yuan Guo crossed M₃ mutant plants of *ft_1* mutant family with the *tfl_2* mutant family and produced F₁ seeds. Followed by this, she selfed the F₁ plants and produced 120 F₂ seeds which were available to me at the beginning of my work (Guo 2015). I sowed these 120 F₂ seeds in the greenhouse to phenotypically characterize double mutant family. I observed that out of 120 seeds, 86 germinated. F₂ plants were analyzed as follows: I isolated DNA from all 86 plants and amplified them using the primers specific to *Bna.FT.C6b* and *Bna.TFL1.A10* (Table S4). Later, I sent all the amplified products for Sanger sequencing using the same primers to analyze the genotypes of each F₂ plant. I got clear sequencing results for 61 plants and therefore, I performed the Chi-square test using these 61 plants. Being a digenic segregating population, I observed that it was structured into nine genotypic classes: *AABB*, *AABb*, *AAbb*, *AaBB*, *AaBb*, *Aabb*, *aaBB*, *aaBb*, *aabb*. I expected a genotypic segregation of 1:2:1:2:4:2:1:2:1 (*AABB*, *AABb*, *AAbb*, *AaBB*, *AaBb*, *Aabb*, *aaBB*, *aaBb*, *aabb*) which was tested by Chi-square test. I observed that χ_{cal}^2 (11.72) was lower than χ_{tab}^2 (15.50) (Table 7). Based on the Chi-square test, I concluded that the difference between the observed and expected number of plants per genotype was not significant and thus, the segregation of F₂ population was in accordance to expected digenic segregation. As the number of plants per genotype was not enough for phenotyping, I selfed the F₂ plants to produce F₃ seeds, and I phenotyped the F₃ plants in the greenhouse.

I observed that the plants carrying splice site mutation in *Bna.FT.C6b* (*aaBB* and *aabb*) flowered later (17 and 44 days, respectively) compared to the plants with wildtype genotypes at both loci (*AABB*) (Figure 19(A)). I also observed that the plants carrying mutations in both loci (*aabb*) showed the least number of seeds per plant as well as seed yield per plant compared to all the other genotypes. Interestingly, the plants carrying a mutation in *Bna.TFL1.A10* (*aaBB*) had higher number seeds and seed yield per plant compared to the plants with wildtype genotypes at both loci (*AABB*) (Figure 19(B & C)).

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Table 7: Genotypic segregation of the F₂ population. The F₂ population was developed by Yuan Guo by selfing the F₁ plants (seed code:122070). F₁ seeds were obtained from a cross between M₃ mutant plants of *ft_1* mutant family with the *tfl_2* family. Genotyping of the plants were done by Sanger sequencing. Allele *A* is wildtype allele of *Bna.FT.C6b*, while allele *a* is a *Bna.FT.C6b* allele with a splice site mutation. Allele *B* is wildtype allele of *Bna.TFL1.A10*, while allele *b* is a *Bna.TFL1.A10* allele with a missense mutation. Chi-square test was performed to check genotypic segregation: df=8, $\alpha=0.05$.

F2 seed code	Number of plants analyzed	Genotypes																		Total		
		<i>AABB</i>		<i>AABb</i>		<i>AAbb</i>		<i>AaBB</i>		<i>AaBb</i>		<i>Aabb</i>		<i>aaBB</i>		<i>aaBb</i>		<i>aabb</i>		Obs.	Exp.	
		Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.					
132360 & 132361	86	1	3.8	10	7.6	4	3.8	10	7.6	10	15.25	7	7.6	7	3.8	11	7.6	1	3.8	61	61	
$\chi_{cal}^2=11.72 > \chi_{tab}^2=15.50$ n.s.																						

df: Degree of freedom

n.s: not significant

Exp.: Expected

Obs.: Observed

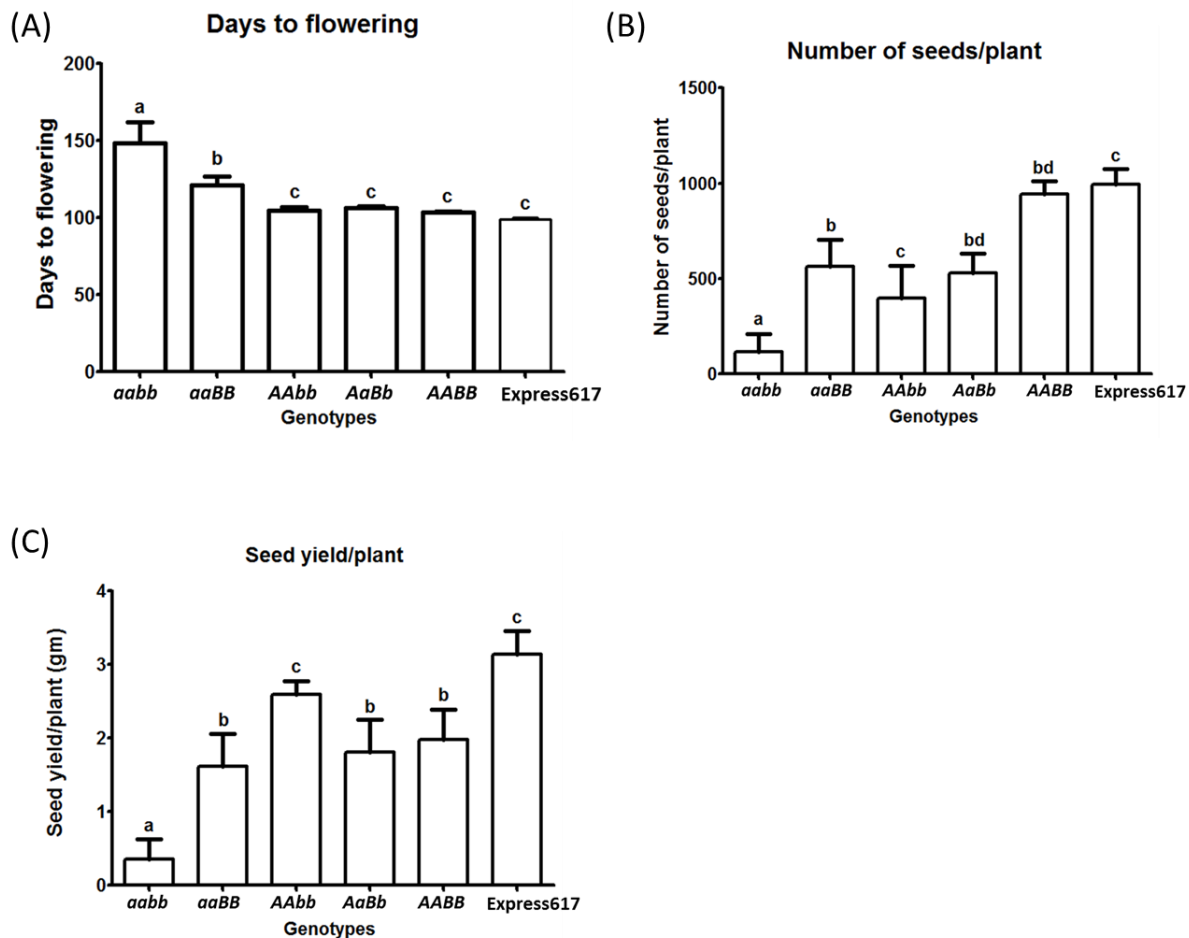


Figure 19: Phenotypic characterization of *ft_1(aa)/tfl_2(bb)* double mutants. (A) Days to flowering (B) Number of seeds per plant (C) Seed yield per plant (g). Allele *A* is wildtype allele of *Bna.FT.C6b*, while allele *a* is a *Bna.FT.C6b* allele with a splice site mutation. Allele *B* is wildtype allele of *Bna.TFL1.A10*, while allele *b* is a *Bna.TFL1.A10* allele with a missense mutation. The phenotypic data was collected from 10 plants per genotype. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Error bars: standard error of the mean for 10 plants. The mean comparison was made using the ANOVA test (P value=0.0001), while the statistical grouping was done using the LSD test ($\alpha \leq 0.05$) using R package 'Agricolae' version 1.2-8.

4.4 Discussion

The current study was structured into four work packages: (1) to shorten the vegetation period of rapeseed grown under greenhouse conditions; (2) to analyze the effect of background mutations on the phenotype of TILLING mutants; (3) to analyze the impact of *Bna.TFL1.A10/Bna.FT.C6b* mutations on the yield performance of their F₁ hybrids under the field conditions; (4) to perform phenotypic analysis *Bna.FT/Bna.TFL1.A10* double mutants.

I propose a rapid cycle protocol for winter type rapeseed in the greenhouse and according to that, the generation time can be reduced by using the smaller pots, reducing the vernalization period to six weeks and increasing the light period. For winter rapeseed accessions it usually takes approximately six months till maturation under greenhouse conditions, which makes backcrossing a very time-consuming process. To make the process of backcrossing quicker by shortening the vegetation period, I performed an experiment with varying pre-culture period, vernalization period and light duration. My data suggests that two weeks of pre-culture, 6 weeks of vernalization and growing the plants under 22

hours light after vernalization shortens the time from sowing to harvest from 6 months to 5 months. It is known from *Arabidopsis* that photoperiod and vernalization pathways promote flowering in response to changes in day length and temperature, respectively (Doyle et al. 2018). The responsiveness to environmental cues increases as the plant ages (Wang et al. 2011b). In other *Brassicaceae* species such as *Brassica oleracea* also vegetative growth for an extended period of several weeks is needed to make them responsive to cold (Wang et al. 2011b). Therefore, rapeseed plants, which are grown under greenhouse conditions, are kept under 22°C constant temperature for 1-2 weeks before transferring them to vernalization. Hence, I checked two different pre-culture periods (1 week and 2 weeks) to see which pre-culture period is optimum for shortening the life cycle. I observed that after both pre-culture periods (1 week and 2 weeks) the plants were matured enough for vernalization response and also to initiate flowering later. Nevertheless, two weeks pre-cultured plants ultimately had the shortest lifecycle. Besides pre-culture, duration of vernalization also plays an essential role in flowering time and overall lifecycle duration of winter rapeseed. The lifecycle of winter rapeseed can be significantly shortened by shortening the vernalization period. A study in winter rapeseed concluded that downregulation of *Bna.FLC* copies allow winter rapeseed to flower without vernalization. And therefore, winter rapeseed could flower as early as the spring varieties (Alvarez-Venegas et al. 2010). Nevertheless, upon unchanged expression of *Bna.FLC*, adequate duration of vernalization is required for winter type plants to flower. None of the previous studies till date have reported about the minimum duration of vernalization period required for the growth of winter rapeseed under the greenhouse conditions. I checked 6 weeks and 8 weeks of vernalization (4°C, 16 h light) and concluded that 6 weeks vernalization is sufficient for winter type rapeseed (Express617) to flower.

The other important factor that influences the flowering time is photoperiod. It was observed that repeated long day cycles provide a high level of *FT* expression for several days and thus can promote flowering in *Arabidopsis* (Krzymuski et al. 2015) Similarly, in the current experiment, rapeseed plants kept under 22h light conditions flowered earlier compared to the plants kept under the 16h light. In another study, it was shown that in bread wheat (*T. aestivum*), durum wheat (*T. durum*), barley (*H. vulgare*) and the model grass *Brachypodium distachyon*, extended photoperiod (22 hours light/2 hours dark) accelerated the growth cycle (Watson et al. 2018). In the same study, it was shown that for spring rapeseed, time to anthesis is reduced ~73 days for the plants grown under 22h light conditions compared to the plants kept under the 12-hour day-neutral photoperiod conditions (Watson et al. 2018). As spring type rapeseed do not require vernalization, overall number of days till flowering cannot be compared between winter type (used in my experiment) and spring type (used by Watson et al. 2018) material. Therefore, results from my experiment illustrate optimum growth conditions for the rapid growth of winter type rapeseed in the greenhouse. In another speed breeding experiment, Watson et al. (2018) also used LED lights in order to reduce the overall operating and cooling costs. For future experiments, I also suggest optimizing light conditions for rapeseed growth in the greenhouse using LED lights which are superior to sodium vapor lamps regarding heat produced and the quality of light.

In work package 2, I analyzed backcross generations (BC₂F₃) of *Bna.FT* and *Bna.TFL1* mutant families in order to check the effect of background mutations on quantitative traits like flowering time and seed yield. I used the protocol for rapid growth cycle to backcross *Bna.FT* and *Bna.TFL1* mutants for two generations. The backcrossed mutant families were phenotyped under greenhouse conditions to validate the functions of *Bna.FT* and *Bna.TFL1* reported by Guo et al. (2014). I observed an apparent effect of background mutation in all four mutant families, as the flowering time phenotype of the backcrossed mutant plants was significantly different compared to the M₄ plants. After two generations of backcrossing, the plants carrying splice site mutation in *Bna.FT.C6b* showed delayed flowering compared to the plants carrying *Bna.FT.C6b* wildtype allele and Express617. This confirmed the

results obtained by Guo et al. (2014), who observed that the same mutant family had 30 days delay in flowering time compared to Express617. Nevertheless, the observed flowering time phenotype for plants carrying missense mutations in *Bna.FT.C6b* and *Bna.TFL1.A10* was in contrast with the observations made by Guo et al. (2014). As Guo et al. (2014) used M₃ plants for phenotyping, the observed phenotype might have hindrance from background mutations. Therefore, phenotyping of the backcrossed mutant families performed in the current experiment depicted a more comprehensive picture of the effect of missense mutations in *Bna.FT.C6b* and *Bna.TFL1.A10* on flowering time. Braatz et al. (2018b) also observed the effect of background mutations on silique resistant phenotype in rapeseed. They demonstrated that due to the effect of background mutations, the plants carrying mutant alleles for *Bna.ALC* gene showed similar silique shatter resistance compared to the plants with wildtype alleles. Ideally, BC₄F₃ or BC₄F₃ generations should have been analyzed in this work package. But due to time limitation, I could perform the backcrossing till BC₂F₃ generation, meaning that still, 12.5% of the mutant background is present in the recipient Express617 genome. As the background mutations affect the phenotype significantly, I suggest backcrossing till BC₄ or BC₅ which can ensure almost complete exclusion of the mutant background. As these backcross populations in the F₂ stage are segregating, development of the F₃ population is required which can be homozygous for wildtype or mutant allele. As an alternative strategy, marker-assisted background selection (Jung 2010) can be a time-saving approach, where backcrossed plants are genotyped with numerous markers to select plants with a high share of the recipient genome. This can be achieved with whole genome sequencing data, rapeseed SNP arrays (Mason et al. 2017) or by AFLP markers (Amplified Fragment Length Polymorphism, Schondelmaier et al. 1996).

The third work package deals with field trials performed in two different locations in Germany to analyze the effect of *Bna.FT* mutations on seed yield heterosis. Hybrids were produced by crossing the *Bna.FT* M₄ mutants with male sterile lines (MSL1 and MSL2) in the field conditions. Though the TILLING mutants are with Express617 background, Express617 MSL line was not used to produce the F₁ seeds. This was because Express617 MSL line is not being currently used by NPZ to produce hybrid seeds. MSL1 and MSL2 are the male sterile lines which are presently being used by NPZ. I conducted field trials in two successive years (2015/16 and 2016/17). In a previous study, it was concluded that EMS mutagenesis reduces the germination rate in rapeseed (Wang et al. 2008). For the field experiment conducted in 2015/16 at Groß Lüsewitz, there was a significant reduction in the germination rate for both, mutant (44%-56%) and F₁ plants (64%-78%). Similarly, Chen et al. (2012a) also observed 40% germination rate in wheat TILLING population. In another study conducted in soybean (Espina et al. 2018), it was also shown that EMS treatment affects the germination rate. They showed decline in the germination rate with increase in the EMS concentration (Espina et al. 2018). This clearly shows the effect of thousands of background mutations present in the genome. The winter survival rate was very low for the mutant (1%-5%) as well as F₁ hybrid plants (8% - 19%). Moreover, the non-mutated Express617 also had a very low winter survival rate (16%). This was mainly because of harsh winter conditions of -20°C without snow cover. Data from both locations showed that Express617 had poor performance compared to other two commercially available cultivars, Penn and Avatar. Apart from Express617 being a very old variety, production of seeds through bagging of plants in the greenhouse can also be a possible reason for poor performance of Express617 under field conditions. Moreover, F₁ hybrids did not have better seed yield per plot compared to the controls (Express617 × MSL1 and Express617 × MSL2), which gives a preliminary indication that a mutation in *Bna.FT.C6a* and *Bna.FT.C6b* may not affect heterosis in rapeseed. Nevertheless, the effect of background mutations cannot be overlooked. As these *Bna.FT* F₁ plants carry thousands of other mutations in the heterozygous stage which may mask the effect of *Bna.FT* mutation on seed yield heterosis.

The last workpackage involves phenotypic characterization of the double mutant plants carrying mutations in both *Bna.FT.C6b* and *Bna.TFL1.A10*. The plants carrying homozygous mutations at both loci flowered later than all other genotypes. In tomato, it was reported that mutation in ortholog of *FT* cause delay in flowering regardless of whether *TFL1* ortholog is mutated (Jiang et al. 2013). I also observed a delay in flowering for the plants carrying mutated *Bna.FT.C6b* and non-mutated *Bna.TFL1.A10* allele. This data confirmed the results from Guo et al. (2014), who concluded that a splice site mutation in *Bna.FT.C6b* causes 29 days delay in flowering. Nevertheless, the plants carrying mutated *Bna.TFL1.A10* allele did not show any delay in flowering time in my experiment. This analysis from the current study is in contrast to the data obtained by Guo et al. (2014), who observed a delay in flowering time in plants carrying a missense mutation in *Bna.TFL1.A10*. Therefore, from the current data of double mutants and backcrossed mutant family, it was evident that missense mutation in *Bna.TFL1.A10* does not play any significant role in flowering time. However, a complete knockdown of all four copies of *Bna.TFL1* simultaneously or individually using CRISPR-Cas technology can provide the final proof for its role in controlling rapeseed flowering time. Besides flowering time, a number of seeds per plant and seed yield per plant was also measured for the double mutant genotypes. The plants carrying missense mutation in *Bna.TFL1.A10* showed an increased number of seeds per plant and seed yield per plant compared to other mutant genotypes and Express617. In tomato, it was demonstrated that a heterozygous mutation in *SFT* in an *SP* (*TFL1* ortholog) mutant background affects tomato yield (Jiang et al. 2013). The data from the current workpackage is based on the mutations in one paralog of each, *Bna.FT* and *Bna.TFL1*. For both genes, there are multiple paralogs in rapeseed, and therefore, a detailed functional analysis of each of those paralogs is needed to fine-tune rapeseed flowering time and seed yield.

In conclusion, phenotypic effects of *Bna.FT* and *Bna.TFL1* mutations were demonstrated in the current study. However, the data must be regarded as preliminary because the backcross mutant families analyzed under the greenhouse conditions still carry 12.5% background mutations, while the field data comes from the F₁ plants generated from M₄ plants carrying thousands of background mutations. In the future, experiments with homozygous mutant and wildtype lines after complete reduction of background mutations are necessary to conclude the role of *Bna.FT* and *Bna.TFL1* paralogs in rapeseed. Moreover, their respective F₁ hybrids should be produced using backcrossed mutant lines and should be tested under field conditions.

4.5 Supplementary data

Table S7: Genotypic segregating of BC2F2 mutant families. Genotyping was done by Sanger sequencing. *aa*- genotype carrying homozygous mutant allele, *AA*- genotype carrying homozygous wildtype allele, *Aa*- genotype carrying heterozygous allele. Chi-square test was performed to check genotypic segregation. Expected genotypic segregation: 1:2:1 (*aa:Aa:AA*), *df*=2, $\alpha=0.05$.

Table S8: Genotypic segregating of BC2F2 mutant families. Genotyping was done by Sanger sequencing. *aa*- genotype carrying homozygous mutant allele, *AA*- genotype carrying homozygous wildtype allele, *Aa*- genotype carrying heterozygous allele. Chi-square test was performed to check genotypic segregation. Expected genotypic segregation: 1:2:1 (*aa:Aa:AA*), *df*=2, $\alpha=0.05$.

Figure S8: Seeds per silique data for the field experiments conducted at Groß Lüsewitz (Fig. A) and Hohenschulen (Fig. B) in 2016/17. For each genotype, average of plants collected from 1 m² area from each of the three replications (plots) was calculated. Error bars: standard error of the mean for 3 replications. Error! Reference source not found.

Figure S9: Plant height data from the field experiments conducted at Groß Lüsewitz (Fig. A) and Hohenschulen (Fig. B) in 2016/17. For each genotype, average of plants collected from 1 m² area from each of the three replications (plots) was calculated. Error bars: standard error of the mean for three replications. The analysis of variance for the investigated traits was performed by ANOVA test (P value=0.0001), while the mean comparison of the genotypes was performed using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

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5 Closing discussion

My study was based on the following hypotheses: (1) Flowering time regulatory network is conserved between Arabidopsis and rapeseed; (2) Plant architecture and yield of rapeseed is affected by a mutation in *Bna.API*; (3) In the case of EMS mutant plants, background mutations have a significant effect on flowering time and yield related traits; (4) Due to heterosis effect, F₁ plants heterozygous for *Bna.FT* and *Bna.TFL1* missense mutations have better yield performance than the individual mutants and (5) Flowering can be severely delayed in the plants carrying mutations in both *Bna.TFL1.A10* and *Bna.FT.C6b*.

5.1 RNA-seq along with GWAS and QTL mapping unveils the flowering time genes in rapeseed

The first research area of my thesis (chapter 2) was based on the question whether flowering time regulatory network is conserved between Arabidopsis and rapeseed or there are new flowering time regulators in rapeseed. RNA-seq analysis in semi-winter line Ningyou7 was performed with the aim to find differentially regulated genes involved in flowering time regulation through the vernalization pathway. I expected that the genes involved in vernalization pathway are differentially expressed before and after vernalization. The power of the transcriptome study to unveil genes involved in the vernalization response has been well documented in previous studies (Greenup et al. 2011; Jones et al. 2018; Li et al. 2016c). Jones et al. (2018) observed variation in expression dynamics of flowering time genes over the period of vernalization in rapeseed. In another study in barley (Greenup et al. 2011), it was concluded through transcriptome analysis that a small number of genes are affected due to prolonged cold, and of which, *VRN1* plays a crucial role in the vernalization response of barley. In the current study, two of the four *Bna.VIN3* paralogs were differentially expressed before and after vernalization, from which *Bna.VIN3.A02* was found associated with flowering time and seed yield. Moreover, the variation observed in the coding sequence of *Bna.VIN3.A02* among winter, semi-winter and spring rapeseed accessions show the involvement of *Bna.VIN3.A02* in the environmental adaptability of rapeseed ecotypes. More detailed functional analysis of these genes involved in environmental adaptability can serve as targets for utilization of exotic germplasms and ultimately to increase genetic diversity in rapeseed (Wei et al. 2017). As mentioned before (Figure 2), understanding of quantitative traits like flowering time in rapeseed is complicated due to the presence of multiple copies of the single gene. Based on the expression profiles from the current transcriptome data and previous QTL studies (Figure 3), I could illustrate a gene regulatory network for flowering time regulation in rapeseed with major flowering time regulators (Figure 20). Nevertheless, this model needs to be further verified by analyzing the expression and functions of the flowering time genes in different tissues and different developmental stages.

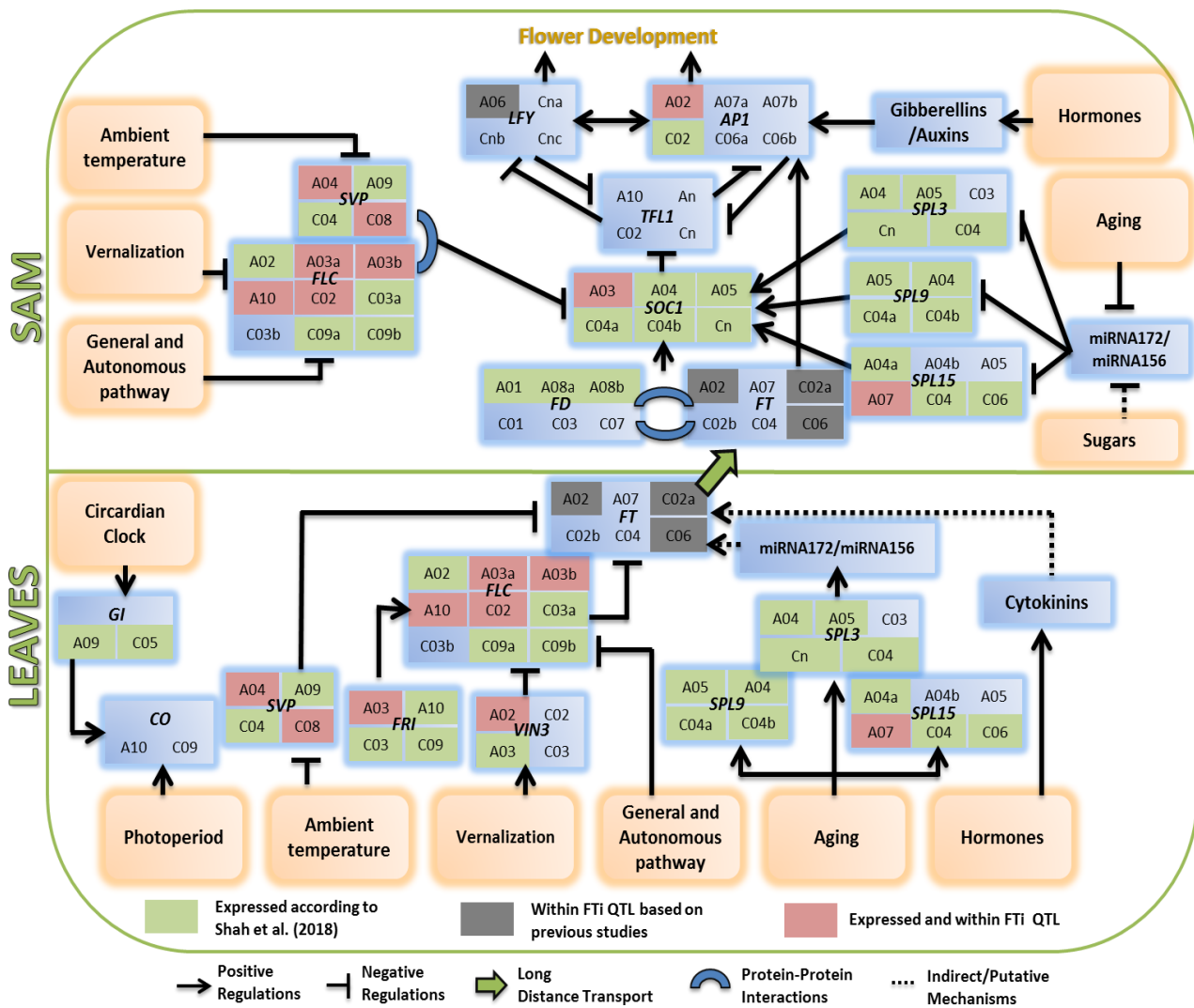


Figure 20: Flowering time gene regulatory network for rapeseed with major flowering time regulators. Arabidopsis gene names are mentioned in the middle of the box. In each box, rapeseed orthologs for each gene are mentioned by chromosome number they are located on. Blue boxes represent major flowering regulators in Arabidopsis, while orange boxes represent environmental as well as endogenous cues regulating flowering time. Ningyou7 leaf transcriptome data (Shah et al. 2018) was used to check the expressed paralogs of flowering time regulators in rapeseed. For checking the genes within flowering time QTL, previous rapeseed QTL studies (Figure 3) were used.

Although the transcriptome analysis performed in the current study gives a hint about sub and neo-functionalization events in rapeseed, a detailed functional analysis of these candidate genes using techniques like CRISPR-Cas can provide a better insight about the functions of these genes. Furthermore, a thorough biochemical analysis of the proteins coded by the genes is needed to distinguish the functions of different paralogs of the gene. It was also evident from the transcriptome study that genes involved in flowering time regulation of rapeseed play a pleiotropic role, and therefore, they are important factors for breeding better adaptive varieties. The 36 candidate genes found in the current study can be useful in rapeseed breeding programmes where the beneficial alleles for flowering time and yield can be screened in the rapeseed gene pool and subsequently it can be integrated into rapeseed lines. Along with the expected outcome of the RNA-seq study, I also found some unexpected results. For instance, orthologs of *FT*, *TFL1*, *API*, and *LFY* were not differentially expressed between early and late developmental stages. Many flowering time regulators are known to be active during a

specific developmental stage and just in a particular tissue of the plant. It was shown in a recent study that in rapeseed, 23% of the flowering time genes are exclusively expressed in the shoot apex, while 7% of them had a leaf-specific expression (Jones et al. 2017). Therefore, the gene network of flowering time regulation in rapeseed can be better understood by performing transcriptome analysis at more developmental stages and in different tissues. To sum up, RNA-seq in combination with other approaches like genetic mapping, GWAS or functional analysis can be a powerful tool to find regulators of quantitative traits.

5.2 Reduction of background mutations is essential for the functional analysis of EMS-induced mutants

Another area of my research was based on the question whether background mutations have a significant impact on days to flowering and yield related traits. The effects of the mutations on flowering time and yield components described by Guo et al. (2014) were measured in the M₃ plants, which presumably carry thousands of background mutations along with the mutations in genes of interest (*Bna.FT.C6b* and *Bna.TFL1.A10*). Therefore, I expected that these background mutations affect the plant phenotype and reduction of background mutation can clearly exhibit the function of *Bna.FT.C6b*, as well as *Bna.TFL1.A10* paralog in rapeseed. Unlike the results of M₃ plants (Guo et al. 2014), I only observed a significant delay in the flowering time in the plants carrying splice site mutation in *Bna.FT.C6b*, while the plants carrying missense mutation in *Bna.FT.C6b* and *Bna.TFL1.A10* exhibited no variation in flowering time compared to the controls. Splice site mutation can cause altered length splice variants of the gene and these variants can cause a frameshift and a premature stop codon (Braatz et al. 2018b). In contrast, a missense mutation causes changes in amino acid, which may or may not lead to variation in protein structure and/or function. This can be the reason why the plants carrying missense mutations analyzed in the current study after two generations of backcrossing does not affect flowering time of rapeseed. However, previous studies have reported the effect of missense mutations on the phenotype of the plants (Guo et al. 2017; Wang et al. 2016). The varying effects of missense mutation might be due to the location of the missense mutation on the gene. In a study conducted in rice, it was shown that different missense mutation sites in a cellulose synthase gene (*OsCESA7*) lead to varying effects on cellulose biosynthesis and plant development (Wang et al. 2016). Besides the position of a missense mutation, type of amino acid change also affects the consequence of missense mutation. Missense mutations substituting the amino acid with another amino acid of varying biochemical property have a significant impact on plant phenotype (Acevedo-Garcia et al. 2017). Contrary to this, both the missense mutants analyzed in the current study (*Bna.FT.C6b.G2122A* and *Bna.TFL1.A10.G52A*) acquired an amino acid substitution with biochemically similar amino acids. Therefore, in spite of the missense mutation being in the exonic regions of *Bna.FT.C6b* and *Bna.TFL1.A10*, they did not have any impact on plant phenotype. Hence, the missense mutant plants which did not show any effect on flowering time in the current study couldn't be considered as the final proof of the involvement of any of the paralogs in flowering time regulation. To further confirm the role of *Bna.FT* and *Bna.TFL1* paralogs, I propose to conduct field trials with homozygous mutant lines without any background mutations. Homozygous mutant lines can be produced by repeated backcrossing till BC₅F₃ generation or by introgressing the mutant allele to the elite line and performing marker assisted selection to select the plants with a higher share of recipient genome.

Although I observed expected flowering delay in the plants carrying splice site mutation in *Bna.FT.C6b*, I did not observe expected early flowering in the plants carrying a mutation in *Bna.TFL1.A10*. *TFL1* being a key flowering time regulator in Arabidopsis, I expected variation in the flowering time in the plants carrying missense mutation in its ortholog in rapeseed. Nevertheless, I did not observe any difference in flowering time, which can also be due to the presence of other non-

mutated copies of *Bna.TFL1*. Therefore, generating mutant plants having mutations in all four copies of *Bna.TFL1* can help to conclude the function of *Bna.TFL1*. Noticeably, in case of the double mutant (*Bna.FT.C6b* & *Bna.TFL1.A10*) analysis, plants carrying missense mutation in *Bna.TFL1.A10* (and non-mutated *Bna.FT.C6b* allele) showed increased seed yield and number of seeds per plant compared to other genotypes. This variation in seed yield related traits can be due to the pleiotropic effect of *Bna.TFL1.A10*, since the beneficial role of mutation in *TFL1* orthologs on yield parameters, have also been reported in other crops (Krieger et al. 2010). It is also demonstrated that the heterozygous stage of an *FT* ortholog in combination with homozygous *TFL1* mutations is more beneficial (Jiang et al. 2013). Therefore, investigating the yield parameters of rapeseed plants having *Bna.TFL1* mutation in a homozygous stage in combination with *Bna.FT* in the heterozygous stage can shed more light on the pleiotropic role of *Bna.TFL1* and *Bna.FT* in rapeseed. These different mutants in homozygous and heterozygous stages can be analyzed to optimize rapeseed yield in a dosage-dependent manner. Moreover, using CRISPR-Cas technology, different mutants can be generated with varying number of mutated *Bna.TFL1* paralogs in combination with *Bna.FT* paralogs. Though CRISPR-Cas technology provides an opportunity to edit gene of interest precisely, a recent decision by the court of justice of the European Union (ECJ) restricts the use of the technology in breeding in Europe, as CRISPR-Cas edited plants will be treated as genetically modified. With these strict regulations in Europe regarding CRISPR-Cas, mutant alleles generated through more conventional methods like chemical or physical mutagenesis become valuable genetic resources for breeding. Nevertheless, these methods have disadvantages of having thousands of background mutations. Additionally, the identification of mutants of interest is a time consuming and laborious process. Moreover, the majority of mutations induced by chemical mutagens like EMS are missense or silent mutations and only a small fraction of these mutations lead to altered plant phenotype (Till et al. 2018). These bottlenecks of conventional mutagenesis methods can be overcome by recent advances in sequences technology, which give a possibility of high throughput selection of mutant lines which has been already shown in rice and wheat (Tsai et al. 2011). So, I anticipate that selecting mutant alleles for paralogs of flowering time genes using TILLING by sequencing approach will provide an opportunity to optimize traits like flowering time, yield and plant architecture in rapeseed.

Moreover, I aimed to answer whether *Bna.FT* mutations have an effect on seed yield heterosis in rapeseed. To answer this question, I conducted field trials with *Bna.FT* F₁ hybrids in two different locations in Germany. This research area had a clear hypothesis: *Bna.FT* mutation affects seed yield heterosis in rapeseed. Therefore, I expected that the F₁ plants carrying *Bna.FT* mutation in heterozygous stage has better yield data compared to the controls. I observed that the F₁ plants had a lower yield per plot compared to the controls. However, the body of data is not sufficient to answer because this data comes from F₁ plants produced by crossing M₄ plants with MSL and therefore, thousands of other background mutations are also in heterozygous stage along with the *Bna.FT* mutation. The heterotic effect of *FT* mutation has been only described in tomato which has determinate growth habit. To expect a similar function of *FT* ortholog in a very different species like rapeseed with a different inflorescence structure is not reasonable. Nevertheless, the role of *Bna.FT* in controlling seed yield heterosis cannot be neglected just based on the current data. Future field experiments are needed using F₁ hybrids produced from homozygous mutant lines to understand the effects of *FT* orthologs on seed yield heterosis in rapeseed.

Besides phenotypic analysis, I also performed expression analysis to check the transcript level of downstream target genes in plants carrying *Bna.TFL1.A10* missense mutation as well as *Bna.API.A02* stop codon mutation. Differential expression patterns and phenotypic analysis of various paralogs of flowering time genes suggest that in a polyploid like rapeseed, multiple copies of genes may regulate flowering time. The presence of multiple gene paralogs in rapeseed complicates the direct transfer of

insights derived from model species like *Arabidopsis* to polyploid crops. For instance, major flowering time regulators of *Arabidopsis*, *FLC*, *FT* and *TFL1* have nine, six and four copies in rapeseed, respectively. From the transcriptome data and expression data of the current study, it was evident that paralogs of these genes have varying expression dynamics among them and thus, may have varying functions. Therefore, a detailed analysis of all the paralogs of flowering time genes is required to understand the complex flowering time network of rapeseed.

5.3 *Bna.AP1.A02* has pleiotropic effects on rapeseed plant architecture and yield related traits

During my studies, I analyzed *Bna.AP1.A02* stop codon mutant families in the greenhouse to answer the question whether *Bna.AP1* has the same meristem identity function in rapeseed as its ortholog *API* has in *Arabidopsis*. As floral meristem identity genes play a crucial role in defining the floral architecture, I expected that analyzing the role of one of such meristem identity genes (*Bna.AP1*) can help to understand the extent till which these genes affect rapeseed flower architecture. Moreover, I also expected to discover if the mutations in *Bna.AP1.A02* confer any other phenotypic changes related to plant architecture or seed yield in rapeseed. I observed that stop codon mutation in one of the six *Bna.AP1* paralogs (*Bna.AP1.A02*) exhibited effects on plant/floral architecture and seed yield related traits. The effect on floral architecture was in accordance with my expectation from *Arabidopsis*, but the variation in seed yield and plant architecture indicated neo-functionalization. However, this data must be regarded as preliminary data because it comes from M₃ plants which carry thousands of background mutations. Moreover, the effect on seed yield cannot be concluded based on the data from a greenhouse experiment. Therefore, I suggest that the role of *Bna.AP1.A02* mutants should be further investigated after backcrossing to verify the function of this paralog in controlling plant architecture and yield related traits. Since *Bna.AP1* affects yield related traits, I also propose that field experiments should be performed in multiple locations and multiple years with backcrossed (at least two generations) *Bna.AP1.A02* mutants. Additionally, I also observed that a splice site mutation in another paralog (*Bna.AP1.C02*) did not show any phenotypic effect. To better understand the functions of both paralogs, I suggest analyzing double mutant plants carrying mutations in both paralogs, simultaneously. In the current study, hand crossing between these two mutant families did not set any filled siliques (data not shown), which can be because of poor pollen germination rate or effect of background mutations. *Bna.AP1.A02* and *Bna.AP1.C02* were chosen for phenotypic analysis because of their transcriptional profiling in the transcriptome analysis, which comes from leaf tissue. Based on the transcriptome data, other four paralogs of *Bna.AP1* did not show any expression. Nevertheless, these four *Bna.AP1* paralogs might be expressed in the shoot apex, and thus, transcriptional profiling of shoot apex can answer the questions regarding functional and transcriptional divergence among different *Bna.AP1* paralogs. Once all the functional paralogs of *Bna.AP1* are identified, different mutants can be generated with individual or combined mutations in different paralogs. Mutants with favourable effects on plant phenotypes can increase the genetic diversity by introducing beneficial alleles, which can be helpful to optimize floral/plant architecture and also seed yield components.

In *Arabidopsis*, the function of floral meristem identity genes have been thoroughly studied and various mutant combinations have been made. It is known that *LFY* and *API/CAL* are part of a positive feedback loop, which ensures that these genes are expressed at optimum levels in early-stage floral primordia (Goslin et al. 2017). Combinations of different mutants in *Arabidopsis* result in more prominent phenotypes compared to the single mutants. For instance, in the *lfy ap1* double mutant, flowers are almost entirely converted into shoots (Weigel and Meyerowitz 1993). For another meristem identity gene, *CAL*, the effect of the mutation is only observed when the mutation in other meristem identity gene is combined. For example, in *ap1 cal* double mutant, floral meristem identity is completely lost, and inflorescence meristems are produced in place of flowers, which results in a

'cauliflower' appearance (Grandi et al. 2012). Similarly, combining mutations in different meristem identity genes in rapeseed can also expand the possibilities of having improved flowering time, plant architecture as well as seed yield. Mutations in different genes can be combined in a dosage-dependent manner to look for a beneficial combination of alleles. Through functional characterization of rapeseed flowering time mutants, elite alleles for early maturity breeding in rapeseed can be added. Detailed knowledge of the active regulatory networks in rapeseed will support future breeding efforts by allowing more precise, paralog-specific crop improvement strategies. Moreover, unveiling the function of various paralogs of flowering time genes, their regulation and the information about how they interact will be a key for crop improvement.

6 Summary

In rapeseed (*Brassica napus*), flowering time is crucial for environmental adaptation and to maximize yield. In the northern hemisphere, late flowering can limit the rapeseed yield. Therefore, breeders try to develop rapeseed varieties with optimum flowering time for the cultivated region. The transition from vegetative to the reproductive stage is a complex biological process and is controlled by multiple flowering genes, which respond to environmental and endogenous signals. Gene regulatory network of flowering time has been studied in detail in the closely related model crop *Arabidopsis thaliana*. However, polyploidy nature of the rapeseed genome makes it difficult to transfer the knowledge from *Arabidopsis* to rapeseed. The objective of my study was to investigate the regulation of the flowering time of oilseed rape by transcriptome analysis as well as the phenotypic characterization of ethyl-methyl-sulfonate (EMS) -induced mutants.

I analyzed the transcriptome data of Ningyou7 leaf material harvested from vernalized and non-vernalized plants at different developmental stages. I investigated the expression profiles of more than 54,000 gene models and selected the differentially expressed flowering time orthologs between different treatments and developmental stages. To further verify the functions of these differentially expressed genes (DEGs), I used the genome wide association study (GWAS) and QTL (quantitative trait loci) mapping data, which was generated by the project partners using Brassica Infinium 60K SNP Chip. I found 36 DEGs to be associated with flowering time, seed yield or both. Among the 36 genes, *Bna.VIN3.A02* showed a DNA polymorphism between winter and spring rapeseed as well as winter and semi-winter rapeseed in a region encoding a functional protein domain. The transcriptome analysis deepens understanding of the interaction of paralogous genes in oilseed rape and points to their pleiotropic effects on seed yield. Moreover, the floral regulators identified from the current transcriptome analysis can be a useful resource for breeding better adapted rapeseed varieties with higher yield.

In another experiment, I investigated the phenotypic effect of mutations in a gene (*Bna.API*) that has high sequence homology to the *Arabidopsis API* gene. For this, I analyzed three stop codon mutant families of *Bna.API.A02* in the greenhouse. One mutant family *api_1* showed altered plant architecture, floral morphology, and yield related traits. This data suggested that *Bna.API.A02* has a pleiotropic effect on seed yield and plant architecture in rapeseed. However, the pleiotropic effects of *Bna.API.A02* on seed yield should be further confirmed in future in the field trials.

For my last experiment, I hypothesized that *Bna.FT* gene in heterozygous stage affect heterosis in F₁ hybrids. To study the effect of *Bna.FT* mutations, I performed field trials in two locations in Germany. I analyzed the seed yield per plot of F₁ hybrids derived from four different *Bna.FT* mutant families. Based on this preliminary data, it was evident that *Bna.FT* missense or splice site mutations have no effect on seed yield heterosis in rapeseed. However, the effect of background mutations on the phenotype of F₁ hybrids was evident. To check the effect of background mutations in EMS-induced mutant plants, I compared phenotypes of non-backcrossed mutants with backcrossed mutants (with Express617) after two generations of backcrossing. I observed that background mutations have a significant influence on quantitative traits like flowering time and seed yield. Therefore, reduction of background mutations is necessary prior to phenotypic characterization of EMS induced mutants.

7 Zusammenfassung

Beim Ölraps (*Brassica napus*) spielt der Blühzeitpunkt eine große Rolle bei der Anpassung an verschiedene Umweltbedingungen sowie bei der Ertragsbildung. In den nördlichen Hemisphären kann ein verzögerter Blühzeitpunkt den Samenertrag einschränken. Daher versuchen Züchter, Rapsorten mit optimalem Blühzeitpunkt für jedes Anbauggebiet zu entwickeln. Der Übergang vom vegetativen zum reproduktiven Stadium ist ein komplexer biologischer Prozess und wird von mehreren Blühgenen reguliert, die auf Umwelt- sowie endogene Signale reagieren. Das Genregulationsnetzwerk des Blühzeitpunkts wurde in der verwandten Modellpflanze *Arabidopsis thaliana* detailliert untersucht. Die Polyploidie des Raps-Genoms macht es jedoch schwierig, das Wissen von *Arabidopsis* auf Raps zu übertragen. Das Ziel dieser Arbeit war es, das Regulationsnetzwerk für den Blühzeitpunkt von Raps durch eine Transkriptomanalyse sowie die phänotypische Charakterisierung von Ethyl-Methyl-Sulfonat (EMS)-induzierten Mutanten zu untersuchen. Dafür wurde die chinesische Semi-Winterraps-Sorte Ningyou7 verwendet.

Für die Analyse des Transkriptoms wurde Blattmaterial von vernalisierten und nicht vernalisierten Pflanzen in verschiedenen Entwicklungsstadien verwendet. Ich untersuchte die Expressionsprofile von mehr als 54.000 Genmodellen und wählte zwischen verschiedenen Behandlungen und Entwicklungsstadien differentiell exprimierte Gene für die weiteren Arbeiten aus, die über hohe Sequenzhomologie zu *Arabidopsis*-Genen verfügen und vermutlich funktionell orthologen Blühzeit-Regulatoren darstellen. Um die Funktionen dieser DEGs weiter zu verifizieren, habe ich Daten aus einer genomweiten Assoziations- (GWAS)- und einer QTL (quantitative trait locus)-Studie verwendet, die von den Projektpartnern mit dem Brassica Infinium 60K SNP-Chip generiert wurden. Die Untersuchung ergab, dass 36 differentiell exprimierte Gene in Genomregionen liegen, in denen QTL für Blühzeitpunkt, Samenertrag oder beide gleichzeitig lokalisiert worden waren. Unter den 36 Genen zeigte *Bna.VIN3.A02* einen DNA-Polymorphismus zwischen Winter- und Sommerraps sowie Winter- und Semi-Winterraps in einem Bereich, die für eine funktionelle Proteindomäne kodiert. Die Transkriptomanalyse vertieft das Verständnis über das Zusammenwirken der paralogen Gene in Raps und deutet auf deren pleiotrope Wirkungen auf den Samenertrag hin. Darüber hinaus können die in der aktuellen Transkriptomanalyse identifizierten Kandidatgene eine nützliche Ressource für die Züchtung besser angepasster Rapsorten mit höherem Ertrag darstellen. In einem weiteren Experiment habe ich die Wirkung von Mutationen in einem Gen (*Bna.API*) untersucht, das hohe Sequenzhomologie zu dem *API* Gen von *Arabidopsis* besitzt. Dazu analysierte ich drei Stop-Codon-Mutanten von *Bna.API.A02* im Gewächshaus. Eine Die Mutante *apl_1* zeigte deutliche Veränderungen bei Pflanzenarchitektur und Blütenmorphologie sowie bei einigen Ertragsmerkmalen. Diese Daten weisen darauf hin, dass *Bna.API.A02* eine pleiotrope Wirkung auf den Samenertrag und die Pflanzenarchitektur beim Raps hat. Die pleiotrope Wirkung von *Bna.API.A02* auf den Samenertrag muss in Zukunft unter Feldversuchen bestätigt werden.

Ich stellte die Hypothese auf, dass heterozygote Sequenzvariationen in Orthologen des *Arabidopsis FT*-Gens die Heterosis in F₁-Hybriden beeinflussen. Um die Wirkung von *Bna.FT*-Mutationen zu untersuchen, führte ich Feldversuche an zwei Standorten in Deutschland durch. Für vier F₁-Hybride aus vier verschiedenen *Bna.FT*-Mutantenfamilien wurde der Samenertrag bestimmt. Basierend auf diesen vorläufigen Daten ließ sich schlußfolgern, dass *Bna.FT missense*- oder *splice-site*-Mutationen keinen Einfluss auf die Heterosis in Raps haben. Die Auswirkung von Hintergrundmutationen auf den Phänotyp von F₁-Hybriden war jedoch deutlich. Um die Wirkung von Hintergrundmutationen in EMS-induzierten mutierten Pflanzen zu überprüfen, verglich ich Phänotypen von Mutanten in der M₄ Generation mit Mutanten, die zweimal mit dem Ausgangsgenotyp Express617 zurückgekreuzt worden waren. Ich beobachtete, dass Hintergrundmutationen einen signifikanten Einfluss auf quantitative

Zusammenfassung

Merkmale wie Blühzeitpunkt und Samenertrag haben. Daher lässt sich schlussfolgern, dass die Reduktion von Hintergrundmutationen vor der phänotypischen Charakterisierung von EMS-Mutanten notwendig ist.

8 Appendix

8.1 Supplementary Tables

Table S1: The *B. napus* mini diversity panel for the initial growth experiment in the climate chamber. For each accession, 20 plants were split into two groups of vernalized and non-vernalized plants. Seeds were provided by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) and Dr. Habibur Rahman (University of Alberta, Canada); DH: doubled haploid line.

Accession name	Growth-type	Origin	Days to flowering (with vernalization / without vernalization)
ROSA	Spring	China	100 / 53
Hi-Q	Spring	Canada	104 / 50
Ningyou7	Semi-winter	China	82 / 100
HAU7	Semi-winter	China	82 / 71
Winter (NPZ)	Winter	Germany	135 / 150
Express617 (DH)	Winter	Germany	135 / 150

Table S2: Total number of RNA probes (rRNA-depleted) for sequencing of 14 Ningyou7 RNA-seq libraries through the Illumina HiSeq2000 platform; RIN: RNA integrity number.

IKMB ID	Experiment- Developmental stage-Biological replicate	Amount of rRNA- depleted RNA (ng) used for sequencing	Quality (RIN)
B1341	V-BBCH20-I	855.0	> 7.0
B1347	V-BBCH20-III	635.7	> 7.0
B1342	V-BBCH30-I	882.7	> 7.0
B1345	V-BBCH30-II	703.3	> 7.0
B1348	V-BBCH30-III	717.6	> 7.0
B1343	V-BBCH50-I	743.6	> 7.0
B1346	V-BBCH50-II	754.0	> 7.0
B1349	V-BBCH50-III	806.0	> 7.0
B1332	NV-BBCH20-I	881.4	> 7.0
B1335	NV-BBCH20-II	517.4	> 7.0
B1338	NV-BBCH20-III	702.0	> 7.0
B1334	NV-BBCH50-I	594.1	> 7.0
B1337	NV-BBCH50-II	422.5	> 7.0
B1340	NV-BBCH50-III	358.8	> 7.0

Appendix

Table S3: Quality filtering and raw read statistics for Ningyou7 transcriptome libraries. Three biological replicates grown under two different conditions (with and without cold treatment) were sequenced from three developmental stages; V: Vernalized plants, NV: Non-vernalized plants.

RNA-seq Library	Biological replicate	BBCH	Growth Condition	No. of raw reads (millions)	No. of high-quality reads (millions)	No. of multiple-mapped reads (millions)	No. of uniquely-mapped reads (millions)
V20-1	1	20	Winter	70.09	52.02	41.67	9.36
V20-3	3	20	Winter	69.81	48.63	39.10	8.69
V30-1	1	30	Winter	73.44	54.16	39.15	13.44
V30-2	2	30	Winter	72.85	49.89	36.01	12.16
V30-3	3	30	Winter	86.34	60.68	45.04	13.84
V50-1	1	50	Winter	72.38	53.43	41.71	10.37
V50-2	2	50	Winter	65.85	45.57	34.09	10.43
V50-3	3	50	Winter	74.08	52.07	40.05	10.57
NV20-1	1	20	Spring	77.12	43.94	34.39	8.70
NV20-2	2	20	Spring	85.97	56.79	41.86	13.66
NV20-3	3	20	Spring	94.93	55.61	42.69	11.70
NV50-1	1	50	Spring	80.46	44.14	35.89	7.22
NV50-2	2	50	Spring	77.82	51.21	37.79	11.84
NV50-3	3	50	Spring	77.45	46.14	34.66	10.35

Table S4: Primers used in this study for screening mutations in Express617 EMS population and for expression analysis by RT-qPCR.

<i>B.napus</i> paralog	Primer name	Sequence	Orientation	Amplicon size (bp)	Annealing temperature (°C)	Application
<i>Bna.API.A02</i>	NE189	CGTGGACCCATTATTATTACAGTTAAGCC	Forward	919	57	TILLING
	NE192	CAGCCAGGAGAACAAAACAATGCATACAG	Reverse			TILLING
	NE190	GATCCCGAGACTCACACACAAGTC	Forward	1323	64	TILLING
	NE194	CATCTAGCTTAGGGTTCTGAGTGCGG	Reverse			TILLING
	NE190	GATCCCGAGACTCACACACAAGTC	Forward	1002	66	TILLING
	NE193	CCAGAAGCAGCTGTACGTTTGAAATTG	Reverse			TILLING
	NE211	GGACCTCTGGGGATCCACGAAATC	Forward	1215	66	TILLING
	NE213	GGTTCTGCAAAGACACACATCACATGATC	Reverse			TILLING
	NE212	CAGACT TAGCTCCAGCTGTTCTTCTTCC	Forward	1153	61	TILLING
	NE213	GGTTCTGCAAAGACACACATCACATGATC	Reverse			TILLING
	NE215	CCATCGCACTACTGCTTGCTT T	Forward	801	60	TILLING
	NE217	CACTGGAACTATAACACCTTACTAGC	Reverse			TILLING
	NE216	CAAAACGTCGTCTCAACTACCTCT	Forward	1122	66	TILLING
	NE218	CCAGTTTCGTCATTCGTTCTCTCCGAA	Reverse			TILLING
<i>Bna.API.C02</i>	NE203	GACATGAAGCTAGCCCTAATTCTG T	Forward	1213	66	TILLING

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	NE205	CAGATGAACAGATCTAGTCGAGGTAGATG	Reverse			TILLING
<i>Bna.TFL1</i> -Conserved	SR206	TGTCTCCTCCAAGCCTAGGGTTG	Forward	185	61	RT-qPCR
	SR207	CTCATAGCTCACCACCTCTTTTCC	Reverse			RT-qPCR
<i>Bna.SEP4</i> -Conserved	SR215	GGCATTGCTAGGTGAAGAGATAGC	Forward	192	63	RT-qPCR
	SR216	CCTCCAACCTTTCTCTTAAGATCTC	Reverse			RT-qPCR
<i>Bna.FUL</i> -Conserved	SR217	GTAGGGTTCAGCTGAAGAGGATAG	Forward	156	63	RT-qPCR
	SR218	CGAAGAGTTTGCCTTTGGAAG	Reverse			RT-qPCR
<i>Bna.TFL1.A10</i>	SR175	GAGCCATTGATAGTGGGAAGAGTGG	Forward	135	62	RT-qPCR
	SR219	CTTGGAGGAGACAGCTAAAGGC	Reverse			RT-qPCR
<i>Bna.TFL1.C03</i>	SR232	GCTACAACATGAAGCAAGTCTCCAAC	Forward	185	63	RT-qPCR
	SR233	GTTTGTTACTATCCAATGCAGGTGC	Reverse			RT-qPCR
<i>Bna.TFL1.Ann</i>	SR234	CGTGAGTTACAACAAGAAGCAAGTCTG	Forward	190	62	RT-qPCR
	SR235	GTTTGTCACAATCCAATGCAGGTG	Reverse			RT-qPCR
<i>Bna.FT.C6b</i>	BnC6FT b_F9	GCTACACACTAAGCCTATTTGTAT	Forward	746	59	Genotyping
<i>Bna.FT.C6b</i>	BnC6FT b_R12	CCACCCTGGTTCATACACC	Reverse			Genotyping
<i>Bna.TFL1.A10</i>	BnTFL1 _F1	TCTCTCTTTCCCTAAGCTCACTT	Forward	1266	59	Genotyping
<i>BnTFL1-2</i>	BnTFL1 _2_R1	CGGGAATTTAATCTCCTATAATGAG	Reverse			Genotyping

Table S5: Details of EMS mutations in *Bna.API.A02* and *Bna.API.C02* paralogs detected by TILLING of Express617.

	<i>Bna.API.A02</i>	<i>Bna.API.C02</i>
Number of TILLING amplicons	3	1
Coding sequence coverage (%)	88.7	55.9
Number of mutations detected by LICOR gels	207	39
Number of mutations verified by sequencing	164	32
Number of nonsense mutations	6	-
Number of UTR mutations	5	-
Number of splice site mutations	-	1
Number of missense mutations	40	4
Number of silent mutations	13	7
Number of intronic mutations	100	20
Mutation frequency (1/kb) ^a	1/17.4 ^b	1/26 ^c
Mutations/1,000 M ₁ /1,000 bp	58	38

^a Mutation frequencies calculated based on the number of mutations/M₁ plant

^b Mutation frequency calculated based on 940 screened M₁ plants

^c Mutation frequency calculated based on 748 screened M₁ plants

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Table S6: Nucleotide position and amino acid changes in different splice site, non-sense and UTR mutants from *Bna.API.A02* and *Bna.API.C02* paralogs. The phenotyping was performed with plants from the M₄ generation. Genotyping of the heterozygous M₃ families was done by Sanger sequencing. *aa*- genotype carrying homozygous mutant allele of *Bna.API.A02* (M₄ generation), *AA*- genotype carrying homozygous wildtype allele of *Bna.API.A02* (M₄ generation), *Aa*- genotype carrying heterozygous allele of *Bna.API.A02* (M₄ generation). Chi-square test was performed to check genotypic segregation. Expected genotypic segregation: 1:2:1 (*aa:Aa:AA*), df=2, $\alpha=0.05$.

M ₂ plant	Mutation	Mutant code	Amino acid change	Zygoty of M ₂	M ₃ seed code	Chi ² test for 1:2:1 ratio (<i>aa: Aa: AA</i>)	M ₄ seed code
48_H3	<i>C1865T*</i>	<i>apl_1</i>	Gln126Stop	heterozygous	160941	$\chi^2 = 4.30 < \chi^2_{tab} = 5.99$ n.s	160952 (<i>aa</i>) 160953 (<i>AA</i>)
50_A8 _c	<i>G2939A*</i>	<i>apl_2</i>	Try186Stop	heterozygous	160942	$\chi^2 = 1.0 < \chi^2_{tab} = 5.99$ n.s	160988 (<i>aa</i>)
50_B8 _c							160989 (<i>AA</i>)
58_F1 _d	<i>C3486T*</i>	<i>apl_3</i>	Gln227Stop	heterozygous	160944	$\chi^2 = 0.5 < \chi^2_{tab} = 5.99$ n.s	160966 (<i>aa</i>)
58_G1 _d							160970 (<i>AA</i>)
42_A1 _b	C2994T		Gln205Stop	heterozygous			
42_B1 _b							
55_E12	C2988T		Gln203Stop	heterozygous			
69_A8 _e	C3024T		Gln215Stop	heterozygous			
69_D8 _e							
56_D4	G-1A		5' UTR	heterozygous			
46_H7	G-29A		5' UTR	homozygous			

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61_E12	G-26A		5' UTR	heterozygous			
56_E10	G-20A		5' UTR	heterozygous			
69_A8 _e	G-8A		5' UTR	homozygous			
69_D8 _e							
53_G1 ^f	G717A		Splice site	homozygous			

^{a,b,c,d,e} : Mutant plants indicated with the same letter belong to the same M₂ family

^f : Mutant family carrying splice site mutation in *Bna.API.C02* paralog

^{n.s} : Non significant

*: Mutant families used in current study for phenotypic evaluation.

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Table S7: Genotypic segregating of BC₂F₂ mutant families. Genotyping was done by Sanger sequencing. *aa*- genotype carrying homozygous mutant allele, *AA*- genotype carrying homozygous wildtype allele, *Aa*- genotype carrying heterozygous allele. Chi-square test was performed to check genotypic segregation. Expected genotypic segregation: 1:2:1 (*aa:Aa:AA*), df=2, $\alpha=0.05$.

Mutant family	No. of plants germinated	Genotypes						χ^2 test (df=2, $\alpha=0.05$)
		<i>aa</i>		<i>Aa</i>		<i>AA</i>		
		Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
<i>ft_1</i>	39	10	9.75	16	19.5	13	9.75	$\chi_{cal}^2 = 1.71 < \chi_{tab}^2 = 5.99$ n.s
<i>ft_2</i>	38	7	9.5	24	19	7	9.5	$\chi_{cal}^2 = 2.41 < \chi_{tab}^2 = 5.99$ n.s
<i>tfl_1</i>	40	12	10	19	20	9	10	$\chi_{cal}^2 = 0.55 < \chi_{tab}^2 = 5.99$ n.s
<i>ft_3</i>	40	11	10	20	20	9	10	$\chi_{cal}^2 = 0.2 < \chi_{tab}^2 = 5.99$ n.s

df: Degree of freedom

n.s: not significant

Exp.: Expected

Obs.: Observed

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Table S8: Germination rate of four mutant families before and after two generations of backcrossing. The phenotypic data was collected from 10 plants per genotype. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks).

Mutant families	<i>ft_1</i>	<i>ft_2</i>	<i>tfl_1</i>	<i>ft_3</i>
Generation				
<i>aa-M</i> ₃	43.3 %	95 %	80 %	43.3 %
<i>aa-BC</i> ₂ <i>F</i> ₃	97.5 %	95 %	100 %	100 %

8.2 Supplementary Figures

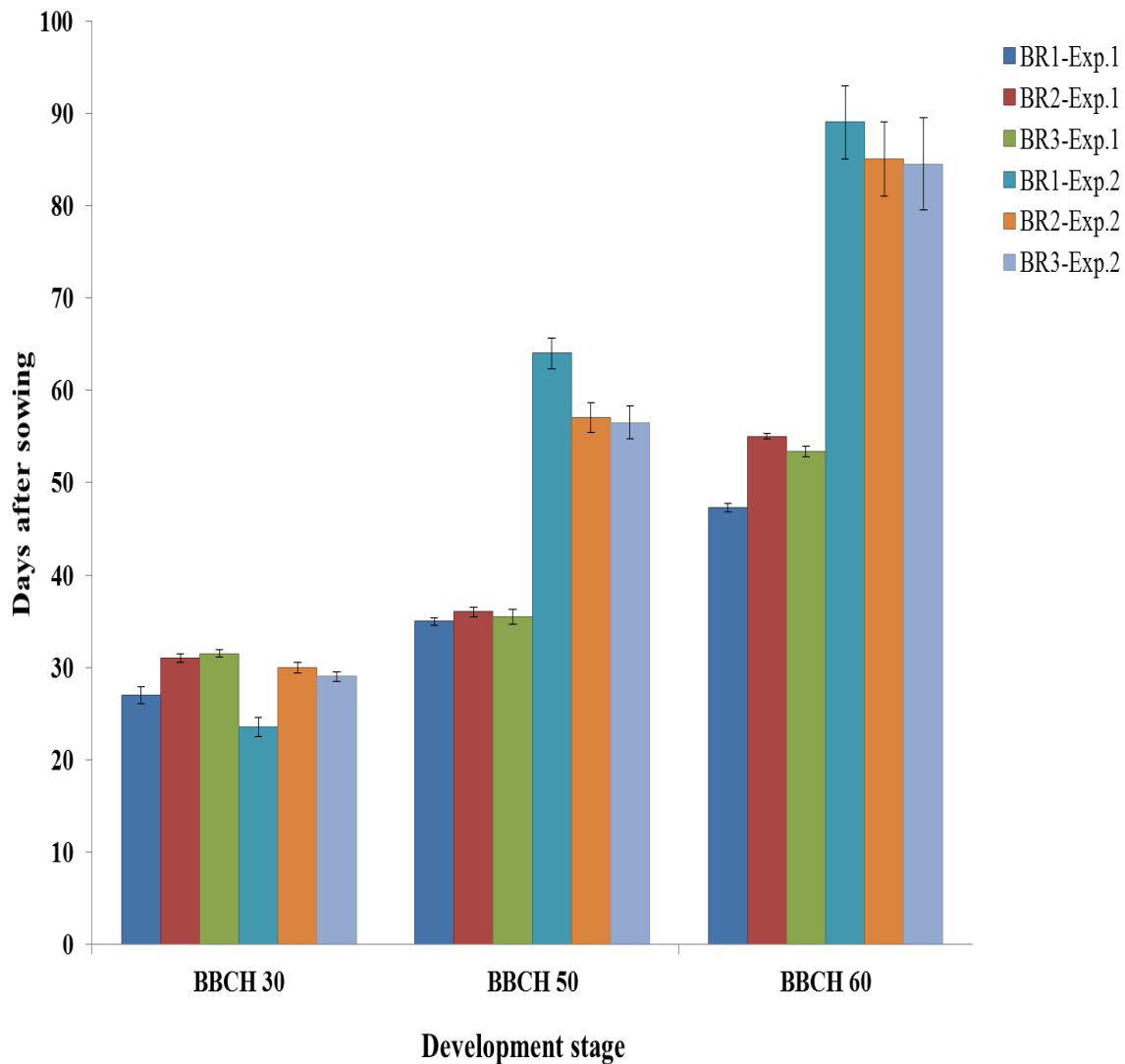


Figure S1: Phenological development of Ningyou7 under greenhouse conditions. Plants were grown in two different experiments 1) Vernalized and 2) Non-vernalized. The number of days to flowering is given as days from sowing to first flower. Each column represents number of days to flowering for each biological replicate which is the mean of four plants; BR: Biological replicate; Exp.1: Experiment 1 (Vernalized conditions); Exp.2: Experiment 2 (Non-vernalized conditions).

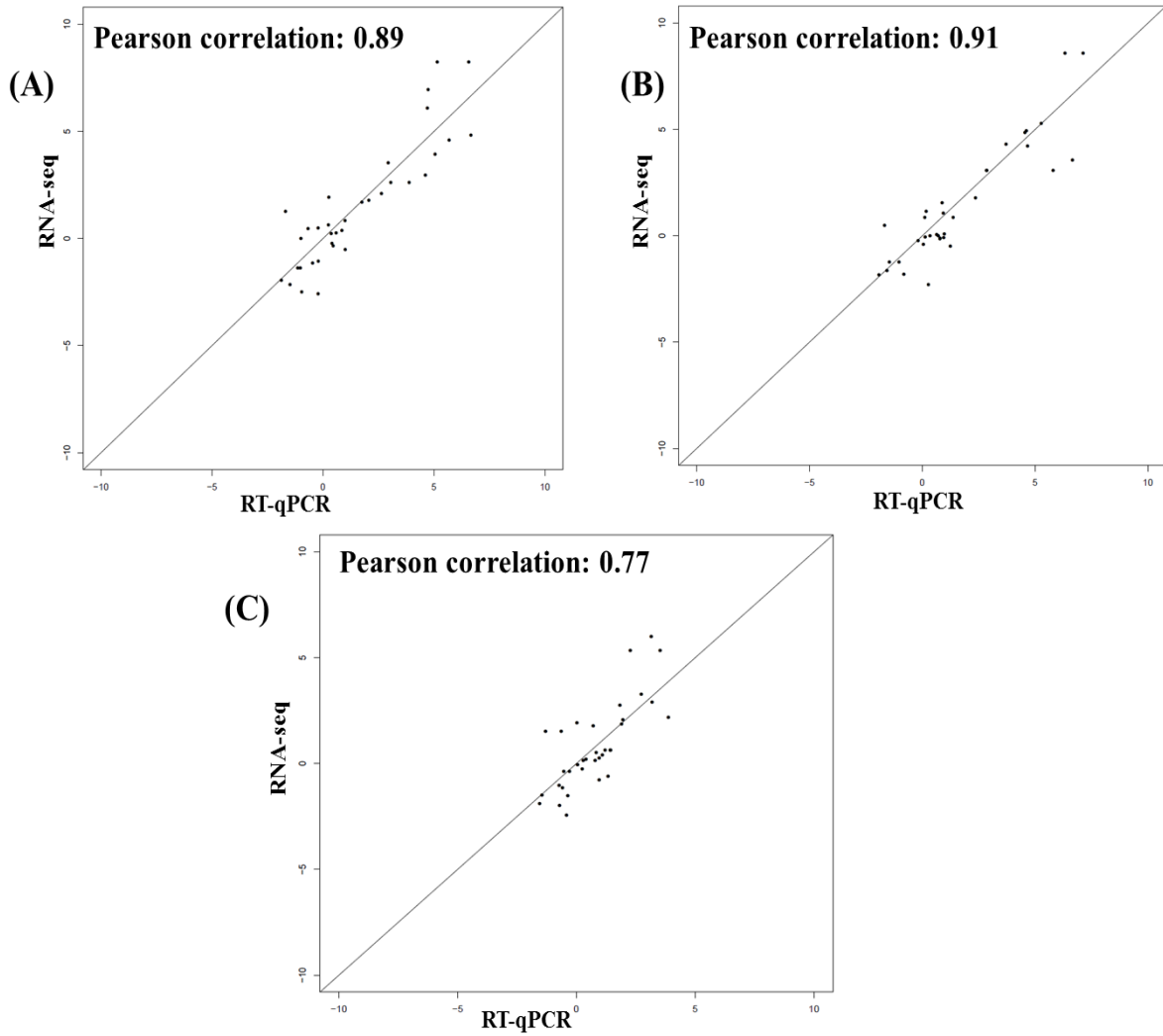


Figure S2: Scatter plot showing correlation between expression data from RNA-seq and RT-qPCR. RT-qPCR values from 63 paralogs of 21 Arabidopsis flowering time orthologs and 14 uncharacterized gene models were used to optimize bioinformatics pipeline. A) log2fold change data between BBCH20 and BBCH50-V; B) log2fold change data between BBCH20 and BBCH50-NV; C) log2fold change data between BBCH20 and BBCH30-V.

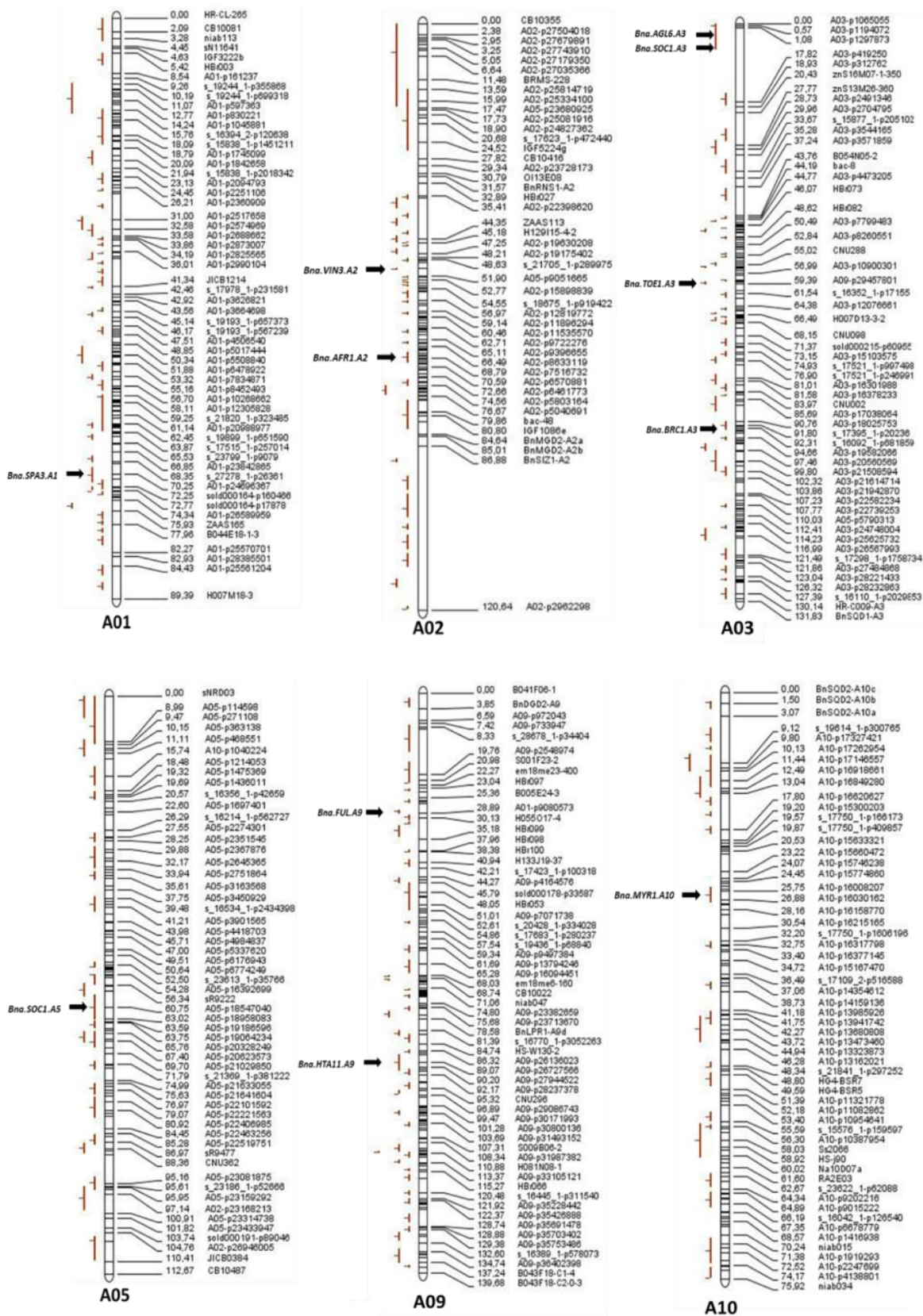


Figure S3: Genetic positions of QTLs related to 22 phenological and yield related traits along with the positions of the candidate genes from current study which mapped to the QTLs. The map was constructed with 202 DH lines of the TN population. Phenotypic traits were measured under 19 environments (Luo et al., 2017). The position of candidate genes

from the current transcriptome study is marked with a black arrow. Vertical lines on the left side of the linkage groups indicate the confidence interval for QTLs; Marker positions mentioned on the right side of the chromosome are in cM.

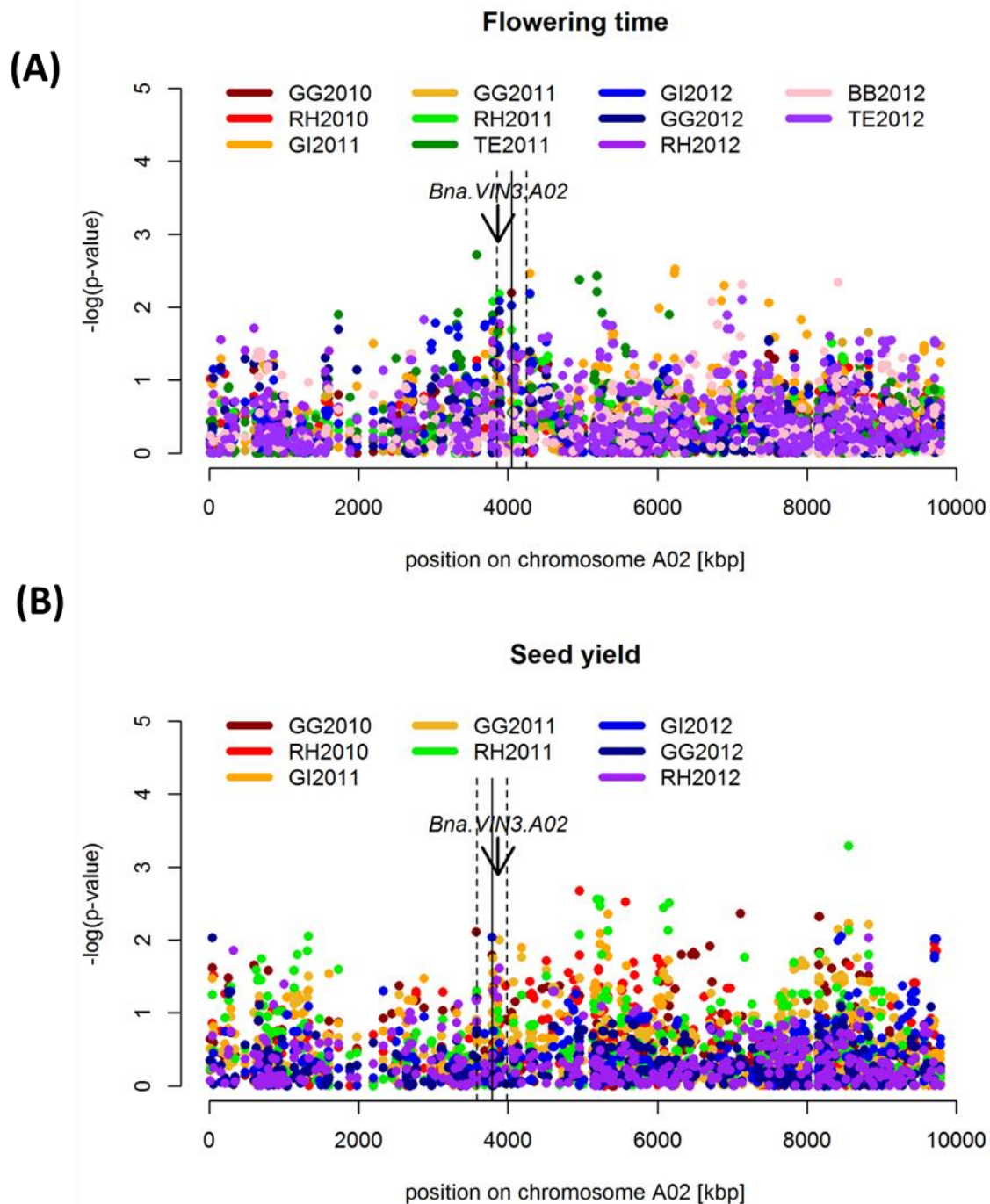


Figure S4: Manhattan plots representing marker-trait associations of *Bna.VIN3.A2* as an example for the candidate genes with absolute values for flowering time (A) and seed yield (B) data from Schiessl et al. (2015). Each color key represents one environment out of 11 different environments from Germany, China and Chile as mentioned by Schiessl et al. (2015). The solid black line represents the position of closest SNP marker to *Bna.VIN3.A2* and the dotted lines on each side represent the region within LD of the gene. The SNP markers within LD and having $-\log_{10}P > 2.0$ for at least one out of 11 environments were considered as associated with trait.

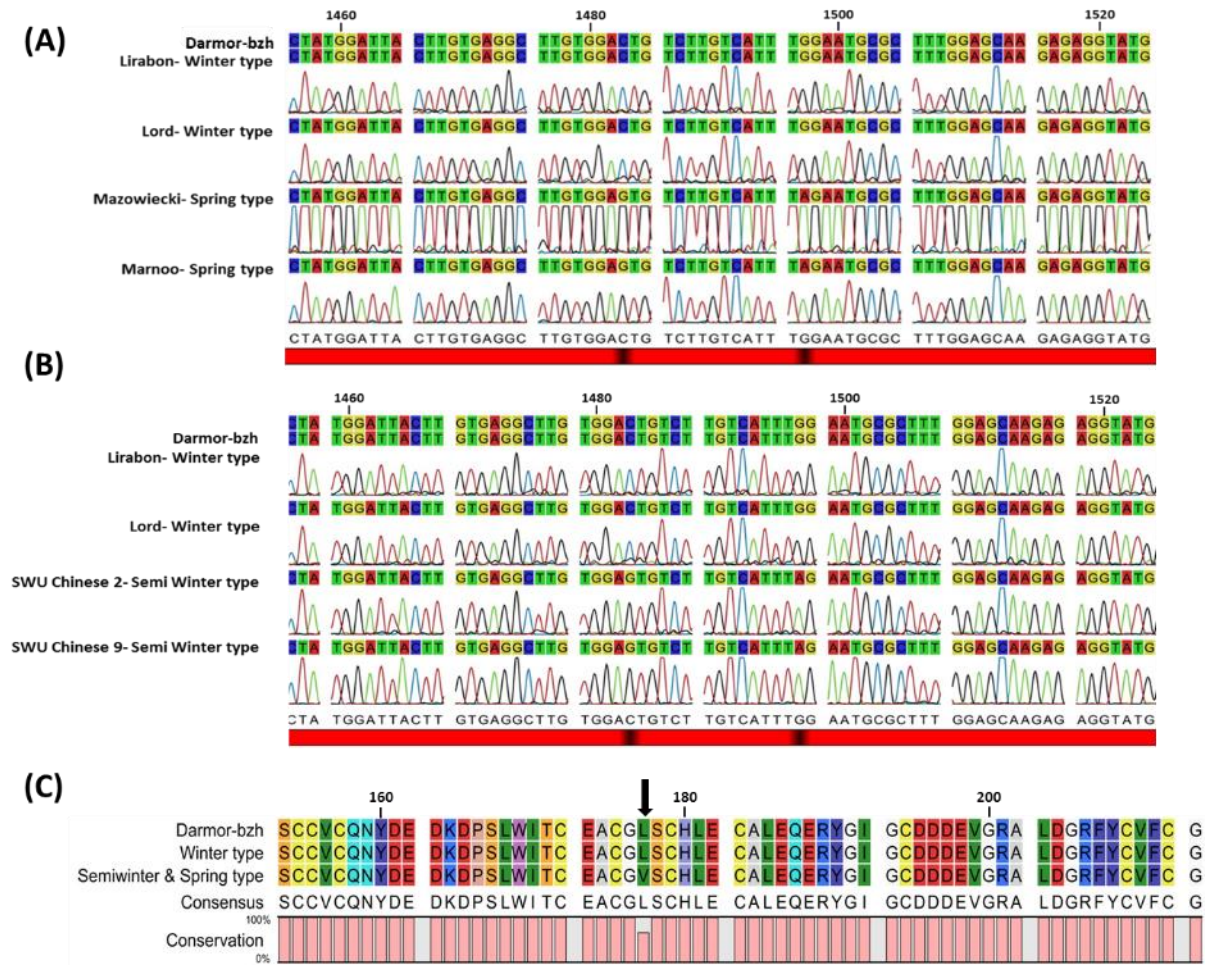


Figure S5: Sequence variation in *Bna.VIN3.A2* among rapeseed accessions with different growth habits. A) Sequence comparison between two winter type and two spring type rapeseed cultivars. B) Sequence comparison between two winter type and two semi-winter type rapeseed cultivars. C) Protein sequence comparison for *Bna.VIN3.A2* PHD zinc finger domain among winter type, semi-winter type, spring type and 'Darmor-bzh'; black arrow indicates sequence level variation. Sequence analysis was performed using CLC Main Workbench.

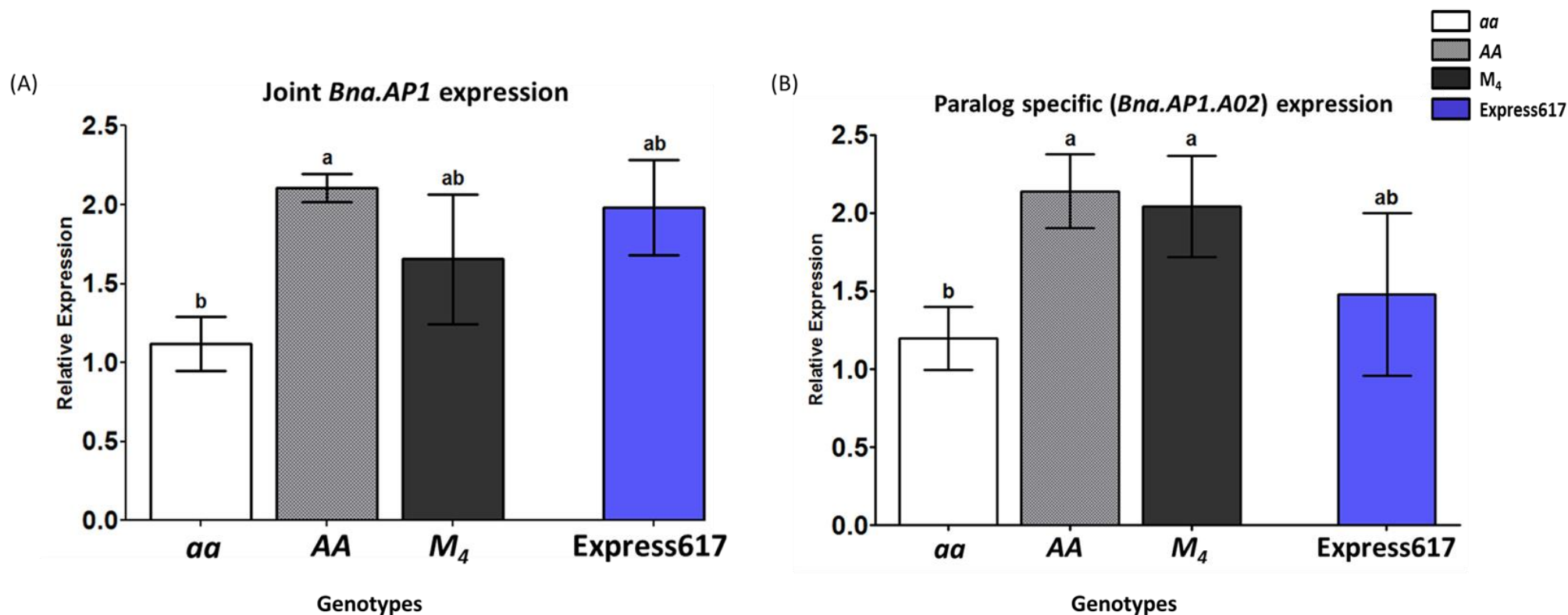


Figure S6: Relative expression of *Bna.AP1* in *Bna.TFL1.A10* missense mutant (Guo et al., 2014) plants. (A) Joint expression of *Bna.AP1* (B) Paralog-specific (*Bna.AP1.A02*) expression. *M*₄: Genotype carrying *Bna.TFL1.A10* missense mutation (*M*₄ generation); *aa*-: Genotype carrying homozygous mutant allele of *Bna.TFL1.A10* in BC₂F₃ generation; *AA*: Genotype carrying homozygous wildtype allele of *Bna.TFL1.A10* in BC₂F₃ generation; Express617: Control. Expression levels of target genes were normalized against *Bna.Actin* total expression. For all genotypes tissue (SAM) sampling was done between zeitgeber 8 h and 9 h. Three biological replicates and three technical replicates were used for each genotype. Error bars: standard error of the mean for biological replicates. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

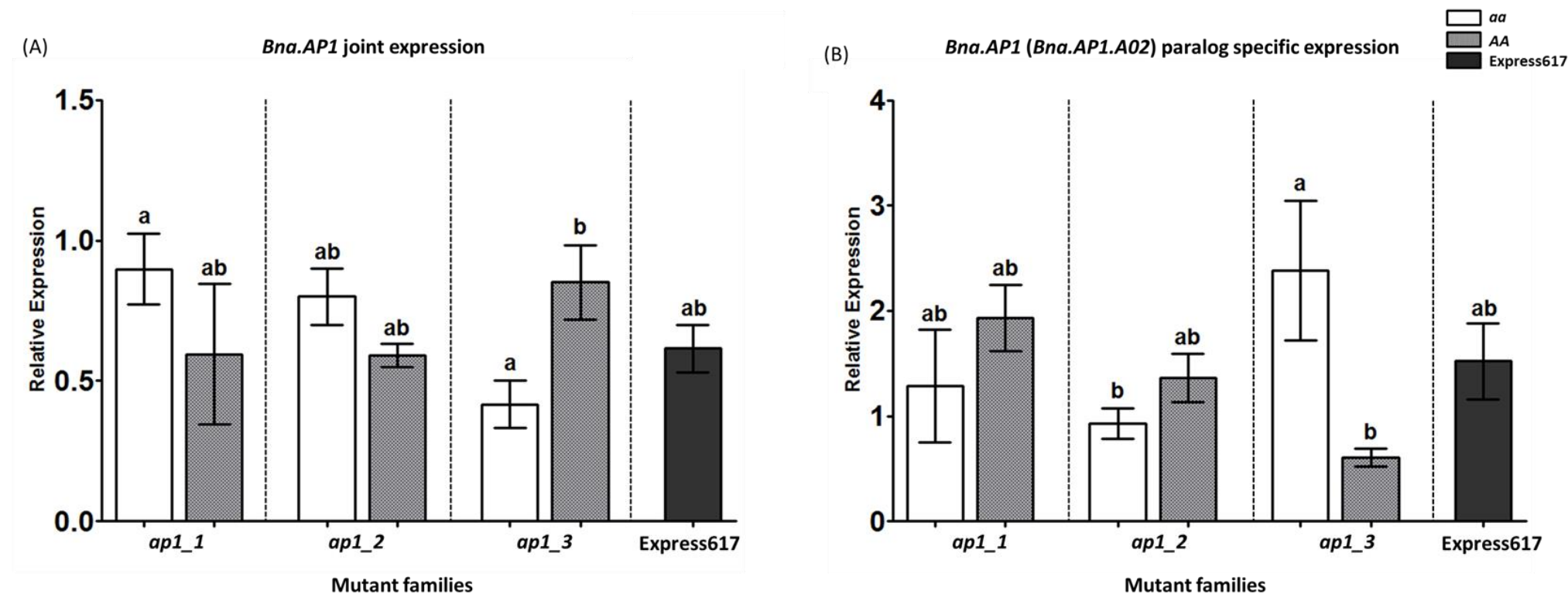


Figure S7: Relative paralog-specific (*Bna.AP1.A02*) (Fig. A) and joint expression of *Bna.AP1* (Fig. B) in *Bna.AP1.A02* stop codon mutant lines. *aa*: Genotype carrying homozygous mutant allele of *Bna.AP1.A02* (M_4 generation); *AA*: Genotype carrying homozygous wildtype allele of *Bna.AP1.A02* (M_4 generation); Express617: Control. Expression levels of target genes were normalized against *Bna.Actin* total expression. For all genotypes tissue (SAM) sampling was done between zeitgeber 8 h and 9 h. Plants were grown in the greenhouse at a constant temperature (22°C), and LD (16 h light) after vernalization (4°C, 16 h light, 8 weeks). Three biological replicates and three technical replicates were used for each genotype. Error bars: standard error of the mean for biological replicates. The mean comparison between the genotypes for the investigated traits was performed by ANOVA test (P value=0.0001), while the grouping was done using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

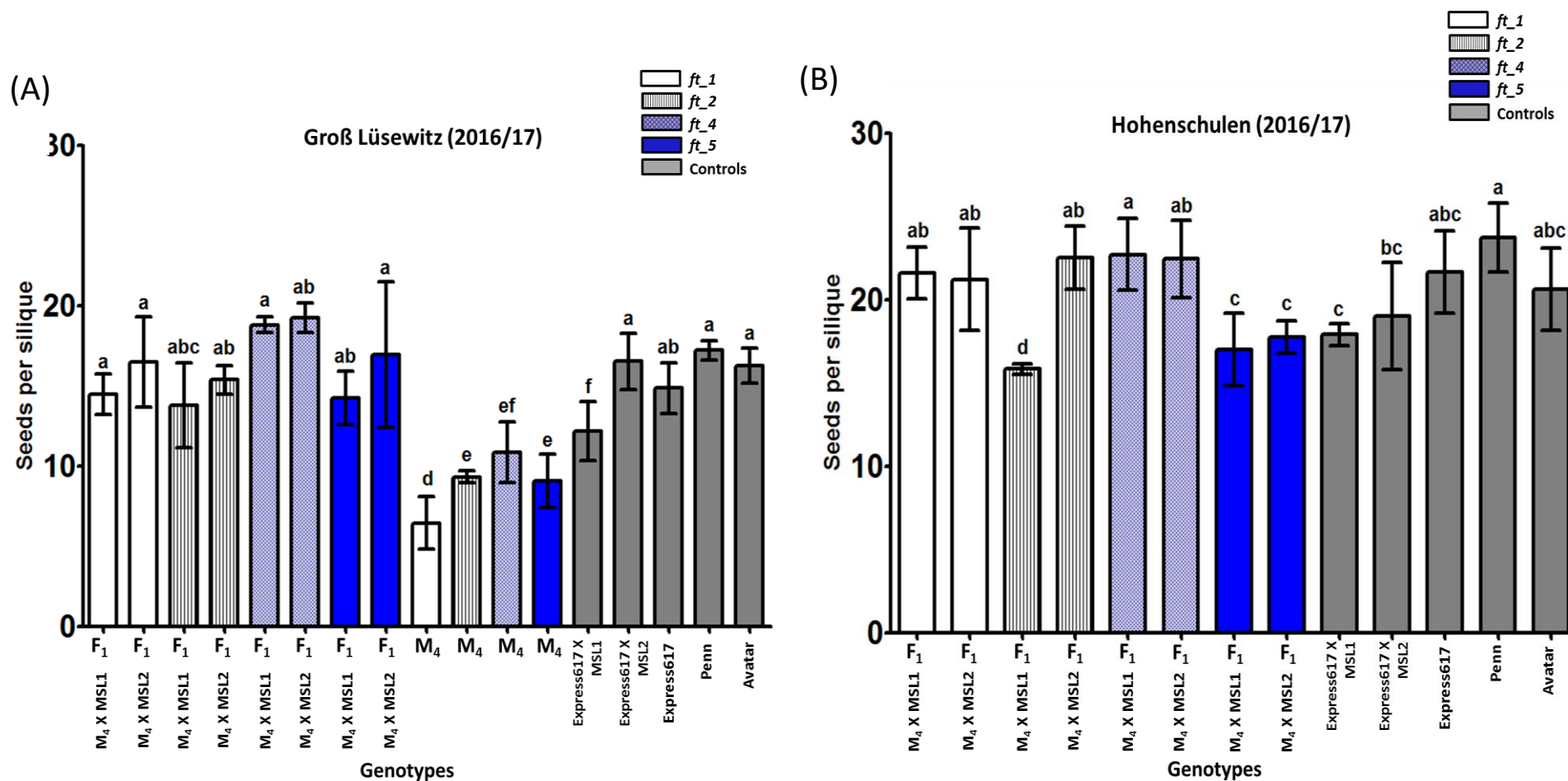


Figure S8: Seeds per silique data for the field experiments conducted at Groß Lüsewitz (Fig. A) and Hohenschulen (Fig. B) in 2016/17. For each genotype, average of plants collected from 1 m² area from each of the three replications (plots) was calculated. Error bars: standard error of the mean for 3 replications. The analysis of variance for the investigated traits was performed by ANOVA test (P value=0.0001), while the mean comparison of the genotypes was performed using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

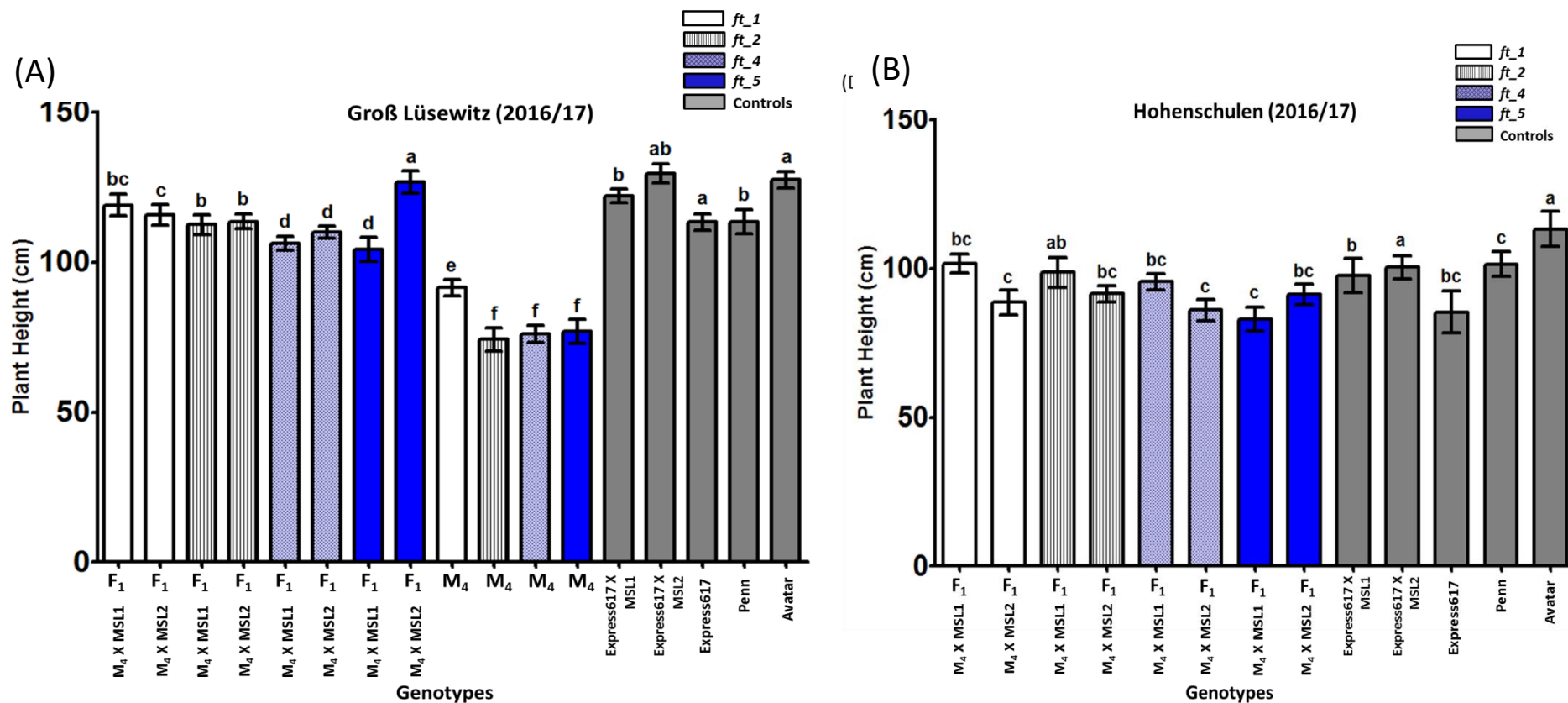


Figure S9: Plant height data from the field experiments conducted at Groß Lüsewitz (Fig. A) and Hohenschulen (Fig. B) in 2016/17. For each genotype, average of plants collected from 1 m² area from each of the three replications (plots) was calculated. Error bars: standard error of the mean for three replications. The analysis of variance for the investigated traits was performed by ANOVA test (P value=0.0001), while the mean comparison of the genotypes was performed using LSD test ($\alpha \leq 0.05$) in R package 'Agricolae' version 1.2-8.

8.3 Supplementary Methods

Method S1: 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). We synthesized the first-strand cDNA using Oligo (dT)18 primers and M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). Quality checks of the synthesized cDNAs were carried out via standard PCR with the housekeeping gene *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH)* in 1/10 dilutions. For each reaction, we arranged cDNA samples from 15 different treatments in a plate considering three technical replicas for each sample. Reactions were performed in a total volume of 20 μ l containing 100 nM of each primer and 2 μ l of diluted cDNA templates. Copy specific primers were designed for 35 groups of Arabidopsis orthologs. We amplified these cDNA samples using the following cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by 95°C for 10 min. A melting curve was generated using a temperature range from 65°C to 95°C with increments of 0.5°C every 5 seconds. For data analysis, we normalized the mean Ct value of the target gene against the average Ct value of *Bna.GADPH-3* (accession number: FJ529182) and *Bna.Actin* (accession number: AF111812), using the Δ Ct method implemented in the CFX manager software (Bio-Rad Laboratories GmbH, München, Germany). Primer efficiencies for the different targets were determined using four two-fold serial dilutions of cDNA and were included in the calculation of relative expression levels in CFX manager.

Method S2: Identification of sequence variations in the candidate gene

To investigate the sequence level variation for the most promising candidate gene in our study, we selected two accessions of each winter (Lirabon and Lord), semi-winter (SWU Chinese 2 & 9) and spring type (Marnoo and Mazowiecki) rapeseed from the ERANET-ASSYST *B.napus* diversity set (Bus, Hecht, Huettel, Reinhardt & Stich, 2012). Genomic DNA was isolated from leaf samples by a standard CTAB method (Saghai-Marooof, Soliman, Jorgensen & Allard, 1984). Locus specific primers were developed for the amplification of the candidate gene and PCR amplicons were sequenced via Sanger sequencing. Sequences were analyzed using the CLC Main Workbench (CLC bio, Aarhus, Denmark).

9 References

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10 Supplementary data on CD/DVD

The following supplemental data are available on CD and can be distributed upon request (contact: Prof. Dr. Christian Jung, c.jung@plantbreeding.uni-kiel.de).

File name	Content	Format
Seed codes.xlsx	Seed codes of produced plant material	.xlsx, Microsoft Excel file
Sequences	Sequences of different genotyping experiments	.clc
Primer list.xlsx	All the primers designed during the thesis	.xlsx, Microsoft Excel file

11 Curriculum Vitae and Publications

11.1 Curriculum Vitae

Personal data

Name Smit Shah
Date of birth 21.07.1989
Place of birth Anand, Gujarat, India
Nationality Indian

Education

2014-2018 **Christian-Albrechts-University of Kiel, Germany**
Doctoral candidate in Plant Breeding and Genetics
Doctoral thesis: Investigations on flowering time genes from oilseed rape (*B. napus*)

2012-2014 **Christian-Albrechts-University of Kiel, Germany**
M.Sc. Agrigenomics
Master thesis: Genetic mapping and transcriptional analysis of candidate genes for root lesion nematode resistance in barley

2006-2011 **Sardar Patel University, V.V.Nagar, Gujarat, India**
M.Sc. (Integrated) Biotechnology with specialization in Industrial Biotechnology
Master thesis: Genetic Diversity Analysis in Nine Cultivars of *Catharanthus Roseus* through RAPD, SSR & ISSR markers & Comparison of Antioxidant Activity

11.2 Publications

Shah S, Weinholdt C, Jedrusik N, Molina C, Zou J, Große I, Schiessl S, Jung C, Emrani N: Whole transcriptome analysis reveals genetic factors underlying flowering time regulation in rapeseed (*Brassica napus* L.). *Plant, Cell & Environment* 2018, 41:1935-1947.

Shah S, Karunarathna N.L, Jung C, Emrani N: An *APETALA1* ortholog affects plant architecture and seed yield component in oilseed rape (*Brassica napus* L.). *BMC plant biology* 2018, 18(1): 380

11.2.1 Oral presentations and posters

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2018) Identification and characterization of flowering time genes in oilseed rape through mutant and transcriptome analysis. Mini-symposium, Kiel Plant Science Network, Kiel, 19th April, 2018, Oral Presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2018) Identification and characterization of flowering time genes in oilseed rape through mutant and transcriptome analysis. III. International 1530 Symposium, Kiel, March 14th to March 16th, 2018, Oral Presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2018) Identification and characterization of flowering time genes in oilseed rape through mutant and transcriptome analysis. German Plant Breeding Conference (GPBC 2018), February 28 – March 02, 2018, Wernigerode, Oral Presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2017) Identification and characterization of flowering time genes in oilseed rape through TILLING and transcriptome analysis. International Workshop on Genome Engineering and Epigenetic Variation of Flowering Time Genes, June 22 - 23, 2017, IPK Gatersleben, Poster Presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2016) Identification and characterization of flowering time genes in oilseed rape through TILLING and transcriptome analysis. PP1530 Workshop on Circadian rhythms, flowering time regulation and crop plant adaptation, January 20-22, 2016, Berlin, Oral Presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2016) A transcriptomic approach to identify flowering time regulators in rapeseed. 2nd Sino-German Rapeseed Symposium: Genetics and Biotechnology of Rapeseed, May 23-26, 2016, Kiel, Germany, Poster presentation

Shah, S., Emrani, N., Guo, Y., Jedrusik, N., Molina, C., Weinholdt, C., Lemnian, I., Grosse, I., Zou J., and Jung, C. (2015) Identification and characterization of flowering time genes in oilseed rape through TILLING and transcriptome analysis. GPZ 2015: New Targets for Crops of the Future, September 22-24, 2015, Düsseldorf, Germany, Poster presentation.

Declaration of co-authorship

12 Declaration of co-authorship

Chapters 3 and 4 were prepared as manuscripts for publication. The respective declarations of co-authorship are presented on the following pages. Meanwhile, chapter 5 includes work of the doctoral candidate alone.

Declaration of co-authorship

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

1. Doctoral candidate

Name: Smit Shah

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



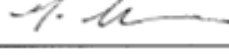
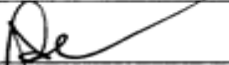

Whole transcriptome analysis reveals genetic factors underlying flowering time regulation in rapeseed (*B. napus* L.)

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

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

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

4. Signature of all co-authors

Date	Name	Signature
22.11.2018	Claus Weinholdt	
22.11.18	Nicole Jedrusik	
22.11.18	Carlos Molina	
	Jun Zou	
22.11.2018	Ivo Große	
22.11.18	Sarah Schiessl	

Declaration of co-authorship

20.11.18	Christian Jung	C. Jung
19.11.2018	Nazgol Emrani	N. Emrani

5. Signature of doctoral candidate		
Date	Name	Signature
19.11.18	Smit Shah	Smit Shah

Declaration of co-authorship

C A U	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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Declaration of co-authorship

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

1. Doctoral candidate

Name: Smit Shah

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

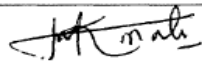
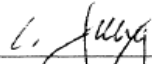
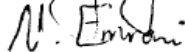
Stop codon mutation in an *APETALA1* homoeolog affects plant architecture and seed yield component in oilseed rape (*Brassica napus* L.)

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

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

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

4. Signature of all co-authors

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20.11.18	Christian Jung	
19.11.18	Nazgol Emrani	

5. Signature of doctoral candidate

Date	Name	Signature
19.11.18	Smit Shah	

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