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der Christian-Albrechts-Universität zu Kiel

**Functional analysis and mutagenesis of GDSL lipase genes for
breeding oilseed rape (*Brassica napus*) with higher oil content**

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List of Abbreviations

%	Percent
°C	Degree Celsius
µl	Microliter
µm	Micrometer
χ^2	Chi square
A	Adenine
<i>A. thaliana</i> , <i>At</i>	<i>Arabidopsis thaliana</i>
<i>ABI3</i>	<i>ABSCISIC ACID-INSENSITIVE3</i>
ACC	Acetyl-CoA Carboxylase
ACP	Acyl Carrier Protein
AFLP	Amplified Fragment Length Polymorphism
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
<i>ALC</i>	<i>ALCATRAZ</i>
ANOVA	Analysis of Variance
Asp, D	Aspartic acid
ATP	Adenosine triphosphate
BC	Backcross
<i>B. juncea</i>	<i>Brassica juncea</i>
BLAST	Basic Local Alignment Search Tool
<i>B. napus</i> , <i>Bn</i>	<i>Brassica napus</i>
<i>B. oleracea</i>	<i>Brassica oleracea</i>
bp	Base pair
<i>B. rapa</i>	<i>Brassica rapa</i>
C	Cytosine
CaMV	Cauliflower Mosaic Virus
Cas	CRISPR-associated
<i>CLV</i>	<i>CLAVATA</i>
CoA	Co-enzyme A
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
CSV	Comma-Separated Values
Ct	Cycle threshold
CTAB	Cetyltrimethyl ammonium bromide
<i>CTS</i>	<i>COMATOSE</i>
D	Aspartate
<i>DA</i>	<i>LARGE IN CHINESE</i>
DAG	Diacylglycerol
DAP	Days after pollination
DAS	Days after sowing
<i>DGAT</i>	<i>DIACYLGLYCEROL ACYLTRANSFERASE</i>

List of Abbreviations

DH	Doubled haploid
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EMS	Ethyl methanesulfonate
ER	Endoplasmic reticulum
FA	Fatty acid
FDR	False discovery rate
F _n	n th generation after cross
FPKM	Fragments per kilobase per million
<i>FUL</i>	<i>FRUITFULL</i>
<i>FUS3</i>	<i>FUSCA3</i>
G	Guanine
GA	Gibberellic acid
GBS	Genotyping by sequencing
GDSL	Gly-Asp-Ser-Leu
Gly, G	Glycine
GMO	Genetically modified organism
<i>GPAT</i>	<i>GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE</i>
<i>GPDH</i>	<i>GLYCEROL-3-PHOSPHATE DEHYDROGENASE</i>
GWAS	Genome-Wide Association Study
H	Histidine
<i>IND</i>	<i>INDEHISCENT</i>
IRD	Infrared Fluorescent Dye
<i>JAG</i>	<i>JAGGED</i>
KASP	Kompetitive Allele Specific PCR
Kb	Kilo base pairs
<i>LACS</i>	<i>LONG-CHAIN ACYL-COA SYNTHETASE</i>
<i>LEC</i>	<i>LEAFY COTYLEDON</i>
Leu, L	Leucine
<i>LPAAT</i>	<i>LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE</i>
MAF	Minor allele frequency
MAGE	Multiplex Automated Genome Engineering
Mb	Mega base pair
Mg	Milligrams
min	Minute
mM	Millimolar
M _n	n th generation after mutagenesis
MNU	1-methyl-1-nitrosourea
MS	Murashige and Skoog medium
MTBE	Methyl tert-butyl ether
N	Asparagine
NaCl	Sodium chloride

List of Abbreviations

NaN ₃	Sodium azide
NCBI	National Center for Biotechnology Information
NIRS	Near-infrared spectroscopy
NJ	Neighbor-joining
NMR	Nuclear Magnetic Resonance
OAC	Oleic acid content
OB	Oil body
PAM	Protospacer adjacent motif
PC (A)	Principal Component (Analysis)
PCR	Polymerase chain reaction
<i>PNC</i>	<i>PYRAZINAMIDASE/NICOTINAMIDASE</i>
QTL	Quantitative trait loci
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restricted Fragment Length Polymorphism
<i>RGA</i>	<i>REPRESSOR OF GAI-3</i>
pmol	picomole
RNAi	RNA interference
RT-qPCR	Real-time quantitative PCR
SCAR	Sequence Characterized Amplified Regions
<i>SDG8</i>	<i>SET DOMAIN GROUP8</i>
<i>SDP1</i>	<i>SUGAR-DEPENDENT1</i>
s/sec	Second
Ser, S	Serine
<i>SFAR</i>	<i>SEED FATTY ACID REDUCER</i>
sgRNA	Single guide RNA
SNP	Single nucleotide polymorphisms
SOC	Seed oil content
SRAP	Sequence-Related Amplified Polymorphism
SSD	Single seed descent
SSR	Simple Sequence Repeat
STS	Sequence-Tagged Sites
T	Thymine
TAG	Triacylglycerol
TaGTEAM	Targeting glycosylase to embedded arrays for mutagenesis
TALEN	Transcription activator-like effector nuclease
TASSEL	Trait Analysis by aSSociation, Evolution and Linkage
TEM	Transmission electron microscope
TF	Transcription factors
TILLING	Targeting Induced Local Lesions IN Genomes
TKW	Thousand Kernel Weight
T _n	n th generation after transformation
TNSV	Total non-synonymous value
TRAP	Target Region Amplification Polymorphism

List of Abbreviations

tracrRNA	Trans-activating crRNA
UAE	Ultrasound-assisted extraction
UV	Ultraviolet light
VCF	Variant Call Format
<i>WR11</i>	<i>WRINKLED1</i>

1 General introduction

1.1 Breeding and cultivation of oilseed rape

Rapeseed or oilseed rape (*Brassica napus* L.) is the third most widely grown oil crop (<http://www.fao.org/faostat/>) with high economic relevance in terms of global production scale. With the reduction of anti-nutritive components like glucosinolates and low erucic acid, rapeseed has become a versatile crop. It is believed that rapeseed was first cultivated in Europe and subsequently spread to other parts of the world (Lu et al. 2019). Today, three main phenological growth types, namely winter, spring, and semi-winter rapeseed, are cultivated in different geographical regions.

Rapeseed belongs to the family *Brassicaceae*, which was formed by a spontaneous hybridization between *Brassica rapa* (AA, $2n = 20$) and *Brassica oleracea* (CC, $2n = 18$) (U 1935) around 7500 years ago (Chalhoub et al. 2014). Rapeseed is an allopolyploid crop (AACC, $2n = 38$) with a genome size of 1130 Mb. Calculated on the basis of the assembled reference genome, the C-subgenome (525.8 Mb) is larger than A-subgenome (314.2 Mb) (Chalhoub et al. 2014). The bioinformatic analysis uncovered about 101,000 gene models based on the cultivar sequenced (Chalhoub et al. 2014; Sun et al. 2017).

During rapeseed breeding, oil and meal quality have been improved substantially. Selection for low erucic acid and low glucosinolates (double low 00-rapeseed), which were introduced from the summer forage variety “Liho” and the Polish summer variety “Bronowski”, respectively, increased usage of rapeseed as food and feed (Friedt et al. 2018). However, the introduction of 00-quality resulted in a reduced genetic variation in rapeseed. Genetic variation within species is a valuable source and a vital basis for breeding and further improvements (Wu et al. 2014a). Therefore, increasing genetic diversity is one of the aims in rapeseed breeding while focusing on increasing yield and oil content. Genetic variation can be introduced by crossing with genetically diverse materials like released cultivar, adapted breeding lines, and landraces. Moreover, interspecific or intergeneric crosses within *Brassicaceae* can be used (Friedt et al. 2018). Resynthesized rapeseed has been produced, as well, by crossing progenitors, *B. rapa* and *B. oleracea*, to extend the existing genetic diversity (Wu et al. 2014a). For instance, clubroot resistance was introduced to rapeseed using interspecific hybridization, and further improvement through crosses and backcrosses led to a clubroot resistant rapeseed cultivar (Diederichsen et al. 2009; Diederichsen and Sacristan 1996). Similarly, resistance to *Verticillium* wilt was introduced to rapeseed (Happstadius et al. 2003). The introduction of new alleles to improve the quality and quantity is a continuous focus in rapeseed breeding.

The choice of the breeding method depends on the reproductive system of crops. Rapeseed is a predominantly self-pollinated crop with 12-47% of outcrossing (Becker et al. 1992). Therefore, either open-pollinated or hybrid rapeseed varieties can be produced. To develop open-pollinated varieties, pure lines are produced using pedigree selection, and to accelerate the breeding process, doubled haploid (DH) technology was employed (Friedt et al. 2018). Homozygous DH lines can also be used as breeding materials for hybrid breeding. Most of the newly registered varieties in central Europe today are hybrids (Friedt et al. 2018), and they are mainly produced using a male sterility system like INRA-Oguru and Male Sterility Lembke (Friedt and Snowdon 2010).

Primary goals of rapeseed breeding include increasing the yield potential, pest and disease resistance, improving seed quality, and agronomic characters like winter hardiness and shatter resistance (Friedt and Snowdon 2010). Seed oil content (SOC) is a major determinant of the rapeseed yield. Over the years, numerous efforts have been made to increase SOC in rapeseed. Generally, SOC in rapeseed varies between 26-50% (Xiao et al. 2019). Oil content is a quantitative trait, which is regulated by many genes (Li-Beisson et al. 2013). Therefore, a better

understanding of genes involved in seed oil regulation in rapeseed is required for further increasing of SOC.

1.2 Lipid biosynthesis and degradation in plants

1.2.1 Fatty acid and lipid biosynthesis

Plant lipids play vital roles as membrane constituents, storage compounds, and in signaling (Graham 2008; Reina-Pinto and Yephremov 2009). Plants produce a broad array of lipids, including triacylglycerols, phospholipids, galactolipids, and sphingolipids (Li-Beisson et al. 2013; Suh et al. 2015). Oil in the form of triacylglycerols (TAG) serves as an energy reserve in plants and is the primary seed storage compound in oil crops and fruits like avocado and olives (Bates et al. 2013; Heldt and Piechulla 2011).

Three fatty acids (FAs) esterified to glycerol are the main components of TAGs. Plant oil biosynthesis is a complex pathway occurring in different cellular compartments engaging different metabolic pathways (Li-Beisson et al. 2013). In plants, *de novo* synthesis of FAs occurs in the plastid. Acetyl-CoA serves as the building block for FA synthesis (Figure 1). In the first step, acetyl-CoA and bicarbonate are converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) (Li-Beisson et al. 2013). Subsequently, malonyl-CoA is transformed to malonyl-ACP by malonyl-CoA: acyl carrier protein (ACP) malonyltransferase. Using acetyl-CoA as the precursor and malonyl-ACP as the provider of two carbon units, first C16:0- and C18:0- ACP are produced. C18:0-ACP is then desaturated to C18:1 and hydrolyzed. Ultimately, C16:0 and C18:1, and limited amounts of C18:0 are produced in the plastid FA synthesis (Figure 1). These reactions are catalyzed by different enzymes (Bates et al. 2013; Li-Beisson et al. 2013). After FA synthesis in plastids, free FAs are transferred into the endoplasmic reticulum (ER) for further modifications like chain elongation and desaturation (Heldt and Piechulla 2011).

Assembly of TAGs occurs in the ER membrane by consumption of glycerol-3-phosphate and acyl-CoA pools (Heldt and Piechulla 2011). Glycerol-3-phosphates are formed through the Kennedy pathway, and the acyl-CoA pools (16:0-CoA, 18:0-CoA, and 18:1-CoA) are imported from the plastid. TAGs are synthesized in a series of reactions (Figure 1) (Bates 2016; Chen et al. 2015). In the end, TAGs are surrounded by a phospholipid monolayer and various proteins forming a structure called oil bodies (OB) or oil droplets (Pyc et al. 2017). Oleosins are the most abundant coating proteins in OBs, and their level correlates with the OB size (Graham 2008). After OBs are formed within the ER membrane bilayer, they bud-off to the cytoplasm (Pyc et al. 2017).

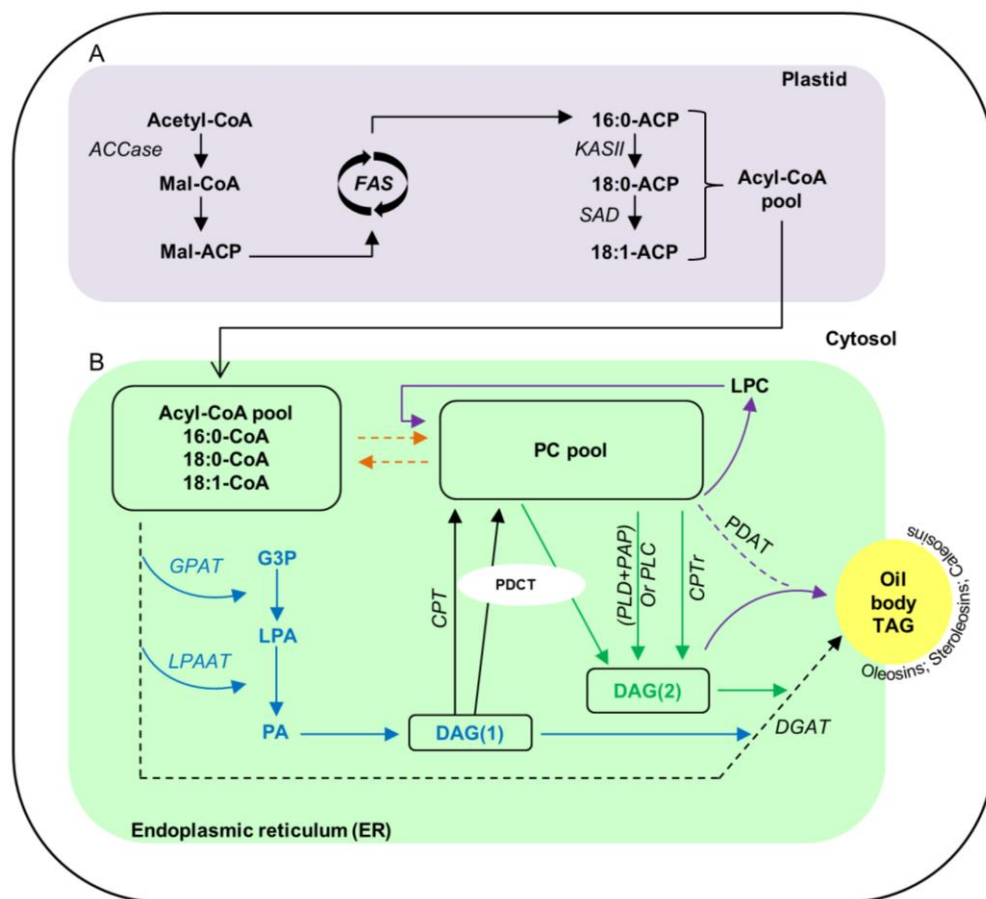


Figure 1: Schematic diagram of major reactions involved in fatty acid (FA) and triacylglycerol (TAG) synthesis. (A) *De novo* FA synthesis in plastids. At the end of plastid FA synthesis, 16:0-, 18:0- and 18:1-Acyl-CoA are produced and transferred to the ER for further modification and TAG synthesis. (B) TAG synthesis in the ER. Dashed lines: Acyl transfer reactions, blue lines: *de novo* synthesis of TAG, green lines: PC-derived DAG synthesis, orange lines: acyl editing, purple lines: phospholipid: diacylglycerol acyltransferase (PDAT). DAG(1) is *de novo* synthesized DAG, and DAG(2) is PC-derived DAG. Substrates are in bold: **ACP**: acyl carrier protein, **DAG**: diacylglycerol, **G3P**: glycerol-3-phosphate, **LPA**: lysophosphatidic acid, **Mal**: malonate, **PA**: phosphatidic acid, **PC**: phosphatidylcholine, **TAG**: triacylglycerol. Enzymatic reactions are in italics: *ACCase*: acetyl-CoA carboxylase, *CPT*: CDP-choline: DAG cholinephosphotransferase, *DGAT*: acyl-CoA:DAG acyltransferase, *FAS*: fatty acid synthase, *GPAT*: acyl-CoA:G3P acyltransferase, *KASII*: ketoacyl-ACP synthase II, *LPAAT*: acyl-CoA:LPA acyltransferase, *PAP*: PA phosphatase, *PDCT*: PC:DAG cholinephosphotransferase, *PLC*: phospholipase C, *PLD*: phospholipase D, *SAD*: Stearoyl-ACP desaturase (modified after Bates et al. (2013)).

1.2.2 Lipid degradation

Lipases are known to initiate the TAG catabolism (Xu and Shanklin 2016). In general, lipases belong to the family of hydrolases, which hydrolyze ester bonds of mono-, di- and triacylglycerols to release FAs and glycerol (Houde et al. 2004). Depending on structure, function, and substrate specificity, lipases are divided into different classes. Many lipases/esterases contain a Gly-x-Ser-x-Gly (GxSxG) motif with active serine (S) in the center. However, this motif is not present in all lipase classes (Akoh et al. 2004). In 1995, a new class of lipases was reported harboring the GDSL motif instead of the GxSxG motif (Upton and Buckley 1995). In addition to these two types, patatin-like lipases were described containing a serine-aspartate dyad (Kelly and Feussner 2016).

GDSL lipases have five consensus sequence blocks, named as block I-V (Upton and Buckley 1995). Of these, a single catalytic residue is mainly conserved in four blocks out of five; serine (S), glycine (G), asparagine (N), and histidine (H) in the blocks I, II, III, and V, respectively (Figure 2). Therefore, these GDSL lipases are also designated as SGNH-hydrolases superfamily

or subfamily (Akoh et al. 2004; Molgaard et al. 2000). The GDSL motif with the active site serine is located near the N-terminus (Akoh et al. 2004). Serine (S) in the block I, glycine (G) in block II, and asparagine (N) in the block III serve as the proton donors. Serine from GDSL motif, histidine (H) and aspartate (D) in the block V (DxxH) belong to the catalytic triad (Chepyshko et al. 2012; Kelly and Feussner 2016).

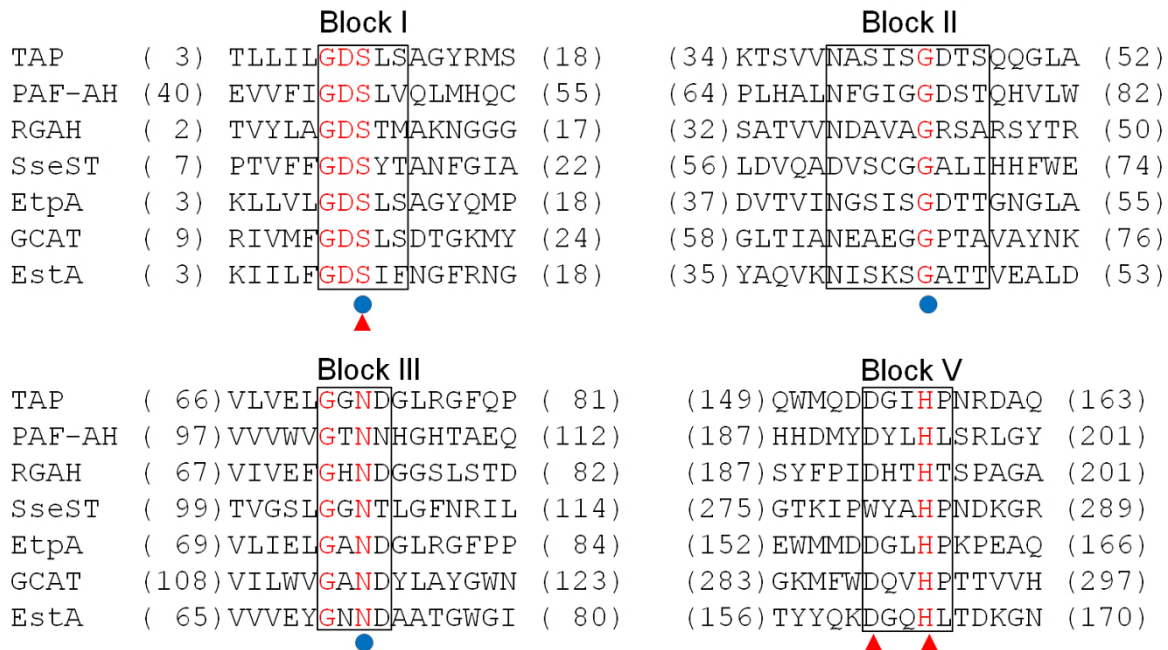


Figure 2: Structural elements of GDSL lipases. Sequence alignments of the conserved amino acid sequence blocks in the GDSL lipases gene family are shown. The black frames indicate the four consensus blocks I, II, III, and V. Conserved amino acids are marked in red. Positions are given in parentheses. Red-filled triangles and blue-filled circles represent the catalytic triad and oxyanion hole, respectively. TAP: *Escherichia coli* thioesterase I, PAF-AH: Bovine brain platelet-activating factor acetylhydrolase, RGAH: *Aspergillus aculeatus* rhamnogalacturonan acylesterase, SseST: *Streptomyces scabies* esterase, EtpA: *Vibrio mimicus* arylesterase, GCAT: *Aeromonas hydrophila* lipase/acyltransferase, EstA: *Lactobacillus helveticus* CNRZ32 arylesterase (modified after Lo et al. (2003)).

In plants, GDSL lipases are involved in the regulation of different biological functions, such as biotic and abiotic stresses, morphological development and secondary metabolism (An et al. 2019; Clauss et al. 2008; Hong et al. 2008; Kim et al. 2013). They are associated with seed oil content in crops such as rapeseed, soybean, and oil palms (Kanai et al. 2019; Kelly and Feussner 2016; Zhang et al. 2018b). Studies showed that they take part in oil degradation during seed maturation (Chen et al. 2012b; Ding et al. 2019). Among them, *SEED FATTY ACID REDUCER (SFAR)* genes belonging to the GDSL lipases have been studied in *Arabidopsis* and found to be regulated by gibberellic acid (GA) signaling.

Knock-outs of *AtSFAR1* (*At1g54790*), *AtSFAR2* (*At1g58430*), *AtSFAR3* (*At2g42990*), *AtSFAR4* (*At3g48460*) and *AtSFAR5* (*At4g18970*) genes resulted in increased total seed FA content in *Arabidopsis* (Chen et al. 2012b). Disruptions of *AtSFAR* genes resulted in a significant increase in total FA from 9.1-16.9%, depending on the gene interrupted. Double mutants also displayed a higher increase in total FA, averaging at 23.7%. Conversely, overexpression of *AtSFAR1-AtSFAR5* showed significantly reduced total FA content in seeds, ranging between 13.4-25.1%. Moreover, overexpression of *AtSFAR1-AtSFAR5* genes changed the FA composition, resulting in an increase of C18:1 and C20:1 and a decrease of C18:2 and C18:3. OB size in seeds of double mutants was larger in comparison to the wild-type or overexpressed plants (Chen et al. 2012b). It is also speculated that *AtSFAR*, especially *AtSFAR4* may play a role in stress response (Chen et al. 2012b; Huang et al. 2015). These data suggest that GDSL lipases regulate the SOC. However, the involvement of GDSL lipases in oil degradation remains to be elucidated.

1.2.3 Genetics of seed oil content in rapeseed

Increasing SOC is a main focus of breeding programs. To meet the rising demand for rapeseed oil and taking into account limited arable land area, further improvements in the enhancement of oil yield are needed. Therefore, it is vital to understand the genetic mechanism underlying seed oil biosynthesis and degradation, as SOC is determined by the balance between metabolism and catabolism. Various approaches have been employed in rapeseed to increase the SOC or understand the functions of the genes important for oil synthesis.

QTL (quantitative trait loci) and genome-wide association (GWAS) studies opened a new insight into the identification of oil content related genes. Moreover, analysis of oil content using self-pollinated and cross-pollinated varieties showed additive and epistatic effects (Wang et al. 2010). Numerous QTL and GWAS studies have been performed to investigate the regulatory loci and genetic architecture underlying SOC at the whole genome level (Table 1). In all 19 linkage groups of rapeseed, QTLs for oil content have been identified (Chao et al. 2017; Delourme et al. 2006; Jiang et al. 2014; Qiu et al. 2006; Sun et al. 2016; Wang et al. 2013). In a recent GWAS study, 17 loci significantly associated with SOC were identified (Xiao et al. 2019). Of these, 12 SNPs on chromosomes A3 and A1 lay within previously identified QTLs, and five SNPs on chromosomes C5 and C7 could not be allocated to known QTLs. Zou et al. (2016) analyzed a biparental DH population and found 27 SNPs associated with SOC based on genotyping with a high-density 60K SNP array. In a validation population of 117 lines, they could predict oil content with an accuracy of 0.73. This demonstrates that the integration of previously identified QTLs into high density marker-assisted genotyping will improve future breeding for oil content in rapeseed.

Some genes linked to the regulation of oil content are involved in transcriptional regulation, primary metabolism, catabolism, transport, or signaling (Table 2). *WRINKLED1* (*WRI1*), *LEAFY COTYLEDON1* and *2* (*LEC1* and *LEC2*), *FUSCA3* (*FUS3*), *ABSCISIC ACID-INSENSITIVE3* (*ABI3*) are major transcription factors (TF) which play significant roles in the oil biosynthesis regulatory network (Zafar et al. 2019). *WRI1* regulates genes involved in FA synthesis leading to an increase in SOC (Adhikari et al. 2016). In rapeseed, seed-specific overexpression of *BnWRI1* increased total FA content and SOC significantly (Wu et al. 2014b). *LEC1*, encoding an NFY-B-type transcription factor, coordinates the expression of FA biosynthesis genes in *Arabidopsis* (Mu et al. 2008). Overexpression of the *BnLEC1* gene in rapeseed cultivar Westar resulted in a 2-20% increase in oil production (Tan et al. 2011).

Table 1: QTL and GWAS studies for rapeseed oil content

Mapping population	Size of the population	Number and type of markers	Method	References
Mansholt's Hamburger Raps x Samourai	151 DH lines	205 RFLP	Linkage analysis	Ecke et al. (1995)
Ceres x Marnoo and Ceres x Westar	128 of BC lines	276 RFLP	Linkage analysis	Butruille et al. (1999)
Victor x Tapidor	22 substitution lines	158 RFLP	Linkage analysis	Burns et al. (2003)
Darmor- <i>bzh</i> x Yudal and Rapid x NSL96/25	445 and a 242 DH lines	540 of RFLP, RAPD, AFLP195 SSR	Linkage analysis	Delourme et al. (2006)
Tapidor x Ningyou7	188 DH lines	277 AFLP, RFLP, SSR, and STS	Linkage analysis	Qiu et al. (2006)
Sollux x Gaoyou	282 DH lines	125 SSR	Linkage analysis	Zhao et al. (2006)
GH06 x P147,	188 RILs	300 SRAP, SSR, AFLP, and TRAP	Linkage analysis	Jin et al. (2007)
High x low oil content lines	150 DH lines	387 SRAP, SSR	Linkage analysis	Chen et al. (2010)
Nine crosses from 10 parents	391 DH lines	253 SNPs	Linkage analysis	Wurschum et al. (2012)
Sollux x Gaoyou	282 DH lines	481 CAPS, SSR, SCAR, STS, and SRAP	Linkage analysis	Zhao et al. (2012)
Tapidor x Ningyou7	202 DH lines, 404 reconstructed F ₂	786 RFLP, SSR, STS, and AFLP	Linkage analysis	Jiang et al. (2014)
160 winter, 200 semi-winter and 110 spring, 1 spring fodder, 1 winter fodder	472 accessions	60K Infinium™ SNP array	GWAS	Li et al. (2014)
Rapeseed cultivars	217 accessions	389 SSR	GWAS	Qu et al. (2015a)
Winter rapeseed accessions Express-617 x V8	89 accessions 29 DH lines	6K Infinium™ SNP array 100 KASP	GWAS Linkage analysis	Gajardo et al. (2015)
Winter rapeseed, winter fodder, swede, semi-winter, spring, spring fodder and vegetable	405 inbred lines	6K Infinium™ SNP array	GWAS	Korber et al. (2016)

Mapping population	Size of the population	Number and type of markers	Method	References
6F313x 51070 and 61616 x 51070 F2 populations	105 individuals per population 227 accessions	60K Infinium™ SNP array	Linkage analysis and GWAS	Sun et al. (2016)
KenC-8 × N53-2	300 DH lines	60K Infinium™ SNP array	Linkage analysis	Sun et al. (2016)
Winter, semi-winter and spring rapeseed	588 accessions	Whole-genome re-sequencing	GWAS	Xiao et al. (2019)

Abbreviations: DH: Doubled Haploid, BC: Backcrossed, RFLP: Restriction Fragment Length Polymorphism, RAPD: Random Amplified Polymorphic DNA, AFLP: Amplified Fragment Length Polymorphism, SSR: Simple Sequence Repeats, CAPS: Cleaved Amplified Polymorphic Sequence, STS: Sequence-Tagged Sites, SCAR: Sequence Characterized Amplified Regions, SRAP: Sequence-Related Amplified Polymorphism, KASP: Kompetitive Allele-Specific PCR, TRAP: Target Region Amplification Polymorphism, GWAS: Genome-Wide Association Study

Another study showed that the constitutive expression of *BnLEC1* in rapeseed enhanced SOC by 7-16%, while downregulation of *BnLEC1* decreased SOC by 9-12% (Elahi et al. 2016). Plant-specific B3 domain-containing *LEC2* is important in embryo and seed development and also for seed oil accumulation (Mendoza et al. 2005; Wojcikowska et al. 2013). Moreover, *LEC2* acts as the main regulator controlling many genes, including the *WRI1*, *LEC1*, *FUS3*, and *ABI3* (Baud et al. 2007; Zafar et al. 2019). Mutations in the transcription factor *AtLEC2* resulted in 30% reduced oil in *Arabidopsis* seeds and altered the chemical composition (Angeles-Nunez and Tiessen 2011). *FUS3* is a member of the plant-specific B3 DNA binding domain family, and B3 domain family genes participate in seed ripening, flowering time control, hormone signaling, and andorgan development (Swaminathan et al. 2008). *BnFUS3* mutant rapeseed lines showed reduced SOC and silique numbers combined with a 3-5% increased protein level (Elahi et al. 2015). *MYB89* belongs to the large *MYB* transcription factor family and acts as a negative regulator of seed oil accumulation in *Arabidopsis* because the total seed FA content was increased in *MYB89* T-DNA inserted and RNAi suppressed *Arabidopsis* mutants (Li et al. 2017).

Overexpression of genes involved in oil biosynthesis also enhanced the SOC in rapeseed. Glycerol-3-phosphate dehydrogenases (*GPDH*), glycerol-3-phosphate acyltransferase (*GPAT*), lysophosphatidic acid acyltransferase (*LPAAT*) and diacylglycerol acyltransferase (*DGAT*) are enzymes involved in the Kennedy pathway (Savadi et al. 2017). Overexpression of all these genes led to an increased SOC in rapeseed by up to 14% (Liu et al. 2015). Overexpression of *BnDGAT1* showed a 2.5%-3.5% increase in SOC under field conditions (Taylor et al. 2009).

Previous studies reported that rapeseed oil content declines during seed maturation (Shahid et al. 2019; Wan et al. 2017), indicating oil breakdown in the last phase of seed development. Therefore, the suppression of oil degradation at the seed maturing stage is expected to increase the SOC. So far, only a few studies in rapeseed have demonstrated that blocking genes involved in the degradation pathway resulted in higher SOC. RNAi suppression of *SUGAR-DEPENDENT1* (*BnSDP1*) triacylglycerol lipase in rapeseed resulted in up to 8% oil increase (Kelly et al. 2013). A recent study showed that RNAi suppression of *BnGDSL1*, which is the homolog of *AtGDSL1* in rapeseed, increased SOC by 3.8-4.6 %, while overexpression of this gene under CaMV 35S promoter reduced SOC (Ding et al. 2019).

Table 2: Functional analysis of genes involved in oil biosynthesis and degradation. SOC: seed oil content, FA: fatty acid, DAG: diacylglycerols, TAG: triacylglycerols, EMS: ethyl methanesulfonate

Gene	Function	Action	Effect on oil yield in rapeseed	References
<i>BnWRI1</i>	Regulation of FA biosynthesis and photosynthesis pathway	Overexpression	8.1% increase in SOC	Wu et al. (2014b)
<i>BnLEC1</i>	Coordinating the expression of FA biosynthesis genes	Overexpression	2-20% increase in SOC	Tan et al. (2011)
		Overexpression	7-16% increase in SOC	Elahi et al. (2016)
		Downregulation	9-12% decrease in SOC	
<i>BnFUS3</i>	Regulation of FA biosynthesis	EMS mutants	Increase in SOC	Elahi et al. (2015)
<i>BnGPDH</i> <i>BnDGAT</i> <i>BnGPAT</i> <i>ScLPAAT</i>	Enzymes of the Kennedy pathway	Overexpression	14.46% increase in SOC	Liu et al. (2015)
<i>BnDGAT1</i>	Catalyzing the final step of the Kennedy pathway, DAG → TAG	Overexpression	2.5%-3.5% increase in SOC	Taylor et al. (2009)
<i>TmLPAAT2</i>	An Enzyme of the Kennedy pathway	Overexpression	25%-29% increase in SOC, changes in FA composition	Woodfield et al. (2019)
<i>BnLPAAT2</i>	Enzymes of the Kennedy pathway	CRISPR-Cas9 gene editing	32% decrease in SOC, changes in FA composition	Zhang et al. (2019a)
<i>BnLPAAT5</i>			29% decrease in SOC, changes in FA composition	
<i>BnFAD2</i> <i>BnFAD3</i>	Converting oleic acid to linoleic acid and linolenic acid	Existing allele introducing	Increase in oleic acid content	Yang et al. (2012) Long et al. (2018)
<i>BnSDP1</i>	Initiation of the oil breakdown	RNAi suppression	8% increase in SOC	Kelly et al. (2013)
<i>BnGDSL1</i>	Oil breakdown	RNAi suppression	3.8-4.64% increase in SOC	Ding et al. (2019)

1.2.4 Methods for lipid analysis

The choice of the lipid analysis method mainly depends on the research goals, i.e. desired yield and purity after extraction. So far, numerous methods for lipid analysis have been described, including destructive and non-destructive methods.

Soxhlet extraction is the standard method for oil extraction based on discontinuous solid-liquid extraction (de Castro and Priego-Capote 2010). Generally, n-hexane is used as the solvent due to its low boiling temperature and easy recovery of oils. Other solvents like methanol, isopropanol, D-limonene have been applied as alternatives (Danlami et al. 2014; Krzyczkowska and Kozłowska 2017; Manirakiza et al. 2001b). The conventional Soxhlet method has been modified using high-pressure, automated, ultrasound-assisted, and microwave-assisted Soxhlet extraction to improve the process (de Castro and Priego-Capote 2010). Soxhlet extraction is an easy and cost-effective method. Major drawbacks are long extraction times leading to thermal decomposition of target compounds, the large volume of solvent used, and several adverse environmental implications due to large amounts of wastes (Danlami et al. 2014; de Castro and Priego-Capote 2010).

Chloroform/methanol extraction is another simple method that uses chloroform: methanol in different ratios (Bligh and Dyer 1959; Folch et al. 1957). This method was improved by replacing the methanol and chloroform with methyl tert-butyl ether (MTBE), n-hexane, cyclohexane, diisopropyl ether, and propan-2-ol (Smedes 1999). The chloroform/methanol method involves solid-liquid extraction similar to the Soxhlet extraction. The main drawbacks of this method are the adverse effect of chlorinated solvent and critical phase separation. To avoid the difficulties in conventional methods, an ultrasonic energy-based technique, so-called Ultrasound-Assisted Extraction (UAE), a solid-liquid extraction was implemented (Shirsath et al. 2012), using different solvents like n-hexane, n-hexane: isopropanol, and chloroform: methanol (Metherel et al. 2009).

Most of the lipid analysis methods require elaborate sample preparation and the use of hazardous solvents and reagents. Some methods are laborious, time-consuming, and low throughput. High throughput non-destructive techniques like Nuclear Magnetic Resonance (NMR) spectroscopy and Near-Infrared Spectroscopy (NIRS) can be used for quantification and determination of molecule structure (Colnago et al. 2007; Prestes et al. 2007). Both methods need extensive sample calibration to correct for matrix effects (Burns et al. 2003).

In some cases, an accurate quantitative measurement of FA composition is a primary concern in rapeseed, as high oleic acid content is favorable for edible oil, while high erucic acid content is important for industrial purposes (Wei et al. 2008). This can be achieved by gas chromatography, allowing precise FA quantification and identification (Zhou et al. 2017).

1.3 Functional gene analysis methods

Mutations are drivers for evolution, domestication of crops, and an increase in genetic diversity. Besides that, they are important tools for unraveling gene functions. Mutations occur naturally (at a rate of 7×10^{-9} in the model plant *Arabidopsis* (Ossowski et al. 2010)) or can be induced artificially. Random mutagenesis and targeted mutagenesis are two main approaches used to change the genome in order to alter the gene activities and have gained increasing importance in plant breeding (Sikora et al. 2011).

1.3.1 Random mutagenesis

Random mutagenesis is a process where mutations are produced randomly distributed in the genome. Chemical, physical, or biological methods have been used to damage DNA (Alonso and Stepanova 2003; Packer and Liu 2015), and for several decades, their application in different organisms making was an attractive approach (Oladosu et al. 2016).

The two main biological mutagenesis methods used in plants were *Agrobacterium*-mediated DNA integrations (T-DNA insertions) and transposons. In both methods, either foreign DNA or a transposon are inserted into a gene, thus disturbing its function (Alonso and Stepanova 2003; Alonso et al. 2003; Duangpan et al. 2013). However, the efficiency of these methods is low because of the relatively low frequency of mutations when compared to chemical and physical mutagenesis (Alonso and Stepanova 2003).

Physical mutagenesis applies electromagnetic radiation like X-rays, γ -rays, UV, and fast neutrons. Deletions are more frequent (Chaudhary et al. 2019; Koornneef 2002), but also insertions, translocations, inversions, and point mutations are induced (Jankowicz-Cieslak and Till 2015). Chemical mutagens have been used successfully, as they are easier to use, and no special equipment is needed (Sikora et al. 2011). Alkylating compounds like ethyl methanesulfonate (EMS) and 1-methyl-1-nitrosourea (MNU) are the major chemical mutagens to induce mutations (Jankowicz-Cieslak and Till 2015). Of these, EMS generates point mutations, primarily G/C to A/T transitions leading to a wide range of mutations like nonsense, missense and splice site mutations (Chen et al. 2014; Tai 2013).

Due to high mutation density and random distribution, highly efficient mutation detecting methods are needed, after chemical or physical mutagenesis. During the last years, numerous methods and protocols have been developed to detect random mutations (Sikora et al. 2011). Targeting Induced Local Lesions IN Genomes (TILLING) is a reverse genetic method for the discovery of induced point mutations (McCallum et al. 2000; Till et al. 2006). In gel-based TILLING, a single gene of interest is amplified by PCR from DNA of a mutagenized population, enzymatic mismatch cleavage is performed at the mutation site, and the fragments are separated on polyacrylamide gels. A major improvement arose from the use of next-generation sequencing, allowing for higher throughput, i.e. the screening of up to 20 genes simultaneously, in TILLING by sequencing (Sashidhar et al. 2019; Tsai et al. 2015; Kim and Tai 2014) and exome capture strategies of mutagenized populations (Krasileva et al. 2017). In rapeseed, different chemically mutagenized mutant populations are available, and numerous studies have been performed to explore the gene functions (Table 3).

Table 3: Overview of rapeseed EMS populations. n.a: data are not available

Mutagen	Variety	Mutation frequency	Mutation detection technology	References
EMS	Ningyou7	1/130.8 kb (0.3% EMS) 1/41.5 kb (0.6% EMS)	Gel-based	Wang et al. (2008)
EMS	huyou-15	n.a.	n.a.	Wu et al. (2008)
EMS	Express-617 YN01-429	1/12 – 1/22 kb 1/27 – 1/60 kb	Gel-based	Harloff et al. (2012)
EMS	Cabriolet	1/75.5 kb (0.4% EMS) 1/39.1 kb (0.6% EMS) 1/20.6 kb (0.8% EMS)	Capillary sequencing	Wells et al. (2014)
EMS	DH12075	1/109 kb	Gel-based and NGS	Gilchrist et al. (2013)
EMS	INRA CZH2	n.a.	n.a.	Channaoui et al. (2019)

A major drawback of chemical and physical random mutagenesis is a high amount of undesirable background mutations (Braatz et al. 2018b). Repeated backcrossing with the EMS donor line or elite material is a common strategy to reduce the background mutation load. However, this is a time-consuming and laborious procedure. To speed up this process, molecular

markers or marker-assisted genomic selection have been used in commercial rapeseed breeding (Nadeem et al. 2018). A variety of markers, like AFLP, KASP, and high-density SNPs arrays (Table 4) have been applied in rapeseed (Obermeier and Friedt 2015).

Table 4: SNPs arrays used for rapeseed.

	8K array	6K array	60K array
Genotypes used	Publicly available <i>Brassica</i> ESTs and Tapidor x Ningyou7 DH population	1,012 <i>B. napus</i> , 46 <i>B. rapa</i> , 4 <i>B. oleracea</i> , and 10 <i>B. nigra</i>	869 <i>B. napus</i> genotypes, 129 <i>B. oleracea</i> and 121 <i>B. rapa</i> samples
Selection criteria	Absence of heterozygosity within genotypes, 2x minimal allele coverage, SNPs between Tapidor and Ningyou7	Total read coverage area ≥ 4 , variant allele has ≥ 2 reads, even spread over 19 chromosomes	No variation within 60 bp on at least one side of the SNP, only bi-allelic SNPs, more than 60% individual heterozygosity per SNP, less than 70% missing individual per SNP, MAF ≥ 0.2
Number of SNPs	7,322	5,306	52,157
Technology used	Illumina Infinium and GoldenGate technology	Illumina Infinium	Illumina Infinium
References	Delourme et al. (2013)	Dalton-Morgan et al. (2014)	Clarke et al. (2016)

1.3.2 Targeted mutagenesis

The availability of genome sequences of many species facilitates targeted mutagenesis, as prior knowledge of the target region is a prerequisite. Targeted mutagenesis or genome editing enables to generate allelic variation at a precise location of the genome. Today, there are two mainly applied methods for targeted mutagenesis TALENs (transcription activator-like effector nucleases) and CRISPR-Cas (Zheng et al. 2017). TALEN and CRISPR-Cas use site-specific endonucleases to induce a double-strand break (DSB) at the target locus. An erroneous DNA repair system then creates mutations at the target site.

TALEN technology is based on DNA binding TALEs domains from *Xanthomonas*, which are fused to the cleavage domain of *FokI* (Christian et al. 2010). TALEN mediated genome editing was used in different crops (Char et al. 2015; Clasen et al. 2016; Khan et al. 2017; Lor et al. 2014; Shan et al. 2013), including rapeseed (Kazama et al. 2019). The mitoTALEN has been used to knock-out *orf125* gene in rapeseed (Kazama et al. 2019). The mitochondrial gene *orf125* is responsible for the Kosena-type cytoplasmic male sterility. Knocking-out of *orf125* restored the plants to fertility and resulted in elongated filaments, mature anthers, and viable pollens. Up to now, a broader application of TALENs in plant genome targeting is impeded by the laborious construction of the TALEN DNA-binding domains and the limited possibility to knock out multiple target sites (Zhang et al. 2019b).

The CRISPR-Cas system, which is based on RNA-guided DNA cleavage, has emerged as an alternative. CRISPR-Cas is an adaptive immune system, which is found in most of the bacteria and archaea (Makarova et al. 2015). The CRISPR-Cas9 originates from the type II CRISPR-Cas

systems, and Cas9 was purified from *Streptococcus pyogenes* (Doudna and Charpentier 2014). The CRISPR-Cas9 system comprises two main components, a single guide RNA (sgRNA) and a Cas9 nuclease. The sgRNA is generated by fusing CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), which enable the binding of the Cas9 nuclease. The crRNA consists of a 20 nucleotide guide sequence, which directs Cas9 to the DNA target (Chen et al. 2019b; Ran et al. 2013). The short sequence next to the targeted DNA (20 bp) in the crRNA, named protospacer adjacent motif (PAM, 5'-NGG), is essential for DNA target recognition (Doudna and Charpentier 2014; Hsu et al. 2014). Altering the 20 nucleotide sequence within the sgRNA next to the PAM allows directing the Cas9 nuclease to any target of interest (Malzahn et al. 2017).

After the discovery of the CRISPR-Cas9 system, it has been widely employed in genome editing because of its high efficiency, easy use, and multiplexing ability. The off-target effects are a major concern when applying CRISPR-Cas9 in plants, especially considering polyploid species. Off-target effect investigations by whole genome sequencing in plants, however, revealed that CRISPR-Cas9 genome editing is highly precise (Braatz et al. 2017; Feng et al. 2014; Li et al. 2019; Nekrasov et al. 2017; Tang et al. 2018b). CRISPR-Cas9 genome editing has been used in rapeseed for functional analysis of genes and trait improvement (Table 5).

Table 5: Traits modified in rapeseed by targeted double-strand break mutagenesis

Donor Genotype	Gene/s	Function	Phenotype modified	Generation investigated	References
SW18	<i>ORF125</i>	Responsible for Kosena-type cytoplasmic male sterility	Restored to fertility, elongated filaments in flowers, mature anthers and viable pollen	T ₀ , F ₁	Kazama et al. (2019)
Haydn	<i>BnALC</i>	Involved in valve-margin development	Shatter resistance	T ₂	Braatz et al. (2017)
Westar	<i>BnRGA</i>	Repressor in gibberellic acid signaling	Dwarf plants	T ₁	Yang et al. (2017)
XY15	<i>BnSDG8</i>	Involved in flowering time, plant size, fertility, shoot branching, and carotenoid composition	Early flowering	BC ₁ F ₂	Jiang et al. (2018)
J9712	<i>BnWRKY70</i>	Involved in jasmonic acid- and salicylic acid-induced resistance to pathogens	Increased resistance to <i>S. sclerotiorum</i>	T ₁	Sun et al. (2018)
J9707	<i>BnCLV2</i>	Control of multilocular trait	Multilocular siliques with two to four locules	T ₁	Yang et al. (2018)
	<i>BnCLV3</i>		Multilocular siliques with two to four locules Increased number of seeds per silique and thousand seed weight	T ₁ , T ₂	
J2016	<i>BnLPAT2</i> <i>BnLPAT5</i>	Enzymes of the Kennedy pathway	Decreased oil content Wizened seeds, enlarged oil bodies, disrupted distribution of protein bodies and increased accumulation of starch in mature seeds	T ₁	Zhang et al. (2019a)

Donor Genotype	Gene/s	Function	References	Generation investigated	References
J9707	<i>BnIND</i> <i>BnALC</i>	Controls both the differentiation of lignified and separation layer Involved in valve-margin development	Increased shatter resistance	T ₂	Zhai et al. (2019b)
ZS6	<i>BnJAG</i>	Key factor regulating fruit dehiscence	Increased replum area, shortened pod length, and enhanced resistance to pod shattering	T ₁ , T ₂	Zaman et al. (2019)
Westar 10	<i>BnWRKY47</i>	Important for responding to boron deficiency	Increased sensitivity to low boron	T ₁	Feng et al. (2019)
J9707	<i>BnTT8</i>	Controls flavonoid accumulation	Seed color, increased SOC and protein content, alteration in FA composition	T ₀ , T ₁ , T ₂	Zhai et al. (2019a)
Spring-type 862	<i>BnMAX1</i>	Regulates plant height and axillary bud growth	Semi-dwarf and increased branching, phenotypes with more siliques	T ₂	Zheng et al. (2019)
Westar	<i>BnFAD2</i>	Desaturation of oleic acid	Increase of oleic acid content	BC ₁ S ₁	Okuzaki et al. (2018)
S-70	<i>BnMLPK</i>	Self-incompatibility response	Breakdown of the self-incompatibility response, abundant pollen germination, pollen tube elongation, and many seeds setting	T ₁	Chen et al. (2019a)
HY	<i>BnLMI1</i>	Determinant of lobed-leaf	Reduced lobed-leaf phenotype	T ₁	Hu et al. (2018)
Nc116	<i>BnAP2</i>	Sepal and petal development	Carpels sepals, missing petals, and a reduced number of stamens	T ₁	Zhang et al. (2018a)
Westar	<i>BnARF2</i>	Repressor in auxin signaling	Short roots, increased seed size and thousand kernel weight	T ₂	Tang et al. (2018a)

1.4 Hypotheses, objectives and scientific questions

Rapeseed SOC declines during the late seed maturity stage (Kelly et al. 2013; Wan et al. 2017). Therefore, my aim in this study was to identify genes involved in rapeseed oil degradation at the seed maturity stage and the functional characterization of those genes.

The hypothesis underlying my thesis was that the functions of oil degradation *SFAR* genes are conserved between *B. napus* and *A. thaliana*, as they belong to the same family *Brassicaceae*, and that knocking out *BnSFARI-BnSFAR5* genes causes higher seed oil content in rapeseed.

I wanted to answer these initial questions:

- How many *SFAR* paralogs are there in the rapeseed genome?
- Do knock-out mutations have an effect on seed oil content, and which *SFAR* genes are suitable as targets for random and targeted mutagenesis?
- Do single mutations have an effect on seed oil content? How many paralogs need to be knocked out to get an increase in seed oil?
- How are CRISPR-Cas mutations inherited to subsequent generations?
- Does marker-assisted genomic background selection improve the reduction of background mutations?

Therefore, the main objectives of this study comprised: identification of *SFARI-SFAR5* homologous in the rapeseed genome, screening of EMS-induced mutations in selected *BnSFAR* genes, targeted mutagenesis in *BnSFARI-BnSFAR5* genes by CRISPR-Cas9 and phenotypic characterization of the obtained mutants in greenhouse trials.

2 Elevating seed oil content in a polyploid crop by induced mutations in *SEED FATTY ACID REDUCER* genes

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2.1 Abstract

Plant-based oils are valuable agricultural products, and seed oil content (SOC) is the major yield component in oil crops. Increasing SOC has been successfully targeted through the selection and genetic modification of oil biosynthesis. The SOC in rapeseed declined during the seed maturation and eventually caused the final accumulated seed oil quantity. However, genes involved in oil degradation during seed maturity are not deeply studied so far. We performed a candidate association study using a world-wide collection of rapeseed germplasm. We identified *SEED FATTY ACID REDUCER* (*SFAR*) genes, which had a significant effect on SOC and fatty acid (FA) composition. *SFAR* genes belong to the GDSL lipases and GDSL lipases have a broad range of functions in plants. After quantification of gene expression using RNA-seq and quantitative PCR, we used targeted (CRISPR-Cas mediated) and random (chemical) mutagenesis to modify turnover rates of seed oil in winter rapeseed. For the first time, we demonstrate a significant increase of SOC in a crop after knocking out members of the *BnSFAR4* and *BnSFAR5* gene families without pleiotropic effects on seed germination, vigor and oil mobilization. Our results offer new perspectives for improving oil yield by targeted mutagenesis.

2.2 Introduction

In the seeds of oil plants, triacylglycerols (TAGs) are the major energy resource required during germination. Seed oil content (SOC) and fatty acid (FA) composition are major determinants of yield and quality. The *de novo* synthesis of FAs occurs in plastids. Then, TAGs are further assembled in the endoplasmic reticulum (Graham 2008; Li-Beisson et al. 2013) and, surrounded by a lipid monolayer and oil body proteins, so-called oil bodies are deposited in the cytosol (Li-Beisson et al. 2013; Xu and Shanklin 2016). TAGs can be degraded into free FAs by various lipases (Li-Beisson et al. 2013). The free FAs are subjected to β -oxidation, a catabolic process by which FA molecules are broken down to produce acetyl-CoA, which is subsequently converted into 4-carbon compounds via the glyoxylate cycle (Borek et al. 2015; Graham 2008; Pracharoenwattana and Smith 2008). Previous studies reported that SOC peaks at seed maturation (Kelly et al. 2013; Wan et al. 2017), and then degrades during seed desiccation indicating that the accumulation of lipids in seeds is not simply a unilateral synthesis process, but a dynamic balance between anabolism and catabolism, influenced by numerous external and internal factors (Kurat et al. 2006; Zhou et al. 2018). Our knowledge about lipid decomposition during seed maturation is comparatively limited (Ding et al. 2019; Kanai et al. 2019; Kelly et al. 2013), and the biological implication of TAG degradation during seed development remains to be elucidated.

GDSL lipases play an important role in TAG degradation. Their name comes from the highly conserved GDSL amino acid motif near the N-terminus. GDSL lipases contain five consensus domain blocks (I-V) forming the catalytical active serine-aspartate-histidine triad with the oxyanion hole residues serine (S), glycine (G) and asparagine (N) (Akoh et al. 2004; Upton and Buckley 1995). GDSL lipases were identified in many species, including microbes, animals, and plants (Akoh et al. 2004; Arif et al. 2004; Brick et al. 1995; Cummins and Edwards 2004; Horne et al. 2009; Pringle and Dickstein 2004). The advancement of genome sequencing projects revealed more than 1,100 members of GDSL lipases from the model plant *Arabidopsis thaliana*, green algae, moss, fern, grapevine, tree, and crop species (Chepyshko et al. 2012; Volokita et al. 2011). The GDSL family from *Arabidopsis* consists of 105 members (Lai et al. 2017). *GDSL* genes have flexible active sites (Akoh et al. 2004; Shakiba et al. 2016), which change

conformation after binding of different substrates. In plants, *GDSL* genes play roles in the regulation of morphological development, pathogen defense, abiotic stress, and secondary metabolism (An et al. 2019; Kim et al. 2013; Lai et al. 2017). However, the functions of most of the *GDSL* enzymes are little understood.

Rapeseed (*Brassica napus*) is the only important oil crop of northern latitudes. The SOC is ranging between 26 and 50% (Xiao et al. 2019). Rapeseed and its closely related oilseed crops *B. juncea* and *B. rapa* belong to the Cruciferae plant family together with *Arabidopsis*. Its allopolyploid genome resulted from a spontaneous hybridization between *B. rapa* and *B. oleracea* (AACC, $2n = 38$) which took place ca. 7500 years ago (Chalhoub et al. 2014). As a consequence, one gene in *Arabidopsis* corresponds to multiple orthologous copies in the rapeseed genome. Therefore, duplicated genes may undergo neo/subfunctionalization (Conant and Wolfe 2008; Xia et al. 2016) and translating knowledge directly from *Arabidopsis* is relatively difficult. The availability of the reference genome ‘Darmor-*bzh*’ facilitates the identification of *Arabidopsis* orthologs in rapeseed and the exploration of their biological function (Chalhoub et al. 2014).

Mutations are instrumental in understanding gene functions. Traditionally, random mutations induced by chemicals or irradiation were mainly used for functional analysis. However, random mutagenesis has several limitations when it comes to practical breeding, especially in polyploid crops like rapeseed, where several genes have to be mutagenized simultaneously to gain the desired phenotype (Jung et al. 2018). During the last decades, targeted genome editing techniques evolved rapidly as a more efficient alternative to classical approaches. In the case of rapeseed, the CRISPR-Cas technology proved to be a powerful tool (Doudna and Charpentier 2014) to create multiple mutations within gene families (Braatz et al. 2017).

This study aimed to elevate the SOC in rapeseed. In the past, numerous studies investigated the increase in SOC by overexpression of genes critical for the lipid biosynthesis pathway (Elahi et al. 2016; Vigeolas et al. 2007; Zafar et al. 2019). So far, little effort was put in preventing the synthesized lipids from decomposing during seed maturation (Ding et al. 2019; Kanai et al. 2019; Kelly et al. 2013). Here, we identified *GDSL* genes in the rapeseed genome for the first time and studied their expression in developing seeds. With a candidate gene association study using a world-wide collection of rapeseed accessions, we identified *SEED FATTY ACID REDUCER* (*SFAR*) genes, which had a significant effect on SOC. We used both chemically induced mutagenesis, and CRISPR-Cas mediated gene editing for the functional characterization of *BnSFAR* genes. We demonstrate that only the simultaneous knock-out of several *BnSFAR4* and *BnSFAR5* genes increases the SOC without adverse effect on seed germination and vigor, thus providing a successful example to increase rapeseed SOC by pyramiding *BnSFAR* knock-out alleles. Furthermore, we demonstrate the superiority of CRISPR-Cas technology over random mutagenesis if the aim is to knock-out multiple gene copies in polyploid species.

2.3 Results

2.3.1 Identification of *GDSL* genes in the rapeseed genome

We performed BLAST using 105 annotated *GDSL* genes from *Arabidopsis* (<http://www.arabidopsis.org/>) against the rapeseed reference genome. We found 222 genes with *GDSL* domains equally distributed between the A and C subgenomes of rapeseed (Supplementary Figure 1). Chromosome A07 has the highest abundance of *BnGDSL* genes (17 out of 222) (Supplementary Figure 1). We applied multi-to-multi, multi-to-one, and one-to-one models for sequence alignment. Each *AtGDSL* gene had one or more orthologs in the rapeseed genome. *GDSL* gene positions across the two genomes and the syntenic relationship between the paralogs and/or orthologs are shown in Figure 3A. Rapeseed orthologs of *AtGDSL* genes showed

high inter- and intra-species sequence similarities confirming a short evolutionary distance between rapeseed and *Arabidopsis*. To further study the relationships between *GDSL* genes from the A and C subgenomes, we performed a phylogenetic analysis using the neighbor-joining method (Figure 3B, Figure 3C). According to the degree of sequence similarity, 111 *BnGDSLs* each from the A and C subgenomes were assigned to 6 and 4 clades, respectively. The cluster analysis of the two subgenomes also uncovered substantial sequence variations between *BnGDSL* genes. The overall sequence diversity of *BnGDSLs* within the C subgenome (Figure 3C) is lower than in the A subgenome (Figure 3B).

We first considered five known *SFAR* genes that function to decrease SOC in *Arabidopsis* and searched for rapeseed homologs (Chen et al. 2012b). BLAST analysis using AtSFAR1–AtSFAR5 protein sequences as queries for the rapeseed database resulted in many hits. We considered different parameters; e-value (0), gene identity (> 80%), and the presence of conserved domains to select the putative rapeseed orthologs. Subsequently, 12 homologous genes were identified, two paralogs in each gene family, except for *BnSFAR4* where four paralogs have been found (Supplementary Table 1). We further divided *BnSFAR4* into subfamily-a and subfamily-b based on their sequence similarity. All the conserved blocks were found in all candidate genes.

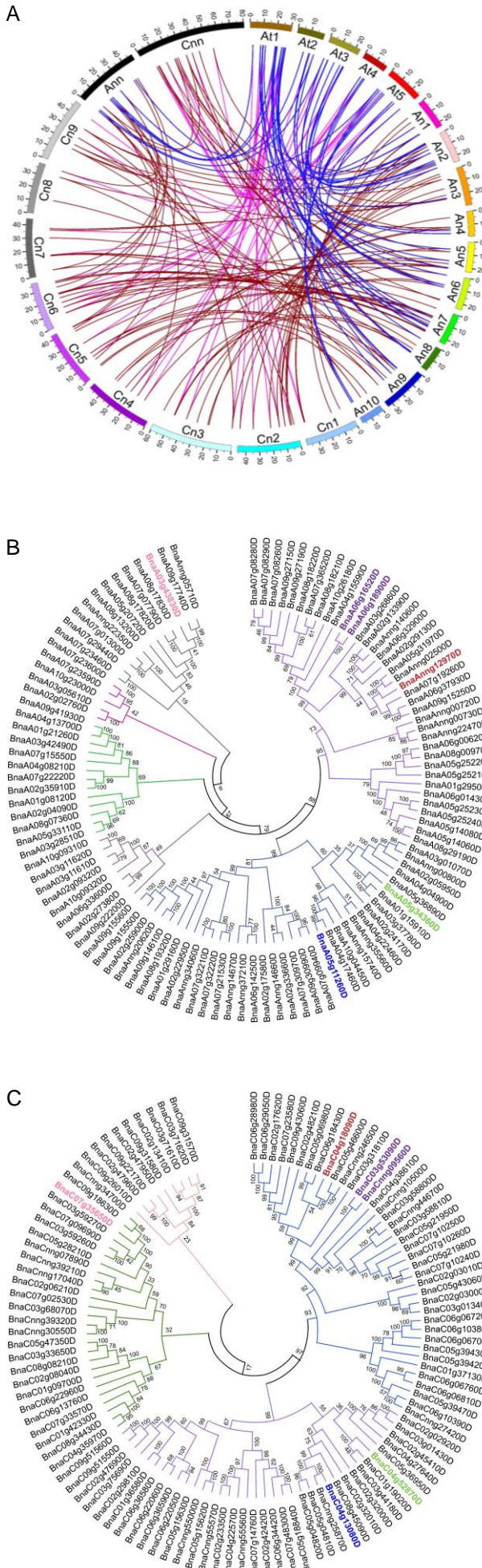


Figure 3: Position of *GDSL* genes in the genomes of *A. thaliana* and *B. napus* and synteny relationships between the paralogous and/or orthologous genes and unrooted phylogenetic trees (A) Chromosomes of *A. thaliana* are named as At1 through At5. Chromosomes of the A and C subgenomes of rapeseed are named as Anx and Cnx, respectively. ‘Ann’ and ‘Cnn’ represent the unmapped sequence reads. The blue lines are connecting the orthologs between *Arabidopsis* and the A subgenome of rapeseed. The pink lines are connecting the orthologs between *Arabidopsis* and the C subgenome of rapeseed. The brown lines are connecting the paralogs between the A and C subgenomes of rapeseed. Phylogenetic trees of 222 *BnGDSL* genes from the A (B) and C (C) subgenomes of rapeseed. *BnGDSL* genes are grouped into six clusters indicated by different colors. The amino acid sequences were aligned using ClustalW2 (default parameters), and the phylogenetic trees were constructed using the neighbor-joining method. *BnSFAR1*-*BnSFAR5* genes are shown in red, blue, green, purple, and pink, respectively.

2.3.2 Sequence variations within *BnSFAR* genes impacting seed oil content

We screened the genomes of 870 rapeseed accessions for SNPs within the twelve paralogs of five *BnSFAR* gene families using our re-sequencing data (Wu et al. 2019) and the ‘Darmor-*bzh*’ sequence as the reference genome. Moreover, the SOC and FA composition of all accessions were determined (Dataset S1). For statistical analysis, homozygous SNP (+/+), heterozygous SNP (+/-), and lack of SNPs (-/-) at a certain position within a *BnSFAR* gene were given a 2, 1, and 0 score value, respectively. Only non-synonymous SNPs were considered for the analysis. A ‘total non-synonymous value’ (TNSV) was defined either as the sum of non-synonymous values at a given position of a single *BnSFAR* gene or as the sum of all non-synonymous values of all *BnSFAR1-BnSFAR5* genes. As a result, accessions with TNSV larger than 20 had significantly higher average SOC than accessions with TNSVs < 20. However, there was no significant difference between accessions if TNSVs were between 10 and 20 or lower than 10 (Figure 4, Dataset S1). We also examined the effect of SNPs on SOC for each *BnSFAR* gene subfamily separately (Dataset S1). We found significant effects of SNPs within *BnSFAR1* and *BnSFAR4* families, respectively (Figure 4). To investigate the role of SNPs in varying FA composition (*SFAR footprint*), we analyzed the seed oleic acid (C18:1) content (OAC) (Dataset S1). Accessions with a TNSV higher than 20 had significantly lower OAC as compared to accessions with a TNSV < 20, indicating a *SFAR*-loss-of-function effect (Supplementary Figure 2). However, no significant difference was obvious if TNSVs were between 10 and 20 or if TNSVs < 10 (Supplementary Figure 2). Studying SNP effects on the individual gene families, resulted in significantly different OAC for *BnSFAR1* ($p = 4.9e-17$), *BnSFAR4* ($p = 2.3e-5$), and *BnSFAR5* ($p = 0.0024$) paralogs (Supplementary Figure 2).

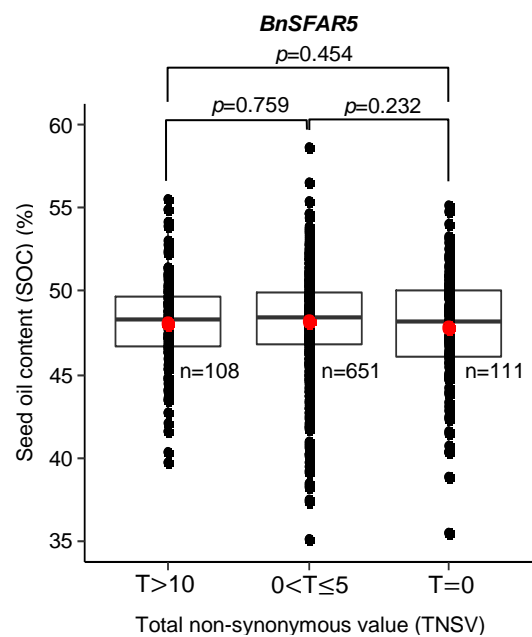
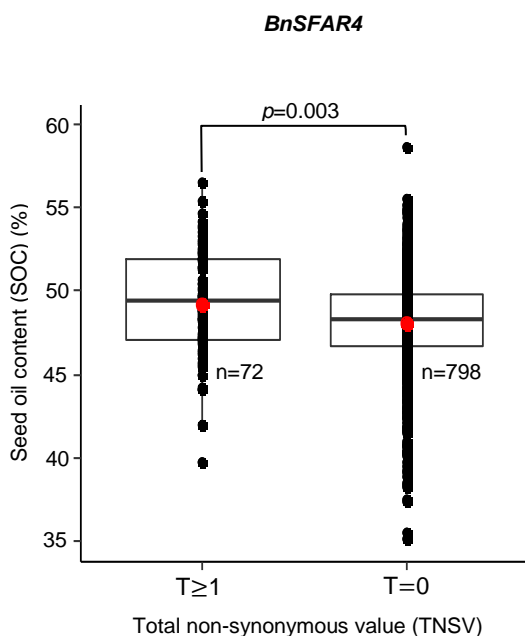
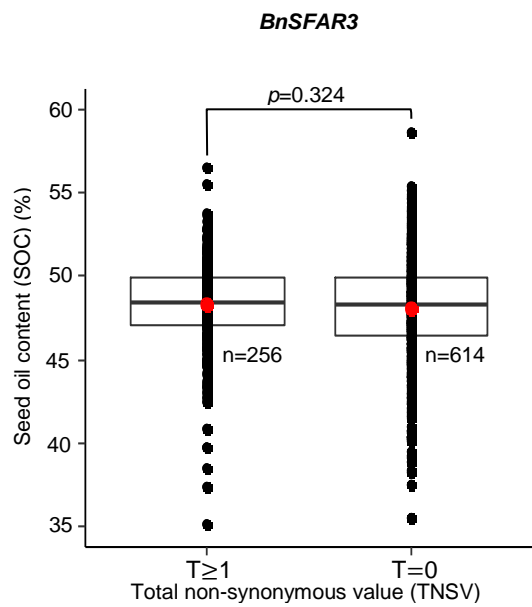
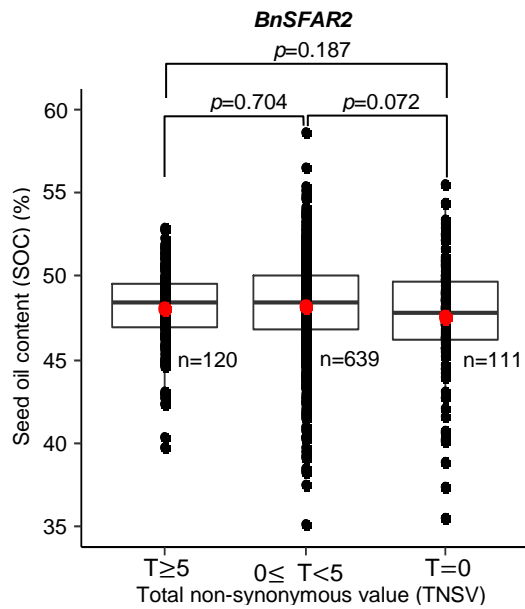
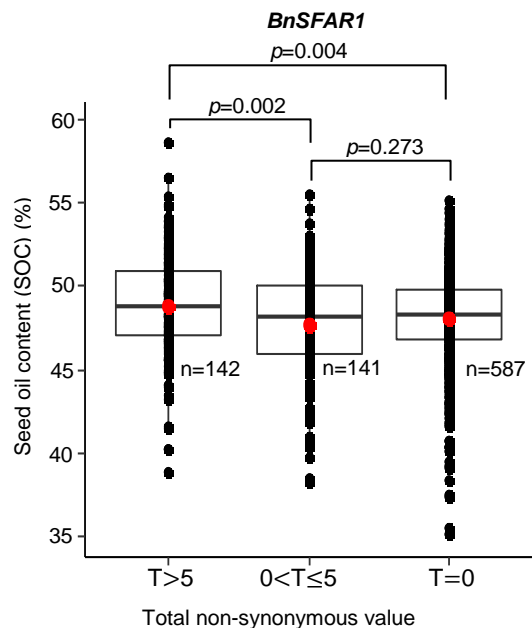
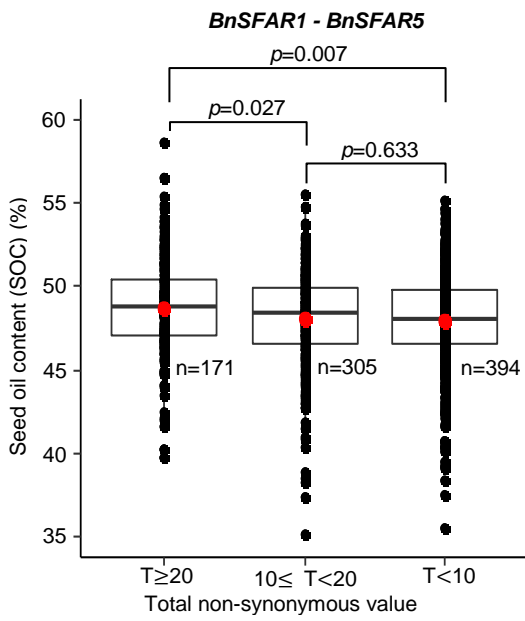


Figure 4: The effect of non-synonymous single nucleotide polymorphisms (SNP) within *BnSFAR* genes on seed oil content in 870 non-related rapeseed accessions. At each position of a given gene, accessions with homozygous and heterozygous non-synonymous SNPs resulting in an amino acid change were given a score of 2 and 1, respectively. Lack of SNPs resulted in a score of 0. A ‘Total Non-Synonymous Value’ (TNSV) was defined either as the sum of non-synonymous values (T) at a given position of a single *BnSFAR* gene or as the sum of all non-synonymous values of all *BnSFAR* genes. n: the number of accessions used for the calculation of mean SOC. The *p* value indicates the significance of pairwise comparisons

2.3.3 Selecting *BnGDSL* genes expressed in developing seeds

We sequenced the transcriptomes of developing seeds 16 and 40 days after pollination (DAP) (Zhou et al. 2017). In total, 105 putative *BnGDSL* genes were found to be expressed in developing seeds, 75 of them were *AtGDSL* orthologs while the rest did not display sufficient homology (FPKM, fragments per kilobase per million >1) (Supplementary Figure 1, Dataset S2). Among the 75 *AtGDSL* orthologs, 14 genes were equally expressed at both developmental stages, 22 and 39 genes were upregulated and downregulated, respectively at 40 DAP (relative to 16 DAP) (Dataset S2). To verify the RNA-seq data, we measured the relative expression of *BnSFAR1-BnSFAR5* by RT-qPCR at five developmental stages in the German winter-type cultivar ‘Express-617’ (15, 25, 35, 45, and 55 DAP) and seven developmental stages in the Chinese semi-winter type cultivar ‘Hu135’ (17, 24, 31, 38, 45, 52, and 59 DAP) (Figure 5, Supplementary Table 2). The results can be summarized as follows: (1) The RT-qPCR results were generally in line with the transcriptome data. (2) Genes from *BnSFAR4* subfamily-a were highly expressed during early stages of seed development (15-35 DAP), while *BnSFAR4* subfamily-b genes were highly expressed at seed maturation and desiccation stages (45-55 DAP). (3) Also, *BnSFAR1* was highly expressed at early stages (25 and 35 DAP), but then their transcriptional activities dropped towards seed ripening (Figure 5). Only minor differences were obvious between winter and semi-winter type cultivars.

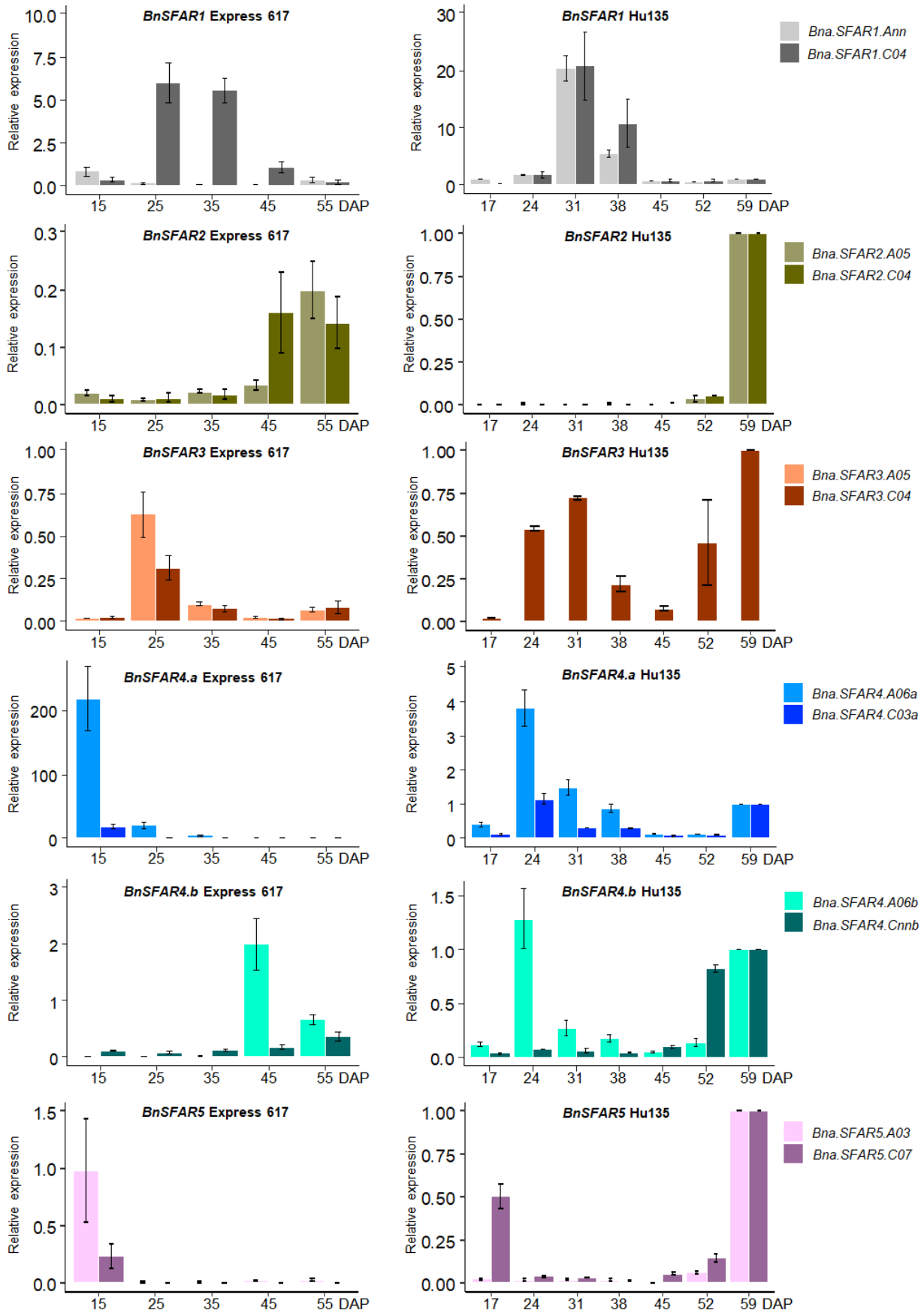


Figure 5: Relative expression of *BnSFAR1-BnSFAR5* genes. Seeds from winter rapeseed variety Express-617 were harvested 15, 25, 35, 45, and 55 days after pollination (DAP). Seeds from the Chinese semi-winter type variety ‘Hu135’ were harvested 17, 24, 31, 38, 45, 52, and 59 DAP. Five biological samples for Express-617 and three biological samples for Hu135 together with three technical replicates were used for analysis. Gene expression was quantified relative to *BnActin*. Error bars were defined by the SEM of five or three biological samples for Express-617 and Hu135, respectively.

2.3.4 SFAR knock-out mutants by CRISPR-Cas and induced mutagenesis

We selected two *BnSFAR1* and four *BnSFAR4* genes because (1) non-synonymous SNPs within these genes had a significant effect on SOC, (2) they were differentially expressed between early and late stages of seed development, and (3) their highest expression was between 35–55 DAP.

Screening of 3,488 M₂ families from our EMS TILLING resource revealed 163 mutations in *BnSFAR1* and *BnSFAR4* genes, corresponding to an average mutation frequency of 1/24.5 kb (Supplementary Table 3). We identified nonsense mutations for five paralogs, except for *Bna.SFAR1.Ann* where we found a missense mutation (*sfar1-2*) in a strictly conserved SGNH amino acid motif and one splice site mutation (*sfar1-3*) (Figure 6, Supplementary Table 3, Supplementary Table 4).

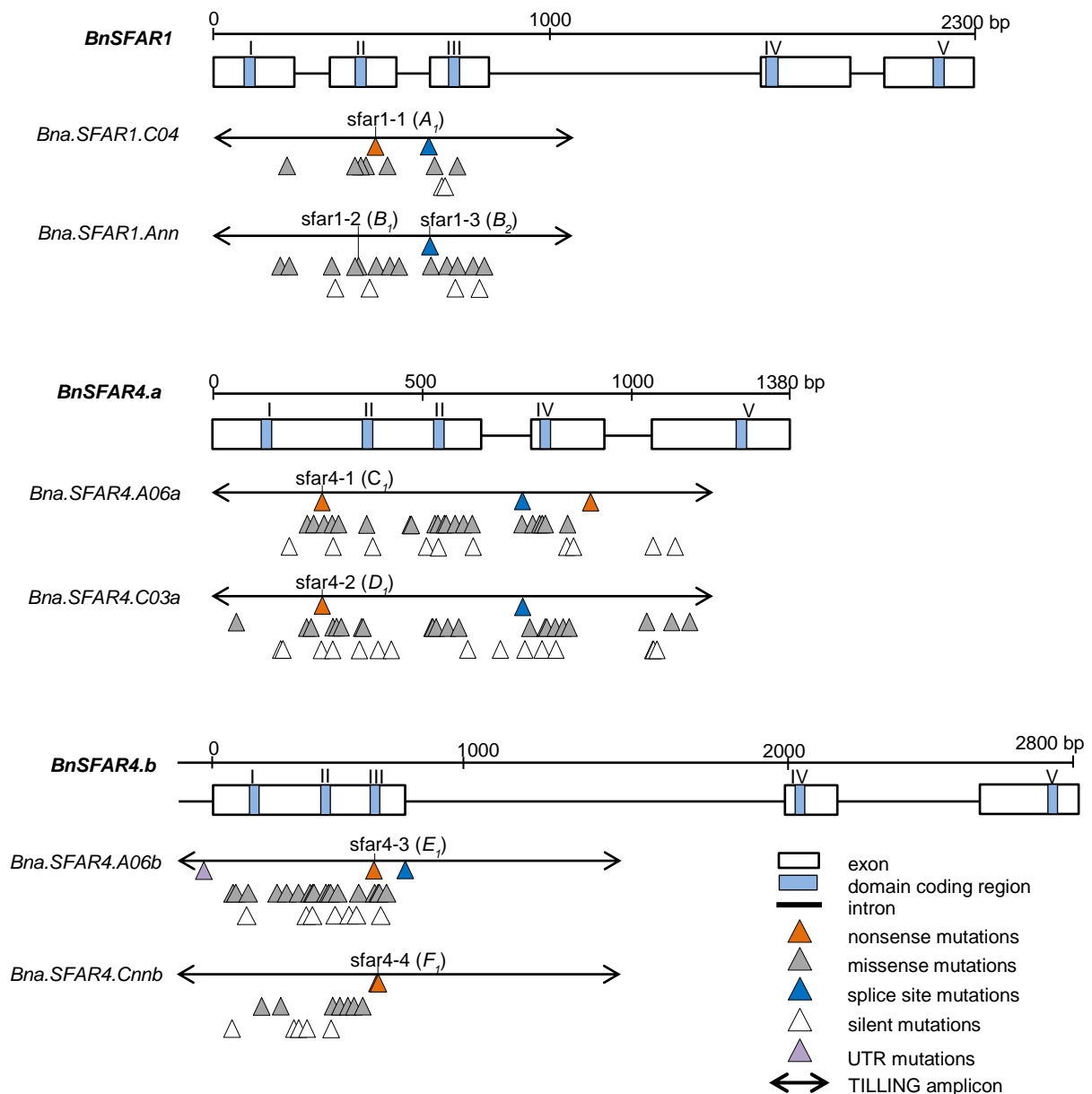


Figure 6: Location of EMS-induced mutations in six *BnSFAR* genes belonging to 3 subfamilies, *BnSFAR1*, *BnSFAR4* subfamily-a, and *BnSFAR4* subfamily-b. Mutations are displayed only if they were confirmed, after Sanger sequencing of M₃ plants. The five GDSL consensus domain blocks are indicated by roman letters (I-V). The mutants are named 'sfar' and the mutant alleles are written in brackets.

We chose seven EMS single mutants for phenotyping. Of these, six mutants were used as parents to produce double mutants (Supplementary Table 5). Because EMS causes a high number of genome-wide random mutations, reduction of the background mutation load is vital. Therefore, we backcrossed mutants with the EMS donor plant Express-617 (Supplementary Figure 3A) and used allele-specific markers (Supplementary Table 2) to isolate homozygous mutant and wild-type plants among segregating F₂ and BC₁F₂ populations.

In polyploid species with high gene redundancy, single gene mutations usually do not have a phenotypic effect. Therefore, we crossed single *BnSFAR1* and *BnSFAR4* mutants to produce double mutants to measure SOC. We crossed either M₃ single mutants directly (M₃ x M₃) or single mutants backcrossed once with the EMS donor Express-617 (M₃-Express-617 x M₃-Express-617) (Supplementary Figure 3B, Supplementary Table 5).

Next, we produced mutants by CRISPR-Cas9 induced mutagenesis to get multiple gene knock-outs simultaneously. We searched for conserved sequences as target sites between four *BnSFAR4* and two *BnSFAR5* genes, separately. Only target sequences with no expected off-target effects were considered (Supplementary Figure 4). Finally, we chose target sites from exon 1 and exon 2 located within (*BnSFAR4*) or 19 bp upstream (*BnSFAR5*) of domain block II (Figure 7A, Figure 7C). To knock out the *BnSFAR1* paralogs, we chose a target site from exon 1 next to the domain block I. The pCas9-TPC construct was introduced into the winter rapeseed line RS306 by *Agrobacterium*-mediated hypocotyl transformation (Braatz et al. 2017b). Transformation of 857 (*BnSFAR1*), 754 (*BnSFAR4*) and 442 (*BnSFAR5*) excised hypocotyls yielded two, five and two transgenic plants (Supplementary Table 6), respectively, equivalent to transformation efficiencies between 0.2 and 1.1%.

We selected two primary T₁ *SFAR4* transformants (bnsfar4-TP3 and bnsfar4-TP4) and the *SFAR5* (bnsfar5-TP1) transformant for further studies. As an initial mutation screening, PCR products enclosing the target region were sequenced from T₁ plants, and we found complete gene editing in all *BnSFAR4* paralogs (Figure 7B). We identified 13 different mutant alleles with single nucleotide insertions and deletions. Two were identical and were assigned the same allele identities (C₂) (Figure 7B, Supplementary Table 4). No wild-type sequence could be detected. Each mutation resulted in a premature stop codon leading to a truncated protein. Hence, we can assume that all are loss-of-function mutations. In contrast, none of the T₁ plants transformed with the *BnSFAR1* construct displayed any mutation within the target sequence.

Sequencing three *in vitro* clones of the *SFAR5* transformant bnsfar5-TP1 (bnsfar5-CP1, bnsfar5-CP2, and bnsfar5-CP3) revealed mutations in both *BnSFAR5* paralogs (Figure 7D, Supplementary Table 4). Three mutations were 1 bp insertions three base pairs upstream of the PAM site, and the other ones were deletions (2 bp-143 bp) between 283-423 bp from the start codon of the *BnSFAR5* genes. Compared to the *BnSFAR4* plants, wild-type alleles were found in all clones, indicating their chimerism (Figure 7D, Supplementary Table 4).

We analyzed the inheritance of CRISPR mutations by genotyping T₂ plants (Supplementary Table 7). All *SFAR4* mutations found in the T₁ plants were confirmed in the T₂ generation, demonstrating that the parents were non-chimeric, and mutations were stably inherited because each locus under study segregated for both mutant alleles detected in the T₁ plants. Taking all four *BnSFAR4* loci together, 81 different T₂ genotypes were expected. For better understanding, we examined the segregation pattern of alleles separately for each gene. In the case of bnsfar4-TP4 (seed code 174250), segregation patterns of all four paralogs were consistent with Mendelian segregation (Supplementary Table 7). Interestingly, the homozygous mutations in

bnsfar4-TP3 were 100% transmitted to the next generation giving rise to a stable mutant line (Supplementary Table 7).

Then, we genotyped *BnSFAR5* T₂ offspring of T₁ clones. As expected, the T₂ generation was segregating for multiple alleles at both loci. Therefore, we analyzed the T₃ generation to select homozygous double mutants. We did not observe Mendelian segregation in transgene, of bnsfar5-CP2 (seed code 174257) and bnsfar5-CP3 (seed code 174258), mutant genes segregated accordingly (Supplementary Table 7). Moreover, the absence of transgene-free offspring in the investigated plants suggests the integration of more than one transgene during transformation.

2.3.5 Mutants with multiple gene knock-outs display increased seed oil content

We performed growth experiments with homozygous *SFAR* mutants to measure yield parameters. We selected two T₂ families (174249 and 174250) and one T₃ line (182975), which were fixed for the mutant alleles. Plants were grown in the greenhouse together with RS306 controls. The plants showed normal growth, flowering, and seed set when compared to wild-type RS306. In *BnSFAR4* mutants, SOC in T₃ and T₄ seeds was significantly increased by 9.7-14.5% and 12.9%, respectively when compared to the donor line RS306 (Figure 7E, Supplementary Table 8), whereas the TKW was not altered in T₃ seeds, but significantly increased in T₄ seeds (182975) (Figure 7F). Likewise, the *BnSFAR5* double mutants displayed a significant seed oil increase by 10.4% and 11.2% in T₃ (174257) and T₄ (183033) seeds, respectively (Figure 7E, Supplementary Table 8). Their TKW and overall plant performance were not significantly different compared to RS306 (Figure 7F, Supplementary Table 8). The erucic acid content was slightly reduced in two *BnSFAR4* lines (174249 and 174250) when compared to RS306, which is a non-quality synthetic rapeseed line. However, the reduction was only significant in 174249 in combination with a non-significant increase in C18:1 and C18:3 contents, while in 174250 only C18:1 content was increased (Supplementary Figure 5).

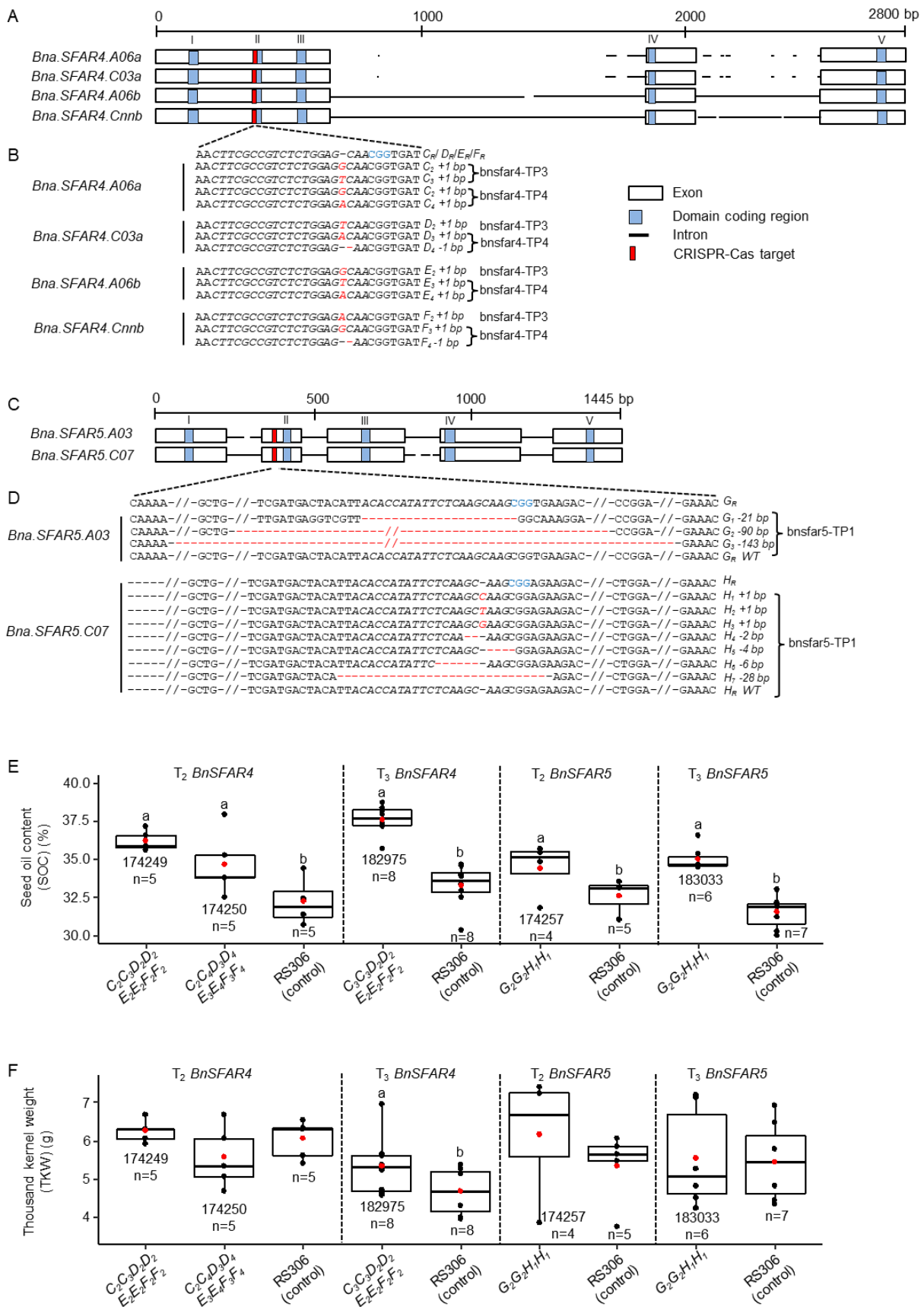


Figure 7: CRISPR-Cas induced mutations in six *BnSFAR* genes and phenotypic analysis of *BnSFAR4* and *BnSFAR5* CRISPR-Cas mutants. (a) A conserved 20 bp target was selected residing within domain coding block II of *BnSFAR4*. (b) Target sites were sequenced from regenerated plants after *Agrobacterium* transformation (T₁ generation). Thirteen mutant alleles were identified in T₁ plants named bnsfar4-TP3 and bnsfar4-TP4 and two of them are identical (C₂) in both plants. (c) Gene structure of *BnSFAR5* genes. A conserved 20 bp region was targeted

on exon 2 upstream of domain coding block II of *BnSFAR5*. (d) CRISPR-Cas induced mutations in the T₁ plants named *bnsfar5*-TP1. The protospacer-adjacent motif (PAM) is written in blue letters, while red color indicates insertions and red dashes ('-') indicate deletions. Mutant alleles are written left of the sequences. Wild-type alleles are carrying a suffix 'R'. (e) Seed oil content (SOC) and (f) Thousand kernel weight (TKW) in T₃ and T₄ seeds of *BnSFAR4* and *BnSFAR5* mutants. Of each genotype, a minimum of four plants was studied. Seed codes and the number of plants used for phenotyping are written below the boxes. Oil extraction was done using n-hexane microextraction. One-way ANOVA test was performed at $p < 0.05$ and grouping was done using a Tukey test at $p < 0.05$ for *BnSFAR4* T₃ mutants and an unpaired *t*-test at $p < 0.05$ was performed for *BnSFAR4* T₄ and *BnSFAR5* mutants. Within each phenotyping experiment (separated by dotted lines), different letters (a, b) indicate significant differences. The red dot in the box plot shows the mean SOC or mean TKW.

Then, we studied the effect of *SFAR* mutations in EMS mutant lines. The plant material included six segregating F₂ populations from crosses between homozygous M₃ plants (M₃xM₃) and Express-617 backcross generations ((M₃ x Express-617) x (M₃ x Express-617)), and backcross populations (F₃ and BC₁F₃) with reduced mutation load (Supplementary Table 5). All plants were genotyped with allele-specific primer combinations (Supplementary Table 2) to distinguish between heterozygous and homozygous genotypes. Therefore, single mutants, double mutants, and EMS treated plants carrying the non-mutated alleles with roughly equal background mutation load could be employed in this experiment (Supplementary Table 5). We did not observe significant differences in SOC between homozygous single mutants and plants with the wild-type allele (171780, 171782, 171784, 171786, 180886-180889) (Figure 8), while the SOC was significantly higher in the non-mutagenized Express-617 (Figure 8A, Figure 8B). Noteworthy, in the backcrossed plants, the SOC of homozygous single mutants (*C₁C₁* (180886), and *F₁F₁* (180889)) was significantly higher (44 - 45 %) demonstrating the bias caused by the mutation load in early mutant generations (Figure 8C, Figure 8D).

Because single mutants did not have elevated SOC, we analyzed *BnSFAR1* double mutants homozygous for the mutant alleles (*A₁A₁B₁B₁*). In two independent populations, no significant differences were found compared to mutant offspring with the Express-617 allele (*A_EA_EB_EB_E*) (Supplementary Table 8). Conversely, SOC was increased by 12.1% (171770), 10.3% (171772), 8.9% (180876), and 8.7% (180877) in *BnSFAR4.a* and *BnSFAR4.b* double mutants as compared to the mutants with the Express-617 alleles (Figure 8). One mutant line (180876) already reached the SOC of the non-mutated Express-617 line despite its high mutation load (Figure 8C).

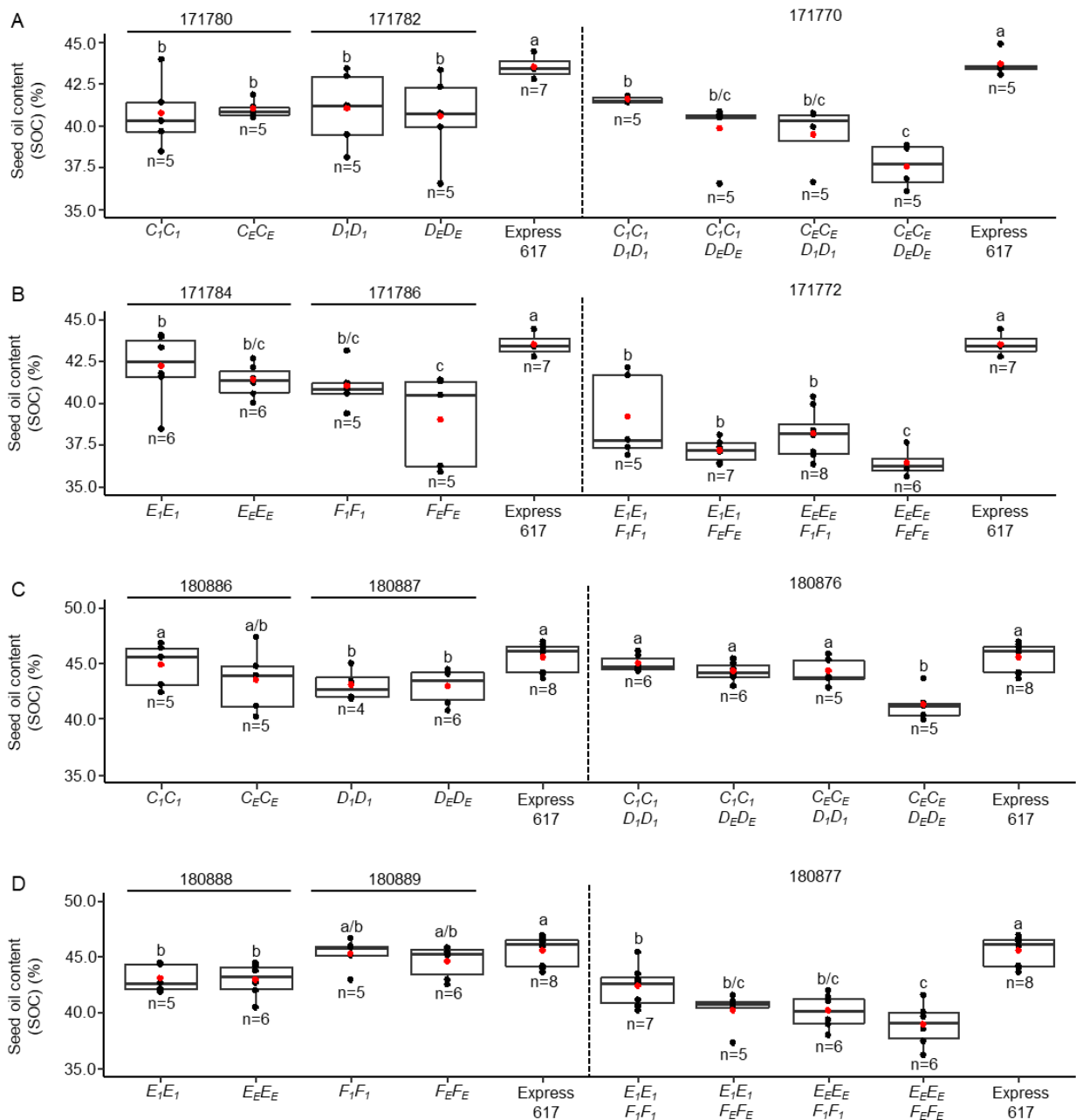


Figure 8: Seed oil content of segregating *BnSFAR4* EMS mutant progenies. (A) and (B) Seed oil content of F_2 populations segregating for single mutations (171780, 171782, 171784 and 171786) and mutant progenies derived from $M_3 \times M_3$ crosses (171770 and 171772) (see supplementary Table 5). (C) and (D) Seed oil content of BC_1F_2 (180886-180889) plants and mutant progenies from $(M_3 \times \text{Express-617}) \times (M_3 \times \text{Express-617})$ crossing generations (180876 and 180877). One-way ANOVA test was performed at $p < 0.05$ and grouping was done using the Tukey test at $p < 0.05$. The red dot in the box plot shows the mean SOC. Within each phenotyping experiment (experiments separated by dotted lines) different letters (a,b,c) indicate significant differences

2.3.6 Loss of *SFAR* function impacts oil body size but not seed vigor

We wanted to know why *SFAR* double mutants have higher SOC. We reasoned that cotyledon cells from developing seeds could contain altered oil bodies (OB) due to *SFAR* mutations resulting in elevated SOC. Therefore, OBs in mature seeds were investigated by transmission electron microscopy (TEM). We found that OBs from the CRISPR-Cas mutant 174249 ($C_2C_3D_2D_2E_2E_2F_2F_2$) were significantly larger than OBs from RS306, indicating less degradation of TAGs during seed maturation (Figure 9A, Figure 9B-G).

Next, we investigated the effects of knock-out mutations on oil accumulation during seed maturation. Seeds from T_3 plants (CRISPR-Cas mutant line 182975, $C_3C_3D_2D_2E_2E_2F_2F_2$) were

harvested from 25-55 DAP. The highest SOC was measured at 45 DAP in both mutant and RS306. The SOC dropped sharply in RS306, whereas a much slower decline was observed in the mutant (Figure 9H). These data confirm the physiological effect of *SFAR* mutations resulting in reduced oil degradation during seed maturation.

However, if seed oil mobilization is also retarded during germination, this could result in reduced seedling vigor. Therefore, we measured SOC at different stages after germination. As expected, oil content decreased with time in all genotypes (182975, 183033, and RS306), however, with an increased rate in RS306 (24.5% and 27% vs. 29.9%) (Figure 9I). It is well known that lipases are important in osmotic stress response. Therefore, we tested the seedling growth of two CRISPR-Cas mutants under mild stress conditions of 50 mM NaCl for five days. We found that *SFAR* mutations had no adverse effect on seed germination, which was in the range of the RS306 donor (Supplementary Table 9). Moreover, root and shoot growth were not significantly different between mutants and RS306 (Figure 9J, Figure 9K, Supplementary Table 9).

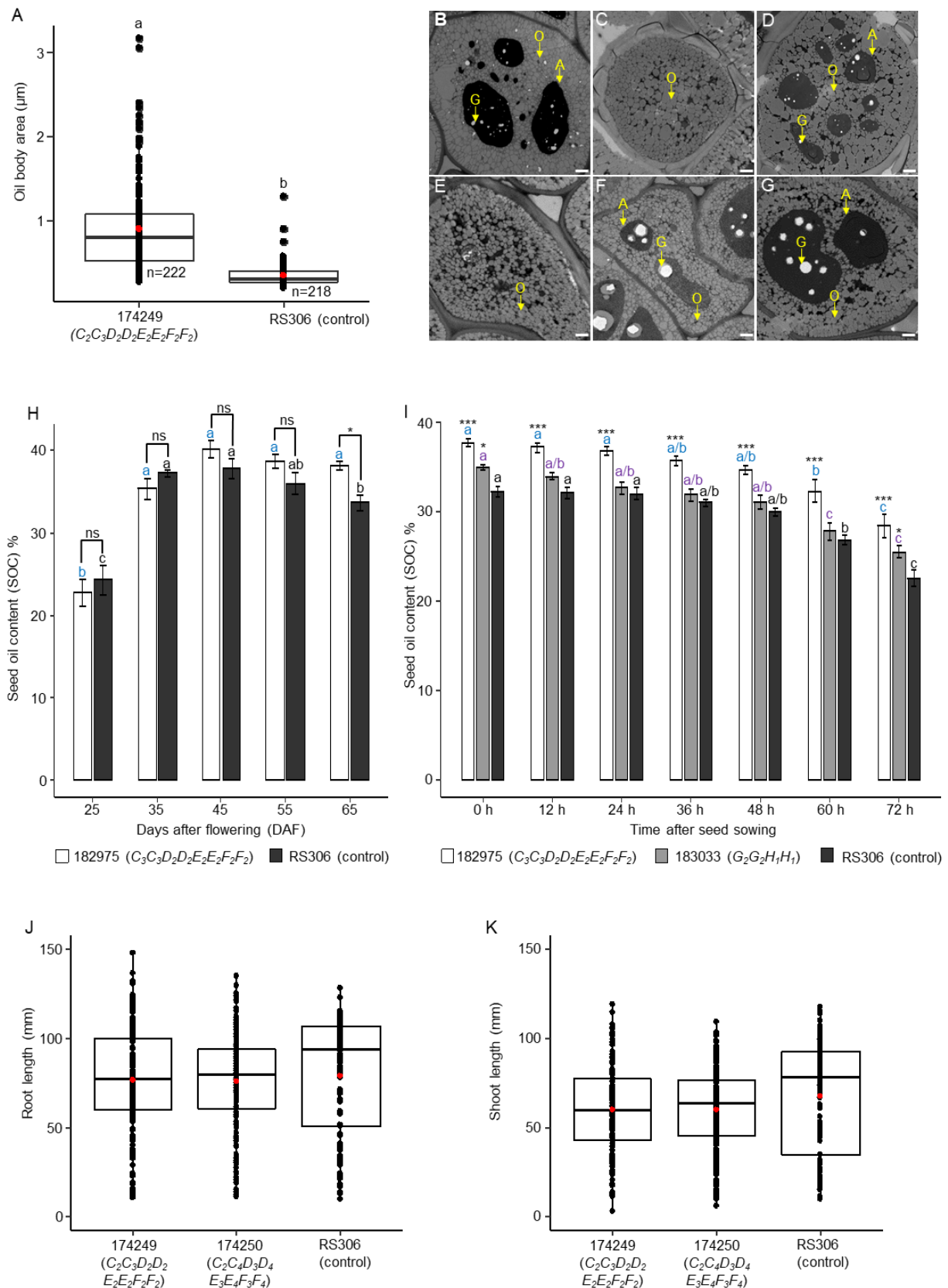


Figure 9: Analysis of seed oil bodies in T_3 seeds, dynamics of seed oil accumulation and mobilization, and assessment of seed vigor in *BnSFAR* CRISPR-Cas mutants. (A) Seed oil body area in seeds of the *BnSFAR4* mutant (174249) and seeds of RS306. Cross-sections of cotyledons were analyzed by transmission electron microscopy. An unpaired *t*-test was performed at $p < 0.05$. Significance levels are indicated by letters. (B)-(D) Oil bodies of *BnSFAR4* mutants and (E)-(G) RS306. Arrows indicate oil bodies (O), aleurone grains (A) and globoids (G). Bar=1 μm . (H) The CRISPR-Cas line 182975 (T_3 , $C_3C_3D_2D_2E_2E_2F_2F_2$) and RS306 were grown in the greenhouse and seeds were harvested at different time points to measure SOC ($n=5$). (I) SOC from germinating seeds at different

time points after sowing ($n = 6$). (H) and (I) The n-hexane micro-extraction method was used for oil isolation. Data are presented as means \pm SEM. One-way and two-way ANOVA were performed at $p < 0.05$, and grouping was done using the Tukey test at $p < 0.05$. Significance levels are indicated by blue (182975), purple (183033), and black letters (control) between different time points. Significant differences between mutants and controls are shown at $p < 0.05$ (*) and $p < 0.001$ (***) (J) Root and (K) shoot growth of two *SFAR* CRISPR mutants five days after sowing (DAS). One-way ANOVA test was performed at $p < 0.05$, and grouping was done using the Tukey test at $p < 0.05$.

2.4 Discussion

Increasing SOC is a major focus in oil crop breeding. In previous years, the TAG biosynthesis pathway has been investigated intensively (Bates 2016; Li-Beisson et al. 2013), and numerous studies have shown an elevation of SOC in rapeseed through the manipulation of transcription factors, FA transporters and promoters or inhibitors of TAG biosynthesis (Peng et al. 2010; Tan et al. 2011). During seed maturation, the balanced activities of seed oil synthesis and degradation genes ensure seed dormancy to prevent early sprouting, a process known as vivipary, which consumes seed reserves, including the storage lipids (Wan et al. 2017). Our study provides the first successful example where knock-out of *BnSFAR4* and *BnSFAR5* lipase genes in an oil crop resulted in higher SOC while germination rates and seed vigor remained unaffected.

We provide the first whole-genome survey of all *BnGDSL* genes in rapeseed. The *BnGDSL*s from the A-subgenome showed more diversity, in accordance with an overall higher genetic diversity within this subgenome. This is explained by the fact that *B. napus* x *B. rapa* crosses were more frequent than crosses with the other founder species, *B. oleracea* (Wu et al. 2019). A candidate gene association study revealed that SOC was positively correlated with the number of non-synonymous SNPs within the coding sequences of *BnSFAR* genes. Therefore, we propose to use natural variation from seed banks to search for new allelic variants within *GDSL* genes in rapeseed and other oilseed crops.

Less than 50% of the *BnGDSL* genes were expressed in developing seeds. The expression of *BnGDSL* genes may be affected by various endogenous and/or exogenous environmental stimuli. The expression profiles did not vary much between the winter and semi-winter cultivar apart from four genes (*Bna.SFAR1.Ann*, *Bna.SFAR4.A06a*, *Bna.SFAR4.A06b*, and *Bna.SFAR5.A03*). This could be explained by different upstream factors that regulate the expression of *BnGDSL*s as was found in *Arabidopsis* where the expression of *AtSFARs* is upregulated by gibberellin signaling (Chen et al. 2012b). The low transcriptional activity at later stages of seed development is probably the reason why *BnSFAR1* mutations did not affect SOC, although a minor effect from the missense mutation cannot be ruled out. Alternatively, these genes may have undergone neofunctionalization. Nevertheless, the knowledge of transcriptional profiles of *GDSL* genes is key to successful knock-out experiments. Lipases initiate lipid mobilization by hydrolyzing storage TAGs into glycerol and FAs to supply precursors for the β -oxidation pathway during seed germination (Graham 2008). Therefore, it was reasonable to investigate the effect of *BnSFAR* mutations on seed germination and seed vigor. It is noteworthy that the mutants did not differ in both characters from their donor genotypes.

We demonstrate that only multiple knock-outs are useful in studying functionally redundant genes typical for polyploids. Although, random mutagenesis was a common method to introduce new allelic variants into plant breeding, gene redundancy in rapeseed makes it challenging to reshape a trait by random mutagenesis (Braatz et al. 2018b; Shah et al. 2018). Conversely, CRISPR-Cas mediated genome editing enables multiple mutations simultaneously. We used both random mutagenesis and site-specific nucleases to induce mutations in *BnSFARs*. As expected, we did not observe significantly increased SOC in single mutants compared to wild-type plants. While pyramiding single mutations in one genotype is cumbersome and laborious, we obtained CRISPR-Cas quadruple *BnSFAR4* knocked out mutants. In less than two years, stably inheriting homozygous T₃ winter-type mutant lines could be selected. As shown in previous studies (Yang et al. 2018; Zhang et al. 2019a), we also found T₁ plants with more than two alleles (haplotypes)

at one locus as clear evidence for chimerism. A possible explanation is the low activity of the Cas9 nuclease caused by partial transgene silencing in the *BnSFAR5* T₁ plant, which carries multiple transgene insertions. Gene silencing in transgenic plants with more than one copy of the transgene is reported for different plant species (Sohn et al. 2011; Tang et al. 2007). However, homozygous mutants could be easily selected in the T₃ generation. The CRISPR-Cas approach was conclusively more efficient than the TILLING approach.

Another shortcoming is the high number of background mutations after EMS mutagenesis. Considering mutations in six genes, each plant from our EMS TILLING population was found to carry ~46,000 mutations, given the rapeseed genome size of 1130 Mb (Chalhoub et al. 2014). Therefore, primary mutants suffer from a high mutation load, resulting in morphological and physiological anomalies. Hence, we reduced the mutation load by backcrossing with the non-mutated donor. Then, the phenotypic effect in double mutants was assessed within segregating populations comparing homozygous genotypes (mutant and wild-type allele) with equal mutation load. In our experiment with an F₂ population segregating for *BnSFAR4.a* and *BnSFAR4.b* mutants, we found that the double mutants had a significantly higher SOC than homozygous genotypes with the wild-type allele which aligns with our CRISPR-Cas results where the same genes were mutated. Moreover, our results suggest the additive action of *BnSFAR4* genes in rapeseed because only multiple mutations affected on SOC.

We used the synthetic rapeseed line RS306 for transformation experiments because explants displayed high shoot regeneration capacity. Typical for synthetic rapeseed, RS306 has a comparatively low SOC compared to modern varieties whose SOC content is in a range between 40 and 50%. There are two lines of evidence why we expect that modern varieties will also display elevated SOC after knock-out of *BnSFAR4* and *BnSFAR5* genes. First, it has been reported that SOC equally declined during seed maturation in high and low SOC inbred lines (Shahid et al., 2019). Therefore, we think that the knocking-out of *BnSFAR* genes will increase SOC even in modern varieties. Second, the variety Express-617 which we used to screen EMS-induced mutations has a comparatively high SOC around 44%. We observed that SOC was significantly higher in *BnSFAR4* double mutants in comparison to the wild type plants with the same mutation load. Even with a high mutation load, one double mutant (180876) reached the SOC of the non-mutated Express-617 line. These results provide evidence that knock-out of *BnSFAR* genes in an elite line will evenly increase SOC.

What are the consequences of *SFAR* knock-out mutations on the cellular and physiological level? In our study, *BnSFAR4* mutants had larger OBs in comparison to the donor RS306. In this respect, ambiguous data have been published in the past. Siloto et al. (Siloto et al. 2006) demonstrated that the knock-out of oleosin genes caused unusually large OBs, which correlated with lower SOC. Contrastingly, in another study OB size in rapeseed was positively correlated with SOC (Tan et al. 2019), where embryos were investigated from 21, 28 and 35 DAP. We reason that the bigger size of OBs in our *BnSFAR4* mutant was due to reduced TAGs decomposition, which has also been observed in previous research in *Arabidopsis* (Chen et al. 2012b).

Perspectives for practical breeding largely depend on the background mutation load and legal scenarios. The high number of background mutations in the EMS mutants is a shortcoming. Therefore, numerous backcrossings are needed to develop a mutant line with enhanced SOC and reduced background mutation load. While backcrossing with winter rapeseed is time-consuming because of the vernalization requirement, the use of spring rapeseed as backcross parent has been proposed to accelerate generation cycles. We are currently producing *BnSFAR4* double mutants by backcrossing with early flowering spring rapeseed in combination with genomic background selection using SNP arrays. This highlights the opportunity to reduce background mutations resulting from EMS mutagenesis with a speed breeding protocol (Watson et al. 2018).

In this way, CRISPR-Cas mutagenesis is clearly superior, because the desired mutation can be directly incorporated into an elite genome. Moreover, multiple mutations can be achieved in a single plant, as demonstrated by this study. However, the application of CRISPR-Cas induced mutations in plant breeding is largely hampered especially in the European Union due to the current legislation where these plants are classified as genetically modified organisms (GMOs) (Kupferschmidt 2018). In contrast, EMS mutants are not considered as GMO and can thus be used by breeders without legal constraints. Therefore, we expect that the *BnSFAR* EMS mutants will be effectively used in Europe, whereas the CRISPR-Cas mutants will be preferred in states with less restrictive legislation like North America or Australia.

To conclude, our results not only shed light on the understanding of seed oil degradation in a polyploid oil crop but also open a new path for breeding for higher SOC. *BnSFAR* mutants will enable increased rapeseed oil yield per unit area, which is an important aim in many (developing) countries such as India, China or Bangladesh which largely depend on seed oil import. Moreover, we propose the application of *SFAR* knock-out in other oil crops.

2.5 Methods

2.5.1 Identification of *GDSL* genes in the rapeseed genome

Sequences and chromosome positions of annotated *GDSL* (*AtGDSL*) genes in *Arabidopsis* were obtained from the TAIR database (<https://www.arabidopsis.org/>). *BnGDSLs* in the rapeseed genome were identified searching for genes with the IPR001087 domain (<http://www.ebi.ac.uk/interpro/entry/IPR001087>) in rapeseed (Genoscope-INRA unmasked v4.1) with an e-value below $1E-30$ using InterProScan 5.36-75.0 (<http://www.ebi.ac.uk/interpro/>) (Jones et al. 2014), and some of them were ruled out (Dong et al. 2016). The sequences and locations of *BnGDSLs* were gained from the rapeseed Genome Browser in GENOSCOPE (<http://www.genoscope.cns.fr/brassicapus/>). Visualization of collinear analyses among the *AtGDSLs* and *BnGDSLs* from the A and C subgenomes were performed using Circos (<http://circos.ca/>) (Krzywinski et al. 2009). Multiple alignments were performed for nucleotide and amino acid sequences. The phylogenetic trees were constructed using the neighbor-joining (NJ) method by ClustalW2 (<http://www.clustal.org/clustal2/>) and visualized in MEGA X 10.0.5 (<http://www.megasoftware.net/>) (parameters; 1000 bootstraps, Poisson model, Pairwise deletion) (Kumar et al. 2018). The distribution of *BnGDSLs* across the rapeseed genome was visualized using Mapchart 2.32 (Voorrips 2002) (<https://www.wur.nl/en/show/Mapchart.htm>).

2.5.2 RNA-seq and data analysis

Developing seeds from the rapeseed cultivar ‘ZY511’ at 16 and 40 DAP were harvested for RNA extraction. Total RNA was extracted using an RNA Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). RNA-seq was performed by Biomarker Technology Co. (Beijing, China). Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit (NEB, Ipswich, USA) following the manufacturer’s manual. The libraries were sequenced on an Illumina HiSeqTM 2500 platform, and paired-end reads were generated. Clean reads were mapped to the rapeseed reference genome using Hisat2 software (Kim et al. 2015). Quantification of transcription levels was estimated by fragments per kilobase per million (FPKM). Differential expression analysis of two samples was performed using the R package DESeq2 (Love et al. 2014). The resulting *p* values were adjusted using Benjamini and Hochberg’s approach to controlling the false discovery rate (FDR). The parameters (FDR<0.05 and $|\log_2^{(\text{fold change})}| \geq 1$) were set as the thresholds for a significantly different expression.

2.5.3 Expression analysis by RT-qPCR

We used the winter rapeseed Express-617 and the semi-winter rapeseed Hu135. Express-617 plants were grown under greenhouse conditions (16 h light/ 8 h dark, ~23-24 °C) while Hu135 were grown in the field (Zhejiang University, Hangzhou), and marked for the pollination date.

Seeds were harvested at 15, 25, 35, 45, and 55 DAP from Express-617 and 17, 24, 31, 38, 45, 52 and 59 DAP from Hu135, and shock-frozen in liquid nitrogen, and stored at -70 °C. We used ~50 mg of seeds for RNA isolation with the peqGold Plant RNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) following the manufacturer's instructions. The quality of the RNA was checked by agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (ThermoFisher Scientific, Waltham, United States). For the expression analysis, we used 2 µl of cDNA (5 ng/µl concentration) synthesized with the First Strand cDNA Kit (Thermo Fisher Scientific, Waltham, United States). Relative expression was measured using *BnACTIN2* and *BnACTIN7* as internal controls for Express-617 and Hu135, respectively (Supplementary Table 2). The difference between the cycle threshold (Ct) of target genes and the Ct of the control gene ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{control}}$) was used to calculate the normalized expression of target genes.

2.5.4 Searching for SNPs within *BnSFAR* genes

A total of 870 rapeseed genomes were sequenced in our previous project (Wu et al. 2019). All clean reads for each accession were mapped to the 'Darmor-*bzh*' genome (v4.1 genome, <http://www.genoscope.cns.fr/brassicnapus/data/>) using the MEM algorithm of Burrows-Wheeler Aligner (Li and Durbin 2009) (BWA v0.7.5a-r405). The mapping results were processed by sorting and duplicate reads marking SAMTOOLS (Li et al. 2009) (v1.1) and PICARD (<http://broadinstitute.github.io/picard/v1.94>). SNPs were called by the HaplotypeCaller module in GATK and were filtered with the parameters (QD < 2.0 || MQ < 40.0 || FS > 60.0 || QUAL < 30.0 || MQrankSum < -12.5 || ReadPosRankSum < -8.0 -clusterSize 2 -clusterWindowSize 5). The SNPs identified by GATK were further filtered; only the SNPs with a minor allele frequency >5% and <50% were considered as high-quality SNPs. The SNP annotation was performed based on the rapeseed v4.1 genome using the snpEff software (Cingolani et al. 2012). SNPs within exons of annotated *BnSFAR* genes were classified as synonymous SNPs and non-synonymous SNPs and the cumulative effects of non-synonymous SNPs were calculated as TNSV.

2.5.5 CRISPR-Cas mutagenesis and mutant detection

We selected conserved 20 bp targets within exons adjacent to the NGG PAM site separately for *BnSFAR4* and *BnSFAR5*. A BLAST search against the rapeseed reference genome (Darmor-*bzh* version 4.1) was performed to identify putative off-targets. We used the binary vector system, pChimera, and pCas9-TPC for *Agrobacterium*-mediated transformation following the protocol previously published (Fauser et al. 2014). The final pCas9-TPC was transformed into the *A. tumefaciens* strain GV3101 pMP90RK for plant transformation.

Rapeseed hypocotyls of the winter type RS306 were transformed following the protocol described previously (Zarhloul et al. 2006) with minor modifications. Here, we applied 400 mg L⁻¹ ticarcillin and clavulanate for the elimination of *Agrobacterium* and 5 mg L⁻¹ of phosphinotricin for transgenic plant selection. Leaf genomic DNA was isolated from transgenic plants using the standard CTAB method. The presence of the transgene was confirmed after performing PCR using Cas1-F and Cas1-R primers (Supplementary Table 2). We also cloned PCR products amplified with paralog-specific primers from T₁ plants into the pGEM-T vector and transformed them into *Escherichia coli* (DH5α cells, DNA Cloning Service, Hamburg). Single colonies were picked for PCR, and mutations were identified by Sanger sequencing using CLC Main Workbench version 7.6.4 (CLC bio, Aarhus, Denmark).

2.5.6 EMS mutant screening by TILLING

We screened 3,840 M₂ plants of our EMS mutated winter rapeseed Express-617 TILLING population for mutations within *BnSFAR* genes. Paralog-specific primers were developed based on the reference genome Darmor-*bzh* (version 4.1), and specificity was confirmed after Sanger sequencing (Supplementary Table 2). We amplified pooled M₂ plant DNA using the primers labeled with infrared fluorescent dyes (IRD) for mutation screening following the protocol

previously described (Till et al. 2006). Subsequently, we checked samples on agarose gels (1%, 100 V, 10 min) for quality. Pools with expected amplicon size were allowed for heteroduplex formation, followed by *CeII* nuclease digestion. Samples were purified using Sephadex dry G-50 powder (GE Healthcare, Chicago, USA) and separated by polyacrylamide gel electrophoresis on a LICOR 4300 DNA analyzer (<https://www.licor.com>). GelBuddy software was used to identify mutations (Zerr and Henikoff 2005).

2.5.7 Plant materials and growth conditions

The re-synthetic winter rapeseed RS306 was used for *Agrobacterium*-mediated plant transformation. Surface sterilized seeds were germinated under low light conditions for 5-7 days at 24 °C, and hypocotyl explants of 0.5-1.0 cm were used for plant transformation. Rooted T₁ plants were transferred into the greenhouse after acclimation. T₁, T₂, and T₃ plants were grown in 11 x 11 cm pots in the greenhouse (16 h light/ 8 h dark at ~23-24 °C) together with RS306 control. All plants were vernalized for ten weeks (16 h light/ 8 h dark at 4 °C).

Selected M₃ EMS mutants were crossed and backcrossed with Express-617 to produce segregating F₂ and BC₁F₂ progenies. We also crossed M₃ single mutants to produce an F₂ population to select homozygous double mutants and wild-types (Supplementary Table 5). All plants were grown in 11 x 11 cm pots under greenhouse conditions along with non-mutated Express-617 controls. Plants were vernalized for eight weeks. Plants selected for selfing were bagged before flowering to prevent cross-pollination and fertilized with 0.5 g of Compo Blaukorn Classic universal fertilizer (Compo, Münster, Germany) before flowering.

2.5.8 Genotyping and phenotyping of mutant plants

Allele-specific markers were developed to genotype segregating EMS mutant progenies. For each paralog, we used a paralog-specific primer pair along with either a mutant- or wild-type-specific primer pair (Supplementary Table 2). After genotyping with allele-specific primers, only the plants homozygous either for mutant or wild-type alleles were Sanger sequenced to verify the mutations. Plants grown under greenhouse condition were used for phenotyping. Important agronomic traits like seed number, seed weight, and plant height were recorded on a single plant basis. Seeds were aliquoted (50-100 seeds/plant) for seed oil extraction and these samples were cooled in liquid nitrogen immediately and stored at -70°C until use.

2.5.9 Oil and fatty acid measurements

According to standard oil extraction protocols (Manirakiza et al. 2001a), an *n*-hexane microextraction method was developed adjusting to the small sample sizes in our experiments. We used 50-70 mg of finely milled seed powder per sample and extracted the oil twice with 1 ml of *n*-hexane. During the first round, seed powder was weighed into a 2 ml glass vial and extracted for 16 hours with 1 ml of *n*-hexane at room temperature in an overhead shaker. The sample was centrifuged at 4000 g for 10 minutes, and the *n*-hexane supernatant was transferred into a new vial. The pellet was extracted for a second time with 1 ml *n*-hexane for one hour, centrifuged as written above and the supernatants of both extracts were combined. The solvent was removed in a rotary evaporator at 40°C (Bachofer vacuum concentrator) for 2 hours. Total SOC was measured by weighing and calculated in percent on a dry weight basis. The extracted oil was stored at -20°C and used for the FA profile measurements using gas chromatography (Chen et al. 2012b). SOC and the seed FA composition were determined in 870 rapeseed accessions using a near-infrared spectroscopy (ANTARIS II, Thermo Scientific™, WI, USA). Three biological replicates of each accession were measured.

2.5.10 Germination and seedling vigor tests

For seed germination tests, we used T₃ CRISPR-Cas mutant seeds. Seeds were sown on ½ MS medium containing 50 mM NaCl and grown in the dark at room temperature. Plates were kept at

a 15° vertical angle in the dark. Plates were opened daily for ca. one minute to take photos, and ImageJ software (<https://imagej.net/>) was used to analyze root and shoot length five days after sowing.

2.5.11 Oil accumulation and mobilization

For the oil accumulation test, we harvested seeds (100-150 mg) from *BnSFAR4* T₃ mutants and RS306 controls at 25, 35, 45, 55, and 65 DAP. Plants were grown under greenhouse conditions. For the seed oil mobilization test, T₄ *BnSFAR4* mutant and wild-type RS306 seeds were sown on wet Whatmann filter paper, and samples were taken 0, 12, 24, 36, 48, 60, and 72 hours after sowing. After sampling, seeds were shock-frozen in liquid nitrogen and stored at -70°C until oil extraction using n-hexane microextraction.

2.5.12 Oil body analysis

OB size was measured in seeds from mutant and wild-type plants. Fully mature seeds from three biological replicates of each genotype were selected for sectioning. Perpendicular transections were produced, and the sections with the largest oval-shaped surface area were selected for quantification. Three cells located in the middle of a section were selected for the measurement of OB size. The cells were photographed using an OLYMPUS SZ 61 stereomicroscope (Tokyo, Japan). For the observation of oil bodies, electron micrographs were taken using a transmission electron microscope (TEM) (JEM-1230, Tokyo Japan) (Chen et al. 2012b). The software ImageJ 1.52p (<https://imagej.nih.gov/>) was used to measure the surface of the oil bodies. The automatic measurements were improved and corrected by manual quantification.

2.5.13 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA), two-way ANOVA, Tukey's test, or two-tailed unpaired *t* test with GraphPad Prism (version 5.00 for windows): GraphPad Software, San Diego, CA, USA

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2.8 Supplementary data

Supplementary Table 1: Features of the *BnSFAR* genes used in this study

Supplementary Table 2: Primers used in this study

Supplementary Table 3: EMS-induced mutations in *BnSFAR1* and *BnSFAR4* genes

Supplementary Table 4: EMS and CRISPR-Cas mutations used for further studies

Supplementary Table 5: Production of EMS mutants by crossing M₃ plants homozygous for the mutant allele

Supplementary Table 6: Results of the *Agrobacterium*-mediated rapeseed hypocotyl transformation

Supplementary Table 7: Inheritance of CRISPR-Cas mutations in *BnSFAR4* and *BnSFAR5*

Supplementary Table 8: Phenotyping data of EMS and CRISPR-Cas *BnSFAR1*, *BnSFAR4* and *BnSFAR5* mutants

Supplementary Table 9: Seed germination, root, and shoot growth 5 DAS in T₃ lines with *BnSFAR* knock-out mutations and in RS306

Supplementary Figure 1: Distribution of *GDSL* genes across the rapeseed subgenomes and their expression in developing seeds

Supplementary Figure 2: The effect of non-synonymous single nucleotide polymorphisms (SNP) within *BnSFAR* genes on oleic acid content in 870 non-related rapeseed accessions

Supplementary Figure 3: Crossing schemes and pedigrees of plant materials used in this study

Supplementary Figure 4: Screening the rapeseed reference genome for putative *BnSFAR4* and *BnSFAR5* off-target sequences

Supplementary Figure 5: Fatty acid profiles in T₃ seeds of two *BnSFAR4* mutants and the RS306 control

Dataset S1: Seed oil content and oleic acid composition of all accessions

Dataset S2: *BnGDSL* gene expression in developing seed

3 Genomic background selection to reduce mutation load using molecular markers and rapid cycle breeding rapeseed (*Brassica napus*)

3.1 Introduction

Rapeseed (*Brassica napus*) as an important oil crop grown worldwide, has adapted to the different climates, forming three main ecotypes, winter, spring, and semi-winter rapeseed. Winter rapeseed is mainly grown in Europe and only flowers after a period of cold exposure. In contrast, spring rapeseed does not need vernalization for flowering, and it is mainly grown in Canada and Australia. Semi-winter rapeseed is widely cultivated in China and requires moderate vernalization. Despite the short domestication history, these three ecotypes have a substantial amount of genetic diversity (Lu et al. 2019; Wang et al. 2014).

The demand for rapeseed increased over the years, as rapeseed is not only used for the production of edible oil, but also as a protein source for feed, biofuel and as industrial raw material (Friedt et al. 2018). As winter and semi-winter rapeseed require vernalization for flowering, the generation cycle takes longer than spring rapeseed. In general, under field conditions, winter rapeseed requires one year to complete its life cycle. However, winter rapeseed needs only six to seven months to complete its life cycle under the greenhouse condition enabling only two generations per year. Conversely, spring rapeseed requires 3-4 months to complete one generation under greenhouse yielding three to four generations per year. Typically, 4-6 generations are a prerequisite to develop a genetically stable line in line breeding to test the agronomically important traits (Lenaerts et al. 2019). Thus, rapid generation cycling should be considered.

Efforts have been made to reduce the generation cycles in *Brassica* crops (Ferrie and Mollers 2011; Williams and Hill 1986). The *Brassicaceae* plant family has around 3,709 species, including economically important crops like *Brassica napus*, *Brassica rapa*, *Brassica oleracea*, and *Brassica juncea*, and also includes the model plant *Arabidopsis thaliana* (Lysak and Koch 2011). Early flowering *Brassica* plants were identified from a worldwide collection of over 2,000 *Brassica* accessions, and plants with shortened generation cycles were selected (Williams and Hill 1986). They were termed rapid-cycling *Brassicaceae*. In that study, *Brassica campestris* plants were found, which could go up to ten generations per year grown at 24°C under continuous high light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$). Notably, *B. napus* plants were identified with 55 days of generation cycle, which can produce six generations per year under greenhouse conditions with high light at 24°C (Williams and Hill 1986).

A recent study claimed to accelerate crop research and breeding by a method called “speed breeding” (Watson et al. 2018). Among other species, they grew canola (*B. napus*) under prolonged photoperiods to a day length of 22 hours in a temperature-controlled glasshouse fitted with high-pressure sodium lamps. With these controlled-environment growth conditions, they were able to reduce generation cycles for canola (73 ± 9 days). Thus four generations per year could be achieved. To accelerate breeding, breeders use different methods and tools like single seed descent (SSD) in pedigree breeding and doubled haploid (DH) technology (Lenaerts et al. 2019). Watson et al. (2018) also evaluated the SSD method for wheat and barley and suggested that integration of speed breeding and SSD can further accelerate the generation cycle.

Recurrent backcrossing is commonly used to transfer genes, which are responsible for favorable agronomic traits from a donor line to the recurrent parent (Hasan et al. 2015). Typically, the recurrent parent is the elite material. Usage of the molecular markers in genomic background selection enables efficient detection of the target gene while retaining the original characters of the recurrent parent (Lenaerts et al. 2019). Applying molecular markers for selection of recurrent parent reduce the time required for backcrossing (Herzog and Frisch 2011). Over the last years, different markers have been used for genomic selection of rapeseed (Obermeier and Friedt

2015). Of these, Amplified fragment length polymorphism (AFLP) markers were developed and employed in rapeseed genotyping (Vos et al. 1995). AFLP is a dominant genetic markers, prior knowledge of sequence information is not a prerequisite, and it is a low-cost method (Bonin et al. 2005).

High throughput genomic technologies with lower sequencing costs started a new era of advanced marker-assisted genomic selection in rapeseed breeding (Snowdon and Luy 2012). Genotyping by hybridization, such as the Infinium array, genotyping by sequencing (GBS), and whole-genome re-sequencing have rapidly developed and become popular techniques in rapeseed marker-assisted selection (Obermeier and Friedt 2015; Snowdon and Luy 2012). First high throughput genotyping reported for rapeseed using an 8K Illumina Infinium array, and 7322 SNPs markers obtained from publically available EST (expressed sequenced tag) data and Tapidor x Ningyou7 DH population, were first used in rapeseed to construct a high-density SNP-based genetic map (Delourme et al. 2013; Delourme et al. 2018). Later, a rapeseed 6K Illumina Infinium™ array was designed using a total of 1,070 samples (1,012 *B. napus*, 46 *B. rapa*, 4 *B. oleracea* and 10 *B. nigra*), including in total 5,306 SNPs, which were equally distributed over the A and C subgenome (Dalton-Morgan et al. 2014). Following this, a high-density *Brassica* 60K Illumina Infinium™ array was designed containing 52,157 SNPs (Clarke et al. 2016; Edwards et al. 2013). Since the release of the commercial 60K Infinium array, it was applied for diverse purposes, genotyping of germplasm collections (Mason et al. 2015), genome-wide association studies for different traits (Hatzig et al. 2015; Li et al. 2014; Wei et al. 2016; Xu et al. 2016) and QTL mapping (Qu et al. 2015b; Zhang et al. 2015).

In this study, we aimed to select mutant plants with reduced background mutations caused by EMS mutagenesis, crossing with a fast flowering spring rapeseed variety. Furthermore, we implemented a marker-assisted genomic background selection using molecular markers. We demonstrate that marker-assisted genomic selection is efficient and accurate for background genomic selection, applying two molecular marker systems: AFLP and a 15K Illumina Infinium™ array.

3.2 Materials and methods

3.2.1 Plant material and crossing scheme

We used EMS induced single mutants (Table 6) and crossed them with Express-617. The F₁ plants were backcrossed twice with spring genotype Peace following the crossing scheme described in Supplementary Figure 6 to produce BC₁ populations. BC₁ from *BnSFAR4* were self-pollinated to produce segregating BC₁F₂ populations, and some plants were further backcrossed with Peace to produce BC₂.

Seed sowing was done in 3 x 3 cm 35-multi-well palettes in the greenhouse. We grew plants in 9 x 9 cm pots for seed production and 11 x 11 cm pots for phenotyping. All these plants were grown under greenhouse conditions (16 h light/ 8 h dark, 23-24°C). Plants requiring vernalization were transferred to the cold chamber (16 h light/ 8 h dark, 4°C) for eight weeks and then transferred back to the previous conditions. Plastic selfing bags were mounted after bolting to control pollination.

Table 6: EMS-induced mutants used for crosses

M ₃ seed code	Gene name	Mutation	Allele name	AA change
170053	<i>Bna.SFAR1.C04</i>	<i>bna.sfar1C04</i> C491T	A ₁	Gln → Stop codon
170043	<i>Bna.SFAR1.Ann</i>	<i>bna.sfar1.Ann</i> G412A	B ₁	Gly → Glu
170061		<i>bna.sfar1.Ann</i> G642A	B ₂	Splice site
170049	<i>Bna.SFAR4.A06a</i>	<i>bna.sfar4.A06a</i> C277T	C ₁	Gln → Stop codon
170063	<i>Bna.SFAR4.C03a</i>	<i>bna.sfar4.C03a</i> C277T	D ₁	Gln → Stop codon
170041	<i>Bna.SFAR4.A06b</i>	<i>bna.sfar4.A06b</i> G540A	E ₁	Trp → Stop codon
170067	<i>Bna.SFAR4.Cnnb</i>	<i>bna.sfar4.Cnnb</i> G539A	F ₁	Trp → Stop codon

3.2.2 DNA isolation and molecular marker analysis

Leaf genomic DNA was isolated by a standard CTAB method (Saghaimarouf et al. 1984). First, we used allele-specific markers mentioned in Supplementary Table 2 for genotyping BC₁ plants to select mutant genotypes. PCR was performed under following cycling conditions: 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 64-66°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 5 minutes final elongation, and PCR products were separated on agarose gels. PCR products carrying the mutant alleles were sequenced by Sanger sequencing to confirm the mutations.

We used an AFLP genotyping method as described by Vos et al. (1995) with minor modifications. First, isolated leaf genomic DNA was normalized to 5 ng/μl and 100 ng of DNA was restricted with *MseI* and *PstI* (ThermoFisher Scientific, Waltham, United States) following the manufacturer's instructions. Next, adaptors (Supplementary Table 10) were ligated by mixing 1 μl of 5 pmol/μl *PstI*-adapter, 1 μl of 50 pmol/μl *MseI*-adapter, 0.1 μl of 100 mM ATP, 0.4 μl of 2U T4 DNA-ligase, 2 μl of 5x RL-buffer and 5.5 μl of H₂O to the reaction mixture, and samples were incubated for three hours at 37°C using an incubator. After the ligation, samples were checked on the agarose gels, and pre-amplification was done using P01, and M01 primers (Supplementary Table 10). For each reaction, we mixed 2 μl of 10x reaction buffers, 2 μl of 2mM dNTPs, 0.6 μl of 50 ng/μl of P01 primer, 0.6 μl of 50 ng/μl of M01 primer, 0.2 μl Taq polymerase, and 5 μl of the sample. PCR was performed with an initial denaturation at 94°C for 30 seconds, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 1 minute, 23 cycles and final elongation at 72°C for 5 minutes. After the PCR, 180 μl of T0,1E buffer was added to the pre-amplified samples. The main amplification was performed using 5 μl of pre-amplified samples as a template. We used six different primers (Supplementary Table 10) for the main amplification, which were previously used in the rapeseed genotyping (Klemm 2017). The *PstI* primers labeled with Infrared Fluorescent Dye (IRD) 700 were used in order to detect the PCR products on the polyacrylamide gel.

After the main amplification, 0.6 μl of samples were mixed 1:1 with formamide loading dye and separated on 6.5% polyacrylamide gels using a LICOR 4300 DNA analyzer (LI-COR Biosciences) for 4:30 hours at 1500 V, 40 mA and 40 W. Gels were analyzed using the GelBuddy software (Zerr and Henikoff 2005). Here, first, gel images were imported, and parameters were adjusted according to the manufacturer's instruction. Then, polymorphic markers between parents were marked, and each individual scored for the presence or absence of the respective markers.

3.2.3 Genotyping with the 15K Illumina Infinium™ SNP array

The Genomic DNA of mutant plants was normalized to a concentration of 50-200 ng/μl. From the normalized DNA, 20 μl aliquots were sent for genotyping (TraitGenetics GmbH, Gatersleben, Germany) using the *Brassica* 15K Illumina Infinium™ SNP array (Illumina, San Diego, CA).

We received the SNP genotyping data of BC₁ plants from TraitGenetics GmbH in the Excel file format (Supplementary data on CD). First, we converted the Excel file format to the Hapmap (Haplotype Map) format by using customized R scripts. Using TASSEL (Trait Analysis by aSSociation, Evolution and Linkage) (Bradbury et al. 2007), the Hapmap file was converted into the VCF (Variant Call Format) format (Danecek et al. 2011). We filtered SNPs for minor allele frequencies lower than 0.001 using the VCFtools (Danecek et al. 2011). With this filtering, we attained a VCF file with high-quality SNPs.

We performed Principal Component (PC) analysis using the SNPRelate package (Zheng et al. 2012) in R 3.6.1 (R Core Team 2019). First, two PCs were plotted using the ggplot2 package in R. Next population structure was analyzed using the ADMIXTURE version 1.3.0 (Alexander et al. 2009). The number of clusters (*k*) was predefined as 2. The default settings of the ADMIXTURE program were used with 1000 bootstraps, and Q matrix containing ancestry coefficients was obtained. Admixture proportions were visualized using the ggplot2 package in R.

Using TASSEL software, we converted all the alleles into the ABH file format, giving ‘A’ allele for Peace, ‘B’ allele for Express-617, ‘H’ for heterozygous alleles, and genotypes were exported to a CSV (comma-separated values) file. The resulting genotype file was used to calculate the share of the Peace genome.

3.2.4 Phenotyping for seed oil content

BC₁ plants selected using AFLP for the highest share of Peace were self-pollinated to produce segregating BC₁F₂ progenies. Allele-specific markers (Supplementary Table 2) were used for genotyping, and only the plants either homozygous for mutant or wild-type allele were selected and grown with Peace and Express-617 controls. Seeds harvested from plants grown under greenhouse conditions (16 h light/ 8 h dark, 23-24°C) were used to measure SOC after n-hexane microextraction. We used 50-70 mg of finely milled seed powder from each sample for n-hexane microextraction, adjusting the previously described protocols (Manirakiza et al. 2001b) to small sample size. First, 1 ml of n-hexane was added into the 2 ml glass vials containing the weighed powdered seed sample and extracted for 16 hours at room temperature in an overhead shaker. Then, samples were centrifuged for 10 minutes at 4000 g, and the supernatant was transferred into a new 2 ml glass vial. Again, 1 ml of n-hexane was added to the pellet for a second extraction and shaken for one hour. Then, the samples were centrifuged, and the supernatant was combined with the first extract. The solvent was evaporated by a rotary evaporator at 40°C (Bachofer vacuum concentrator) for 2 hours. By weighing, total SOC was measured and calculated in percentage on a dry weight basis.

3.3 Results

3.3.1 Production of backcross generations

We identified 163 EMS induced mutations in *BnSFAR1* and *BnSFAR4* genes (Supplementary Table 11). Of these, we considered only seven different EMS induced single mutants (Table 6) of *BnSFAR1* (*Bna.SFAR1.C04* and *Bna.SFAR1.Ann*) and *BnSFAR4* (*Bna.SFAR4.A06a*, *Bna.SFAR4.C03a*, *Bna.SFAR4.A06b*, and *Bna.SFAR4.Cnnb*) for crossing with the spring-type cultivar, Peace. For the initial crossing with Peace, we used F₁ hybrids resulting from a cross with Express-617 wild-type (Supplementary Figure 6) because these plants had reduced background mutations. We wanted to know whether F₁ plants from a single cross with Peace

would flower without undergoing vernalization conditions. Therefore, we grew the F_1 of this cross with and without vernalization. We tested 20 plants from each mutant genotype under both conditions. F_1 plants grown under the greenhouse condition (16 h light/ 8 h dark, 23-24°C) without vernalization did not flower even after four months. After eight weeks of vernalization (16 h light/ 8 h dark, 4°C), all F_1 plants flowered between two to three weeks after transferring to greenhouse conditions. Mutant F_1 plants were backcrossed with Peace, and the resulting BC_1 plants (Table 7) grown under greenhouse conditions (16 h light/ 8 h dark, 23-24°C) flowered without vernalization.

3.3.2 Genomic background selection using AFLP markers

We used plants from BC_1 generation for genomic background selection by AFLP markers to select the plants with a high genomic background of Peace. We chose seven EMS mutants mentioned in Table 7 for AFLP marker genotyping. First, 30 BC_1 plants from each mutant genotype were genotyped using allele-specific markers (Supplementary Table 2) to select plants with the mutant allele, because the BC_1 population segregates for heterozygous and wild-type alleles. We further confirmed the mutations after the sequencing of PCR products from heterozygous plants by Sanger sequencing. We analyzed the inheritance of the mutant allele in the BC_1 generation and observed that segregation was as expected (Table 7). In total, 104 heterozygous mutant plants were identified in seven different mutant genotypes (Table 7), and these plants were genotyped by AFLP markers (Figure 10).

Table 7: BC₁ plants used for AFLP marker genotyping. The respective alleles from Peace marked as suffix 'P'.

BC ₁ seed code	F ₁ seed code	<i>B. napus</i> paralog	Genotype of the heterozygous plants	Total number of genotyped BC ₁ plants		Number of plants		χ^2 value	Number of analyzed AFLP markers	Number of polymorphic markers	Range of Peace genome %	Range of Express-617 %
				BC ₁ plants	Wild-type	Wild-type	Heterozygous					
180898	171795	<i>Bna.SFARI.C04</i>	<i>A₁A_p</i>	30	14	14	16	0.13 ^a	26	26	64.0-80.8	19.2-36.0
180899	171796	<i>BnaA.SFARI.Ann</i>	<i>B₁B_p</i>	30	20	20	10	3.33 ^a	26	26	60.0- 80.8	19.2-40.0
180900	171797		<i>B₂B_p</i>	30	20	20	10	3.33 ^a	26	26	65.4-76.0	24.0-34.6
180901	171798	<i>Bna.SFAR4.A06a</i>	<i>C₁C_p</i>	30	12	12	18	1.20 ^a	25	25	58.3-79.2	20.8-41.7
180902	171799	<i>Bna.SFAR4.C03a</i>	<i>D₁D_p</i>	30	11	11	19	2.13 ^a	25	25	50.0-80.0	20.0-50.0
180903	171800	<i>Bna.SFAR4.A06b</i>	<i>E₁E_p</i>	30	15	15	15	0.00 ^a	20	20	57.9-80.0	20.0-42.1
180904	171801	<i>BnaC.SFAR4.Cnb</i>	<i>F₁F_p</i>	30	14	14	16	0.13 ^a	20	20	40.0-75.0	25.0-60.0

^a1:1 segregation, χ^2 (df=1 at $p = 0.05$) = 3.84

Four primer combinations yielded in a total of 26 informative AFLP fragments, which were polymorphic between Express-617 and Peace. Among them, 17 were specific for Peace, while 11 were specific for Express-617 (Supplementary Table 12). However, five polymorphic fragments either to Peace or Express-617 did not segregate in the progenies. In other words, these five AFLP fragments were present in all the BC₁ plants.

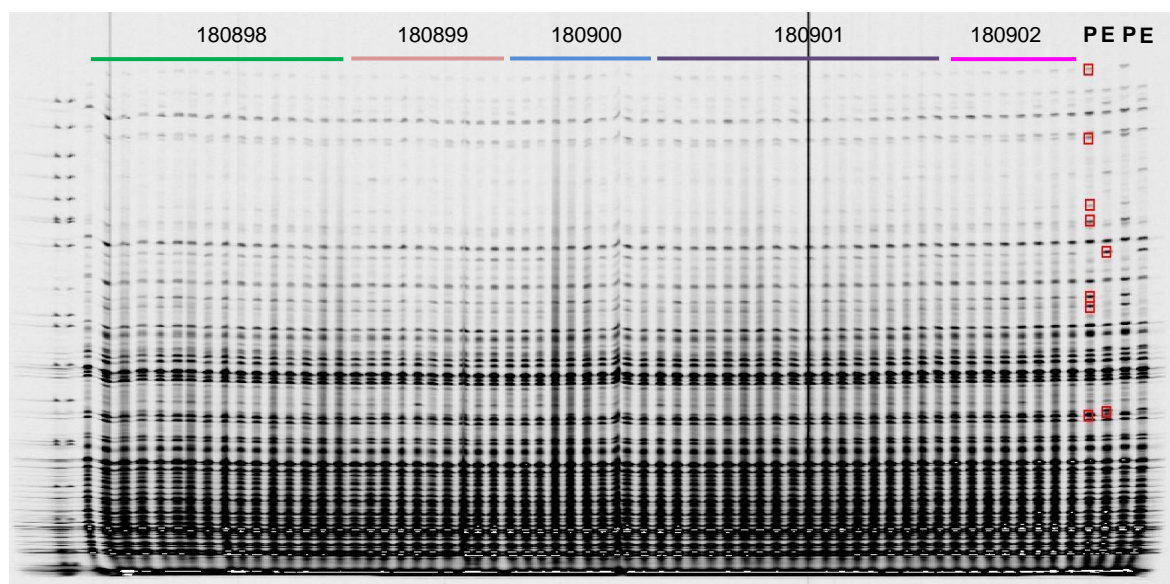


Figure 10: Amplification pattern obtained for BC₁ plants using the M31+P32 primer combination. Only part of the samples was separated on this polyacrylamide gel using the LICOR 4300 DNA analyzer. Each lane represents one single plant of the respective seed codes. P: Peace, E: Express-617. Red boxes indicate the dominant fragment, either for Peace or Express-617.

We scored each BC₁ plant for the presence or absence (Figure 10) of the specific AFLP markers. The Peace fragment was marked as allele ‘A’, the Express-617 fragment as allele ‘B’. We calculated the share of Peace or Express-617 as the percentage of total informative markers (Table 7). We expected to have individual BC₁ plants with more than 75% share of Peace genomic background and indeed observed individuals with 80.8% of Peace genomic background (Table 7).

3.3.3 15K rapeseed SNP chip array for marker-assisted background selection

Since AFLP genotyping is laborious and did not yield enough markers, we chose the 15K Illumina InfiniumTM SNP array to genotype BC₁ and tested a higher number of plants. We exclusively selected *BnSFAR4* subfamily-a single mutants because *BnSFAR4.a* double mutants showed the highest increase in oil content in our study (Figure 8). First, we selected BC₁ plants carrying the mutant alleles using allele-specific markers (Supplementary Table 2) as in AFLP genotyping. In total, 85 and 109 heterozygous plants with mutant alleles were identified by genotyping 180 and 300 of BC₁ plants for *Bna.SFAR4.A06a* and *Bna.SFAR4.C03a*, respectively. We examined the inheritance of mutant alleles in BC₁ generation for both mutant alleles and observed that the segregation pattern was in accordance with Mendelian segregation for *Bna.SFAR4.A06a* while *Bna.SFAR4.C03a* showed deviating segregation (Supplementary Table 13). We genotyped 85 and 99 plants of *Bna.SFAR4.A06a* and *Bna.SFAR4.C03a*, respectively, using the 15K Illumina InfiniumTM array along with M₃ mutant parents, Express-617 as the EMS donor and Peace as the recurrent parent. Backcrossed plants of *Bna.SFAR4.A06a* are named as progeny A (seed code: 180901) and backcrossed plants of *Bna.SFAR4.C03a* are named as progeny C (seed code: 180902).

The Illumina 15K InfiniumTM array carries in total 13,714 SNPs. Of these, in total 13,416 SNPs were functional in this genotyping. We filtered SNPs for minor allele frequency (MAF) for 0.001

to exclude sequence artifacts and monomorphic markers and obtained 7,686 informative SNPs (Table 8). Only 6,887 SNPs were polymorphic between Peace and Express-617.

Table 8: SNP information

	Total number of SNPs
Number of markers on the array	13,714
Number of functional markers for BC ₁ genotyping	13,416
Number of markers after filtering for MAF 0.001	7,686

Then, we performed a principal component analysis (PCA), in order to understand the population structure. PCA was carried-out separately for both progenies. The first PCA component (PC1) explained 45.62% of the total genetic variation, while the second PCA component (PC2) explained only 8.90% of the total genetic variation in the progeny A (Figure 11A). In progeny C, PC1 and PC2 explained 44.21% and 8.60% of the total variance, respectively (Figure 11B). We observed four main clusters for Express-617, Peace, parents, and BC₁ plants in both progenies. As expected, backcrossed plants and recurrent genotype Peace had less distance, indicating that backcrossed plants carried a higher percentage of Peace background. Express-617 and M₃ parents were clustered along with the second principal component representing low genetic variation between these genotypes. We observed a higher variation among parent A (170049) samples compared to parent C (170063), Express-617, or Peace (Figure 11A). This may be due to the variation among the technical replicates.

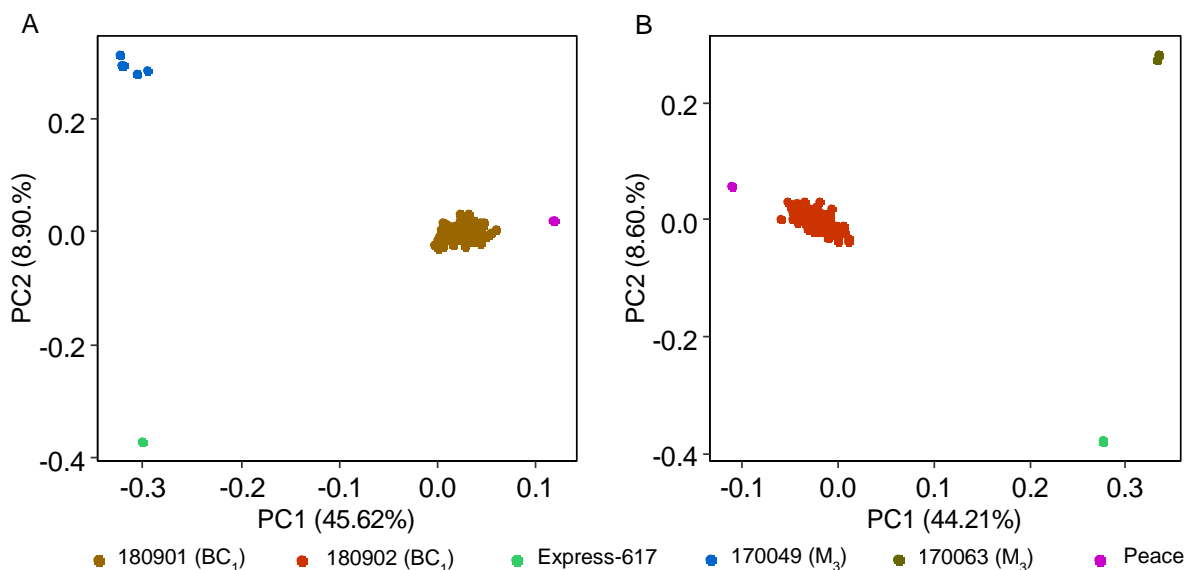


Figure 11: Principle component analysis (PCA) for progeny A (seed code: 180901) (A) and progeny C (seed code: 180902) (B). PCA was performed for both progenies separately, and PC1 and PC2 were displayed using ggplot2 package in R. 170049, and 170063 are the mutant parent for progeny A and progeny C, respectively.

The population structures of the progenies were calculated using 7,686 SNPs by ADMIXTURE, and clustering was performed with predefined $k = 2$ (Figure 12). We observed a clear separation of individuals according to their share of the parent genome. We further calculated the proportion of accumulated Peace and Express-617 background for every single mutant plant as follows.

$$\% \text{ of parent genome} = \frac{(\text{homozygous allele} \times 2) + (\text{heterozygous allele} \times 1)}{\text{Total number of alleles}} \times 100$$

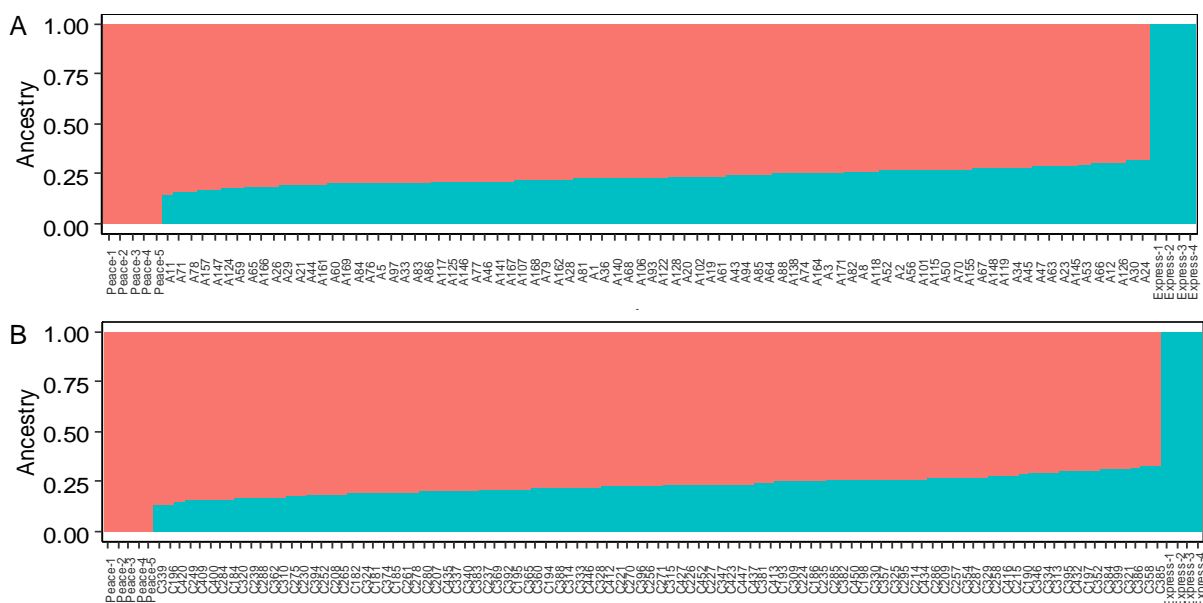


Figure 12: Population structure based on $k = 2$ for progeny A (180901) (A) and progeny C (180902) (B). Each individual is represented by a vertical bar and color segment showing the proportion of the donor and recurrent parent genome

The Peace genomic background for mutant progeny A and mutant progeny C in individual plants ranged between 68.3 - 83.7% and 65.7 - 84.5 %, respectively, while Express-617 background varied between 16.3 - 31.7% and 15.5 - 34.3%. As expected, we observed an average Peace genomic background for progeny A and progeny C of 75.4% and 74.8%, respectively, and an average Express-617 genomic background for progeny A and progeny C of 24.6% and 25.2%, respectively. In total, 50 plants in progeny A and 48 plants in progeny C had more than 75% of recurrent parent (Peace) genomic background (Table 9). These plants are suitable for further crossing and backcrossing

Table 9: 10 BC₁ plants with the highest share of the Peace genome

Seed codes	% of Peace genome	% of Express-617 genome	Seed codes	% of Peace genome	% of Express-617 genome
Progeny A (180901)			Progeny C (180902)		
190860	83.7	16.3	190997	84.5	15.5
190893	82.8	17.2	190948	83.7	16.3
190897	82.2	17.8	191027	82.7	17.3
190927	82.2	17.8	191023	81.8	18.2
190930	81.8	18.2	191022	81.7	18.3
190883	81.3	18.7	190980	81.5	18.5
190918	80.9	19.1	191006	81.2	18.8
190888	80.8	19.2	190964	81.0	19.0
190934	79.8	20.2	190963	80.6	19.4
190869	79.5	20.5	190983	80.5	19.5

3.3.4 Seed oil measurements in single mutants

Based on the AFLP marker genotyping, BC₁ mutant plants of all *BnSFAR4* paralogs with the highest share of the Peace genomic background were selected. These plants were self-pollinated

to produce segregating BC₁F₂ populations. Then, we selected homozygous single mutants and plants with the wild-type alleles carrying the same background mutation load, as well as Peace and Express-617 for SOC measurements. We did not observe significant differences in SOC between homozygous single mutants and plants with wild-type alleles for all the mutant genotypes (Figure 13). SOC did not significantly vary between homozygous single mutants and Peace controls, but between Peace and Express-617.

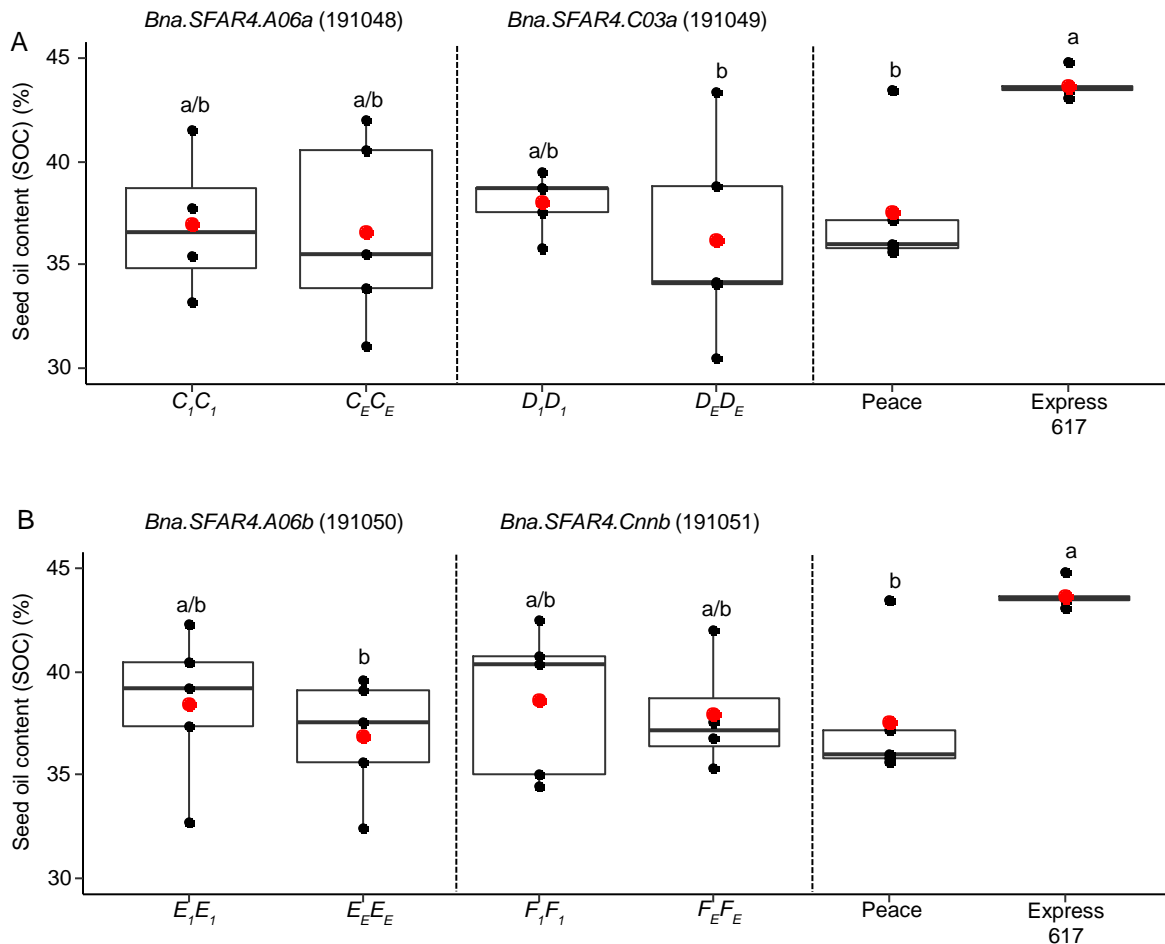


Figure 13: Seed oil content (SOC) in segregating BC₁F₂ *BnSFAR4* subfamily-a (A) and subfamily-b (B) mutants. One-way ANOVA was performed at $p < 0.05$, and grouping was done using the Tukey test at $p < 0.05$. The red dot in the box plot shows the mean SOC. Different letters (a,b) indicate significant differences.

3.4 Discussion

A long generation time of crop species is a bottleneck in breeding and applied research. We produced mutant plants with short generation time by crossing with fast flowering spring rapeseed and used molecular marker-assisted genomic selection in BC₁ to select mutant plants with a high share of the recurrent parent genome. In this study, we demonstrated the potential of genomic background selection to reduce EMS-induced background mutations and accelerate the generation cycle in rapeseed.

The problem arising with our EMS mutagenized population is a high mutation background load in plants, and this is a severe drawback for functional analysis. Therefore, background mutations should be eliminated with subsequent backcrossing either with EMS donor or elite germplasm. In our study, EMS donor is a winter rapeseed type and requires a minimum of six months to complete one generation under the greenhouse condition. Repeated backcrossing is required to produce a mutant line with a low mutation background load, and conventional methods take time. Here, we produced BC₁ plants after crossing with a spring type recurrent parent within nine months, and with this, we could save three months compared to a cross with a winter-type.

Applying the single seed descent (SSD) method under highly controlled growth conditions has reduced generation time in wheat and barley (Watson et al. 2018). Harvesting and germination of immature seed and thereby shortening the generation cycle have been proven for pigeon pea, faba bean, and wheat (Mobini et al. 2015; Saxena et al. 2019; Watson et al. 2018). Hence, there is a potential for further reducing the generation cycle, at least by three to four weeks in our rapeseed populations by the combination of SSD with immature seed harvesting and germination under greenhouse conditions.

After the first cross with Peace, the F₁ plants still required vernalization for flowering. These results indicate that flowering time alleles of winter rapeseed are dominant in F₁ plants. In our experiment, we provided eight weeks of vernalization at 4°C and long-day conditions (16 h light/8 h dark). In *Arabidopsis*, the regulation of vernalization temperature (0-14°C) and duration (4-12 weeks) resulted in shifting flowering time, depending on the accessions used (Duncan et al. 2015). Thus, a change of the temperature regime during vernalization might accelerate the flowering of F₁ plants. A prolonged photoperiod or continuous light supply has also reduced the generation time in canola (Ghosh et al. 2018; Watson et al. 2018; Williams and Hill 1986). Adapting these conditions would help to reduce the generation time of F₁ plants and further generations. However, BC₁ plants did not require vernalization for flowering. Spring rapeseed cultivars have been tested for vernalization response at 4°C (Murphy and Scarth 1994) and found that early flowering occurred when spring rapeseed was vernalized. Hence, this strategy can also be applied to shorten the flowering time in BC₁ and subsequent generations.

We used two different marker systems, PCR-based AFLP markers and hybridization-based SNPs to genotype mutant BC₁ plants. First, we selected for the mutant plants in the progenies using allele-specific markers. This was necessary, as BC₁ plants segregated for mutant and wild-type genotypes. Recovery of the recurrent parent genome depends on the number of markers used (Frisch and Melchinger 2005). Also, equal distributions of the markers are essential for efficient selection. We could select plants with 81% of the Peace genomic background with AFLP marker genotyping. However, the result might be biased due to the low number of AFLP markers. Therefore, we chose the 15K Illumina Infinium™ SNPs array for genotyping. SNPs arrays are more informative in comparison to AFLP, as we could genotype each individual in the BC₁ progenies for heterozygosity and homozygosity of Peace or Express-617 alleles by 15K Illumina Infinium™ SNPs array, whereas analysis of zygosity was not possible with AFLP genotyping. Furthermore, SNPs array genotyping is less laborious compared to AFLP markers, as in the latter restrictions, PCR and polyacrylamide gel separation were required for AFLP genotyping. Sample preparation for SNPs array is relatively easy and simple.

The theoretically expected value of the recurrent parent genome in the BC₁ progeny is an average of 75%. In our study, we observed average shares of the Peace genome of 75.4% and 74.8% for progeny A and progeny C, which is in accordance with expected values and between 68.3 – 83.7 % and 65.7 – 84.5% of individuals which demonstrated the selection success. It also suggested that no self-pollination occurred during the backcrossing, as we obtained a 75% average of the Peace genome in both populations. Finally, we already could select plants with 84% of the recurrent parent genome in BC₁. Plants with the highest share of the Peace genome can be used for further studies. Thus, we demonstrated a novel use of rapeseed SNP array for genomic background selection, in addition to its application in genome-wide association mapping (Li et al. 2014; Xu et al. 2016) or QTL mapping (Fletcher et al. 2015; Qu et al. 2017).

The combination of winter and spring rapeseed can be employed as a strategy to accelerate generation time in rapeseed. Moreover, marker-assisted genomic selection is highly efficient in the selection of the recurrent parent genome and, SNP arrays provide more accurate information than AFLP genotyping.

3.5 References

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3.6 Supplementary Data

Supplementary Table 10: Adaptor sequences used for AFLP

Supplementary Table 11: EMS mutations detected in six of *BnSFAR1* and *BnSFAR4* paralogs. Positions of the mutations are given related relative to the adenine of the start codon “ATG”.

Supplementary Table 12: Markers used for AFLP genotyping

Supplementary Table 13: Inheritance of *Bna.SFAR4.A06a* and *Bna.SFAR4.C03a* mutant alleles. BC₁ plants were analyzed for inheritance.

Supplementary Figure 6: Crossing scheme for single and double mutant production

4 Closing discussion

Increasing seed oil content (SOC) is a major aim in rapeseed breeding, and it is a quantitative trait controlled by many genes. Both seed oil biosynthesis and degradation determine the final oil amount in the seeds. Therefore, it is essential to study not only the synthesis but also the oil degradation pathway. In this study, the main focus was to increase SOC by knocking-out genes involved in lipid degradation and characterize the resulting phenotypes under greenhouse conditions.

The results of my study provide a major contribution to understanding the genes involved in oil degradation during seed maturity of rapeseed. I studied the GDSL lipase genes *BnSFAR1*, *BnSFAR2*, *BnSFAR3*, *BnSFAR4*, and *BnSFAR5* in rapeseed. For the first time, I demonstrated that loss of functions of lipid degrading enzymes (*BnSFARs*) increases SOC significantly in an oil crop without conferring adverse effects on seed germination, seed vigor, and oil mobilization. Moreover, my investigation of oil bodies in mutant and wild-type seeds showed that oil body size increased in mutants due to the reduced oil (triacylglycerol) degradation. I showed that CRISPR-Cas was more efficient than random mutagenesis if the aim is to knock-out multiple genes in a polyploid crop like rapeseed (Chapter 2). An EMS-induced mutated rapeseed population is an excellent resource to introduce new alleles for breeding in countries where the use of GMOs is legally constrained. However, a high number of background mutations is one of the major drawbacks. I demonstrated that the use of spring rapeseed as the recurrent parent in a backcrossing program could accelerate breeding by reducing the generation cycle. Furthermore, the application of a molecular marker-assisted genomic background selection was an efficient way to select progeny with reduced background mutations within a short period (Chapter 3).

4.1 Identification of *BnSFAR1-BnSFAR5* homologs

At the beginning of my work, I searched for homologous genes of *AtSFAR1-AtSFAR5* in the rapeseed genome. I used both genomic DNA and amino acid sequences from *Arabidopsis* as queries to perform a BLAST search against the rapeseed genome database. Amino acid sequences are more conserved than nucleotide sequences because of the degeneracy of codons, in which various codon combinations can code for the same amino acid. As *SFAR* genes belong to a large family of GDSL lipases, with 105 genes in *Arabidopsis* (Lai et al. 2017), the BLAST search resulted in many hits. Considering different criteria, such as 0 e-value, > 80 % gene identity, and the presence of conserved domains, 12 paralogs were identified in the rapeseed genome, which were homologous to the *AtSFAR1-AtSFAR5* genes in *Arabidopsis*. Gene copy number variation between different rapeseed cultivars has been reported in previous studies (Schiessl et al. 2017). As I used Darmor-*bzh* as the reference for homolog gene findings, there was a risk of losing *BnSFAR* homologs present in other genotypes. Therefore I performed the BLAST search also at the NCBI database, where the reference genome is the semi winter rapeseed cultivar ZS11, to avoid the risk not to find all the homologous genes for *AtSFAR1-AtSFAR5*.

Not all selected 12 homologs might be functional, i.e. expressed, in rapeseed. The expression profile of *AtSFAR1-AtSFAR5* genes in *Arabidopsis* at different stages of seed development had been published (Chen et al. 2012b). All 5 *SFAR* genes of *Arabidopsis* were expressed during seed maturation with a maximum at 14 DAP corresponding to 45 DAP in rapeseed. For my study, it was important to figure out which genes were highly expressed in the different seed developmental stages to select the best candidates for random and targeted mutagenesis. According to my hypothesis, I expected the highest *BnSFAR* gene expression at the onset of seed desiccation. I found all kinds of patterns starting from early expressed *Bna.SFAR4.A06a* and *Bna.SFAR4.C03a* to low expression at the end or low expression at the beginning for *Bna.SFAR4.A06b* and *Bna.SFAR4.Cnnb* to highest expression at the end of the seed development. Two *BnSFAR1* homeologs also showed relatively high expression levels compared to other paralogs of *BnSFAR2*, *BnSFAR3*, and *BnSFAR5*. The high RNA expression does not

necessarily result in a high protein concentration or enzyme activity. In this case, the expression pattern was more important. Based on the expression level and the pattern, six paralogs were selected for gel-based mutation screening. A recently published transcriptome data of low and high oil content in semi-winter-type inbred lines (Shahid et al. 2019), showed expression patterns similar to the ones I observed in the winter rapeseed, Express-617. Interestingly, in that study, *BnSFAR1* and *BnSFAR4* gene expressions were higher in the low oil content inbred line as compared to the high oil content inbred line.

It is speculated that there are around 25 *SFAR* genes in *Arabidopsis* (Lixi Jiang, Zhejiang University, personal communication), which is expected to result in many homologous genes (minimum 50) in the rapeseed genome. Conserved sequence motifs have been used to find the genes conserved across species and within the same genome (Wong et al. 2015). Therefore, I suggest to search for conserved motifs within known *SFAR* genes, and this would help to find new *SFAR* genes among the GDSL lipases family. The main question is that are all of these genes involved in seed oil degradation, or have these genes gained new functions? Therefore, it is important to characterize their functions in rapeseed. Candidate gene association study would help to identify genes, which have a significant impact on SOC. Moreover, a whole-genome transcriptome analysis of developing seeds and siliques of low and high oil content cultivars would assist in choosing the genes associated with oil degradation. Previous GWAS studies focused on the final oil content (Korber et al. 2016; Lu et al. 2019). I propose to perform a GWAS for measuring oil degradation, by phenotyping SOC at different seed development stages to identify candidate genes for oil degradation. Furthermore, I suggest a functional analysis using targeted mutagenesis by CRISPR-Cas9 with a multiplexing approach to knock-out multiple gene families simultaneously.

4.2 EMS and CRISPR-Cas9 induce mutations in *BnSFAR* genes

I successfully induced targeted mutations in *BnSFAR4* and *BnSFAR5* genes using the CRISPR-Cas9 mediated gene targeting with a transformation efficiency of 0.2 - 1.1%. *BnSFAR4* T₁ plants were edited in all four paralogs. I detected only homozygous and biallelic mutations in the leaf DNA of T₁ and a stable Mendelian inheritance into T₂. Also, I found five independent T₁ plants with gene editing in all four paralogs. Therefore, I suggest an early mutagenesis event in the tissue culture phase and a very efficient gene editing. Conversely, the *BnSFAR5* T₁ plant was chimeric, and ten distinct alleles were identified, including the wild-type alleles. This indicates that gene editing occurred at later developmental stages resulting in a chimeric plant. In addition to small insertions (1 bp) and deletions (1-28 bp), I also identified larger deletions that are 90 bp and 143 bp deletions for one target. Although such large deletions result in mainly from multiplex gene targeting (Zhou et al. 2014), larger deletions by single sgRNA were also reported in mouse and *Arabidopsis* (Fauser et al. 2014; Kosicki et al. 2018). To my knowledge, so far, larger deletions by single sgRNA have not been reported for crop plants.

I analyzed the inheritance of the CRISPR-Cas9 induced mutations and found that all the mutations found in the *BnSFAR4* and *BnSFAR5* were inherited to the T₂ generation. I did not detect transgene-free *BnSFAR5* mutant plants in the T₂ generation. This may be due to two or more transgene integrations. Previous studies have reported multiple transgene insertions and gene silencing because of multiple integrations (Sohn et al. 2011; Tang et al. 2007). Based on the result of the mentioned studies, I speculate that low Cas9 activity and editing efficiency resulted in chimeric plants in the case of *BnSFAR5*, which might be attributed to multiple transgene insertions. To eliminate the transgene, crossing of these *BnSFAR5* mutants with the donor RS306 or another genotype is required.

In this study, 163 EMS-induced mutated alleles were identified for *BnSFAR1* and *BnSFAR4* paralogs. Chemical mutagenesis with EMS resulted in different mutation frequencies in the *Brassicaceae* family, for instance, *Arabidopsis* 1/345 kb (Lai et al. 2012), rapeseed 1/109 kb (Gilchrist et al. 2013), *B. rapa* 1/56 kb, (Stephenson et al. 2010) and *B. oleracea* 1/447 kb,

(Himelblau et al. 2009). Apart from the *Brassicaceae* family, 1/47 kb mutation frequency in hexaploid wheat (Chen et al. 2012a) and 1/300 kb mutation frequency in rice (Till et al. 2007) were reported. These studies suggest that mutation frequency in the diploid species is expected to be lower than the mutation frequency in the polyploid species, indicating genome redundancy in polyploids increases tolerance to high mutation frequencies (Tsai et al. 2013). Mutation frequencies of six *BnSFAR* paralogs ranged between 1/18.6 kb to 1/34.6 kb which was in the range of the mutation frequencies reported for the same EMS mutated population (Braatz et al. 2018a; Emrani et al. 2015; Harloff et al. 2012; Sashidhar et al. 2019). I found missense mutations in functionally conserved amino acids of domain coding blocks. These mutants can be used to study the substrate specificity of the *BnSFAR* genes.

4.3 Loss of function in *BnSFAR* genes increases seed oil content without pleiotropic effect on seed germination

I studied the effect of *BnSFAR1*, *BnSFAR4*, and *BnSFAR5* mutations on SOC, TKW, fatty acid profile, oil bodies, seed oil accumulation, oil mobilization, seed germination, and vigor, using EMS-induced mutations in *BnSFAR1* and *BnSFAR4*, and Cas9-induced mutations in *BnSFAR4* and *BnSFAR5*. Out of three double mutants, only two double mutants in which two premature stop codon mutations were combined (*BnSFAR4.a* and *BnSFAR4.b*) showed a significant SOC increase. The combination of a premature stop codon and a missense mutation in *BnSFAR1* did not increase SOC. It is known that in GDSL lipase genes, serine (domain block I), glycine (domain block II), asparagine (domain block III), histidine and aspartate (domain block V) are essential for enzyme activity (Kelly and Feussner 2016). Glycine in domain block II acts as a proton donor (Akoh et al. 2004). The missense mutation affected a glycine to glutamic acid substitution on domain block II of *Bna.SFAR1.Ann*, introducing a negatively charged amino acid for a neutral one. Although this led to the expectation that by this substitution, the function as proton donor was disrupted, the phenotypic result implied either minor importance of the amino acid in this position for the catalytic activity or functionality of glutamic acid. Another possibility would be that a *BnSFAR1* knock-out in rapeseed has no effect on SOC, which is contradicting with GWAS data. This hypothesis can be tested in the future by using a different putative knock-out mutant for this gene, in this case, a splice site mutation.

I selected T₂ and T₃ generations to study the *BnSFAR4* and *BnSFAR5* Cas9-induced mutant effect. I observed a significant SOC increase in both T₃ and T₄ seeds of *BnSFAR4*, which was in line with EMS mutants. *BnSFAR5* knock-outs also increased SOC in both T₃ and T₄ seeds. Overall plant performance was not significantly different in both *BnSFAR4* and *BnSFAR5* when compared to wild-types. Loss of functions of these genes had no impact on seed germination, vigor, or oil mobilization. These findings highlight that mutations on *BnSFAR4* and *BnSFAR5* genes can be used for breeding as they do not have adverse effects on yield parameters and seedling establishment. Nevertheless, I suggest field experiments to evaluate seed germination and vigor, as those traits are highly influenced by environmental factors (Hatzig et al. 2015).

My results clearly showed that only multiple gene knock-outs are important for functional analysis in a polyploid, as there are functionally redundant genes. Single mutants did not show phenotypic effects, while pyramiding of single mutants or multiple gene knock-outs displayed significant SOC increase. However, gene pyramiding is time-consuming, especially in randomly mutagenized plants, because, at first, the background mutation load should be eliminated. Otherwise, a combination of mutations increases the load of undesirable background mutations. In contrast, CRISPR-Cas9 targeted mutagenesis is more efficient as multiple gene knock-outs can be achieved easily, and stably inherited homozygous mutant lines can be produced in less than two years, as I demonstrated in this study.

I demonstrate that *BnSFAR4* and *BnSFAR5* genes are involved in seed oil degradation in rapeseed. Huang et al. (2015) investigated the effect of *AtSFAR4* on genes involved in the β -oxidation pathway and the glyoxylate cycle. *AtSFAR4* was expressed during early seed

germination. They found that *AtSFAR4* upregulated the expression of *CTS*, *LACS6*, *LACS7*, *PNC1* and *ICL* genes during *Arabidopsis* seed development. *CTS* gene is responsible for transporting fatty acid into peroxisomes, and *LACS6* and *LACS7* deliver β -oxidation substrates. *PNC1* is essential for supplying peroxisomes with ATP while *ICL* is important for post-germinative growth and seedling establishment (Huang et al. 2015). Therefore, studying the downstream gene of *BnSFAR* genes is essential to get a better understanding of *BnSFAR* function in rapeseed. Expression analysis should be done especially for genes important for the β -oxidation pathway, using mutant and wild-type plants. Moreover, *BnSFAR* enzyme activity and localization studies would be necessary for a better understanding of gene function and regulation.

In Figure 14, I propose a model for oil storage in rapeseed based on the consideration of steady-state equilibria between oil synthesis and oil degradation processes at different stages of seed development. The sizes of the arrows in the figure follow the assumed metabolic activity. In the case of degradation, they respect known *BnSFAR4* and *BnSFAR5* expression profiles during seed development. For oil synthesis, I assumed that it correlated with photosynthetic activity. In stage I, there is still the high activity of *BnSFAR* genes in milky, white seeds, while in stage II, *BnSFAR* expression decreases, and green seeds add to photosynthetic energy production resulting in a maximum oil synthesis rate. Stage III marks the saturation when synthesis equals degradation. Due to *BnSFAR* knock-out, degradation should generally be decreased in mutants, leading to a higher maximum than in wild-type plants ($\Delta 1$). During seed desiccation, photosynthetic activity ceases, and I hypothesize that energy for vital processes in embryo development is mainly supplied from the oil reserve (Chia et al. 2005). In mutants, less oil will be consumed compared to the wild-type leading to a higher seed oil content in the mature seeds (IV, $\Delta 2$). As *SFAR4* also plays a role during seed germination in *Arabidopsis* (Huang et al. 2015), I suggest that the difference in seed oil mobilization in stage V results from reduced GDSL- (SFAR-) lipase activity ($\Delta 3$ – $\Delta 2$). The dynamic model proposed here visualizes the potential for future oil increase in rapeseed by further decreasing lipase activity, i.e. pyramiding more *BnSFAR* knock-out mutations. A selection of genes mainly expressed in stages I-III might be essential to avoid negative effects on embryo development and early seedling establishment.

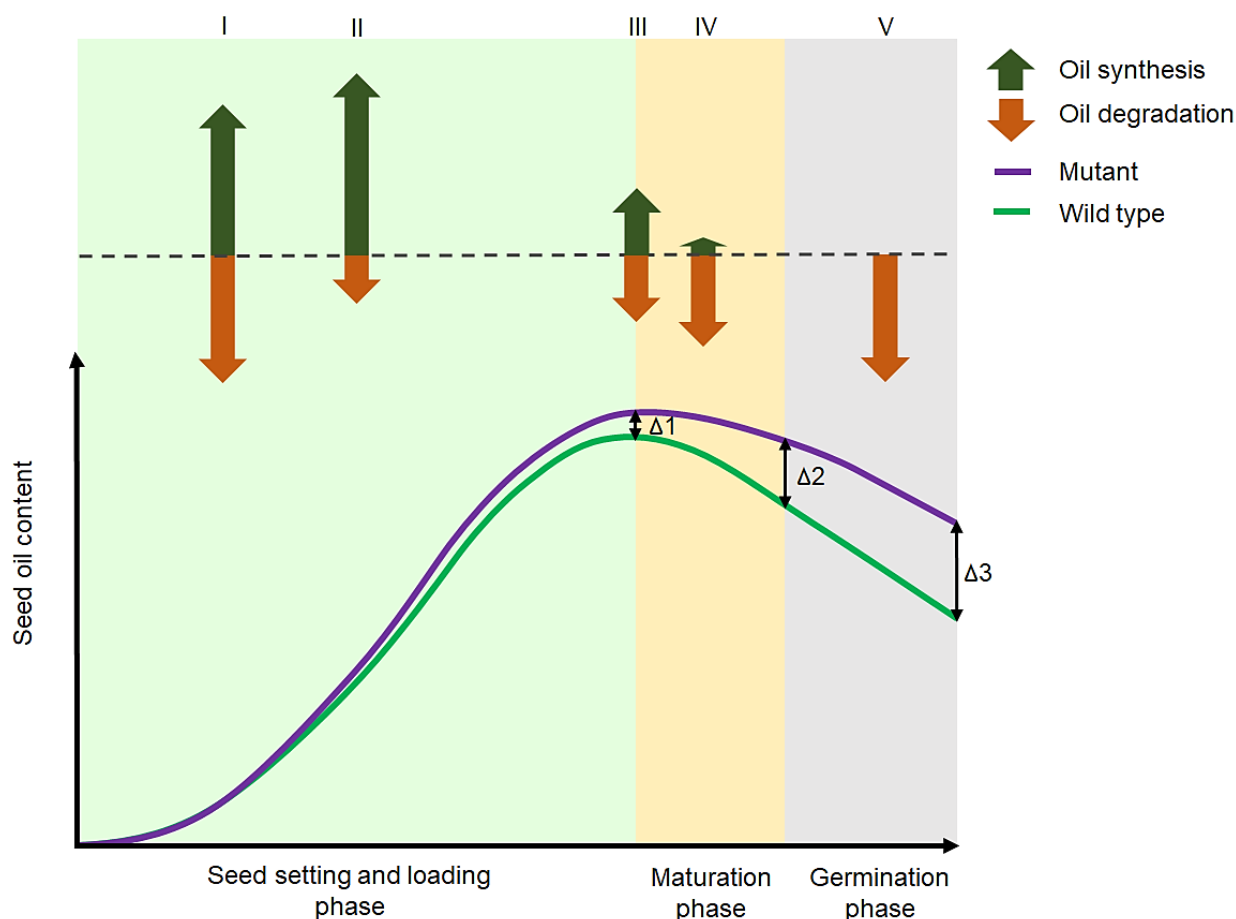


Figure 14: Model for oil storage in rapeseed on the basis of equilibrium between synthesis and degradation processes. I: start of oil accumulation, II: maximum of oil deposition, III: maximum of oil content, IV: oil reduction during maturation, V: seed germination. $\Delta 1$, $\Delta 2$, $\Delta 3$ differences between mutant and wild-type at maximal oil content, end of maturation, and after 3 days of germination, respectively.

4.4 Speed breeding is an option to reduce background mutations in EMS induced mutations

It should be noted that EMS causes random mutations all over the genome, and based on the average mutation rate in *BnSFAR* genes, it is expected to have ~46,000 mutations in a single plant. Undesirable mutation load is a severe drawback resulting in growth retardation, abnormal inflorescence, late flowering, and reduced seed set and can obscure or bias the true effect of a gene of interest (Braatz et al. 2018b; Shah et al. 2018). Hence, the reduction of background mutations caused by EMS is necessary for *BnSFAR* mutants, either crossing with EMS donor or elite material. The shortcoming of using winter rapeseed is the requirement of vernalization for flowering, which causes longer generation cycles and elimination of background mutations, thereby time-consuming. Therefore, I chose the spring rapeseed variety Peace as the recurrent parent to backcross with EMS mutants. I was able to shorten the generation time of *BnSFAR* mutants by three months after crossing with spring-type. When EMS mutants are backcrossed with their donor Express-617, which is winter rapeseed, it takes a minimum of six months for one generation under greenhouse conditions. In contrast to Express-617, Peace offspring takes only 3-4 months to complete one generation. Further acceleration of generation time of backcrossed plants can be achieved, employing a temperature-controlled glasshouse with a prolonged photoperiod (Watson et al. 2018).

I used marker-assisted genomic selection in BC_1 to reduce the number of necessary backcrossing generations (Jung 2010). I tested AFLP markers for genomic selection in the BC_1 generation. The shortcomings of AFLP markers are that they are dominant, less informative (Vignal et al.

2002), and laborious. Therefore, I chose a 15K Illumina InfiniumTM SNP array, which is easy to use, informative, and are high-throughput (Mason et al. 2017). On average, 75% of the recurrent parent genome is expected to be present in the BC₁ generation (Hasan et al. 2015). Interestingly, I observed BC₁ plants already with 83-84% of the Peace genomic background. These plants can be used for further backcrossing and a combination of mutants for double mutant production. Recovery of the recipient genome by marker-assisted backcrossing relies on three main factors, 1) the number of the backcross generation, 2) marker density, and 3) population size. In general, higher recovery of the recipient genome can be obtained in early BC generations using high marker densities and large populations (Herzog and Frisch 2013). Therefore, a 60K chip could provide higher peace genome recovery with a large population. However, resource usage should be optimized for the cost-effectiveness of backcross programs.

Taking all together, I could increase SOC in rapeseed by knocking-out of genes using targeted and random mutagenesis. These mutations can be introduced into a commercial breeding program. EMS-induced mutations can be used in countries of the European Union where releasing commercial GMO cultivars are extremely difficult. CRISPR-Cas9 mutants can be introduced to breeding programs in Canada or China where GMO legislation is flexible.

All the phenotyping experiments were carried out under greenhouse conditions. SOC is a yield-related parameter and a quantitative trait, which is often influenced by genotype, environment, and genotype and environment interaction. Therefore, I propose performing field experiments in multiple environments to obtain more robust data. EMS mutants should be backcrossed at least one time before doing field experiments.

5 Summary

Rapeseed is the third-largest oil crop grown worldwide. Seed oil accumulation is a complex process, which is controlled by many genes. Final oil content in seeds is determined by the balance between both anabolic and catabolic pathways. Therefore, studying the catabolic pathway is important as many researchers until today mainly focused on the synthesis pathway to increase seed oil content (SOC). In this study, *SEED FATTY ACID REDUCER (SFAR)* genes known to be involved in seed oil degradation in *Arabidopsis* were knocked out by two different approaches, CRISPR-Cas9 targeted mutagenesis and EMS random mutagenesis.

I identified 12 homologous genes for the five known *Arabidopsis SFAR* genes, *AtSFAR1-AtSFAR5*. Each gene family had two paralogs in rapeseed except *BnSFAR4*, which had four paralogs. The *BnSFAR4* gene family was subdivided into subfamily a and b, based on their sequence similarity. Their expression was studied during seed development in Express-617 at 15, 25, 35, 45 and 55 days after pollination. Based on relative expression data, I selected *BnSFAR1*, *BnSFAR4*, and *BnSFAR5* copies for functional analysis.

I screened an EMS mutagenized winter rapeseed population using a TILLING platform to identify mutations in *BnSFAR1* and *BnSFAR4* paralogs. In total, 163 mutations were found in six paralogs comprising 7 nonsense, 103 missense, 46 silent, 6 splice site, and 1 UTR mutations. I found nonsense mutations in all genes except for *Bna.SFAR1.Ann*. I considered a splice site and one missense mutation in a functional amino acid for the *Bna.SFAR1.Ann* paralog, and nonsense mutations for all other five paralogs for further analysis. Crosses and backcrosses were performed to reduce background mutation load and to produce double mutants. By CRISPR-Cas9, I knocked out all paralogs in *BnSFAR4* and *BnSFAR5* simultaneously using specific target regions. After *Agrobacterium*-mediated hypocotyl transformation of winter-type RS306, I obtained two, five and two transgenic T₁ plants for *BnSFAR1*, *BnSFAR4* and *BnSFAR5*, respectively, representing 0.2 - 1.1% transformation efficiency. I selected two T₁ plants from *BnSFAR4* and the single *BnSFAR5* for further studies. I found complete gene editing on *BnSFAR4* T₁ plants resulting in quadruple mutants. *BnSFAR5* T₁ was chimeric, but the mutations were fixed in T₂. T₂ and T₃ generations were used for phenotypic analysis.

Six segregating F₂ populations derived from crosses either between M₃ single mutant plants or M₃ single mutant plants backcrossed with Express-617 were analyzed for SOC. In both crossing populations, SOC was increased in *BnSFAR4.a* double mutants by 12.1% and 8.9% and in *BnSFAR4.b* double mutants by 10.3% and 8.7% when compared to wild-type plants segregating within the progenies. However, SOC was not significantly different between *BnSFAR1* double mutants and wild-type plants in both populations. Moreover, single mutants did not show a significant increase in SOC either for *BnSFAR1*, *BnSFAR4.a*, and *BnSFAR4.b*. In T₂ and T₃ *BnSFAR4* and *BnSFAR5* CRISPR-Cas9 mutants, SOC was increased significantly by 14.5% and 12.9% for *BnSFAR4* and 10.4% and 11.2% for *BnSFAR5*, as compared to the wild-type. Analysis of fatty acid composition in *BnSFAR4* T₂ showed a significant reduction of erucic acid (C22:1) content and a non-significant increase of C18:1 and C18:3.

Oil body size in CRISPR-Cas9 T₂ mutants was significantly larger than in wild-type RS306. Seed oil accumulation in CRISPR-Cas9 T₃ mutants reached a maximum at 45 DAP in both mutant and wild-type of 40.3% and 37.8%, respectively, but declined more in wild-type (33.7%) as compared to mutants (38.2%) during the seed maturity. In germinating seeds, oil was mobilized with a higher rate in RS306 than in *BnSFAR4* and *BnSFAR5* T₄ mutants. Seed germination rate and seed vigor were not significantly affected T₂ *BnSFAR4* CRISPR-Cas9 mutants and revealed no pleiotropic effects.

Background mutation load was reduced by backcrossing EMS single mutants with fast flowering spring rapeseed variety 'Peace', and the reduction was monitored using AFLP markers and a 15K Illumina InfiniumTM SNP array. I could select backcrossed plants (BC₁) with 84% share of

Summary

the Peace genomic background. Single mutants selected from SNPs array should be combined to produce double mutants for further studies.

In summary, I can conclude that loss of function in *BnSFAR4* and *BnSFAR5* increases SOC in rapeseed. *BnSFAR4* loss of function seems to have no adverse effect on seed germination and vigor. It is vital to combine mutations within *BnSFAR* gene families. Otherwise, a single gene knock-out can be restored from its functional homoeolog. Backcrossing of EMS mutated winter rapeseed with a spring variety can be applied to reduce undesirable mutations rapidly using marker-assisted genomic background selection.

6 Zusammenfassung

Raps ist die dritt wichtigste Ölfrucht weltweit. Die Ölspeicherung im Samen ist ein komplexer Prozess, der durch viele Gene reguliert wird. Der dabei erreichte Ölgehalt wird durch ein Gleichgewicht zwischen Aufbau- und Abbauprozessen bestimmt. Bis heute konzentrierte sich die Forschung hauptsächlich auf Aufbauprozesse, um den Ölgehalt im Samen zu erhöhen. Aus diesem Grund ist es wichtig, nun auch die Abbauprozesse genauer zu untersuchen. In dieser Arbeit wurden die sogenannten *SEED FATTY ACID REDUCER (SFAR)* Gene ausgeschaltet, von denen bekannt ist, daß sie in Arabidopsis am Ölabbau beteiligt sind. Dazu wurden zwei Ansätze, CRISPR-Cas und EMS Mutagenese gewählt.

Für die fünf bekannten *SFAR* Gene *AtSFAR1-5* aus Arabidopsis konnte ich 12 homologe Gene identifizieren. Jede Genfamilie bestand aus zwei Paralogen außer *BnSFAR4*, die vier Paraloge besaß. Die *BnSFAR4*-Genfamilie konnte aufgrund von Sequenzhomologien in zwei Unterfamilien aufgeteilt werden. In Express-617 wurde die Genexpression während der Samenreife 15, 25, 35, 45 und 55 Tage nach Bestäubung gemessen. Aufgrund der relativen Genexpression wählte ich die Genfamilien *BnSFAR1*, *BnSFAR4* und *BnSFAR5* für die funktionelle Analyse aus.

Um Mutationen in den Paralogen von *BnSFAR1* und *BnSFAR4* zu identifizieren, untersuchte ich eine Winterrapspopulation, die chemisch mit EMS mutagenisiert worden war. Ich fand insgesamt 163 Mutationen in allen 5 Paralogen, darunter 7 Nonsense-, 103 Missense-, 46 Silent-, 6 Splice Site-Mutationen und 1 UTR-Mutation. Nonsense-Mutationen gab es in allen Genen außer in *Bna.SFAR1.Ann*. Ich wählte für dieses Paralog eine Splice Site- und eine Missense-Mutation in einer funktionellen Aminosäure aus, für die anderen 5 Paraloge jeweils eine Nonsense-Mutation. Zur Erzeugung von Doppelmutanten wurden die Einzelmutanten in jeder Genfamilie gekreuzt. Mittels CRISPR-Cas schaltete ich alle Paralogen in *BnSFAR4* und *BnSFAR5* gleichzeitig mit spezifischen Zielsequenzen aus. Nach Hypokotyltransformation des Winterraps RS306 mit Agrobakterium erhielt ich zwei transgene T₁ Pflanzen für *BnSFAR1*, fünf für *BnSFAR4* und zwei für *BnSFAR5*, was einer Transformationseffizienz von 0,2 - 1,1 % entsprach. Ich wählte zwei *BnSFAR4* T₁ Pflanzen und die einzelne *BnSFAR5* T₁ Pflanze für weitere Untersuchungen aus. Ich fand vollständige Gen-Editierung in *BnSFAR4*, was Vierfachmutanten entsprach. Die *BnSFAR5* T₁ Pflanze war chimär, aber die Mutationen konnten in der T₂ Generation fixiert werden. Die T₂ und die T₃ generation wurden für die phänotypische Analyse verwendet.

Der Ölgehalt im Samen wurde in sechs spaltenden F₂ Populationen von Kreuzungen zwischen M₃-Einzelmutanten bzw. Kreuzungem zwischen einmal mit Express-617 rückgekreuzten M₃-Einzelmutanten bestimmt. In beiden Kreuzungsnachkommenschaften war der Ölgehalt in *BnSFAR4.a* Doppelmutanten um 12,1% bzw. 8,9% erhöht, in *BnSFAR4.a* Doppelmutanten um 10,3 % bzw. 8,7%, wenn man mit spaltenden Wildtyppflanzen verglich. In *BnSFAR1* Doppelmutanten fand sich keine signifikante Erhöhung in beiden Kreuzungspopulationen. Einzelmutanten zeigten in keiner Population einen signifikanten Effekt. In *BnSFAR4* und *BnSFAR5* CRISPR-Cas9 Mutanten war der Ölgehalt in der T₂ und T₃ Generation verglichen mit dem Wildtyp für *BnSFAR4* um 14,5% und 12,9% erhöht, für *BnSFAR5* um 10,4% und 11,2%. Eine Analyse des Fettsäuremusters zeigte in *BnSFAR4* T₂ eine signifikante Senkung des Erucasäuregehalts (C22:1), verbunden mit einer nicht-signifikanten Erhöhung von C18:1 und C18:3.

Die Öltröpfchengröße war in CRISPR-Cas9 T₂ Mutanten signifikant größer als im Wildtyp RS306. Die Öllakkumulation erreichte nach 45 Tagen ihr Maximum sowohl im Wildtyp (37,8%) als auch in der Mutante (40,3%), nahm aber anschließend bis zur Samenreife im Wildtyp stärker ab als in den Mutanten (33,7% vs. 38,2%). Während der Samenkeimung wurde das Öl in RS306 schneller mobilisiert als in *BnSFAR4* und *BnSFAR5* T₄ Mutanten. Die Keimungsrate und die frühe Keimlingsentwicklung waren in den Mutanten nicht signifikant verändert und zeigten keine pleiotropen Effekte.

Die Anzahl der Hintergrundmutationen wurde durch Rückkreuzungen mit der frühblühenden Sommerrapsorte Peace verringert und mit AFLP Markern und einem 15K Illumina Infinium™ SNP Array überprüft. In BC₁ konnte ich Pflanzen mit einem Anteil von 84% des Peace-Genoms selektieren. Einzelmutanten, die mittels SNP Array selektiert wurden, sollen für weitere Untersuchungen zu Doppelmutanten gekreuzt werden.

Zusammenfassend läßt sich sagen, daß ein Funktionsverlust von *BnSFAR4* and *BnSFAR5* zu einer Erhöhung des Ölgehalts in Rapssamen führt. In *BnSFAR4* scheint dieser Funktionsverlust keine negativen Auswirkungen auf die Samenkeimung und frühe Keimlingsentwicklung zu haben. Da Einzelmutanten keine Effekte zeigten, weil ihre Wirkung durch funktionelle Homologe ausgeglichen wird, ist ihre Kreuzung zu Doppelmutanten notwendig. Durch eine markergestützte genomische Hintergrundselektion in Rückkreuzungen des EMS mutagenisierten Winterraps mit einem Sommerraps können unerwünschte Mutationen schnell entfernt werden.

7 Appendix

Supplementary Table 1: Features of the *BnSFAR* genes used in this study

<i>Arabidopsis</i> gene	<i>B. napus</i> sequence annotation	<i>B. napus</i> paralogs	Genomic sequence length (bp)	Exon/ Intron structure		Coding region (bp)	Protein size (aa)	Genome sequence identity with <i>AtSFAR</i> (%)	Amino acid sequence identity with <i>AtSFAR</i> (%)
				Exons	Introns				
<i>AtSFAR1</i>	<i>BnaC04g18090D</i>	<i>Bna.SFAR1.C04</i>	2310	5	4	1149	382	90.2	89.6
	<i>BnaAnng12970D</i>	<i>Bna.SFAR1.Ann</i>	2316	5	4	1149	382	83.0	90.1
<i>AtSFAR2</i>	<i>BnaA05g11260D</i>	<i>Bna.SFAR2.A05</i>	2006	4	3	1308	435	83.8	77.1
	<i>BnaC04g13080D</i>	<i>Bna.SFAR2.C04</i>	1254	3	2	1083	360	82.8	77.6
<i>AtSFAR3</i>	<i>BnaA05g34360D</i>	<i>Bna.SFAR3.A05</i>	1143	2	1	1053	350	86.5	90.6
	<i>BnaC04g52870D</i>	<i>Bna.SFAR3.C04</i>	1145	2	1	1053	350	84.9	93.5
<i>AtSFAR4</i>	<i>BnaA06g18900D</i>	<i>Bna.SFAR4.A06a</i>	1587	3	2	1149	382	85.6	84.8
	<i>BnaC03g53090D</i>	<i>Bna.SFAR4.C03a</i>	1460	3	2	1149	382	87.0	87.0
	<i>BnaA06g16520D</i>	<i>Bna.SFAR4.A06b</i>	2793	3	2	1149	382	85.6	82.3
	<i>BnaCnng09560D</i>	<i>Bna.SFAR4.Cnmb</i>	2803	3	2	1149	382	82.5	81.9
<i>AtSFAR5</i>	<i>BnaC07g35650D</i>	<i>Bna.SFAR5.C07</i>	1429	5	4	1083	360	88.8	90.7
	<i>BnaA03g43830D</i>	<i>Bna.SFAR5.A03</i>	1563	5	4	1086	361	88.5	90.9

Supplementary Table 2: Primers used in this study. Red color indicates the mismatches introduced to increase the specificity of primers according to (Liu et al. 2012)

<i>B. napus</i> paralog	Primer name	Primer sequence	Orientation	Primer combination	Purpose
<i>Bna.SFAR1.C04</i>	NK005	CAT GCT CTC TCG AAC AAA TTT CAA CCG	forward	NK005+NK032	RT-qPCR (Express-617)
	NK032	CTCCACCAATTCCACAACAGACCA	reverse		
	NK051	CAACCATATGGCTCCCACAGTAAAC CAT	forward	NK051+NK052	TILLING
	NK052	ACGTGAGTGCAACACGTATGAAAG GTT	reverse		
	NK131	AGATTTGAACCGAATAAATTGAGAGAT GTA	reverse	NK051+NK131	Genotyping
	NK139	AGATTTGAACCGAATAAATTGAGAGAT GTG	reverse	NK051+NK139	Genotyping
	HW001	TTCTTGCCTCTATTCCTCCAC	forward	HW001+HW002	RT-qPCR (Hu135)
	HW002	GTTTGGCTGCTTGGTTATGG	reverse		

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<i>Bna.SFAR1.Ann</i>	NK042	TGCCCTCTCGAACAAATTTCAAGCC	forward	NK042+NK012	RT-qPCR (Express-617)
	NK012	CCACCAGTTCACAACAGAGCT	reverse		
	NK055	CAACCCTATGGCTCCCCTGTAAACCAG	forward	NK055+NK056	TILLING
	NK056	ACGTGAGTGCAACACATATGAAACCG	reverse		
	NK132	CCGGCTGCTGCAAAGTTACA ^{ACT}	reverse	NK055+NK132	Genotyping
	NK140	CCGGCTGCTGCAAAGTTACA ^{ACC}	reverse	NK055+NK140	Genotyping
	HW003	CAACACAGGACCGTTAGGATG	forward	HW003+HW004	RT-qPCR (Hu135)
	HW004	GTTTGGCTGCTTGGTTATGG	reverse		
<i>Bna.SFAR4.A06a</i>	NK033	GTGTCAAGCGACACTATTAGAGAA ^{GTT}	forward	NK033+NK023	RT-qPCR (Express-617)
	NK023	CATCTTCTGCAGCCAGCGACA ^{AT}	reverse		
	NK063	CAACCGCGG TCTCCGTCGC	forward	NK063+NK064	TILLING
	NK064	GTAGCCGACCCACACGTCTCG ^{AAC}	reverse		
	NK134	CGACTCACCATTGACTTCGTGG ^{AGT}	forward	NK134+NK023	Genotyping
	NK142	CGACTCACCATTGACTTCGTGG ^{AGC}	forward	NK142+NK023	Genotyping
	HW005	ACTGCCACGGATAGTTACGG	forward	HW005+HW006	RT-qPCR (Hu135)
	HW006	GGTTCGTTCCAAGTGTCTCC	reverse		
<i>Bna.SFAR4.C03a</i>	NK020	CTGTGTCAAGCGACACTATTAGAG ^{GAT}	forward	NK020+NK021	RT-qPCR (Express-617)
	NK021	CATCTTCTGCAGCCAGCGACT ^{TTC}	reverse		
	NK060	CAACCGCCGTCTCCGT ^{GGG}	forward	NK060+NK062	TILLING
	NK062	GTTAATGTACCGACTCGGGTCCTTAG ^{GAC}	reverse		
	NK136	GCGACTCACCATTGACTTCGTGG ^{AGT}	forward	NK136+NK021	Genotyping
	NK144	GCGACTCACCATTGACTTCGTGG ^{AGC}	forward	NK144+NK021	Genotyping
	HW007	ACTGCCACGGATAGTTACGG	forward	HW007+HW008	RT-qPCR (Hu135)
	HW008	GGTTCGTTCCAAGTGTCTCC	reverse		
<i>Bna.SFAR4.A06b</i>	NK037	CGC AAC CGC CGT CTC CAC	forward	NK037+NK038	RT-qPCR
	NK038	GTCGATGGTGAGTCGACCGTCAG	reverse		
	NK066	CAGGGATCTGATCTGCCATA ^{TTCG}	forward	NK066+NK068	TILLING
	NK068	CGACTGCTCGAGGTATCTTAAGCATGA ^{CTA}	reverse		
	HW009	CTCCCTTATCCTCGCAACC	forward	HW009+HW010	RT-qPCR (Hu135)

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	HW010	CTGTGTCGGTGAATGAGTCG	reverse		
<i>Bna.SFAR4.Cnnb</i>	NK040	TCGCAACCGCCGTCTCCAT	forward	NK040+NK041	RT-qPCR (Express-617)
	NK041	CGATGGAGAGTCGACCGTCGC	reverse		
	NK072	GCATGCACTGTTGCTTGAAGAGCATATC	forward	NK072+NK073	TILLING
	NK073	CGACTGCTCGAGGTATCTTAAGCATGAGTT	reverse		
	NK138	CATAGTCATTTACTCCAATTTCTCCGAGCT	reverse	NK072+NK138	Genotyping
	NK146	CATAGTCATTTACTCCAATTTCTCCGAGCC	reverse	NK072+NK146	Genotyping
	HW011	ACGATGACTCTGGCTGATGA	forward	HW011+HW012	RT-qPCR (Hu135)
	HW012	GTGGCGTTCGGATACTTGAT	reverse		
<i>Bna.SFAR5.A03</i>	NK030	CAACGATCTCATCAACCGATACGCC	forward	NK030+NK031	RT-qPCR
	NK031	GCATTGATGTAAGTGAACCTTAGCGTCTCCT	reverse		
	NK048	CTTCACGTTCTCAGACAAGTCTCTCTCCT	forward	NK048+NK049	Genotyping
	NK049	GTTGCTCGGTGTATCGGTTGATGAGA	reverse		
	NK107	AGAGTACTGGTTACCGGTGGAGTAGGAT	reverse	NK048+NK107	Genotyping
	HW013	TCGGTTTGGGAAGTAACGAC	forward	HW013+HW014	RT-qPCR (Hu135)
	HW014	CTCGGTGTATCGGTTGATGA	reverse		
<i>Bna.SFAR5.C07</i>	NK028	CAACGACCTTATCAACCGCTACGCT	forward	NK028+NK028	RT-qPCR (Express-617)
	NK029	GCATTGATGTAAGTGAACCTTAGCATCTGCA	reverse		
	NK045	CTTCACGTTCTCAAAGCAGTCTCTCTCCA	forward	NK045+NK046	Genotyping
	NK046	GTTGCTCAGTGTAGCGGTTGATATGG	reverse		
	NK104	AGAGTACTGGTTCCCGGTGGAGTAATAG	reverse	NK045+NK107	Genotyping
	HW015	TGCCAACGACCTTATCAACC	forward	HW015+HW016	RT-qPCR (Hu135)
	HW016	GTCCCGTCTCTGCTGTTCTG	reverse		
<i>Bna.SFAR2.A05</i>	NK080	CTCCATCGTGTCCGAATCGCT	forward	NK080+NK081	RT-qPCR (Express-617)
	NK081	CCTTTACCTCTTGTTTTGCTACTGCAAG	reverse		
	HW017	GACCTAACGACCTCTCCACT	forward	HW017+HW018	RT-qPCR (Hu135)
	HW018	GCTTCTTGCTCTCCACGAT	reverse		
<i>Bna.SFAR2.C04</i>	NK085	GCCATTCTAATCTTCGGCGACTCG	forward	NK085+NK088	RT-qPCR (Express-617)
	NK088	GACGAGCAATGTAATTCCTGAACATCGTA	reverse		

	HW019	CCTAACCAGCCTCTCCACTC	forward	HW019+HW020	RT-qPCR (Hu135)
	HW020	GCTTTCTTGTCTCCCACGAT	reverse		
<i>Bna.SFAR3.A05</i>	NK013	CCCGGCCTATCTTGATCCCTCC	forward	NK013+NK015	RT-qPCR (Express-617)
	NK015	GAAGTCGTTGGTTCCTATGCTGACTAG	reverse		
	HW021	CGTGTTGTGGAAGTGGATTG	forward	HW021+HW022	RT-qPCR (Hu135)
	HW022	TTAGTCCTCTCGGTCGGATG	reverse		
<i>Bna.SFAR3.C04</i>	NK017	CCCGGCCTATCTGGATCCGTCT	forward	NK017+NK019	RT-qPCR (Express-617)
	NK019	GAAGTCGTTGGTTCCTATGCTGACCAA	reverse		
	HW023	AACGGGAGGTTGAGGAGATT	forward	HW023+HW024	RT-qPCR (Hu135)
	HW024	CACAACACGCAGAAGTTCGAT	reverse		
<i>Bna.ACTIN2</i>	Act-1	TCTGGTGATGGTGTGTCTCA	forward	Act-1+Act-2	RT-qPCR (Express-617)
	Act-2	GGTGAACATGTACCCTCTCTCG	reverse		
pCas9-TPC <i>bar</i> cassette	Cas1-f	CAGTCTTTCACCTCTCTTTGG	forward		Transgene verification

Supplementary Table 3: EMS-induced mutations in *BnSFAR1* and *BnSFAR4* genes. For each gene, one TILLING amplicon was used

	<i>Bna.SFAR1.</i> <i>C04</i>	<i>Bna.SFAR1.</i> <i>Ann</i>	<i>Bna.SFAR4.</i> <i>C03a</i>	<i>Bna.SFAR4.</i> <i>A06a</i>	<i>Bna.SFAR4.</i> <i>A06b</i>	<i>Bna.SFAR4.</i> <i>Cnnb</i>
Coding sequence coverage (%)	53.0	53.0	87.1	83.8	56.1	56.1
Number of M ₂ pools used for mutation screening	4	8	8	10	10	4
Nonsense mutations	1	0	1	2	1	2
UTR mutations	0	0	0	0	1	0
Splice site mutations	1	1	2	1	1	0
Missense mutations	7	15	26	23	25	7
Silent mutations	2	4	15	12	8	5
Mutation frequency (1/kb) ^a	1/27.1	1/25.4	1/18.6	1/22.4	1/25.6	1/34.6

^aMutation frequencies were calculated as the number of mutations per M₁ plant based on the analyzed M₂ families.

Supplementary Table 4: EMS and CRISPR-Cas mutations used for further studies

<i>B. napus</i> gene name	M ₃ /T ₁ mutant code	Mutant type	Amino acid change	Allele
EMS induced mutations				
<i>Bna.SFAR1.C04</i>	sfar1-1	C to T transitions	Glutamine to stop	A ₁
<i>Bna.SFAR1.Ann</i>	sfar1-2	G to A transitions	Glycine to glutamic acid	B ₁
<i>Bna.SFAR1.Ann</i>	sfar1-3	G to A transitions	Splice	B ₂
<i>Bna.SFAR4.A06a</i>	sfar4-1	C to T transitions	Glutamine to stop	C ₁
<i>Bna.SFAR4.C03a</i>	sfar4-2	C to T transitions	Glutamine to stop	D ₁
<i>Bna.SFAR4.A06b</i>	sfar4-3	G to A transitions	Tryptophan to stop	E ₁
<i>Bna.SFAR4.Cnnb</i>	sfar4-4	G to A transitions	Tryptophan to stop	F ₁
<i>Bna.SFAR1.C04</i>	wild-type			A _E
<i>Bna.SFAR1.Ann</i>	wild-type			B _E
<i>Bna.SFAR4.A06a</i>	wild-type			C _E
<i>Bna.SFAR4.C03a</i>	wild-type			D _E
<i>Bna.SFAR4.A06b</i>	wild-type			E _E
<i>Bna.SFAR4.Cnnb</i>	wild-type			F _E
CRISPR-Cas mediated mutations				
<i>Bna.SFAR4.A06a</i>	bnsfar4-TP3	G insertion	Truncated protein (145/382 aa)	C ₂
	bnsfar4-TP3	T insertion	Truncated protein (145/382 aa)	C ₃
	bnsfar4-TP4	G insertion	Truncated protein (145/382 aa)	C ₂
	bnsfar4-TP4	A insertion	Truncated protein (145/382 aa)	C ₄
<i>Bna.SFAR4.C03a</i>	bnsfar4-TP3	T insertion	Truncated protein (145/382 aa)	D ₂
	bnsfar4-TP4	A insertion	Truncated protein (145/382 aa)	D ₃
	bnsfar4-TP4	1 bp deletion	Truncated protein (131/382 aa)	D ₄
<i>Bna.SFAR4.A06b</i>	bnsfar4-TP3	G insertion	Truncated protein (145/382 aa)	E ₂
	bnsfar4-TP4	T insertion	Truncated protein (145/382 aa)	E ₃
	bnsfar4-TP4	A insertion	Truncated protein (145/382 aa)	E ₄

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<i>Bna.SFAR4.Cnnb</i>	bnsfar4-TP3	A insertion	Truncated protein (145/382 aa)	F_2
	bnsfar4-TP4	G insertion	Truncated protein (145/382 aa)	F_3
	bnsfar4-TP4	1 bp deletion	Truncated protein (131/382 aa)	F_4
<i>Bna.SFAR5.A03</i>	bnsfar5-TP1	21 bp deletion	Truncated protein (355/360 aa)	G_1
	bnsfar5-TP1	90 bp deletion	Truncated protein (94/360 aa)	G_2
	bnsfar5-TP1	143 bp deletion	deletion spanning intron if only exon 2 lost, 320/ 360 aa	G_3
<i>Bna.SFAR5.C07</i>	bnsfar5-TP1	C insertion	Truncated protein (122/360 aa)	H_1
	bnsfar5-TP1	T insertion	Truncated protein (122/360 aa)	H_2
	bnsfar5-TP1	G insertion	Truncated protein (122/360 aa)	H_3
	bnsfar5-TP1	2 bp deletion	Truncated protein (121/360 aa)	H_4
	bnsfar5-TP1	4 bp deletion	Truncated protein (101/360 aa)	H_5
	bnsfar5-TP1	6 bp deletion	Truncated protein (358/360 aa)	H_6
	bnsfar5-TP1	28 bp deletion	Truncated protein (93/360 aa)	H_7
<i>Bna.SFAR4.A06a</i>	wild-type			C_R
<i>Bna.SFAR4.C03a</i>	wild-type			D_R
<i>Bna.SFAR4.A06b</i>	wild-type			E_R
<i>Bna.SFAR4.Cnnb</i>	wild-type			F_R
<i>Bna.SFAR5.A03</i>	wild-type			G_R
<i>Bna.SFAR5.C07</i>	wild-type			H_R

All EMS mutant alleles were combined to produce double mutants, except the B_2 mutant allele. The respective alleles from Express-617 (EMS donor) and RS306 (CRISPR-Cas donor) are named as suffix 'E' and 'R', respectively.

Supplementary Table 5: Production of EMS mutants by crossing M₃ plants homozygous for the mutant allele

Crossing type	<i>B. napus</i> gene combination/ gene	M ₃ Mutants	Amino acid change	F ₂ Seed code	Genotype used for F ₂ phenotyping
M ₃ x M ₃	<i>Bna.SFAR1.Ann</i> x <i>Bna.SFAR1.C04</i>	sfar1-1 x sfar1-2	Glutamine to stop x Glycine to glutamic	171769	<i>A₁A₁B₁B₁, A₁A₁B_EB_E, A_EA_EB₁B₁, A_EA_EB_EB_E</i>
M ₃ x M ₃	<i>Bna.SFAR4.A06a</i> x <i>Bna.SFAR4.C03a</i>	sfar4-1x sfar4-2	Glutamine to stop x Glutamine to stop	171770	<i>C₁C₁D₁D₁, C₁C₁D_ED_E, C_EC_ED₁D₁, C_EC_ED_ED_E</i>
M ₃ x M ₃	<i>Bna.SFAR4.A06b</i> x <i>Bna.SFAR4.Cnnb</i>	sfar4-3 x sfar4-4	Tryptophan to stop x Tryptophan to stop	171772	<i>E₁E₁F₁F₁, E₁E₁F_EF_E, E_EE_EF₁F₁, E_EC_ED_ED_E</i>
(M ₃ -Express) x (M ₃ -Express)	<i>Bna.SFAR1.Ann</i> x <i>Bna.SFAR1.C04</i>	sfar1-1 x sfar1-2	Glutamine to stop x Glycine to glutamic	180875	<i>A₁A₁B₁B₁, A₁A₁B_EB_E, A_EA_EB₁B₁, A_EA_EB_EB_E</i>
(M ₃ -Express) x (M ₃ -Express)	<i>Bna.SFAR4.A06a</i> x <i>Bna.SFAR4.C03a</i>	sfar4-1x sfar4-2	Glutamine to stop x Glutamine to stop	180876	<i>E₁E₁F₁F₁, E₁E₁F_EF_E, E_EE_EF₁F₁, E_EC_ED_ED_E</i>
(M ₃ -Express) x (M ₃ -Express)	<i>Bna.SFAR4.A06b</i> x <i>Bna.SFAR4.Cnnb</i>	sfar4-3 x sfar4-4	Tryptophan to stop x Tryptophan to stop	180877	<i>C₁C₁D₁D₁, C₁C₁D_ED_E, C_EC_ED₁D₁, C_EC_ED_ED_E</i>
M ₃ x Express (F ₂)	<i>Bna.SFAR4.A06a</i>	sfar4-1	Glutamine to stop	171780	<i>C₁C₁, C_EC_E</i>
M ₃ x Express (F ₂)	<i>Bna.SFAR4.C03a</i>	sfar4-2	Glutamine to stop	171782	<i>D₁D₁, D_ED_E</i>
M ₃ x Express (F ₂)	<i>Bna.SFAR4.A06b</i>	sfar4-3	Tryptophan to stop	171784	<i>E₁E₁, E_EE_E</i>
M ₃ x Express (F ₂)	<i>Bna.SFAR4.Cnnb</i>	sfar4-4	Tryptophan to stop	171786	<i>F₁F₁, F_EF_E</i>
F ₁ x Express (BC ₁ F ₂)	<i>Bna.SFAR4.A06a</i>	sfar4-1	Glutamine to stop	180886	<i>C₁C₁, C_EC_E</i>
F ₁ x Express (BC ₁ F ₂)	<i>Bna.SFAR4.C03a</i>	sfar4-2	Glutamine to stop	180887	<i>D₁D₁, D_ED_E</i>
F ₁ x Express (BC ₁ F ₂)	<i>Bna.SFAR4.A06b</i>	sfar4-3	Tryptophan to stop	180888	<i>E₁E₁, E_EE_E</i>
F ₁ x Express (BC ₁ F ₂)	<i>Bna.SFAR4.Cnnb</i>	sfar4-4	Tryptophan to stop	180889	<i>F₁F₁, F_EF_E</i>

Alleles from the non-mutated donor genotype Express-617 are named as suffix 'E'

Supplementary Table 6: Results of the *Agrobacterium*-mediated rapeseed hypocotyl transformation

Targeted <i>B. napus</i> paralogs	No. of hypocotyl explants	No. of regenerated shoots	No. of transgenic plants	No. of gene-edited plants
<i>BnSFAR1</i>	857	291	2	0
<i>BnSFAR4</i>	442	393	5	5
<i>BnSFAR5</i>	754	416	2	1

Supplementary Table 7: Inheritance of CRISPR-Cas mutations in *BnSFAR4* and *BnSFAR5*. We analyzed T₂ offspring of four T₁ plants, bnsfar4-TP4, bnsfar4-TP3, bnsfar5-CP2 and bnsfar5-CP3 (seed codes below). O: observed number, E: expected number, H_R and G_R: wild type alleles from the donor genotype RS306. Allele names are given in Supplementary Table 4.

Plant	Transgene		<i>sfar4</i> Genotypes												
			<i>Bna.SFAR4.A06a</i>			<i>Bna.SFAR4.C03a</i>			<i>Bna.SFAR4.A06b</i>			<i>Bna.SFAR4.Cnnb</i>			
	Transgenic	Non-transgenic	C ₂ C ₂	C ₂ C ₄	C ₄ C ₄	D ₃ D ₃	D ₃ D ₄	D ₄ D ₄	E ₃ E ₃	E ₃ E ₄	E ₄ E ₄	E ₃ E ₃	E ₃ E ₄	E ₄ E ₄	
bnsfar4-TP4 (174250)	O	11	7	4	7	7	4	12	2	5	7	6	6	11	1
	E	13.5	4.5	4.5	9	4.5	4.5	9	4.5	4.5	9	4.5	4.5	9	4.5
	χ ²		1.9 ^a		1.9 ^b		2.4 ^b		1.0 ^b					3.7 ^b	
bnsfar4-TP3 (174249)				C ₂ C ₂	C ₂ C ₃	C ₃ C ₃									
	O	9	1	2	4	4									
	E	7.5	3.5	2.25	4.5	2.25									
	χ ²		1.2 ^a		1.2 ^b										
		Transgene		<i>sfar5</i> Genotypes											
	Transgenic	Non-transgenic	G ₂ G ₂	G ₂ G ₂	G ₂ G ₂	G ₂ G _R	G ₂ G _R	G ₂ G _R	G _R G _R	G _R G _R	G _R G _R				
			H ₁ H ₁	H ₁ H _R	H _R H _R	H ₁ H ₁	H ₁ H _R	H _R H _R	H ₁ H ₁	H ₁ H _R	H _R H _R				
bnsfar5-CP2 (174257)	O	36	0	5	6	2	2	8	1	4	4	4			
	E	27	9	2.25	4.5	2.25	4.5	9	4.5	2.25	4.5	2.25			
	χ ²		12.0 ^a				10.89 ^c								
bnsfar5-CP3 (174258)				G _R G _R	G _R G _R	G _R G _R									
	O	23	0	3	14	6									
	E	17.25	5.75	5.75	11.5	5.75									
	χ ²		7.7 ^a		1.87 ^b										

^a3:1 segregation, χ² (df =1 at p = 0.05) = 3.84, ^b1:2:1 segregation, χ² (df =2 at p = 0.05) = 5.99, ^c 1:2:2:1:4:1:2:2:1 segregation, χ² (df =8 at p = 0.05) = 15.5. Here we assume that T₁ parents were non-chimeric.

Supplementary Table 8: Phenotyping data of EMS and CRISPR-Cas *BnSFAR1*, *BnSFAR4* and *BnSFAR5* mutants. T₂ and T₃ generations were derived from the selfing of T₁ plants. F₁ plants are offspring from M₃ mutants crossed with the EMS donor Express-617. TKW: Thousand Kernel Weight, N: number of plants investigated. All data are means ± SEM.

Mutant crossings	Generation	Seed code	Parental generation	Genotype	N	Seed oil content	TKW
EMS induced mutations							
<i>Bna.SFAR1.C04</i> x <i>Bna.SFAR1.Ann</i>	F ₃	171769	M ₃	<i>A₁A₁B₁B₁</i>	7	39.05±0.63	3.50±0.28
				<i>A₁A₁B_EB_E</i>	5	38.67±1.64	3.27±0.20
				<i>A_EA_EB₁B₁</i>	6	38.98±0.83	3.63±0.29
				<i>A_EA_EB_EB_E</i>	6	40.38±1.41	3.75±0.19
				Express-617	7	44.66±0.45	3.14±0.12
<i>Bna.SFAR4.A06a</i> x <i>Bna.SFAR4.C03a</i>	F ₃	171770	M ₃	<i>C₁C₁D₁D₁</i>	5	41.70±0.17	2.53±0.26
				<i>C₁C₁D_ED_E</i>	5	39.82±0.83	2.47±0.28
				<i>C_EC_ED₁D₁</i>	5	38.22±1.45	2.87±0.16
				<i>C_EC_ED_ED_E</i>	5	37.04±0.74	2.44±0.20
				Express-617	5	43.68±0.30	2.39±0.13
<i>Bna.SFAR4.A06b</i> x <i>Bna.SFAR4.Cnnb</i>	F ₃	171772	M ₃	<i>E₁E₁F₁F₁</i>	5	39.17±1.12	4.25±0.32
				<i>E₁E₁F_EF_E</i>	7	36.84±0.40	3.87±0.14
				<i>E_EE_EF₁F₁</i>	8	38.16±0.51	3.65±0.11
				<i>E_EC_ED_ED_E</i>	6	35.50±0.65	3.68±0.31
				Express-617	7	44.66±0.45	3.14±0.12
<i>Bna.SFAR4.A06a</i>	F ₃	171780		<i>C₁C₁</i>	5	40.75±0.94	3.34±0.34
				<i>C_EC_E</i>	5	40.97±0.24	2.96±0.33
<i>Bna.SFAR4.C03</i>	F ₃	171782		<i>D₁D₁</i>	5	41.02±1.01	3.08±0.14
				<i>D_ED_E</i>	5	40.54±0.94	3.33±0.22
<i>Bna.SFAR4.A06b</i>	F ₃	171784		<i>E₁E₁</i>	6	42.16±0.86	3.22±0.20
				<i>E_EE_E</i>	6	41.34±0.40	3.64±0.20

<i>Bna.SFAR4.Cnnb</i>	F ₃	171786		F_1F_1	5	41.00±0.60	3.62±0.30
				F_EF_E	5	39.04±0.12	3.13±0.12
<i>Bna.SFAR1.C04</i> x <i>Bna.SFAR1.Ann</i>	F ₃	180875	F ₁ (M ₃ -Express)	$A_1A_1B_1B_1$	5	41.25±0.59	4.06±0.34
				$A_1A_1B_EB_E$	6	41.51±0.51	4.15±0.25
				$A_EA_EB_1B_1$	5	40.23±1.21	3.81±0.24
				$A_EA_EB_EB_E$	7	41.50±0.75	3.65±0.22
<i>Bna.SFAR4.A06a</i> x <i>Bna.SFAR4.C03a</i>	F ₃	180876	F ₁ (M ₃ -Express)	$E_1E_1F_1F_1$	6	44.96±0.31	3.92±0.12
				$E_1E_1F_EF_E$	6	44.23±0.35	3.72±0.28
				$E_EE_EF_1F_1$	5	44.24±0.57	3.62±0.29
				$E_EC_ED_ED_E$	5	41.28±0.65	3.47±0.28
<i>Bna.SFAR4.A06b</i> x <i>Bna.SFAR4.Cnnb</i>	F ₃	180877	F ₁ (M ₃ -Express)	$C_1C_1D_1D_1$	7	42.33±0.69	4.67±0.25
				$C_1C_1D_ED_E$	5	40.18±0.74	4.12±0.32
				$C_EC_ED_1D_1$	6	40.11±0.65	3.96±0.20
				$C_EC_ED_ED_E$	6	38.91±0.79	3.71±0.32
			Express-617	8	45.50±0.48	3.78±0.09	
<i>Bna.SFAR4.A06a</i>	F ₃	180886		C_1C_1	5	44.82±0.90	4.55±0.25
				C_EC_E	5	43.42±1.29	4.00±0.44
<i>Bna.SFAR4.C03</i>	F ₃	180887		D_1D_1	4	43.00±0.74	4.00±0.17
				D_ED_E	6	42.91±0.64	3.87±0.17
<i>Bna.SFAR4.A06b</i>	F ₃	180888		E_1E_1	5	43.05±0.56	3.91±0.11
				E_EC_E	6	42.88±0.63	3.46±0.16
<i>Bna.SFAR4.Cnnb</i>	F ₃	180889		F_1F_1	5	45.31±0.63	3.84±0.20
				F_EC_E	6	44.57±0.60	3.74±0.32
CRISPR-Cas9 mediated mutations							
<i>BnSFAR4</i>	T ₂	174249		$C_2C_3D_2D_2E_2E_2F_2F_2$	5	36.21±0.30	6.25±0.12
<i>BnSFAR4</i>	T ₂	174250		$C_2C_4D_3D_4E_3E_4F_3F_4$	5	34.66±0.92	5.56±0.36

				RS306	5	31.61±0.88	6.04±0.22
<i>BnSFAR4</i>	T ₃	182975		<i>C₃C₃D₂D₂E₂E₂F₂F₂</i>	8	37.58±0.34	5.33±0.28
				RS306	8	33.29±0.49	4.48±0.27
<i>BnSFAR5</i>	T ₂	174257		<i>G₂G₂H₁H₁</i>	4	34.44±0.87	6.15±0.82
				RS306	5	31.19±0.93	5.35±0.41
<i>BnSFAR5</i>	T ₃	183033		<i>G₂G₂H₁H₁</i>	6	35.04±0.33	5.52±0.53
				RS306	7	31.66±0.35	5.45±0.38

Supplementary Table 9: Seed germination, root, and shoot growth 5 DAS in T₃ lines with *BnSFAR* knock-out mutations and in RS306. All data are means ± SEM.

Seed code	Genotype	Germination rate (%)	Root growth (mm)	Shoot growth (mm)
174249	<i>C₂C₃D₂D₂E₂E₂F₂F₂</i>	97.2±2.6 ^a	75.7±7.3 ^a	59.0±6.4 ^a
174250	<i>C₂C₄D₃D₄E₃E₄F₃F₄</i>	97.8±1.0 ^a	76.5±5.8 ^a	60.0±5.2 ^a
RS306 (control)	<i>C_EC_ED_ED_EE_EE_EF_EF_E</i>	96.4±2.1 ^a	76.0±13.0 ^a	62.8±13.2 ^a

Supplementary Table 10: Adapters and primers used for AFLP genotyping

Enzyme name	Primer name	Sequence
<i>MseI</i>	<i>MseI</i> -Forward	GACGATGAGTCCTGAG
<i>MseI</i>	<i>MseI</i> -Reverse	TACTCAGGACTCAT
<i>PstI</i>	<i>PstI</i> -Forward	CTCGTAGACTGCGTACATGCA
<i>PstI</i>	<i>PstI</i> -Reverse	TGTACGCAGTCTAC
<i>MseI</i>	M01	GATGAGTCCTGAGTAAA
<i>MseI</i>	M31	GATGAGTCCTGAGTAA + AAA
<i>MseI</i>	M32	GATGAGTCCTGAGTAA + AAC
<i>MseI</i>	M33	GATGAGTCCTGAGTAA + AAC
<i>MseI</i>	M36	GATGAGTCCTGAGTAA + ACC
<i>PstI</i>	P01	GACTGCGTACATGCAGA
<i>PstI</i>	P32	GACTGCGTACATGCAG + AAC
<i>PstI</i>	P36	GACTGCGTACATGCAG + AAC

Supplementary Table 11: EMS mutations detected in six of *BnSFAR1* and *BnSFAR4* paralogs. Positions of the mutations are given related relative to the adenine of the start codon “ATG”.

<i>B. napus</i> paralog	Single mutant plant	Mutation	AA substitution	M ₂ genotype	Mutant code	Amino acid Changes
<i>BnaC.SFAR1.a</i>	60_H06	G229A	D77N	Homozygous	<i>bnaC.sfar1.a</i> G229A	Aspartic acid to Asparagine
	58_A03	G431A	A109T	Homozygous	<i>bnaC.sfar1.a</i> G431A	Alanine to Threonine
	52-E05	G450A	P115L	Heterozygous	<i>bnaC.sfar1.a</i> G450A	Proline to Leucine
	55-A10	C465T	S120F	Heterozygous	<i>bnaC.sfar1.a</i> C465T	Serine to Phenylalanine
	48-A02	C491T	Q129*	Homozygous	<i>bnaC.sfar1.a</i> C491T	Glutamine to stop codon mutation
	45-G02	G530A	E14K	Heterozygous	<i>bnaC.sfar1.a</i> G530A	Glutamine to Lysine
	47-F06	G641A		Homozygous	<i>bnaC.sfar1.a</i> G641A	Splice site mutation
	57-A5	C672T	S672F	Heterozygous	<i>bnaC.sfar1.a</i> C672T	Serine to Phenylalanine
	47-E04	C741T	S181F	Heterozygous	<i>bnaC.sfar1.a</i> C741T	Serine to Phenylalanine
<i>BnaA.SFAR1.a</i>	60-B04	G189A	K189R	Homozygous	<i>bnaA.sfar1.a</i> G189A	Lysine to Asparagine
	49-B05 49-C05	G215A	G72E	Homozygous	<i>bnaA.sfar1.a</i> G215A	Glycine to Glutamic acid
	69-F11 69-G11	G411A	G103R	Homozygous	<i>bnaA.sfar1.a</i> G411A	Glycine to Arginine
	60-H10	G412A	G103E	Heterozygous	<i>bnaA.sfar1.a</i> G412A	Glycine to Glutamic acid
	51-F08	G423A	D127N	Heterozygous	<i>bnaA.sfar1.a</i> G423A	Aspartic acid to Asparagine
	56-G12	C478T	S125F	Homozygous	<i>bnaA.sfar1.a</i> C478T	Serine to phenylalanine
	41-G02	G478A	S125F	Heterozygous	<i>bnaA.sfar1.a</i> G478A	Serine to phenylalanine
	60-A11 60-B11 60-C11	G520A G345A	R139K D81N	Heterozygous	<i>bnaA.sfar1.a</i> G520A	Arginine to Lysine Aspartic acid to Asparagine
	65-D05	G545A	G148R	Heterozygous	<i>bnaA.sfar1.a</i> G545A	Glycine to Arginine
	67-A01	G642A		Heterozygous	<i>bnaA.sfar1.a</i> G642A	Splice site mutation

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	67-B01					
	58-H05	G643A	G148E	Heterozygous	<i>bnA.sfar1.a</i> G643A	Glycine to Glutamic acid
	45-B07	G690A	G164R	Heterozygous	<i>bnA.sfar1.a</i> G690A	Glycine to Arginine
	53-B08 53-C08	G720A	D174N	Heterozygous	<i>bnA.sfar1.a</i> G720A	Aspartic acid to Asparagine
	62-G08 62-H08	C769T	S190F	Heterozygous	<i>bnA.sfar1.a</i> C769T	Serine to Phenylalanine
	41-F05	G801A	G201E	Homozygous	<i>bnA.sfar1.a</i> C769T	Glycine to Glutamic acid
<i>BnaA.SFAR4.a</i>	41-G12	C245T	S82F	Homozygous	<i>bnA.sfar4.a</i> C245T	Serine to Phenylalanine
	62_A3	C260T	T87I	Heterozygous	<i>bnA.sfar4.a</i> C260T	Threonine to Isoleucine
	45-B01 45-C01	C260T	T87I	Heterozygous	<i>bnA.sfar4.a</i> C260T	Threonine to Isoleucine
	46-D06	C277T	Q93*	Heterozygous	<i>bnA.sfar4.a</i> C277T	Glutamine to stop codon mutation
	42-A12 42-B12	G285A	M95I	Heterozygous	<i>bnA.sfar4.a</i> G285A	Methionine to Isoleucine
	72_G02	C304T C623T	P102S S208L	Heterozygous	<i>bnA.sfar4.a</i> C304T & C623T	Proline to Serine Serine to Leucine
	74_F12	G320A	R107K	Heterozygous	<i>bnA.sfar4.a</i> G320A	Arginine to Lysine
	79_G05	C389T	T120M	Homozygous	<i>bnA.sfar4.a</i> C389T	Threonine to Methionine
	71_B01	G497A G764A	G166E	Heterozygous	<i>bnA.sfar4.a</i> G497A & G764A	Glycine to Glutamic acid and splice site mutation
	53_E4 53_G4	C493T	L165F	Heterozygous	<i>bnA.sfar4.a</i> C493T	Leucine to Phenylalanine
	67_H01	G554A	G185E	Heterozygous	<i>bnA.sfar4.a</i> G554A	Glycine to Glutamic acid
	55_C03 55_D03	G562A	D188N	Heterozygous	<i>bnA.sfar4.a</i> G562A	Aspartic acid to Asparagine
	70_A9	G577A	V193I	Homozygous	<i>bnA.sfar4.a</i> G577A	Valine to Isoleucine

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	70_B9			Heterozygous		
	67_D7	G580A	G194R	Homozygous	<i>bnA.sfar4.a</i> G580A	Glycine to Arginine
	47_A08 47_C8 47_D8	C602T	T201I	Heterozygous	<i>bnA.sfar4.a</i> C602T	Threonine to Isoleucine
	43-B05 43-C05 43-D05	G643A	E245L	Heterozygous	<i>bnA.sfar4.a</i> G643A	Glutamic acid to Lysine
	60_C7	C789T	H224W	Homozygous	<i>bnA.sfar4.a</i> C789T	Histidine to Tryptophan
	50_D1 50_E1	G805A	G229E	Heterozygous	<i>bnA.sfar4.a</i> G805A	Glycine to Glutamic acid
	67_A11	C811T	P231L	Heterozygous	<i>bnA.sfar4.a</i> C811T	Proline to Leucine
	53_F07 53_G07	G820A	G234E	Heterozygous Homozygous	<i>bnA.sfar4.a</i> G820A	Glycine to Glutamic acid
	67_A01	G820A	G234E	Heterozygous	<i>bnA.sfar4.a</i> G820A	Glycine to Glutamic acid
	65_B10	G873A	G252R	Heterozygous	<i>bnA.sfar4.a</i> G873A	Glycine to Arginine
	41-H11	C924T	Q269*	Homozygous	<i>bnA.sfar4.a</i> C924T	Glutamine to stop codon mutation
<i>BnaC.SFAR4.a</i>	49-F08	C245T	S82W	Heterozygous	<i>bnA.C.sfar4.a</i> C245T	Serine to Tryptophan
	43-B01	G254A	R85Q	Heterozygous	<i>bnA.C.sfar4.a</i> G254A	Arginine to Glutamine
	44-H12	G254A	R85Q	Heterozygous	<i>bnA.C.sfar4.a</i> G254A	Arginine to Glutamine
	54-E10	C308T	S127F	Heterozygous	<i>bnA.C.sfar4.a</i> C308T	Serine to phenylalanine
	50-C09	C316T C183T	L106F F61F	Heterozygous	<i>bnA.C.sfar4.a</i> C316T & C183T	Leucine to Phenylalanine
	50-D09	C316T	L106F	Heterozygous	<i>bnA.C.sfar4.a</i> C316T	Leucine to Phenylalanine
	53-C03	C326T	T109I	Heterozygous	<i>bnA.C.sfar4.a</i> C326T	Threonine to Isoleucine
	68-F02	C277T	Q93*	Heterozygous	<i>bnA.C.sfar4.a</i> C277T	Glutamine to stop codon mutation
	68-F03	G376A	V126I	Heterozygous	<i>bnA.C.sfar4.a</i> G376A	Valine to Isoleucine

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	68-H03					
	62_D8 62_E8 62_F8	G545A	G182E	Homozygous Heterozygous	<i>bnac.sfar4.a</i> G545A	Glycine to Glutamic acid
	59-G04	G547A	E183R	Heterozygous	<i>bnac.sfar4.a</i> G547A	Glutamic acid to Arginine
	49-G04	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	72-F04	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	72-A09	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	72-B09	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	72-C09	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	72-D09	G554A	G185E	Heterozygous	<i>bnac.sfar4.a</i> G554A	Glycine to Glutamic acid
	41-C03	G581A	G194E	Heterozygous	<i>bnac.sfar4.a</i> G581A	Glycine to Glutamic acid
	48-B10	G581A	G194E	Heterozygous	<i>bnac.far4.a</i> G581A	Glycine to Glutamic acid
	67-B11	G608A	R609K	Heterozygous	<i>bnac.sfar4.a</i> G608A	Arginine to Lysine
	54-C02	G777A	G221D	Heterozygous	<i>bnac.sfar4.a</i> G777A	Glycine to Asparagine
	71_B11 71_C11	G760A		Heterozygous	<i>bnac.sfar4.a</i> G760A	Splice site mutation
	70-H12	G816A	G272Q	Heterozygous	<i>bnac.sfar4.a</i> G816A	Glycine to Glutamine
	49-F02	G819A	C235W	Heterozygous	<i>bnac.sfar4.a</i> G819A	Cysteine to Tryptophan
	55-B04	C837T	S241L	Heterozygous	<i>bnac.sfar4.a</i> C837T	Serine to Leucine
	46-C05	G858A	R248Q		<i>bnac.sfar4.a</i> G858A	Arginine to Glutamine
	51-D01	G870A	G252E	Homozygous	<i>bnac.sfar4.a</i> G870A	Glycine to Glutamic acid
	41-A7	G870A	G252E	Heterozygous	<i>bnac.sfar4.a</i> G870A	Glycine to Glutamic acid
	53-B09 53-C09	C1055T	P280S	Heterozygous	<i>bnac.sfar4.a</i> C1055T	Proline to Serine
	63-A04	C1116T	S300F	Heterozygous	<i>bnac.sfar4.b</i> C1116T	Serine to Phenylalanine

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<i>BnaA06.SFAR4.b</i>	72-F02	C65T	T22I	Homozygous	<i>bnA.sfar4.b</i> C65T	Threonine to Isoleucine
	69-F09	C74T	S25F	Homozygous	<i>bnA.sfar4.b</i> C74T	Serine to Phenylalanine
	65-C12	G113A	R38K	Heterozygous	<i>bnA6Asfar4.b</i> G113A	Arginine to Lysine
	76-A08 76-B08 76-D08	G213A	M71I	Homozygous Homozygous Heterozygous	<i>bnA.sfar4.b</i> G213A	Methionine to Isoleucine
	66-E02 66-F02	C245T	S82F	Heterozygous	<i>bnA.sfar4.b</i> C245T	Serine to Phenylalanine
	75-C07	G285A	M95I	Heterozygous	<i>bnA.sfar4.b</i> G285A	Methionine to Isoleucine
	73-E12	C323T	S108F	Homozygous	<i>bnA.sfar4.b</i> C323T	Serine to Phenylalanine
	67-H07	G332A	G111D	Heterozygous	<i>bnA.sfar4.b</i> G332A	Glycine to Aspartic acid
	61-F09	G335A	S112N	Homozygous	<i>bnA.sfar4.b</i> G335A	Serine to Asparagine
	76-C11	C380T	S127F	Homozygous	<i>bnA.sfar4.b</i> C380T	Serine to Phenylalanine
	74-D08	C389T	T130M	Heterozygous	<i>bnA.sfar4.b</i> C389T	Threonine to Methionine
	55-D01	G391A	V131M		<i>bnA.sfar4.b</i> G391A	Valine to Methionine
	79-G07	G391A	V131M	Heterozygous	<i>bnA.sfar4.b</i> G391A	Valine to Methionine
	79-A06 79-B06	G415A	E139L	Heterozygous	<i>bnA.sfar4.b</i> G415A	Glutamic acid to Lysine
	76-G03	G487A	E163K	Homozygous	<i>bnA.sfar4.b</i> G487A	Glutamic acid to Lysine
	57-A09 57-B09	G487A	E163K	Heterozygous	<i>bnA.sfar4.b</i> G487A	Glutamic acid to Lysine
	51-C02	G540A	W180*	Heterozygous	<i>bnA.sfar4.b</i> G540A	Tryptophan to stop codon mutation
	64-F12	G545A	G182E		<i>bnA.sfar4.b</i> G545A	Glycine to Glutamic acid
	70-H04	G554A	G185E		<i>bnA.sfar4.b</i> G554A	Glycine to Glutamic acid
	68-G10	G556A	V186I	Heterozygous	<i>bnA.sfar4.b</i> G556A	Valine to Isoleucine
	42-F01	T557A	V186E		<i>bnA.sfar4.b</i> T557A	Valine to Glutamic acid
	68-E09	G646A		Heterozygous	<i>bnA.sfar4.b</i> G646A	Splice site mutation

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<i>BnaC.SFAR4.b</i>	64-F12	G148A	G50R	Homozygous	<i>bnaC.sfar4.b</i> _{G148A}	Glycine to Arginine
	70-F06	G213A	M71I	Heterozygous	<i>bnaC.sfar4.b</i> _{G213A}	Methionine to Isoleucine
	45-G09	G391A	V131M	Homozygous	<i>bnaC.sfar4.b</i> _{G391A}	Valine to Methionine
	71-G04	G415A	E139L	Heterozygous	<i>bnaC.sfar4.b</i> _{G415A}	Glutamic acid to Lysine
	71-E10 71-F10	C442T	P148S	Homozygous Heterozygous	<i>bnaC.sfar4.b</i> _{C442T}	Proline to Serine
	61-F09	G460A	E154L	Homozygous	<i>bnaC.sfar4.b</i> _{G460A}	Glutamic acid to Lysine
	64 C9 64 D9	C491T	T164I	Heterozygous	<i>bnaC.sfar4.b</i> _{C491T}	Threonine to Isoleucine
	71_B10	G539A	W180*	Heterozygous	<i>bnaC.sfar4.b</i> _{G539A}	Tryptophan to stop codon mutation
	47-H10	G539A	W180*	Homozygous	<i>bnaC.sfar4.b</i> _{G539A}	Tryptophan to stop codon mutation

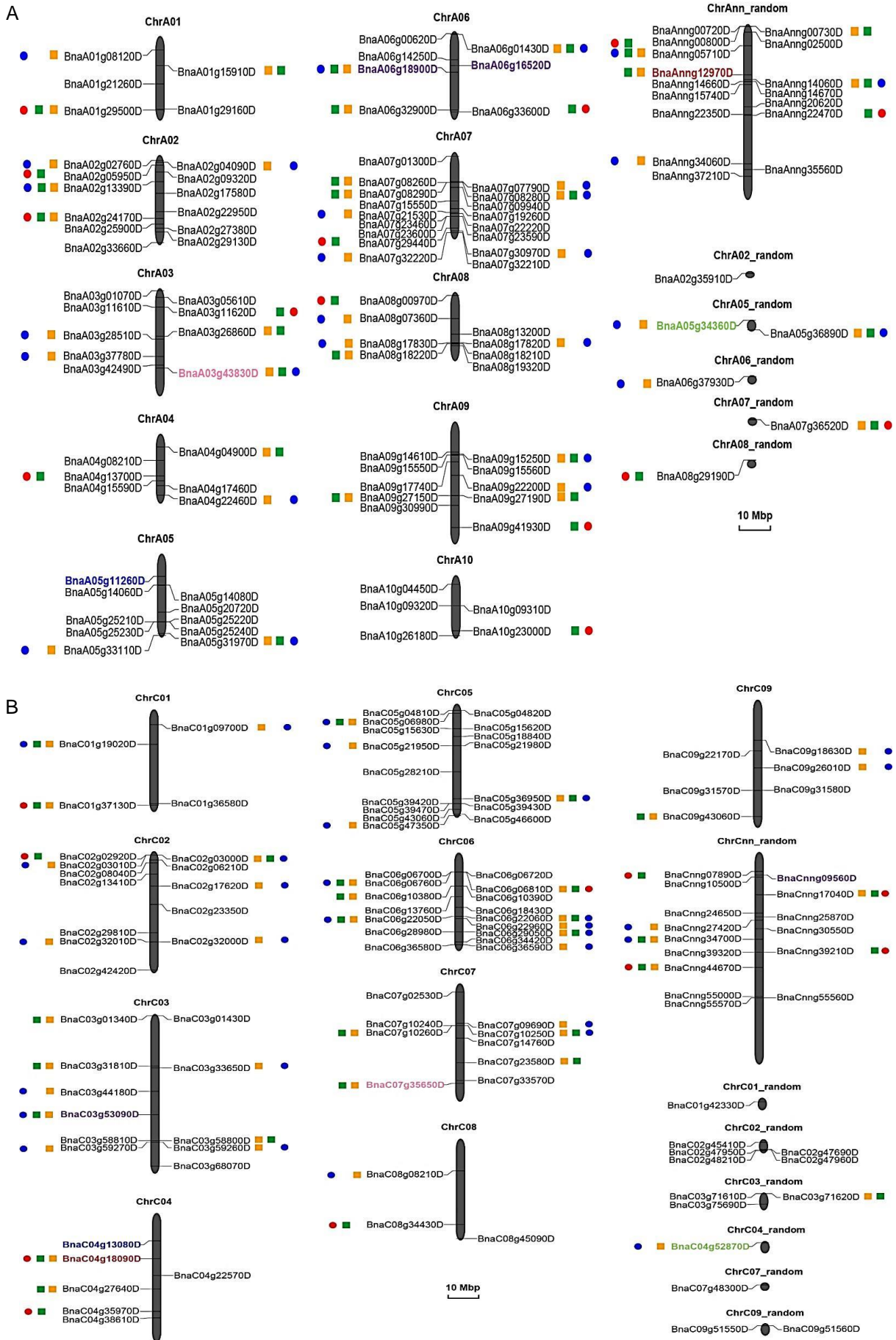
Supplementary Table 12: Markers used for AFLP genotyping

Primer combination	Size of the marker (bp)	Primer combination	Size of the marker (bp)
M31+P32	237	M33+P32	164
	241		218
	376		261
	390		389
	452		412
	498		434
	522	M32+P32	119
	627		180
	763		245
M36+P35	265		276
	315		311
	324		458
	416		
	428		

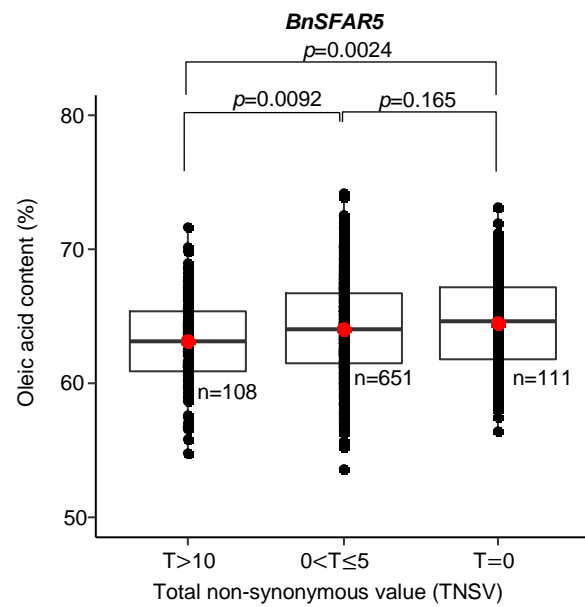
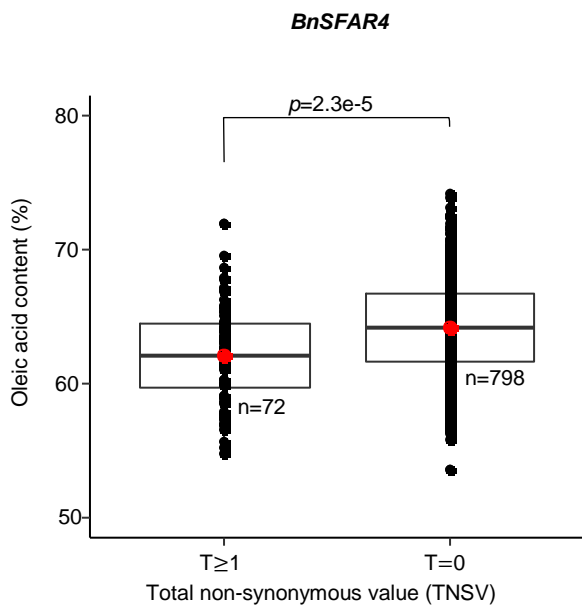
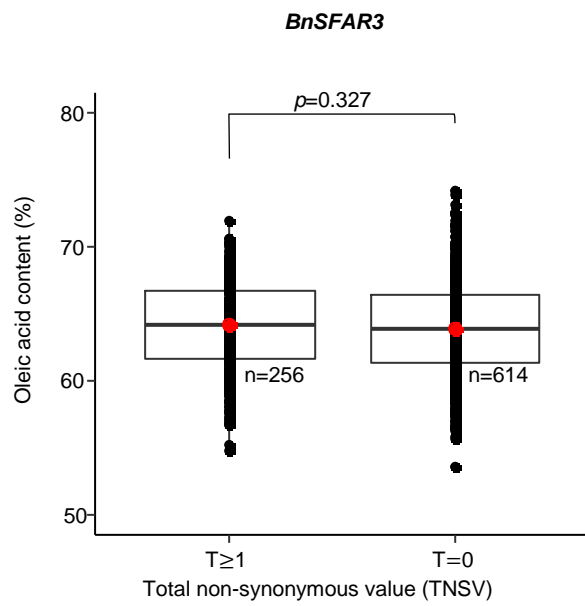
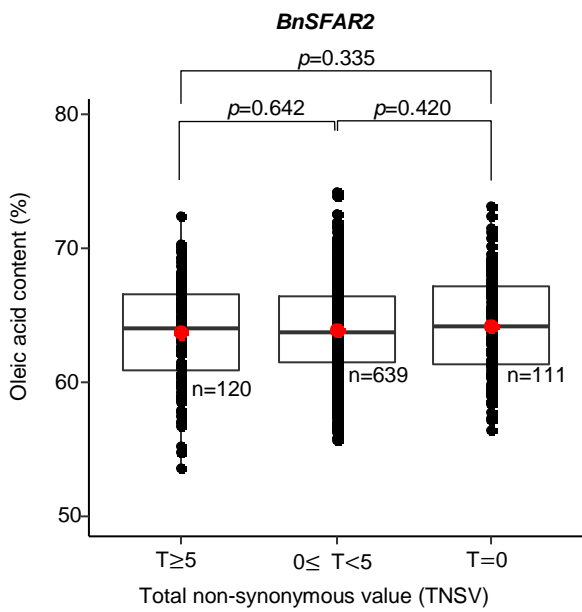
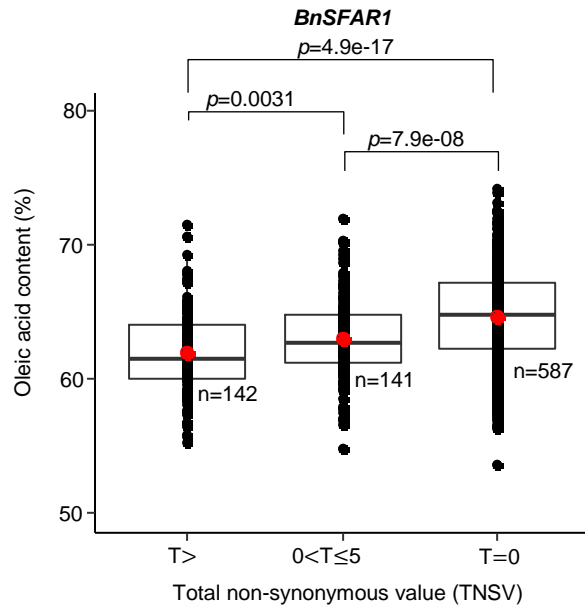
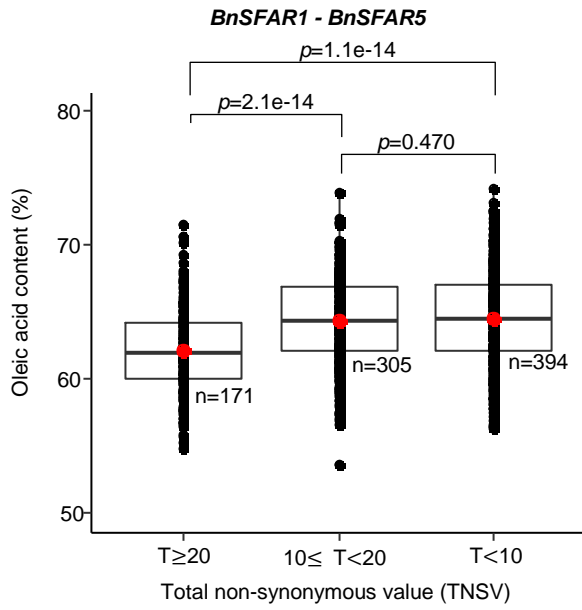
Supplementary Table 13: Inheritance of *Bna.SFAR4.A06a* and *Bna.SFAR4.C03a* mutant alleles. BC₁ plants were analyzed for inheritance.

BC ₁ seed code	<i>B. napus</i> paralog	Genotype of the heterozygous plants	Total number of genotyped BC ₁ plants	Number of plants		χ^2 value
				Wild-type	Heterozygous	
180901	<i>Bna.SFAR4.A06a</i>	C_1C_p	180	95	85	0.59 ^a
180902	<i>Bna.SFAR4.C03a</i>	D_1D_p	300	191	109	22.41 ^a

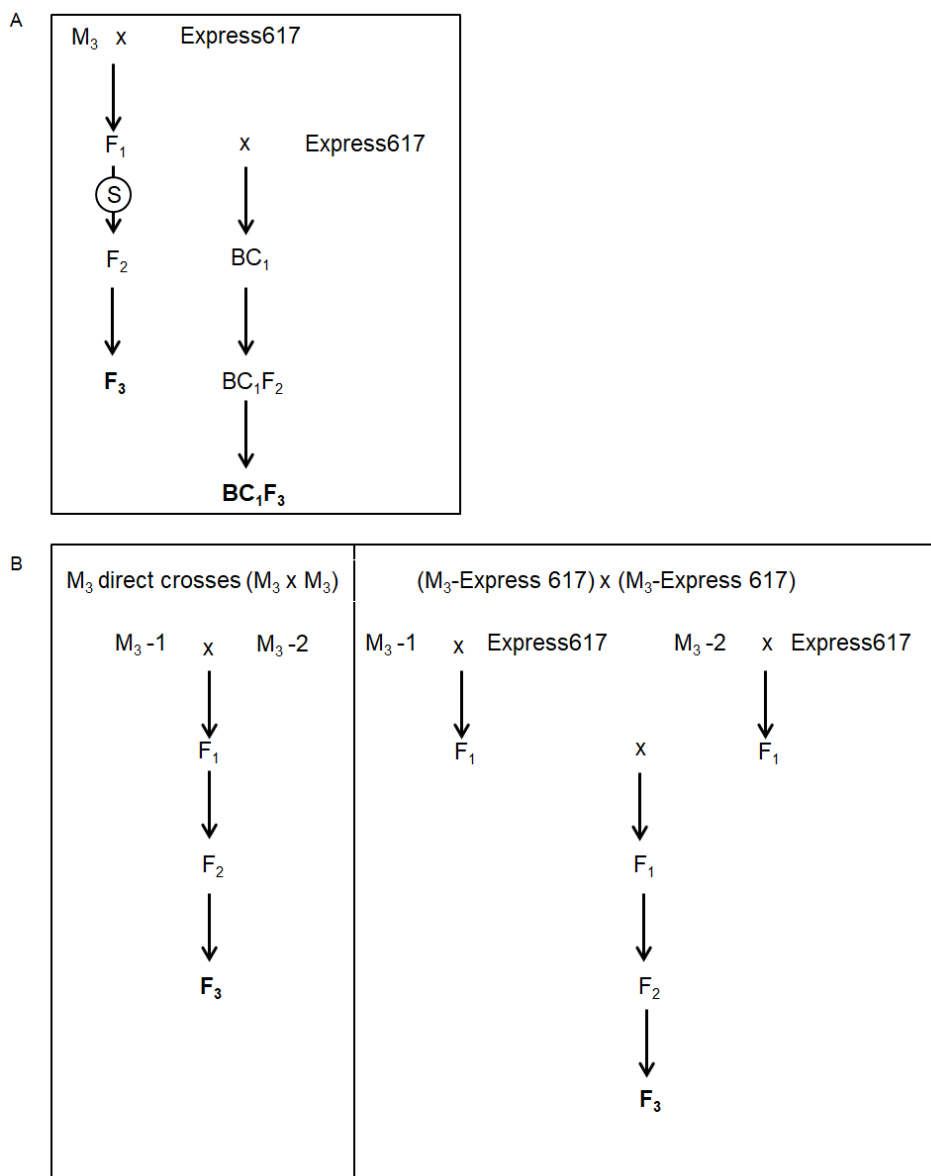
^a1:1 segregation, χ^2 (df=1 at $p = 0.05$) = 3.84



Supplementary Figure 1: Distribution of *GDSL* genes across the rapeseed subgenomes and their expression in developing seeds. Altogether 111 *BnGDSL* genes were mapped to each of the two subgenomes A (A) and C (B). 'Chr.Ann' or 'Chr.Cnn' indicates unmapped sequences. Genes labeled with 'random' have unknown chromosome positions. The *BnSFAR1* to *BnSAFR5* genes are written in red, blue, green, purple and pink colors, respectively. The orange or green squares on the right and left sides of a gene indicate the gene expression as revealed by RNA-seq (FPKM>1) at 16 and 40 DAP, respectively. The red and blue dots indicate up- or down-regulation of the gene at 40 DAP relative to 16 DAP, as defined by the log₂ expression folds >1 or <1.



Supplementary Figure 2: The effect of non-synonymous single nucleotide polymorphisms (SNP) within *BnSFAR* genes on oleic acid content in 870 non-related rapeseed accessions. At each position of a given gene, accessions with homozygous and heterozygous (non-synonymous SNPs) resulting in an amino acid change were given a score of 2 and 1, respectively. Lack of SNPs resulted in a score of 0. A 'Total Non-Synonymous Value' (TNSV) was defined either as the sum of non-synonymous values at a given position of a single *BnSFAR* gene or as the sum of all non-synonymous values of all *BnSFAR1*-*BnSFAR5* genes. n: number of accessions used for the calculation of mean oleic acid content. The *p* value indicates the significance of pairwise comparisons.



Supplementary Figure 3: Crossing schemes and pedigrees of plant materials used in this study (A) M₃ single mutants were crossed and backcrossed with the EMS donor Express-617 to reduce mutation load. (B) M₃ single mutants were crossed to produce double mutants. Homozygous M₃ plants were either crossed directly with each other (M₃ x M₃) or they were first crossed with Express-617 (M₃-Express-617) x (M₃-Express-617).

References

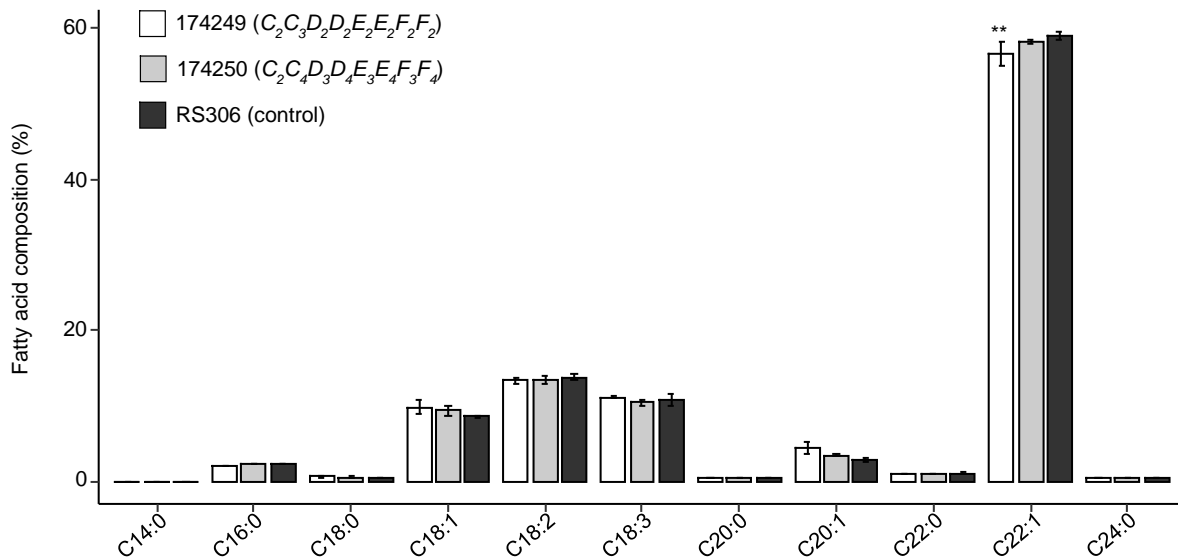
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Bna.SFAR4.CR2  c t t c g c c g t c t c t g g a g c a a c g g
chrCnn_random  C T T C G C C G T C T C T G G A G C A A C G G ←
chrC03         C T T C G C C G T C T C T G G A G C A A C G G ←
chrC03         C T T C T C C G C C T C T G G A G C A A C
chrC03         T C T C C A T C T C T G G A G C A A C G
chrA06         C T T C G C C G T C T C T G G A G C A A C G G ←
chrA06         C T T C G C C G T C T C T G G A G C A A C G G ←
chrA10         T C G C C G T C T C T G G A G C
chrAnn_random  T C T C C A T C T C T G G A G C A A C G
chrC09         G C C G T C T C T G G A G C A
chrC09         G C C G T C T C T G G A G C A
chrC08         C C G T C T C T G G A G C A A
chrC06         C G T C T C T G G A G C A A C
chrC01         C T T C G C C G T C T C T G G
chrA09         G C C G T C T C T G G A G C A
chrA09         T T C G C C G T C T C T G G A
chrA07         C T T C G C C G T C T C T T T A G C A A
chrA05         C T T C G T C - T C T C T G G A G C A A C G
chrA04         T C T C C A T C T C T G G A G C A A C G
chrA04         T C T C C A T C T C T G G A G C A A C G
chrA03         T C T C C A T C T C T G G A G C A A C G

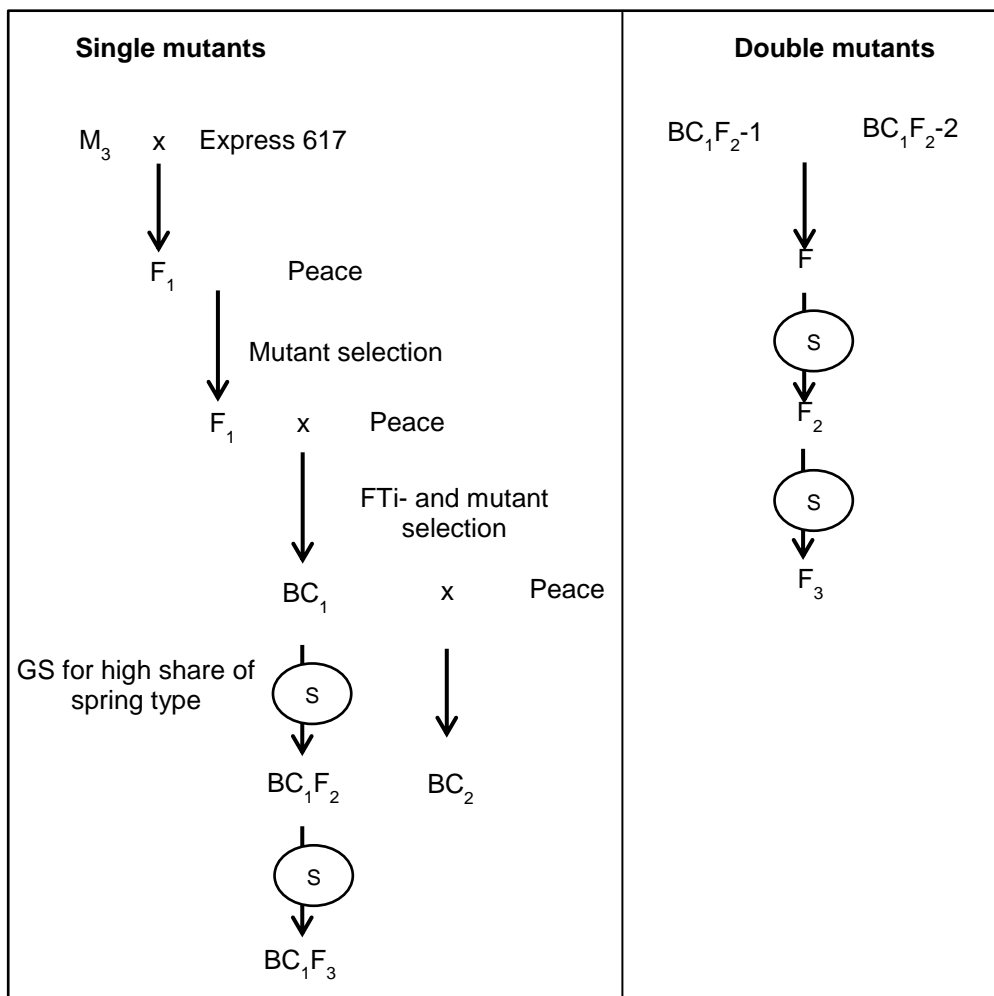
Bna.SFAR5.CR5  a c a c c - a t a t t c t c a a g - c a a g c g g
chrC07         A C A C C - A T A T T C T C A A G - C A A G C G G ←
chrA03         A C A C C - A T A T T C T C A A G - C A A G C G G ←
chrC06         C C - A T A T T C T C A A G - C A A G
chrA02         C C - A T A T T C T C A A G - C A A G
chrC05         C C - A T A T T C T C A A G - C A A
chrC05         A C C - A A A T A C T C A A G - C A A G C G
chrC05         A T A T T C T C A A G - C A A G
chrC05         T A T T C T C A A G - C A A G C
chrCnn_random  A C A C C T A T A T T C T C A A G - C A A
chrC03_random  C A C C - A T A T T C T C A A G A C A A G
chrC04         C C - A T A T T C T C A A G - C A
chrC04         C C - A T A T T C T C A A G - C A
chrC03         C - A T A T T C T C A A G - C A A
chrC02         A T A T T C T C A A G - C A A G
chrA07         T A T T C T C A A G - C A A G C
chrA05         T A T T C T C A A G - C A A G C

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Supplementary Figure 4: Screening the rapeseed reference genome for putative *BnSFAR4* and *BnSFAR5* off-target sequences, which could be altered by CRISPR-Cas induced mutagenesis with the single guide RNAs used in this study. The 20 bp nucleotide sequences, including the PAM (marked with blue line) sites were used to BLAST against the rapeseed genome using the CLC main workbench 7.6.4 (CLC bio, Aarhus, Denmark). The target sequences are marked by arrows, the PAM sequence is marked by a blue line.



Supplementary Figure 5: Fatty acid profiles in T₃ seeds of two *BnSFAR4* mutants and the RS306 control (n = 5). Fatty acid profiles were determined using gas chromatography. ***p* < 0.01 using a two-way ANOVA test, significant differences as compared to the RS306 controls. Data are presented as means ± SEM.



Supplementary Figure 6: Crossing scheme for single and double mutant production. GS=genomic selection, BC₁F₂-1: homozygous single mutant 1, BC₁F₂-2: homozygous single mutant 2

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9 Supplementary data on CD/DVD

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

File name	Content	Format
Reference sequences	<i>BnSFAR1</i> – <i>BnSFAR5</i> annotated gene sequences	.clc
Seed codes.xlsx	Seed codes of the produced plant material	Microsoft Excel file
Polyacrylamide gels.zip	Polyacrylamide gels for <i>BnSFAR1</i> and <i>BnSFAR4</i>	.xml and .tif, to be analyzed with GelBuddy TILLING Gel Analysis Tool
AFLP genotyping.xlsx	Genotyping data using AFLP markers	Microsoft Excel file
SNP genotyping.xlsx	Genotyping data using SNP array	Microsoft Excel file
Source file.xlsx	Source data for chapter 2 and chapter 3 figures	Microsoft Excel file

10 Curriculum Vitae and Publications

10.1 Curriculum vitae

Personal Data

Name: Nirosha Lakmali Karunaratna, Dewatha Pedige

Date of birth: 14th August 1987, Kegalle, Sri Lanka

Place of birth: Kegalle, Sri Lanka

Nationality: Sri Lankan

Education

02/2016 - 02/2020 Ph.D. student at the Plant Breeding Institute, University of Kiel.
Topic: Functional analysis and mutagenesis of *GDSL* genes for breeding oilseed rape (*B. napus*) with higher oil content

10/2013 - 10/2015 Master of AgriGenomics, University of Kiel
Master's thesis at the Plant Breeding Institute, University of Kiel
The topic of master's thesis: Screening for mutations in different *BnAPI* paralogs in rapeseed by TILLING

08/2008- 10/2012 Bachelor of Science in Agriculture Technology and Management,
Faculty of Agriculture, University of Peradeniya, Sri Lanka
The topic of bachelor's thesis: Varietal evaluation of greenhouse Bell Pepper (*Capsicum annuum* L.) in response to organic and inorganic fertilizer in the temperate climate

10.2 Publications

10.2.1 Articles

Karunarathna, N. L., Wang, H., Harloff, H.-J., Jiang, L. and Jung, C. (2020) Elevating seed oil content in a polyploid crop by induced mutations in *SEED FATTY ACID REDUCER* genes. *Plant Biotechnol. J.*, <https://doi.org/10.1111/pbi.13381>

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

10.2.2 Oral presentations and posters

Karunarathna N.L., Harloff, H.-J., Jung, C. (2019), Gene knock-out by CRISPR-Cas9 and EMS-induced point mutations on *SEED FATTY ACID REDUCERS* increase the seed oil content in rapeseed (*Brassica napus*), International Rapeseed Conference, Berlin, 16-19th June, 2019. Oral presentation

Karunarathna N.L., Harloff, H.-J., Jung, C. (2019), Knock-out of *SEED FATTY ACID REDUCER4* by CRISPR-Cas9 increases seed oil content in rapeseed (*Brassica napus*), Kiel Plant Center Mini-Symposium, Kiel, 17.01.2019. Oral presentation

Karunarathna N.L., Wang H., Harloff, H.-J., Jiang L., Jung, C. (2018), Functional analysis and mutagenesis of *GDSL* genes for breeding oilseed rape (*Brassica napus*) with higher oil content, GPBC2018, Wernigerode, 28.02-02.03.2018. Poster presentation

Karunarathna N.L., Wang H., Harloff, H.-J., Jiang L., Jung, C. (2018). Functional analysis and mutagenesis of *GDSL* genes for breeding oilseed rape (*Brassica napus*) with higher oil content III. International PP1530 Symposium: Genetic Variation of Flowering Time Genes and Applications for Crop Improvement, Kiel, 14-16.02.2018. Poster presentation

Karunarathna N.L., Wang H., Harloff, H.-J., Jiang L., Jung, C. (2017), Functional analysis and mutagenesis of *GDSL* genes for breeding oilseed rape (*Brassica napus*) with higher oil content, Botanikertagung 2017, Kiel, 17.-21.09.2017. Poster presentation

Karunarathna N.L., Wang H., Harloff, H.-J., Jiang L., Jung, C. (2017). Functional analysis and mutagenesis of *GDSL* genes for breeding oilseed rape (*Brassica napus*) with higher oil content PP1530 International Workshop: 'Genome Engineering', Gatersleben, 22.-23.06.2017. Poster presentation

11 Declaration of own contribution

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

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: Nirosha L. Karunarathna

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


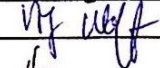
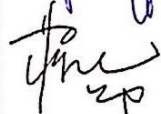

Elevating seed oil content in a polyploid crop by induced mutations in *SEED FATTY ACID REDUCER* genes

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


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

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

4. Signature of all co-authors

Date	Name	Signature
28.11.2019	Haoyi Wang	
29.11.2019	Hans-Joachim Harloff	
28.11.2019	Lixi Jiang	
29.11.19	Christian Jung	

5. Signature of doctoral candidate

Date	Name	Signature
29.11.2019	Nirosha L. Karunarathna	

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