Characterization of a thermosensitive male sterility system in *Brassica napus*

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Petra Schuhmann

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Erstgutachter: Prof. Dr. Jürgen Soll

Zweitgutachter: Prof. Dr. Peter Geigenberger

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Summary

Heterosis is an important trait used in plant breeding to obtain a higher yield, since the F1 generation is outperforming their parental lines. For obtaining heterosis or hybrid vigor, male sterile lines are used to prevent self pollination. Using male sterile lines comes with other problems. The most important one is the generation of seeds for these lines. Depending on the ms system (cytoplasmic (CMS) or genic male sterility (GMS)) different breeding options are used. For the GMS system, only a two line system is necessary, because most GMS sterile lines can be converted to fertility by different environmental changes. However, the process behind it is still not understood in plants.

In this thesis, the male sterility system LEMBKE (MSL), a system in *Brassica napus*, is used, in which the sterile C545 line can be reverted into fertility by heat treatment at 37° C. This treatment is rather energy consuming and requires a large effort by breeding companies. The MSL system is regulated by two genes, the restorer gene, which was identified as BnTic40C9 and when present restores fertility. The Tic40 protein is known to play a role in protein import into chloroplasts and the *attic40* knock out shows a pale, but fertile phenotype. The other component for coordinating the MSL system is the so called male sterility gene, which causes the male sterile phenotype.

The first part of this work includes the analysis of several candidates for the ms gene and the final determination of this gene, as well as its chloroplastic localisation. Characterization of *A. thaliana* mutants, carrying the proposed ms gene from *B.napus* showed a sterile phenotype. Due to the same localisation of the ms gene and the restorer gene, a possible interaction between the according proteins was investigated using the yeast-two hybrid system. Furthermore, possible differences concerning the sequence and function of the restorer gene compared to the other *B.napus* Tic40s, were analysed.

In the second part of this thesis, different treatment strategies were used for one of the sterile lines of the MSL system (C545), to establish under which condition this line can obtain fertility. The treatments, which were able to revert the sterile phenotype at least partially to fertility, were further analysed via transcriptome analysis. Therefore RNA sequencing was performed, also in combination with laser capture microdissection to examine only microspores. With this approach it can be hypothesised, that genes associated with pollen development, especially related to sporopollenin biosynthesis are differentially expressed after heat treatment. Furthermore, stress induced gene ontology (GO) terms which are related to the ubiquitin-proteasome system as well as polyamines seem to be involved.

Zusammenfassung

Der Heterosis-Effekt ist ein wichtiges Merkmal für die Pflanzenzucht, um den Ertrag zu erhöhen, da die F1-Generation ihre Elternlinien übertreffen. Zur Erzielung von Heterosis (oder *hybrid vigor*) werden männlich-sterile (ms) Linien verwendet, um die Selbstbestäubung zu verhindern. Die Benutzung von männlich-sterilen Linien ist allerdings nicht unproblematisch. Das größte Problem ist herbei die Gewinnung von Samen für diese Linien. Abhängig davon, welches ms System benutzt wird (zytoplasmatisch oder genetisch männlich steriles System), werden unterschiedliche Zuchtansätze benutzt. Charakteristisch für das GMS System ist ein Zwei-Linien-System, da die meisten sterilen Linien durch verschiedene Umweltveränderungen zu fertilen Linien umgewandelt werden können. Der genaue Prozessablauf in Pflanzen kann allerdings immer noch nicht erklärt werden. Im CMS System können Samen nur durch Kreuzen mit einer Linie mit dem gleichen genetischen Hintergrund, aber fertilen Zytoplasma (*maintainer* Linie), erhalten werden.

Für diese Arbeit wurde das männliche Sterilitätssystem LEMBKE (MSL), ein System in *B. napus*, verwendet bei dem die sterile C545 Linie durch die Hitze Behandlung bei 37 °C fertil wird. Diese Behandlung verbraucht viel Energie und benötigt einen hohen Aufwand von Seiten der Zuchtbetriebe. Das MSL System wird von zwei Genen reguliert. Das erste Gen ist das Restorer-Gen, welches als *Bn*Tic40 C9 identifiziert wurde und bei dessen Anwesenheit die Fertilität bewahrt wird. Das Tic40 Protein ist bekannt für seine Rolle im Protein-Import in Chloroplasten. Die *attic40* knockout Mutante zeigt einen blassen, aber fertilen Phänotyp. Die andere Komponente, die für die Koordinierung des MSL Systems verantwortlich ist, ist das sogenannte männliche Sterilitätsgen, welches den männlich-sterilen Phänotyp verursacht. Der erste Teil dieser Arbeit beinhaltet die Analyse mehrerer Kandidaten für das ms Gen und die letztendliche Bestimmung dieses Gens, sowie dessen Lokalisierung in Chloroplasten zu beweisen. Die Charakterisierung von *A. thaliana* Mutanten mit dem wahrscheinlichen ms Gen aus *B. napus* weisen einen sterilen Phänotyp auf. Aufgrund derselben Lokalisierung des ms Gens und des Restorer-Gens in Chloroplasten wurde eine mögliche Interaktion zwischen den beiden Proteinen mit Hilfe des Hefe-zwei-Hybrid Systems untersucht. Außerdem wurden mögliche Unterschiede in der Sequenz und Funktion des Restorer-Gens im Vergleich zu den anderen *B. napus* Tic40 analysiert.

Im zweiten Teil dieser Arbeit wurden verschiedene Behandlungsstrategien für die C545 Linie des MSL Systems getestet, um herauszufinden unter welchen anderen Konditionen diese Linie fertil wird. Die Behandlungen, welche zumindest zum Teil den sterilen Phänotyp zur Fertilität verändern können, wurden mittels Transkriptomanalysen ausgewertet. Dafür wurden die Methoden der RNA Sequenzierung aber auch Lasermikrodissektion, um nur Mikrosporen untersuchen zu können, eingesetzt. Mit diesem Ansatz kann die Hypothese aufgestellt werden, dass Gene, die mit der Pollenentwicklung, insbesondere im

Zusammenhang mit der Sporopollenin-Biosynthese, assoziiert sind, nach der Wärmebehandlung unterschiedlich exprimiert werden. Darüber hinaus scheinen stressbedingte *Gene ontology* (GO) Begriffe, welche zum Ubiquitin-Proteasom System gehören, sowie Polyamine beteiligt zu sein.

Abbreviations

(E)GMS	(environmental sensitive) genic male sterility
A. thaliana/At	*Arabidopsis thaliana*
A. tumefaciens	*agrobacterium tumefaciens*
ADC	arginine decarboxylase
ALB4	Albino4
AMS	aborted microspores
AOX	alternative oxidase
ARF	auxin responsive factor
ATP	adenosine triphosphate
B. napus/Bn	*Brassica napus*
Bark1	BAK1-Associating Receptor-Like Kinase 1
BCA assay	bicinchoninic acid assay
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
CMS	Cytoplasmic male sterility
Col-0	*A.thaliana* ecotype Columbia
cTP	chloroplastic transit peptide
DEGs	differentially expressed genes
dNTP	nucleoside triphosphate
DTT	Dithiothreitol
DYT1	dysfunctional tapetum 1
E.coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FDR	false discovery rate
g	relative centrifugal force (RCF)
GAPB	Glyceraldehyde-3-phosphate dehydrogenase B
gDNA	genomic DNA
GFP	green fluorescent protein
GO	gene ontology
GST	glutathione S-transferase
GTP	guanosine triphosphate
Hip	Hsp70 interacting protein
Hop	Hsp70/Hsp90 organizing protein
Hsp	heat shock protein
IEM	inner envelope membrane
I-NAA	1-naphthaleneacitic acid
IPTG	Isopropyl ß-D-1-thiogalactopyanoside
kDa	kilodalton
ko	knock out
LMD	laser microdissection
LSM	laser scanning microscopy
ms	male sterility
MS	Murashige and Skoog
MSL	male sterility system LEMBKE

N. benthamiana	*Nicotiana benthamiana*
NADH	nicotinamide adenine dinucleotide
NPZ	Norddeutsche Pflanzenzucht Hans-Georg Lembke KG
O. sativa	*Oryza sativa*
OCD	ornithine decarboxylase
OEM	outer envelope membrane
orf	open reading frame
P. sativum	*Pisum sativum*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCA	principle component analysis
PCD	programmed cell death
PCR	polymerase chain reaction
PMC	pollen mother cell
PMSF	phenylmethylsulfonyl fluoride
PPR	pentatricopeptide repeat
PVDF	Polyvinylidene difluoride
PVP	polyvinylpyrrolidone
qRT-PCR	quantitative real time PCR
RACE	rapid amplification of cDNA-ends
RING	really interesting new gene
RNAi	RNA interference
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SPP	stromal processing peptidase
SRP	signal recognition particle
TAE	Tris-acetate-EDTA
TAT	Twin Arginine Translocase
TCA	trichloroacetic acid
TDF1	tapetal development and function 1
TIC /TOC	translocon of the inner/outer chloroplast
TMD	transmembrane domain
UPS	ubiquitine-proteasome system
XTH16	xyloglucan endotransglucosylase-hydrolase
YTH	Yeast two hybrid

1 Introduction

1.1 Male sterility and pollen development

Male sterility (ms) plays an important role in plant breeding for obtaining heterosis or hybrid vigor during hybrid seed production. Heterosis results in a higher yield of the heterozygous offspring compared to their homozygous parental lines (Tester and Langridge, 2010). To obtain this outcome it is important to avoid self-pollination in order for crossing to be even possible. Because of the laborious work in manual emasculation, male sterile lines are used. Male sterility results in the plants inability to produce functional anthers and therefore failure of pollen release while leaving the female organs intact. Hence, understanding the process of anther and pollen development is crucial for gaining insights into the processes which take place in male sterile plants.

1.1.2 Anther and pollen development

Anther development starts with the generation of three layers: L1, L2 and L3 (outer to inner layer) which differentiate further into four cell layers, surrounding the four anther lobes. The innermost of the four cell layers of the anther wall surrounding the developing pollen is called the tapetum. The tapetum is surrounded by the middle layer, the endothecium while the outer layer is called the epidermis (Goldberg *et al.*, 1993) (Figure 1). The degeneration of the tapetum cells is initiated via programmed cell death (PCD). The time frame for PCD is species dependent (Gómez *et al.,* 2015; Browne *et al*., 2018) but any shift in the timing can lead to male sterility (Kawanabe *et al*., 2006). PCD for tapetal breakdown is necessary for anther dehiscence and normal pollen development (Gómez *et al*., 2015). Pollen development can be sectioned into three stages, which are named microsporogenesis, post-miotic development of microspores and microspore mitosis (Chaudhury *et al*., 1993). It all starts with the pollen mother cell (PMC) (Figure 1) which develops after two miotic steps into the tetrad form of microspores. Pollen mother cells are surrounded by a callose wall, which consists of a ß-1,3-glucan polymer. The callose wall aids in building the pollen wall, which breaks down after tetrad formation to release the free microspores into the anther locules (Wilson and Zhang, 2009). This is initiated by release of the enzyme callase from the tapetum, which happens at the end of miosis (Scott *et al.*, 2004). However, the callose breakdown does not appear in the mutant of *tapetal development and function 1 (tdf1)*. TDF1 encodes for a R2R3 transcription factor, which is mainly expressed in microspores and the tapetum. The knockout of this gene leads to a male sterile phenotype due to the defects during the tapetal development (Zhu *et al*., 2008). The tapetum plays an essential role in formation of the pollen wall. The pollen wall is composed of the outer exine and the inner intine layers. *ARF17*, an auxin responsive factor, is important for primexine development,

which is regulated by the callose wall, and hence plant fertility (Yang *et al*., 2013). Both, exine as well as intine, consist of two layers. For intine they are called exintin and enditine and in the case of exine they are named nexine and sexine. The sexine can also be divided into the tectum and bacula, while the nexine includes nexine I and nexine II (Jiang *et al*., 2013). The composition and structure of the intine inner layer is mainly synthesized by microspores while the exine layer consists mainly of sporopollenin, which is released from the tapetum (Piffanelli *et al.*, 1998; Wilson and Zhang, 2009). The so called Ubisch bodies are small granules and thought to play a role in the sporopollenin synthesis, by transport of components from the tapetum to the still developing microspores in rice and other cereals (Wang *et al*., 2003). However, they are not detected in *A. thaliana* (Zhang *et al*., 2011). Due to its high resistance to external conditions, the sporopollenin acts as a protective layer for the pollen grains (Meuter-Gerhards *et al*., 1999). A mitotic division of the microspores results in a vegetative cell containing a smaller generative cell (Figure 1). The second mitosis gives rise to two sperm cells (Jiang *et al*., 2013).

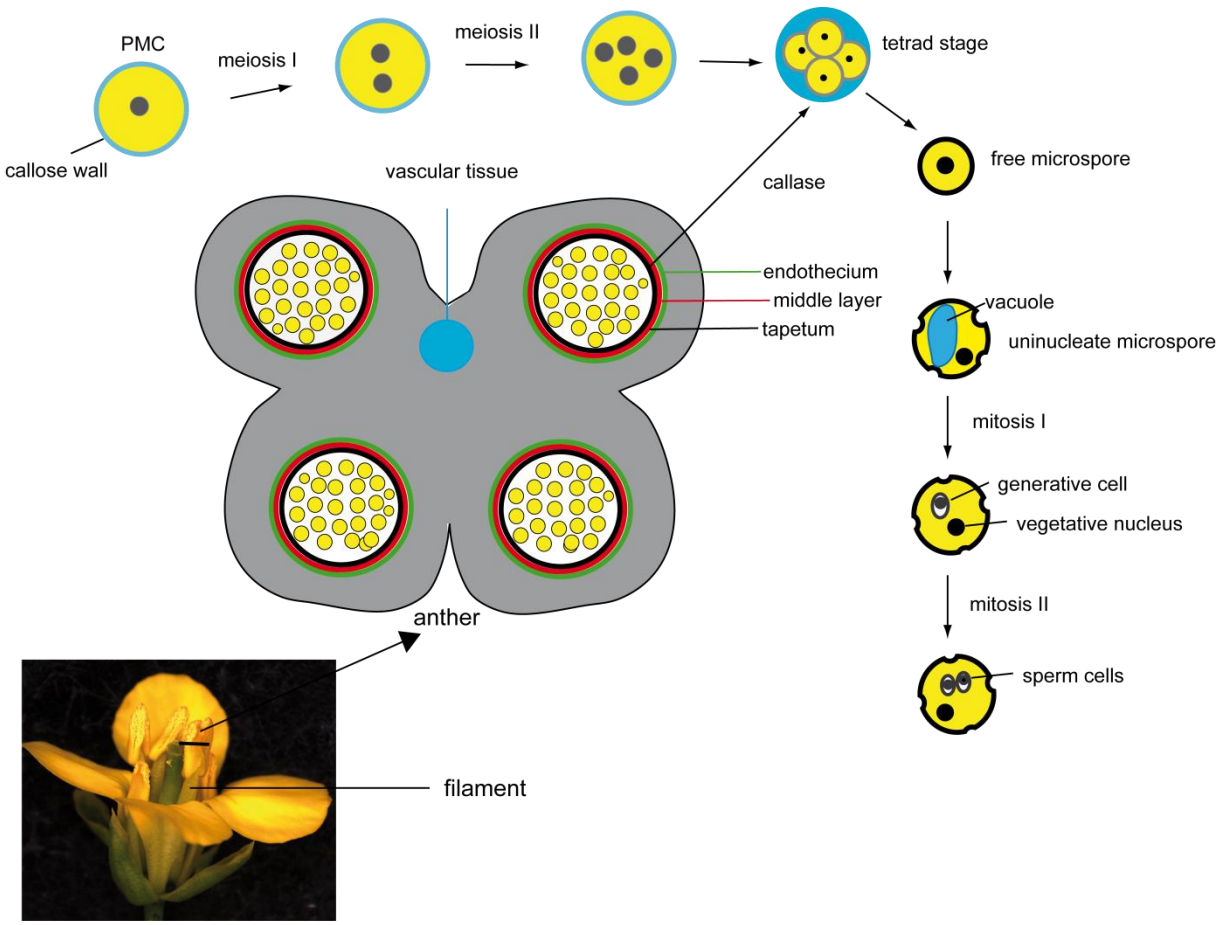

Figure 1: Overview demonstrating pollen development and anther structure. The pollen mother cell (PMC) is surrounded by the callose wall. After two miotic steps, the tetrad stage is formed. The enzyme callase is released from the tapetum which destroys the callose wall to free the microspores. The next stage, the uninucleate microspore, forms via mitosis I into a vegetative cell containing a generative cell. After a second mitotic process two sperm cells are formed which results in mature pollen. (Adapted from Honys *et al*., 2006).

Then, after anther elongation and flower opening, the pollen are released. When the pollen come in contact with the stigma and become hydrated, the pollen tube is formed (Zheng *et al*., 2018). Any disturbance during these anther and pollen developmental steps can lead to sterility of the plant. Besides of the already mentioned genes, there exist several other transcription factors in *A. thaliana*, which are involved in the regulation of anther and pollen development and are therefore responsible for a fertile phenotype. This results in a strongly regulated and complex process for pollen development. However, only a few selected transcription factors are going to be mentioned.

Dysfunctional Tapetum 1 (*DYT1*) encodes for a proposed basic helix-loop-helix (bHLH) transcription factor expressed in the tapetum and seems to have a regulation function concerning tapetal genes. The *A. thaliana* mutant *dyt1* is male sterile and shows defects in tapetum development and function (Zhang *et al*., 2006). *Aborted microspores* (*AMS*) is also expressed in the tapetum, belongs to the bHLH proteins and is important for tapetum development. The *ams* mutant shows failure of pollen development and therefore also exhibits a sterile phenotype (Sorensen *et al*., 2003). The *Male Sterility 1* (*MS1*) gene encodes a plant homeodomain transcription factor and the *ms1* mutant does not produce viable pollen and shows defects in PCD of the tapetum (Wilson *et al*., 2001; Vizcay-Barrena and Wilson, 2006). The gene *MALE STERILITY 2* (*MS2*) encodes for a possible fatty acid reductase and the Arabidopsis *ms2* mutants are not able to produce viable pollen and therefore show a male sterile phenotype. The expression of MS2 occurs in the tapetum during microsporogenesis (Aarts *et al*., 1997). MYB103/MYB80 are also important for tapetum development in *A. thaliana* (Zhang *et al*., 2007) and, as well as AMS and MS1, expressed in the tapetal cells. Furthermore these three transcription factors also seem to play a major role in obtaining a functional pollen wall (Quilichini *et al*., 2015).

1.1.3 Male sterility in crops

Male sterility is mainly represented by cytoplasmic male sterility (CMS) and genic male sterility (GMS) systems. CMS is triggered by mitochondrial genes in combination with nuclear genes and inherited maternally while in GMS, only nuclear genes are involved. CMS genes, which are causing ms are usually specified as open reading frames (*orf*). An example of a CMS gene is URF13 which was found in the maize CMS-T and exhibits a chimeric structure (Dewey *et al*., 1987). The CMS genes are often described as chimeric genes, which are genes assembled from different mitochondrial genes combined with unknown sequences (Pelletier and Budar, 2007). However, not all chimeric genes which can be found in the plant mitochondrial genome seem to function as CMS genes (Marienfeld *et al*., 1997; Pelletier and Budar, 2007) and not all CMS genes are of a chimeric structure, for example the truncated

version of Cox2 which was discovered in the sugar beet CMS-G system (Ducos *et al*., 2001). The antagonist of the CMS gene is the restorer-of fertility gene (restorer gene).

For the CMS system the majority of restorer genes encode pentatricopeptide repeat (PPR) proteins (Chen and Liu, 2014) like Rf1a in rice (Akagi *et al*., 2004) or Rfn in *B. napus* (Liu *et al*., 2017). These PPR proteins belong to a huge family of RNA binding proteins. The main characteristic of these proteins is a degenerated 35-amino acid motif. The *A. thaliana* genome encodes for about 450 PPR proteins and most of them are localized in the mitochondria or chloroplasts (Lurin *et al*., 2004)

A three line system is necessary for obtaining seed material of the sterile line and producing hybrid vigor in the CMS system. These lines include the sterile CMS line which contains the CMS gene and the non-functional restorer gene. The maintainer line has the same nuclear genome as the CMS line but is fertile because of the different cytoplasm. The function of the maintainer line is to maintain the sterile CMS line. This is achieved by crossing the CMS line with the maintainer line which results in seeds for the sterile CMS line. The restorer line contains the restorer gene and the crossing between the sterile CMS line and the restorer line leads to the fertile F1 generation of hybrid plants. Male fertility in the hybrids is restored by the restorer gene. Seed production for the maintainer line and the restorer line occurs by self-pollination of these two lines (Chen and Liu, 2014).

The GMS system is caused solely by nuclear genes (Chen and Liu, 2014) and usually controlled by a recessive gene (Singh *et al*., 2019). When the sterile line can retain fertility under different growth conditions like changes in light and temperature, it is labelled an environmental sensitive GMS (EGMS) system. In this case the GMS system can work with two lines, because the maintainer line is not necessary for obtaining hybrid seeds, which reduces costs and facilitates seed production (Chen and Liu, 2014). MS systems can be found in many crops like rice, maize, wheat, tomato, cotton, sunflower and different Brassica species (Akter *et al*., 2016; Rieseberg *et al*., 1994; Chen and Liu, 2014). For *Brassica napus* (*B. napus),* there exist different cytoplasm leading to CMS, like Ogura (*ogu*), Polima (*pol*), *nap, cam* and *hau* (Singh *et al*., 2019). *B. napus* (rapeseed) plays an important role in the production of vegetable oil as well as biodiesel and is grown worldwide. Cultivation started in Europe during the middle ages (McVetty *et al.,* 2016). *B. napus* (AACC) is an allopolyploid species and developed after hybridization of *Brassica rapa* (AA) and *Brassica olearacea* (CC) (Parkin *et al*., 1995; Chalhoub *et al*., 2014).

The Chinese ms system 7-7365ABC in *B. napus* is a genic male sterility system, which is composed of three lines. The 7-7365A line is sterile, while 7-7365B is the fertile wildtype line and the 7-7365C line as the fertile temporary maintainer line. This system is controlled by BnaMs3/Bnams3 and BnaMs4^{b} genes (Zhou *et al*., 2012, Dun *et al*., 2011, Xia *et al*., 2016). Histological analysis of the 7-7365 A and B- and C-line of anthers in different developmental

stages revealed that for the wild type (7-7365B) and the other fertile 7-7365C line, the tapetum started to degenerate normally and the pollen mother cells developed into free microspores. In contrast the tapetum cells of the 7-7365A line started to grow larger and lead to degradation of the anther without releasing functional microspores (Zhu *et al*., 2010, Zhou *et al*., 2012). It was also demonstrated that some sterile flowers of the sterile line 7-7365A could be restored to being fertile after heat shock treatment (35-45 °C) for one day (Zhu *et al*., 2010). The BnaMs3 gene was identified as BnTic40C9, which can restore fertility and is believed to be important for pollen development (Dun *et al*., 2011; Dun *et al*., 2014; Zhou *et al*., 2012). Tic40 is a chloroplast localised protein and plays a role in importing proteins into chloroplasts (Stahl *et al.*, 1999; Chou *et al*., 2003). For another GMS system (9012AB), the BnMs3 gene was also identified as BnTic40C9 via a map-based cloning approach (Li *et al*., 2012). The difference between BnaMS3 and the Bnams3 (*Bntic40c9*) gene in the described T45 maintainer line was identified as a two base pair (bp) deletion (Li *et al*., 2012), while for the 7365ABC system a three bp deletion accompanied with an insertion of nine nucleotides and several single nucleotide substitutions (Dun *et al*., 2011).

It was shown, that BnTic40C9 (BnaMS3) is highly expressed in seedlings, anthers, 20-day old seeds, inflorescence tissue, stem and root (Dun *et al*., 2011; Dun *et al*., 2014), but also in the tapetum and microspores (Dun *et al.,* 2011). Although there are four BnTic40 proteins in *B. napus* (BnTic40 C9, A10, C2 and A2, named after their chromosomal localisation), only one of them was shown to be able to restore fertility in *B. napus* (Dun *et al.,* 2011, Li *et al*., 2012; Dun *et al*., 2014). Furthermore, it was speculated, that the new function of the BnTic40C9 as restorer gene for fertility in the 7-7365A line arose during the evolution of the ma gene. Therefore the restorer gene might interact with the ms gene (Dun *et al*., 2014).

Later on, the ms gene for the 9012AB and 7-7365ABC MS system was identified (Deng *et al*., 2016; Xia *et al*., 2016). Both groups describe a similar chimeric gene, while the determined localization differs. This ms gene (Bnams4^{b}) is proposed to be localized either in the nucleus (Deng *et al*., 2016) or the chloroplast (Xia *et al*., 2016).

1.2 Protein import into chloroplasts

Due to an endosymbiotic event, a eukaryotic host already containing mitochondria engulfed an ancestral cyanobacterium (Gould *et al*., 2008, Timmis *et al*., 2004). On the basis of this event most of the chloroplast genome was transferred to the host nucleus. Of the approximately 3000 chloroplastic proteins, only ~100 proteins are still encoded in the chloroplast genome (Timmis *et al.,* 2004). Hence, the chloroplast proteins which are now encoded in the nucleus and translated in the cytosol, need to be imported into the chloroplast. This led to the development of a protein import system which is named TIC/TOC

(translocon of the inner and outer chloroplast membranes) complexes localised in the two chloroplast envelope membranes according to their names (Sjuts *et al*., 2017) (Figure 2). The chloroplast proteins, which are translated in the cytosol, carry a cleavable N-terminal sequence. This so called transit peptide (TP) directs the translated proteins to the chloroplast. The size of TPs range between 10 and 150 amino acids (Teixeira and Glaser, 2013) and contain a low amount of acidic residues which leads to an overall positive charge (Heijne *et al*., 1989). The TP is cleaved off by the stromal processing peptidase (SPP) after the protein has entered the stroma (Richter and Lamppa, 1998).

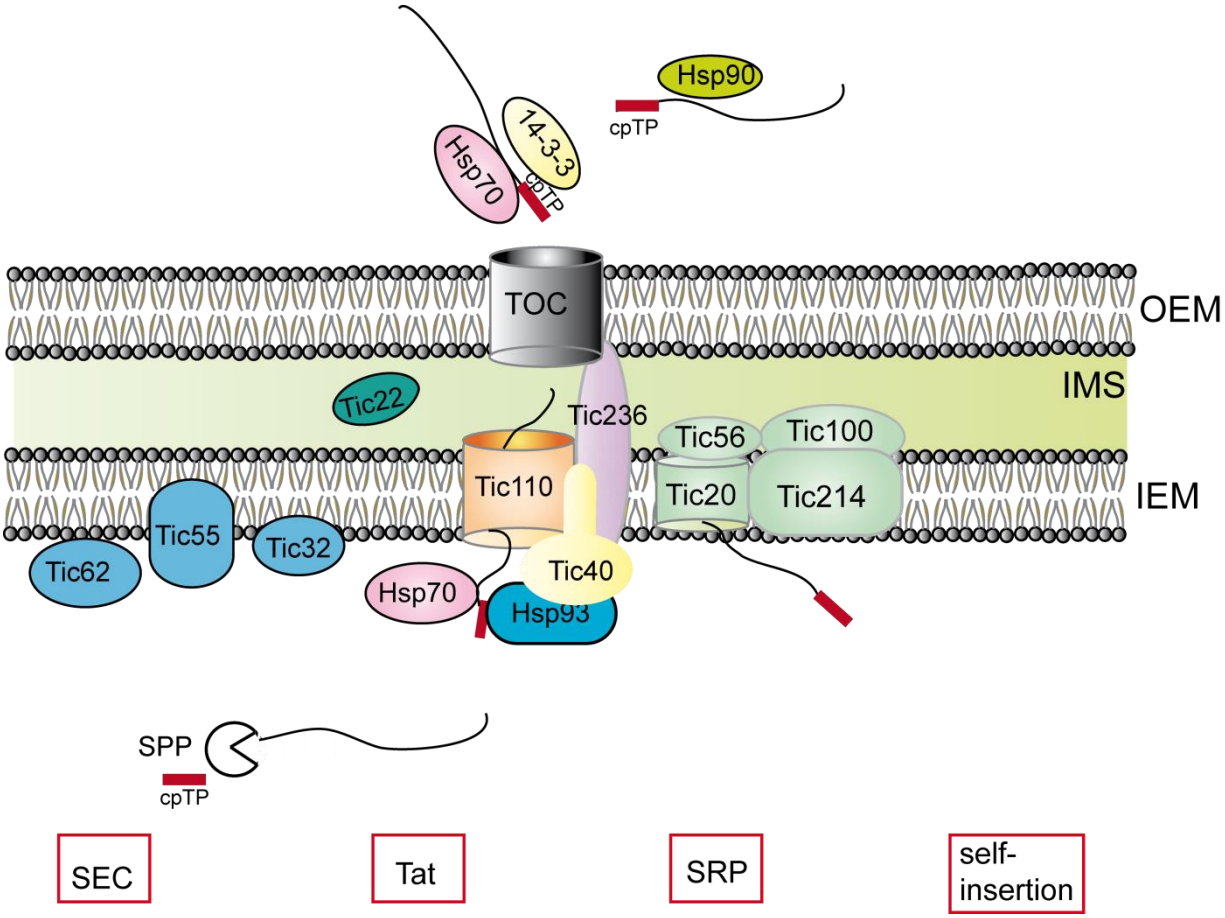

Figure 2: Model of the protein import system through the chloroplast membranes. Pre-proteins are chaperoned to the TOC complex in the outer envelope membrane (OEM). After crossing the OEM, the pre-proteins are guided with the help of the Tic components Tic22 and Tic236, which both interact with the TIC and TOC complex. Tic110 and Tic20 are the proposed channel proteins. Tic110 in combination with Tic40 allows the pre-proteins to pass the inner envelope membrane (IEM), with the help of different chaperones. The redox regulon is composed of Tic62, Tic55 and Tic32, which interact with Tic110. The other channel protein Tic20 in combination with Tic100, Tic56 and Tic214 assemble into the so called 1 MDa complex, which might offer an alternative route for protein import. After passing the two membranes, the cpTP is cleaved of by the SPP. Proteins localised in the thylakoids, can be either inserted by the SEC, Tat and SRP pathway or via self-insertion.

First of all, the cytosolic translated proteins carrying the transit peptide need to be directed towards the chloroplast outer membrane. This task involves different chaperones and is either carried out by the cytosolic Hsp90 (Qbadou *et al*., 2006) or the so called guidance

complex, which involves Hsp70 in combination with 14-3-3 proteins (May and Soll, 2000). The main Toc complex consists of the proteins Toc75, Toc159, and Toc34. The pre-proteins involved with the chaperones bind to either of the two GTPases Toc159 or Toc34 which are important for pre-protein recognition and function as the main pre-protein receptors in the outer membrane (Becker *et al*., 2004). Pre-proteins bound to Hsp90 connect with Toc64 from where the pre-proteins are transferred to Toc34. Toc64 interacts with Hsp90 via its TPR domain (Qbadou *et al.,* 2006). The major transport channel is built by the ß-barrel protein Toc75 (Hinnah *et al*., 2002) and the knockout of AtToc75-III in *A. thaliana* is embryo lethal (Baldwin *et al*., 2005). After passing the Toc complex, the pre-proteins need to cross the inner membrane space (IMS) to reach the Tic complex.

The protein Tic22 is exposed to the inner membrane space (Rudolf *et al*., 2013) and until recently, the only identified protein in this compartment involved in protein import. Because of its interaction with pre-proteins it is thought to be associated with protein import by aiding pre-proteins from the TOC to the TIC complex (Kouranov *et al*., 1998). For the inner membrane proteins, Tic110 was the first component which was observed (Schnell *et al.,* 1994). Tic110 homozygous *A. thaliana* mutants are lethal, which implies an essential function (Inaba *et al*., 2005). Tic110 is hypothesized to build the main import channel for the inner chloroplast membrane (Balsera *et al*., 2009b; Kovacheva *et al*., 2004). In combination with Tic40 and Hsp93, Tic110 plays a role for the protein import through the inner chloroplast membrane (Kovacheva *et al*., 2004; Chou *et al*., 2006). Tic40 acts as a co-chaperone during protein import (Chou *et al*., 2003) and interacts with Hsp93 leading to ATP hydrolysis (Chou *et al.,* 2006). It was later demonstrated that Hsp70 as well as Hsp90 are also involved in the protein import process (Chou *et al*., 2006; Inoue *et al.,* 2013), especially because double mutants of tic40 and cpHsp70 are lethal (Su and Li, 2010). However, recently it was proposed, that Hsp93 might rather play a role in the quality control during protein import, than in energy supply (Flores-Pérez *et al*., 2016).

The three components Tic55, Tic62, Tic32 were identified as a redox regulon for regulating the protein import through the inner membrane. This regulation seems to be dependant on the $NADP^+$/NADPH ratio (Stengel *et al*., 2009). Tic55 is a Rieske iron-sulfur protein, while Tic62 and Tic32 are dehydrogenases and both proteins have NADP(H)-binding sites (Caliebe *et al*., 1997; Hörmann *et al.,* 2004; Stengel *et al.,* 2008).

However, not only Tic110, but also Tic20 was also shown to be a channel protein in the inner chloroplast membrane (Kovács-Bogdán *et al*. 2011). Recently, Tic20 was confirmed to be part of a 1 MDa Tic complex as the core protein. Next to Tic20 three further proteins were identified in this complex: Tic56, Tic100 and Tic214 (Kikuchi *et al*., 2013). Tic214 is also known as Ycf1 and encodes for an essential protein in *A.thaliana* (Sjuts *et al.,* 2017). Neither Tic110 nor Tic40 were found to be associated with this complex (Kikuchi *et al*., 2013). To this

point it is still under debate, which is the main import complex or if Tic110 and Tic20 just offer alternative routes.

Recently, another Tic component was identified and named Tic236, according to the size of the mature protein (Chen *et al*., 2018). Reduced import of different pre-proteins was shown in *tic236* mutant plants. Besides, Tic236 seems to be associated with Toc75, Tic110, Tic20 and Tic40. (Chen *et al*., 2018) and therefore might play a role in the transfer of pre-proteins from the TOC to the TIC complex (Schnell, 2019).

After the imported mature protein reaches the stroma, it will either be folded and inserted into the inner chloroplast membrane or redirected to the thylakoids, by stromal chaperones. For the proteins localised in the thylakoids four different pathways exist to redirect them to either the thylakoid membrane or lumen. These are called Signal Recognition Particle (SRP) dependent pathway, the chloroplastic Twin Argenine Translocase pathway (cpTAT), or Secretory (cpSEC) pathway as well as self-insertion (Schünemann *et al*., 2007; Albiniak *et al*., 2012).

1.2.1 The Tic40 protein

The Tic40 protein was first identified in *B. napus* in 1994 (Wu *et al*., 1994) as Cim44/Com44, which were described as inner and outer envelope proteins with a size of 44 kDa. It was renamed later to Tic40 and analysed in *P. sativum* with a mature protein size of 40 kDa (Stahl *et al*., 1999). Furthermore, evidence was presented which indicated that Tic40 is an inner envelope protein and part of the protein import apparatus (Stahl *et al*., 1999). One transmembrane domain (TMD) was predicted for Tic40 (Stahl *et al*., 1999; Chou *et al*., 2003). It was shown during protease digestion that the major part of Tic40 is present in the stroma, while it is anchored with the TMD in the inner envelope membrane (Chou *et al*., 2003; Li and Schnell, 2006). The Tic40 section directed to the stroma (C-terminus) consists of a degenerated TPR domain as well as a Sti1/Hop/Hip (Hop: Hsp70 and Hsp90 organizing protein; Hip: Hsp70 interacting protein) domain (Chou *et al*., 2003; Chou *et al*., 2006). The TPR domain interacts with Tic110, while Hsp93 binds to the Sti1 domain. However, the interaction of Tic110 with Hsp93 was shown to be stronger compared to Tic40 in combination with Hsp93 (Flores-Pérez *et al*., 2016). Furthermore Tic40 might also interact with stromal Hsp70s (Shi and Theg, 2010; Shi and Theg, 2013). For *A. thaliana tic40* ko mutants complemented with Tic40 constructs lacking either the transmembrane domain, the TPR domain or Sti1 domain, it was shown that they could not complement the *tic40* phenotype (Bédard *et al*., 2007). This implicates, that the mentioned Tic40 domains are important for its function (Bedard *et al*., 2007). However, after analysis of the amino acid sequence, it was proposed, that Tic40 might be composed of two Sti1 domains instead (Balsera *et al.,* 2009a). Tic40 in *A. thaliana* is encoded by one single gene. The T-DNA insertion mutants of *tic40* in

A. thaliana were described as pale coloured and showed a slower development compared to the wildtype. Furthermore, electron microscopy pictures showed reduced grana stacking in mutant chloroplasts compared to the wildtype. It was also shown, that the import rate into chloroplasts of *tic40* mutants was reduced (Chou *et al*., 2003), but no effects on pollen development or fertility were observed (Dun *et al*., 2011). The viability of the mutant plants indicates a rather accessory function in the import mechanism instead of an essential role (Li and Schnell, 2006). The *A. thaliana* Tic40 protein is expressed in seedlings and rosette leaves, as well as in flowers and roots on a lower level (Kovacheva *et al*., 2004). With the help of *A. thaliana* mutant plants and protein import studies, it was proposed that Tic110, Tic40 and Hsp93 might function together during protein import into chloroplasts while interacting with each other (Kovacheva *et al*., 2004, Chou *et al*., 2006). Furthermore, a co-chaperone function of Tic40 during protein import was proposed (Bédard *et al*., 2007). In vitro it was shown that Tic40 is first processed by the SPP which leads to a stromal intermediate form. Apparently a second peptidase is necessary to obtain the mature protein after insertion into the inner chloroplast membrane (Tripp *et al*., 2007). Furthermore it was suggested, that Tic40 also plays a role in the insertion of proteins into the inner membrane from the stroma, itself particularly, but also Tic110 and Pic1 (Chiu *et al*., 2008). In a recent discovery, it was proposed that Tic40 might be important for the import mechanism of proteins with specific TPs (Lee and Hwang, 2019). This research group also speculated that there must be a Tic40-dependent and independent import pathway. In 2017, while screening for suppressors of tic40, the proteins STIC1 and STIC2 were identified (Bedard *et al.*, 2017). Stic1 actually correlates with ALB4 (ALBINO4), which belongs to the Alb3/Oxa1/YidC family, which are membrane protein insertases (Benz *et al*., 2009; Trösch *et al*., 2015). These findings lead to the assumption, that Tic40 might rather be involved in thylakoid biogenesis and protein insertion than the actual import process (Bedard *et al*., 2017; Chiu *et al*., 2008).
In conclusion, the exact role of Tic40 in protein import into chloroplasts still seems to be unclear in current research.

1.3 The MSL (Male Sterility Lembke) system

The genic male sterility system used for this thesis, was developed by the German breeding company Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) in the early 1980s (Frauen *et al*., 2003). More than 40 MSL hybrids exist and are cultivated in Europe (Frauen *et al*., 2007). In different trials the F1 hybrids of the MSL system show higher yield compared to other open-pollinated cultures (Frauen *et al*., 2003, Frauen *et al*., 2007). The system which is used in this thesis consists of two sterile, thermosensitive mutants (A- and C545-line), a maintainer line (B-line) and a restorer line (Monty) (Figure 3). However, restorer lines for this

ms system can be all known conventional *B. napus* lines (Frauen and Paulmann, 1999). The sterile lines have a special trait: they can be reverted to fertility with heat treatment. For the C545-line, this can be accomplished by treating the plants during their flowering period with 37 °C for three days. These systems are called thermosensitive genic male sterility systems (TGMS) (Tang *et al*., 2019). Hence, seeds for the sterile line can be obtained without the involvement of the restorer or maintainer line (as can be seen in Figure 3).

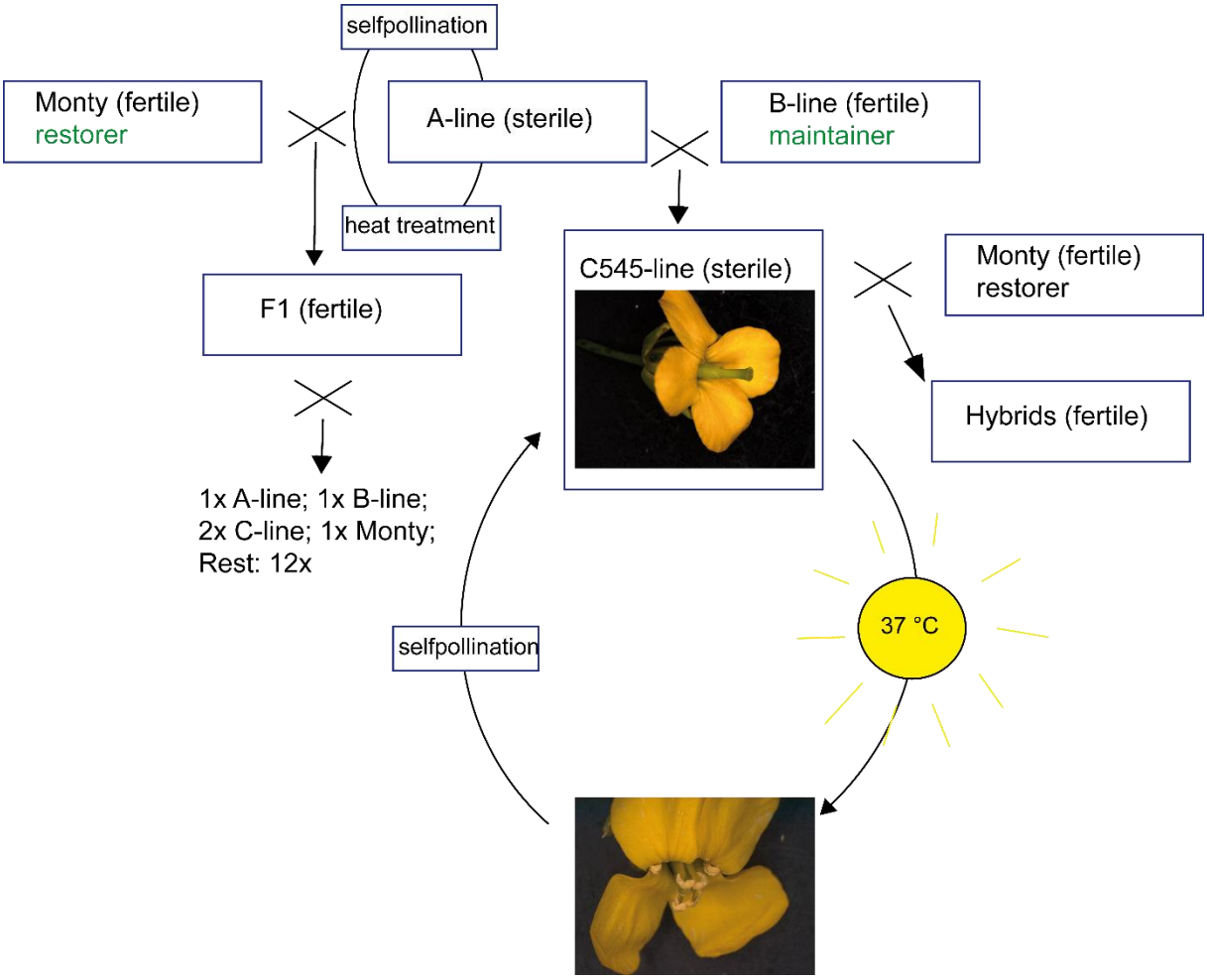

Figure 3: Overview of the crossing mechanism and seed production for the MSL system. Crossing of the sterile A-line with the restorer line results in the F1 generation. When the A-line is crossed with the maintainer line, the C545 line arises, which is sterile like the A-line. Crossing of the C545 line with the restorer line leads to the wanted hybrids. The C545 line can produce seeds after heat treatment at 37 °C for 3 days. The sterile A-line can also be reverted to fertility with heat treatment for a longer period of time. Another possibility to produce seeds from all the pictured lines in this ms system, can be achieved by crossing the F1 generation. The figure is based on data from the NPZ.

However, the treatment needs to be done at a certain developmental stage of the flower buds and is rather energy costly. The restorer gene was identified as *BnTic40C9* via mapping by the NPZ, similar to the description for the Chinese ms system (Dun *et al*., 2011,

2014). Although this gene is also named *BnaMs3* (Dun *et al*., 2011, Dun *et al*., 2014), in this thesis it will be labelled as restorer gene or *BnTic40C9*, since it is thought to restore fertility. The gene causing sterility is called the ms gene. Sequencing analysis for the MSL system revealed that only the restorer line (Monty) carries the WT form of the restorer gene.

In the A-, B- and C545-lines a mutated version of the restorer gene exists which leads to early stop codon and most likely to a non-functional protein. For the ms gene a certain region of the A07 chromosome was located via mapping by the NPZ resulting in several possible ms gene candidates.

In 2018 (Luo *et al*., 2018), the genic male sterile lines of the MSL system and 9012A, which belongs to a Chinese ms system, were compared. Cytological analysis revealed premature or retarded degradation of the tapetum for the sterile lines of both systems. Furthermore, anther development for the sterile MSL line could only be observed until the tetrad stage, while anther development was possible until the microspore stage for the 9012A line (Luo *et al*., 2018).

1.4 Aim of this thesis

There exist many different male sterility sytems in *B. napus* which are already intensively studied and described. For the MSL system, the restorer gene was confirmed via a mapping approach executed by the NPZ. Therefore, one important part of this thesis was determining the subsequent ms gene. Furthermore it was necessary to discover, if the restorer gene is interacting with the male sterility gene. This can only be the case if the ms gene is also localised within the chloroplast. Although four Tic40s exist in *B. napus* with a high sequence similarity, only one of them can restore fertility in the MSL system. To understand why, the difference of the restorer gene compared to the other Tic40 had to be analysed.

The male sterile lines of the MSL system are temperature sensitive. This leads to one major question: why does heat treatment of the sterile line lead to fertility of the plants without a fully functional restorer gene and which mechanisms in the plant are responsible? To this point the molecular regulations occurring in TGMS lines are not yet fully understood. This thesis will aim to answer these questions.

For breeding companies, it is indispensable to find a way to obtain seeds from their sterile lines and therefore hybrid plants. A better understanding about the molecular mechanisms of the ms system is required in order to facilitate its practical application for successful breeding and future hybrid development resulting in yield gain. The increase in yield of crops is not only important in an economical point of view, but also as food source for the growing world population.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

If not mentioned otherwise all chemicals were ordered from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma Aldrich (Taufkirchen, Germany) and Thermo Fischer Scientific (Braunschweig, Germany).

2.1.2 Oligonucleotides

All primers were ordered by Metabion (Martinsried, Germany). The oligonucleotides used in this work are listed in Table 9 in the appendix.

2.1.3 Plasmids

Table 10 is listing every generated plasmid/constructs used in this thesis and is located in the appendix. The C-terminus of the Chimera construct in pEX-K4 was synthesized by Eurofins (Planegg, Germany).

2.1.4 Antibodies

Purified protein (~ 1 mg) was sent to Pineda Antibody-service (Berlin, Germany) for immunisation of rabbits to generate an antibody against BnTic40sol (Tic40C9). Antisera directed against GFP was purchased from Roche (Penzberg, Germany) while the c-myc Antisera was obtained from Santa Cruz Biotechnology (Texas, USA). The secondary antisera rabbit horseradish peroxidase and mouse horseradish peroxidase were obtained from Sigma Aldrich. The first antibodies were used in a 1:1000 dilution in 3% milk or 5% BSA (anti-Chimera) in TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), while the secondary antibodies were diluted 1:10000 in TBST.

2.1.5 Enzymes

Restriction enzymes were either ordered form New England BioLabs (Frankfurt am Main, Germany) or Thermo Fisher Scientific. Phusion DNA Polymerase and the Q5 SDM kit were obtained from New England Biolabs. T4 DNA ligase was obtained from Thermo Fisher Scientific, Taq DNA Polymerase from Bioron (Ludwigshafen, Germany). BP and LR Clonase II (for Gateway Cloning) were purchased from Thermo Fisher Scientific.

2.1.6 Membranes

The PVDF membrane (Immobilon-P membrane, 0.45 µm) for western blotting was ordered from Macherey-Nagel (Düren, Germany), while the blotting paper was purchased from Millipore (Darmstadt, Germany).

2.1.7 Bacterial and Yeast strains

Top 10 (F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$) or **DH5alpha** (F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ$^-$)) cells were used for cloning methods obtaining DNA. **BL21 (D3)** (B F$^-$ *ompT gal dcm lon hsdS$_B$*($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S)) or **BL21 (D3) pLysS** (B F$^-$ *ompT gal dcm lon hsdS$_B$*($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S) pLysS[*T7p20 ori*$_{p15A}$](Cm^R)) strains were used for protein overexpression. The *Agrobacterium tumefaciens* strains used in this thesis were **AGL1** (C58 RecA (rif R/carbR) Ti pTiBo542DT-DNA Succinamopine) for transient transformation of *Nicotiana tabacum* and **GV3101** (C58 (rif r) Ti pMP90 (pTiC58DT-DNA) (gentr) Nopaline) for stable transformation of *Arabidopsis thaliana*. To create the Yeast-two-Hybrid (YTH) assay the strain **Y2HGold** (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS–Gal1TATA–His3,GAL2UAS–Gal2TATA–Ade2 URA3 : : MEL1UAS–Mel1TATA AUR1-C MEL1)* was used.

2.1.8 Accession numbers

The following accession numbers in Table 1 apply to the genes investigated in this thesis:

Table 1.: Accession numbers of the used genes in this thesis

AtTic40	At5g16620
Chimera male sterility gene	KX756984
BARK1	At3g23750
DNA-Methyltransferase	At3g23740
BnTic40A2	BnaA02g03180D
BnTic40A10	BnaA10g17870D
BnTic40C2	BnaC02g06750D
BnTic40C9	BnaC09g41260D
GAP B	At1g42970
AOX	X68702

2.1.9 Molecular weight markers

For determination of protein sizes on SDS-PAGEs the peqGOLD protein marker I (VWR, Ismaning, Germany) or the prestained protein latter PageRuler (Thermo Scientific) were utilized. Lambda phage DNA (New England Biolabs (NEB), Frankfurt am Main, Germany) was digested with PstI (NEB) to be used as a marker for DNA agarose gels.

2.2 Methods

2.2.1 Molecular biological methods

General methods concerning experiments for this thesis were performed according to (Sambrook *et al*., 1989) and are not subject to further explanations.

2.2.1.1 Molecular cloning strategies

Several different strategies were performed for cloning the different constructs used in this thesis.

The Gateway cloning system (Invitrogen) was used for cloning the PCR product from the entry vector pDONR207 into pk7GWIWG2(I) vector to construct a RNAi line and pH2GW7 for complementation of *A. thaliana.* The method was conducted according to the manufacturer's instructions.

Another cloning approach was construct assembly with the GoldenGate system (as described in Binder e*t al.*, 2014). The assembly of constructs for this system is based on the restriction enzymes BsaI and BpiI, depending on the different construct levels. For this thesis constructs up to LIII were designed, which contain two different genes with their according promoter with the plant resistance marker.

For base pair (bp) mutations in the DNA, the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used according to the manufacturer's instructions. Primers for site directed mutagenesis were designed using the NEB primer design software (www.NEBaseChanger.neb.com). The correct mutation was confirmed via sequencing.

RACE-PCR for cloning the ms gene from the sterile *B. napus* A/C545-line was done with the SMARTer RACE 5'/3' Kit (Clontech, Mountain View, USA) according to the manufacturer's instructions. The primers were either designed according to the manual or taken from (Xia *et al*., 2016). Therefore, RNA was isolated and used for synthesis of 5' ad 3' RACE cDNA. Nested PCR had to be performed to obtain the sequence, which was used to assemble the

ms gene via the GoldenGate system. The C-terminus could not be fully obtained with the RACE-PCR approach and was synthesized by a company (Eurofins, Planegg, Germany).

2.2.1.2 Polymerase chain reaction (PCR)

PCR was conducted on different templates (cDNA, gDNA or plasmid DNA) depending on the further procedure. Plasmid DNA was either used for further cloning or colony PCR. CDNA was necessary for cloning procedures, while for genotyping the extraction of gDNA was necessary. Phusion and Q5 polymerase with a proofreading function were chosen for cloning and site-directed mutagenesis. The Taq polymerase was used for colony PCR and all PCRs with gDNA as a template. The cycling conditions for each polymerase were chosen according to the manufacturer's instructions, with adaptation to the annealing temperature and elongation time. The PCR products were run on a 1% agarose gel in TAE buffer (40 mM Tris, 2.5 mM EDTA, 1% acetic acid) and extracted with the NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel).

2.2.1.3 Sequencing

The sequencing of the plasmids used in this work was conducted by the Sequencing Service (Genetics) of the LMU (Martinsried). Therefore 130-300 ng of plasmid or ~100 ng of PCR-product with the corresponding oligonucleotides was sent for the sequencing process.

2.2.1.4 Transformation of E.coli

Between 10-100 ng of plasmid DNA was transformed into 50 µl competent cells by performing heat shock at 42 °C for one min and cooling down the cells on ice afterwards. Thereafter, 250 µl of LB-medium (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl) was added to the cells to allow them to to grow for one hour at 37 °C. The cells were plated out on LB medium with appropriate antibiotics. Competent *E.coli* cells were made according to (Hanahan *et al.*, 1991)

2.2.1.5 Transformation of Agrobacterium tumefaciens

5 µl (approximately 2 µg) of plasmid DNA was transformed in 100 µl of AGL1 or GV3101 cells by incubating the cells on ice for 5 min, followed by an additional 5 min in liquid nitrogen and 5 min at 37 °C. 800 µl of LB were added to the cells. After 4 h incubation at 28 °C, the cells containing the plasmid were plated out on LB plates containing the appropriate antibiotics. To confirm the correct transformation with the right plasmid sequencing was performed.

2.2.1.6 DNA isolation from bacteria

Plasmid isolation was done with the nuclear spin Kit on a small scale or the midi prep kit for larger amount of DNA (both Macherey-Nagel, Düren, Germany)
Isolation of *A. tumefaciens* for sequencing was also done with the nuclear spin kit to confirm the intake of the correct plasmid before stable or transient transformation.

2.2.1.7 Isolation of genomic DNA for genotyping PCR of *A. thaliana*

Leaf samples were grinded with a TissueLyser (Quiagen) in High Purity extraction buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 50 mM EDTA, pH 8, 1% (w/v) polyvinylpyrrolidone) for 3 min. After addition of 66 µl 10% (w/v) SDS and 166 µl of a potassium acetate solution (5M potassium acetate, 11.5% (v/v) glacial acetic acid), the plant extract was centrifuged for 15 min at full speed. 0.7 volumes of isopropanol were added to the supernatant. The sample was incubated at -20 °C for 15 min or overnight, which resulted in the precipitation of the DNA. After another centrifugation step the pellet was washed with ethanol, dried at 37°C and resuspended in 40-50 µl H_20_{dd}.

2.2.1.8 Isolation of genomic DNA of *B. napus*

Genomic DNA isolation of *B. napus* leaves to use for the PCR was done with the innuPREP Plant DNA kit (Analytikjena, Jena, Germany) according to the manufacturer's instructions.

2.2.1.9 Isolation of total RNA from *A. thaliana* and *B. napus*

To isolate the RNA, the RNeasy Plant Mini Kit (Quiagen, Hilden, Germany) was used according to the manufacturer's instructions. For qRT-PCR, the DNase treatment was done after obtaining the RNA. Therefore the Turbo DNase I Kit (Ambion, Thermo Fisher Scientific) was used according to the manual. The same approach was used to obtain RNA from whole flower buds (2 mm size) of *B. napus* for RNA-sequencing analysis. The flower buds for RNA-sequencing were collected directly after treatment. The samples for RNA-sequencing were analysed with a nano photometer (Implen, München, Germany) to check the concentration and the A260/280 ratio of the RNA.

2.2.1.10 RNA isolation of extracted microspores

The total RNA extraction of the brassica microspore samples was accomplished with the Arcturus PicoPure RNA isolation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions by Dr. Chris Carrie. For each treatment, the microspores from all 10 buds were pooled for the RNA isolation and the sample divided in four replicates.

2.2.1.11 RT-PCR and qRT-PCR

The synthesis of cDNA from 500 ng RNA for real-time PCR was performed with the iScript cDNA synthesis Kit (BIORAD, Hercules, USA) as instructed by the manufacturer's manual.

In a 20 µl reaction volume, 10 µl LightCycler FastStart Essential DNA Green Master mix (Roche), 2 µl diluted cDNA (10-fold in H_20) and 1 µM Oligonucleotides were added. The reaction was conducted with the LightCycler 96 (Roche). Therefore the following program was used: one cycle at 95 °C for 60 s, 45 cycles of a three step amplification (95 °C, 10 s/60 °C, 10 s/72 °C, 10 s), 1 melting cycle (95 °C, 20 s/ 65 °C, 60 s/ 97°C, 1 s) and in the end 30 s of cooling at 37°C. The $2^{-\Delta\Delta\text{-ct}}$ method (Pfaffl, 2001) was applied to perform the expression analysis.

2.2.2 Biochemical methods

2.2.2.1 Heterologous protein overexpression

One bacterial colony, after transformation of the plasmid, was inoculated overnight in 20 ml LB media with the corresponding antibiotics for overexpression of the protein of interest. The cells were grown at 37 °C. In the morning the overnight culture was diluted 1:5 in LB and grown at 37 °C to an OD_{600} of 0.5 and induced with 1 mM of IPTG. Three hours after induction the cells were harvested by centrifugation (4000 rpm; 15 min, 4 °C).

2.2.2.2 Purification of soluble proteins (BnTic40 for antibody production)

Following overexpression, the harvested cells were resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 1 mM DTT, pH 7.3, 1 mM PMSF) and broken with a Microfluidizer (Microfluidics, USA). After a centrifugation step (20000 g, 30 min, 4 °C) the supernatant was incubated with Glutathione sepharose (GE healthcare, München, Germany) for 2 h at 4 °C. For GST-tagged proteins Glutathione sepharose was equilibrated and used for binding of the protein via the tag. After incubation with the sepharose, the protein was washed (500 g, 2 min, 4 °C) with splitting buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT). The protein was eluted off the sepharose by cutting off the GST-tag with the PreScission protease. Therefore, the protein-sepharose solution was incubated with the protease in splitting buffer over night at 4 °C while rotating. The elution fraction was incubated with Nickel-Sepharose (Macherey & Nagel) for 1 h at 4 °C to remove the His-tagged protease. After separation of the Nickel-Sepharose from the purified BnTic40 protein with a protein column, the elution was sent for antibody production.

2.2.2.3 Determination of protein concentration

Measuring of protein concentration was done by using Bradford protein assay (Bradford, 1976) with 3-5 µl of protein in water in a 1:5 dilution with the Bradford reagent (Biorad, München, Germany) and then measured at OD_{596}. Another method was used to determine the protein concentration: Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific) handled according to the manufacturer's instructions.

2.2.2.4 Trichloroacetic acid (TCA) protein precipitation

TCA was added in a final concentration of 10-15% to precipitate the protein. The protein solution was then incubated on ice for 30 min, followed by a centrifugation step (20000 g, 15 min, 4 °C). 500 µl of acetone was added to the pellet. After 10 min of incubation on ice the protein solution was centrifuged again (20000 g, 15 min, 4 °C) and the pellet dried before resuspension in an appropriate amount of protein sample buffer.

2.2.2.5 Pulldown of BnTic40C9 and BnTic40C9$_{S\text{-}D}$ mutant

Both BnTic40C9 constructs in the pGEX6p1 vector were transformed into BL21 cells. The cells were grown in 1L LB medium with 100 mg/ml ampicillin to an OD_{600} of 0.5 and induced with 1 mM IPTG. After growing at 37 °C for 3 h, the cells were harvested at 4000 rpm, for 15 min and 4°C. The pellets were either stored at -20 °C or directly used.

The pellet of 2 L of overexpressed protein was resuspended in 30 ml PBS (140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 1 mM DTT, pH 7.3, 1 mM PMSF). The cells were disrupted with a microfluidizer (microfluidics, Westwood, USA) and then centrifuged (30 min, 4 °C, 18 000 g). The supernatant (load) was mixed with 300 µl equilibrated Glutathione (GST) sepharose and incubated at 4 °C for 2 h while rotating. The sepharose was pelleted with centrifugation (500 g, 4 °C, 2 min) and washed with 20 ml PBS. This step was repeated three times in total. Protein from *B. napus* cotyledons was isolated as described in section 2.2.2.15. The rapeseed extract was diluted 1:10 in PBS to reduce the NP40 reagent to 0.1%. Thereafter, ~54 mg of rapeseed protein from cotyledons was added to each GST-Sepharose-Tic40 and incubated for one hour at 4 °C while rotating. The sepharose was pelleted again and incubated with ≥ 3 ml of splitting buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT). After another centrifugation step (500 g, 1 min, 4 °C), 200 µl of PreScission protease (constructed in the group of Dr. Bettina Bölter) was added to the Sepharose-Protein bed volume and then filled up to 7 ml with splitting buffer and incubated over night at 4 °C while rotating. The Tic40 proteins and the PreScission protease were separated from the GST-sepharose with a protein column. The flow through was concentrated down to 4 ml with an amicon filter (Merck Milipore, Darmstadt, Germany) and

TCA precipitated (section 2.2.2.4). The proteins were run on a 8.5 % SDS gel and stained with colloidal coomassie. The lanes of interest were cut out of the gel with a scalpel and send for mass spectrometry analysis.

2.2.2.6 Mass spectrometry

The mass spectrometry was done by the proteomics core unit (group of Axel Imhof) at the LMU (München, Germany).

2.2.2.7 Production of competent *Saccharomyces cerevisiae* cells

Yeast cells were grown at 30 °C and harvested (3000 rpm, 3 min, 4 °C) after reaching an OD_{600} between 0.5-0.6. The pellet was washed with sterile water and resuspended in sterile filtrated LiSorb (100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 M sorbitol). Following another centrifugation step (3000 rpm, 3 min, 4 °C) the yeast cells were resuspended in an appropriate amount of LiSorb and Carrier DNA was added (7 µl of 2 mg/ml for each 50 µ of yeast suspension). After this process, the cells were aliquoted and stored at -80 °C.

2.2.2.8 Transformation of yeast competent *S. cerevisiae* cells

For the Yeast-Two-Hybrid (YTH) interaction studies, 500 ng of each plasmid (one bait, one prey plasmid) were transformed simultaneously in 50 µl of competent yeast cells. After adding 300 µl of sterile filtrated LiPEG (100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA, 40% PEG3350) the cells were vortexed and incubated for 20 min at room temperature (RT). 35 µl DMSO was added and the transformation was incubated for 15 min at 42 °C. After centrifugation (4000 rpm,1 min, 4 °C), the pellet was resuspended in 0,9% NaCl and plated out on SD (0.7% yeast nitrogen base without amino acids, 0.2% dropout mix containing the appropriate amino acids, 2% glucose monohydrate, 2% bacto agar) without trp/leu and SD-trp/leu/his/ade. The plates were incubated for 3 days at 30 °C to allow the yeast cells to grow.

2.2.2.9 Isolation of protein from *S. cerevisiae* for immunoblotting

With immunoblotting the expression of the proteins of interest after YTH was verified. Therefore, several colonies of the mated interaction partners were added to SD-trp/leu medium and incubated over night at 30 °C. The cells were centrifuged (700 g, 5 min) and washed in 1 mM EDTA. The pellet was resuspended in 2 M NaOH before an equal amount of 50% TCA was added. After centrifugation (14000 g, 20 min, 4 °C), ice-cold acetone was used to break the pellet. Following another centrifugation step, 5% SDS was added to the pellet with an equal amount of SDS protein sample buffer. After vortexing, tris base was

added if the sample turned yellow. The extract was incubated at 37 °C for 15 min while shaking, centrifuged (14000 g, 5 min) and the supernatant was either stored at -20 °C or directly used for SDS-PAGE.

2.2.2.10 Sodium dodecyl sulfate (SDS)- PAGE

Separation of proteins was conducted using the negative charge provided with the treatment of the detergent SDS. According to their size, proteins move toward the positive charged site during polyacrylamide gel electrophoresis (Laemmli, 1970). The gels were either stained with Coomassie solution or used for western blot.

2.2.2.11 Coomassie staining of protein gels

The coomassie staining was done utilizing a staining solution (0.18% Brilliant Blue R 250, 50% methanol (v/v), 7% acetic acid (v/v)) until the gels were completely stained. The destaining was done in several steps in destaining solution (40% (v/v) methanol, 7% (v/v) acetic acid) and the gels were watered and vacuum dried.
The SDS-gel for the pulldown experiment, which was send for mass spec analysis, was stained in colloidal coomassie (0.12% Coomassie Brilliant Blue G-250 (w/v), 10% ammoniumsulfate (w/v), 10% phosphoric acid (v/v), 20% methanol (v/v)). The solution was produced according to (Candiano *et al*., 2004). The gel was stained overnight and destained for 2x30 min with H_2O.

2.2.2.12 Immunoblotting

Proteins were transferred on a PVDF membrane either via semi-dry or wet blotting. Therefore the membrane was activated with 100% methanol before use. The gel was put on the membrane between three layers (semi-dry) or one layer (wet blot) of whatman paper and blotted in transfer buffer (25 mM Tris/HCl pH 8.3, 192 mM glycine, 0.1% SDS, 20% methanol). The transfer was done for 1 h with 0.8 mA/cm2 gel (semi-dry) or 12 h with 50 mA at 4 °C (wet). After transfer, the membrane was activated with methanol again, stained with a Ponceau solution (5% acetic acid, 0.3% Ponceau S) and destained in water. The membrane was blocked with 3% low fat milk powder in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). The first antibody was incubated for 2 h at RT or overnight at 4 °C in blocking solution in a dilution of 1:1000. The membrane was washed three times with TBST before incubation with the second antibody (anti-mouse) in TBST (1: 10000) for 1 h. The washing process was repeated prior to detection.
The enhanced chemiluminescence (ECL) reader (ImageQuant LAS 4000, GE Healthcare, München, Germany) was used for antibody detection. Thereby Solution I (100 mM Tris-HCl pH 8.5, 1% Luminol (w/v), 0.44% (w/v) Coomaric Acid) and Solution II (100 mM Tris-HCl pH

8.5, 0,018% H_2O_2) were mixed in equal amounts and applied on the membrane for a minute before detection.

2.2.2.13 Isolation of protoplasts from *N. benthamiana*

For localisation studies of GFP tagged proteins, protoplasts were isolated after transformation into *N. Benthamiana.* One transformed leaf was cut into small pieces with a razor blade in 10 ml of enzyme solution (1% Cellulase R10, 0.3% Mazerozym R10 in F-PIN solution, heated for 10 min to 55 °C. After cooling down to RT, 0.1% BSA was added). Vacuum was applied to the leaves in solution for 30 sec and then left for 90 min at 40 rpm in the dark. After releasing the protoplasts (80 rpm, 1 min), the solution was filtered (100 µM gauze) and over-layed with 2 ml F-PCN solution in 15 ml centrifugation tubes. After the first centrifugation step (70 g, 10 min with low acceleration and brake) the intact protoplasts were collected between the different phases and washed in 5 ml W5 solution. After pelleting (50 g, 10 min), the protoplasts were resuspended in a small amount (<0.5 ml) of W5 solution and directly used for Laser scanning microscopy.

F-PIN: 10 mM KNO_3, 3 mM $CaCl_2$ x 2 H_2O, 1.5 mM $MgSO_4$ x7 H_2O, 1.3 mM KH_2PO_4, 5 ml of 2 M NH_4-Succinat, 20 mM MES/KOH pH 5.8, 0.5 ml of 1000x Micro-MS, 1 ml of 500x PC vitamins and ~ 55 g sucrose to 550 mOsm, sterile filtrated (45 µM)

F-PCN: 10 mM KNO_3, 3 mM $CaCl_2$ x 2 H_2O, 1.5 mM $MgSO_4$ x7 H_2O, 1.3 mM KH_2PO_4, 5 ml of 2 M NH_4-Succinat, 20 mM MES/KOH pH 5.8, 0.5 ml of 1000x Micro-MS, 1 ml of 500x PC vitamins and ~ 40 g glucose to 550 mOsm; sterile filtrated (45 µM)

5 W: 150 mM NaCl, 125 mM CaCl, 5 mM KCl, 2 mM MES; pH 5.8 with KOH; 550 mOsm; sterile filtrated (45 µM)

2 M NH_4-Succinate: 2 M succinic acid, 2 M NH_4Cl; pH 5.8 with KOH; sterile filtrated (45 µM)

1000x Micro-MS (100 ml): 75 mg KJ, 4 g EDTA, 300 mg H_3BO_3, 1 g $MnSO_4$ x H_20, 200 mg $ZnSO_4$ x 7 H_2O, 25 mg Na_2MoO_4 x 7 H_2O, 2.5 mg $CuSO_4$ x 5 H_2O, CaCl2 x 6 H_2O

500x PC-Vitamins (100 ml): 10 g Myo-Inosit, 100 mg pyridoxine HCl, 50 mg thiamine HCl, 100 mg nicotinic acid, 1 g biotin, 100 mg calcium pantothenate

2.2.2.14 Protein isolation from *N. Benthamiana*

Leaves from the same transformed plants used for protoplast isolation were grinded in liquid nitrogen to analyze the expression of the GFP-fused proteins. The extraction was carried out as described in (Lyska *et al*.,2013). For small SDS-gels and Western blotting, an amount of 25 µg protein per sample was used.

2.2.2.15 Protein isolation from *B. napus*

The isolation of proteins from cotyledons, leaves and flowers was always done with the same procedure. The plant tissue was grinded thoroughly in liquid nitrogen and then thawed in an appropriate amount of extraction buffer (50 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 0.1 mM PMSF). The tissue solution was incubated on ice for 30 min and then centrifuged (16000 g, 30 min, 4 °C). The amount of protein in the supernatant was detected via BCA-test.

2.2.2.16 Protein isolation from *A. thaliana* leaves

200 mg of leaf material was grinded in liquid nitrogen and let thaw in an equal amount of extraction buffer (0.05 M Tris-HCl pH 8, 2% LDS, 0.1 mM PMSF). After 30 min incubation time on ice, the lysate was centrifuged (15 min, 16000 g, 4 °C). The protein concentration of the supernatant was measured via BCA-test. Afterwards the protein solution was supplied with 50 mM EDTA and 10 mM DTT and stored at -20 °C.

2.2.2.17 In vitro transcription, translation and detection of radiolabeled proteins

For transcription, 1 µg of the linearized plasmid (T7 or SP6 promoter) was added to a mixture (50 µl end volume) of 2% BSA, 100 mM DTT, 2.5 mM CAP, 0.4 mM ACU, 50 u RNase inhibitor, 30 units RNA polymerase in transcription buffer. After an incubation time of 15 min at 37 °C, 1.2 mM GTP was added. The transcription reaction was set for 120 min at 37 °C.
The produced mRNA was verified by running on an agarose gel,
In vitro translation mix (50 µl) consisted of ~2 µg transcription product, 70% rabbit reticulate (Promega) lysate, 20 µM amino acid mixture without methionine, 22 µCi ^{35}S-methionine (Perkin Elmer, Walluf, Germany), 40 u RNase inhibitor and was incubated at 30 °C for 1 h. The translation product was run on a SDS-gel and exposed on a phosphor imaging plate (Fujifilm) The radiolabeled proteins were analyzed by utilizing a Typhoon scanner (GE healthcare, Chicago, USA).

2.2.2.18 Isolation of chloroplasts from *P. sativum*

Approximately 200 g of 7-9 days old pea leaves were harvested in the dark period and transferred to a blender. The leaves were homogenized with 330 ml of isolation medium (330 mM sorbitol, 20 mM MOPS, 13 mM Tris-HCl pH 7.6, 3 mM $MgCl_2$ and 0.1% BSA). After filtering the leaf solution through 4 layers of mull and 1 layer of gauze, the extract was centrifuged (3400 rpm, 1 min, 4 °C). The resuspended pellet (in a small amount of isolation medium) was placed on top of a discontinuous percoll gradient of 12 ml 40% percoll (in 330 mM sorbitol, 50 mM HEPES-KOH pH 7.6 and 3 mM $MgCl_2$) and 8 ml of 80% percoll (in 330 mM sorbitol, 50 mM HEPES-KOH pH 7.6 and 3 mM $MgCl_2$) and centrifuged (700 rpm, 5 min, 4 °C). Intact chloroplasts were removed from the percoll layers and washed twice (3400 rpm,

1 min, 4 °C) in washing buffer (300 mM sorbitol, 50 mM HEPES-KOH pH 7.6). The pellet was resuspended in an appropriate amount of washing buffer.
In order to determine the chlorophyll concentration, 1 µl of chloroplast solution was resuspended in 1 ml of 80% acetone and the optical density was measured at 663 nm, 645 nm and 750 nm. The concentration was calculated according to (Arnon, 1949) with the following formula: mg chlorophyll/ml = 8.02 x (E663-E750) + 20.2 x (E645-E750).

2.2.2.19 Isolation of mitochondria from *A. thaliana*

Approximately two week old plants from either ½ MS plates or liquid culture were grinded with a mortar and pestle in grinding buffer (0.3 M sucrose, 25 mM $Na_4O_7P_2$, 2 mM EDTA, 10 mM KH_2PO_4, 1% (w/v) PVP-40, 1% BSA, 20 mM sodium ascorbate, 20 mM L-cysteine). The solution was filtered and centrifuged (2450 g, 5 min, 4 °C). Supernatant was transferred to a new centrifuge tube and centrifuged (17400 g, 20 min, 4 °C). The pellet was resuspended in wash buffer (0.3 M sucrose, 10 mM TES, 0.1% BSA, pH 7.5) and the last two centrifugation steps were repeated again. The resuspended mitochondria were placed on a continuous gradient consisting of 35 ml of 28% percoll and 4.4% PVP in wash buffer and 35 ml of 28% percoll without PVP in wash buffer and centrifuged (40000 g, 40 min, 4 °C). The mitochondria accumulating at the bottom of the tube, were collected and washed twice with wash buffer. The protein concentration was determined with the Bradford assay.

2.2.2.20 In vitro Protein import into chloroplasts

In vitro protein import was directly carried out after isolation of intact chloroplasts. 10 µg of chlorophyll was combined with 20 mM methionine, 10 mM cysteine, 0.2% BSA, 4 mM ATP, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 330 mM sorbitol, 50 mM HEPES-KOH pH 7.6, 3 mM $MgCl_2$, 3-5% translation product in a final volume of 100 µl at 25 °C. After 15 min, the import reaction was added on top of a 40% percoll solution in washing buffer (330 mM sorbitol, 50 mM HEPES-KOH pH 7.6, 3 mM $MgCl_2$) and centrifuged (7000 rpm, 5 min, 4 °C). The pellet containing chloroplasts and import product were washed twice (3400 rpm, 1min, 4°C) with washing buffer. The chloroplast treatment with thermolysin was done in the next step to remove all translated and not imported proteins. Therefore, the pellet was resuspended with 1.5 µg thermolysin in 330 mM sorbitol, 50 mM HEPES-KOH pH 7.6, 0.5 mM $CaCl_2$ and the chloroplasts incubated on ice for 20 min. After adding 10 mM EDTA to stop the reaction, the reaction mixture was centrifuged (3200 rpm, 1 min, 4 °C). The pellet was resuspended in wash buffer, containing 5 mM EDTA instead of $MgCl_2$, centrifuged (3200 rpm, 1 min, 4 °C) and resuspended in SDS loading buffer to be run on a SDS gel.

2.2.2.21 *In vitro* Protein import into mitochondria

100 µg protein of isolated mitochondria were added to the import mixture (100 mM MgCl2, 10 mM methionine, 10 mM ADP, 10 mM ATP, 50 mM succinate, 50 mM DTT 50 mM NADH, 10 mM GTP, ± 1 µM valinomycin) and incubated on ice for 3 min. 10 µl of translational product was added and the reaction took place at 26 °C, for 20 min at 350 rpm. For proteinase K treatment, 0.032 mg proteinase K was added and the reaction was incubated on ice for 30 min. In the end 1 mM PMSF was added to stop the reaction. Mitochondria with their import product were centrifuged (16100 g, 3 min, 4 °C) and resuspended in SDS loading buffer.

2.2.2.22 Auxin treatment of *B. napus*

The flowers and buds of plants with an age of 7.5-8.5 weeks were sprayed (0.03% silvet L-77, 10 µM 1-Naphthaleneacetic acid (I-NAA), 100 µM ethephon) four times within two weeks. During that time the plants were kept at 28 °C with 70% humidity, (highlight) and a day/night rhythm of 20 h/4 h. After the auxin treatment, plants were put back into the green house. Siliques could be counted six weeks after the treatment at the earliest time.

2.2.2.23 Pollen staining of flower buds

The Alexander staining solution (Alexander; 1969) was used to stain the viable pollen in *A. thaliana*. Whereby *A. thaliana* flower buds, which had not opened yet, were dissected under the stereo microscope to extract the anthers. The anthers were put on a microscope slide and covered in the staining solution (10% ethanol (v/v), 0.01% Malachite green (w/v), 25% glycerol (v/v), 5% phenol (w/v), 5% chloral hydrate (w/v), 0.05% acid fuchsin (w/v), 0.005% orange G (w/v) . The incubation was done for 2-3 hours at RT and for the assessment a light microscope was used (2.2.4.1). Viable pollen regained a purple colour, while sterile pollen stained green.
B. napus pollen were stained with acetocarmine solution. Therefore, pollen grains were collected on a microscopy slide and covered in the staining solution. The pollen in the staining solution were carefully heated over a Bunsen burner and directly examined under the light microscope.

Acetocarmine solution:

1% Acetocarmine in 45% acetic acid was refluxed in a reflux apparatus for 24 h. Then the solution was filtered and stored at 4 °C.

2.2.3 Plant methods

2.2.3.1 Plant materials

The *Brassica napus* lines were part of the MSL system. The seeds were provided by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) (Hohenlieth, Germany). For *Arabidopsis thaliana*, Columbia-0 ecotype (Col-0, Lehle Seeds, Round Rock, USA) was used as control and background for stable transformations. *Pisum sativum* ("Arvica") for the chloroplast isolation were obtained from Bayrische Futtersaatbau (Ismaning, Germany). The used T-DNA insertion lines (SALK_076646, SALK_042862, SALK_057111) were ordered from NASC (Nottingham, GB).

2.2.3.2 Plant growing conditions

A. thaliana plants were grown either on soil under greenhouse conditions or long day conditions (16 h light/ 8 h dark, 21 °C, 100 µmol photons/m^2s). Furthermore they were grown on ½ MS plates (1% sucrose, 0.05% MES, 0.237% MS (Murashige and Skoog) salts, pH 5.8 supplied with 0.75% agar) in a climate chamber, before transfer to soil. Seeds growing on sterile ½ MS plates were sterilized for 10 min with 0.05% Triton X-100 in 70% ethanol. After two washing steps (70% ethanol and 100% ethanol), the seeds were allowed to dry under the sterile hood, before placing them on the plates. The seeds (on soil and on plate) were vernalized at 4°C for 1-2 days before putting them into the growing chambers. The *A. thaliana* mutant plants, which contained BASTA resistance were grown on soil, sprayed with BASTA and the living plants were collected and transferred to fresh pots.
N. benthamiana for transient transformation was grown under greenhouse conditions.
P. sativum was grown on vermiculite, after watering the seeds over night, at 12 h light/ 12 h dark, 21°C, 100 µmol photons/m^2s.
B. napus was either grown under greenhouse conditions or in a climate chamber at 28 °C with 70% humidity and highlight (approx. 300-350 µmol photons/m^2s) for 20 h light/4h dark. Heat treatment with 37 °C for real time PCR was performed in a growing chamber (Percival Scientific) adjusted to 20 h light/4 h dark (for flower phenotyping and RNA sequencing of whole buds). High humidity (80-90%) was obtained by putting a tray with water into the chamber.

2.2.3.3 Stable transformation of *Arabidopsis thaliana* with Agrobacteria

5 ml of LB medium with appropriate antibiotics was inoculated with *A. tumefaciens* (GV3101) and grown for two days at 28°C. This culture, containing the plasmid of interest, was transferred into 500 ml of LB medium with appropriate antibiotics (50 µg/ml rifampicin, 10 µg/ml gentamycin and 100 µg/ml spectinomycin) and grown over night. The *agrobacteria*

were centrifuged at 2500 g for 20 min and the pellet resuspended in 500 ml of 5% Sucrose and 0,03% silvet L-77. The bolts of the Arabidopsis plants were clipped one week before dipping with *agrobacteria*. The flowers of 12 or 24 plants were then dipped for 10 seconds in the sucrose media. The plants were covered overnight and the transformation was repeated after one week. The selection of transformants was either done with hygromycin (Tic40 complementation), kanamycin (*BARK1* RNAi lines) or BASTA (chimeric gene mutants).

2.2.3.4 Transient transformation of *N. benthamiana*

For localization studies of GFP-fused proteins tobacco leaves were infected with *A. tumefaciens* (AGL1). Therefore 3 ml LB medium containing appropriate antibiotics (100 µg/ml carbenicillin and either 100 µg/ml Spectinomycin or 50 µg/ml Kanamycin, depending on the plasmid) and the plasmid containing cells were grown over night at 28 °C while shaking. After this, 27 ml of LB were added to the culture and grew further for 4 h at 28°C. After a centrifugation step (4000 rpm, 20 min, RT) the cells were resuspended in 30 ml of induction medium (10 mM MES pH 6, 10 mM $MgCl_2$, 200 µM acetosyringone). After 2 h at 28 °C, the cells were centrifuged again (4000 rpm, 20 min, RT) and resuspended in 5 ml of a sucrose solution (5% sucrose, 200 µM acetosyringone). The leaves were infiltrated with a 1 ml syringe on their abaxial side. The infected plants were set aside for 3 days before detection of the fluorescence signal by confocal microscopy.

2.2.3.5 Crossing of *A. thaliana*

Double mutant plants of At5g16620 and At3g23740 were obtained by crossing the two according lines. Therefore, pollen from the anthers of one mutant were used for pollinating the stigma of the flowers of the other parent plant. By using genotyping, homozygous lines were obtained after several generations. These double mutant plants were constructed in the group of Dr. Chris Carrie.

2.2.4 Microscopy methods

2.2.4.1 Plant documentation

Plant pictures of whole plants were taken with the Canon EOS 70D camera. The flower buds and siliques were documented with the stereo-microscope Zeiss Stemi 2000-C and the camera Leica DFC320. The pictures of the stained pollen were taken with the Leica DM 1000 light microscope adapted with the Leica DFC 7000T camera.

2.2.4.2 Laser scanning microscopy for localization studies

The GFP-fluorescence images in this work were taken with the Leica TCS SP5 CLSM according to the manufacturer’s instructions.

2.2.4.3 Sample preparation for Laser microdissection and RNA sequencing analysis

Plants of two different *Brassica napus* lines of the Lembke system were used for the experiments mentioned above: Monty and C545. The heat treated plants were kept for three days at 37 °C before the buds were harvested. The heat treatment of the plants was done by Prof. Dr. Margret Sauter (Christian-Albrechts Universität, Kiel). Each sample and treatment contained 10 flower buds in the same development stage (see overview in Table 2).

Table 2: Overview of the different lines, treatments and amount of flower buds used for laser microdissection and RNA seq.

	untreated	**3 days, 37 °C**
Monty	10 flower buds (size: 2 mm)	10 flower buds (size: 2 mm)
C545	10 flower buds (size: 2 mm)	10 flower buds (size: 2 mm)

For the RNA sequencing of whole *B. napus* flower buds, buds with a size of 2 mm and approximately 100 ng in weight material were collected 1-3 days after the end of each treatment. For each treatment or sample type, three replicated were prepared.

2.2.4.4. Embedding of *B. napus* flower buds

The embedding was done according to (Schmid *et al*., 2012).
Flower buds were fixed in an ethanol:acetic acid ratio of 3:1. Vacuum was applied for 10 min, then the buds were kept overnight at 4 °C in the fixation medium. Afterwards the tissue was treated with different ethanol steps at RT (70% ETOH for 1 h, 90% for 1 h (repeated 3 times), 100% ETOH for 1 h (repeated 3 times), before exchanging it with xylol for 1 h (repeated 3 times). Paraplast X-tra embedding media was heated over night at 58 °C to become soluble. The flower buds were selected in xylol under the hood. Xylol was removed and exchanged with liquid paraplast for one hour at 58 °C. The paraplast was exchanged two more times to remove residual xylol. After the last exchange, the buds were kept in paraplast for 3 h at 58 °C before moving them to room temperature to allow the embedding medium to become solid. The embedded buds were kept at 4 °C till further use.
This method was conducted by Dr. Chris Carrie.

2.2.4.5 Laser microdissection

Using the microtome (Leica; Wetzlar; Germany) the brassica flower buds were dissected in 3-6 µm thick slices and placed on nuclease free FrameSlides (Leica,, PET-Membrane 1,4 µm; Wetzlar, Germany) in water (Ampuwa, Fresenius Kabi, Bad Homburg, Germany). The slicing of the samples was done with the help of Dr. Irene Gügel. The nuclease free slides

with the samples were dried overnight at 42 °C. In the next step, the slides were incubated in 100% Xylol for 5 min twice and then air-dried. This resulted in de-waxing of the flower bud samples.

By using the Laser microdissection system (LMD6500) (Leica; Wetzlar; Germany), the microspores of the samples were dissected and collected in the lid of a 0.5 ml reaction tube. All microspores that could be distinguished from the tapetum were collected from each flower bud. 20 µl of extraction buffer (Arcturus PicoPure RNA isolation Kit, Thermo Fisher Scientific) were added to the samples, heated up to 42 °C for 30 min and then stored at -80 °C until RNA extraction was performed. The isolation of RNA is described in section 2.2.1.9 and 2.2.1.10.

2.2.5 RNA Sequencing and analysis

The sequencing of whole flower bud RNA was done by Novogene (Peking, China) by using Illumina High-Seq Pair-end150. Briefly, before starting the libraries, the quality of the RNA was verified by using a nanodrop, agarose gel and Agilent 2100. The sequencing of the RNA samples was performed in several steps. First of all the RNA underwent a quality control, before the library was constructed. In the next step, the mRNA was enriched and randomly fragmented. With the help of random hexamers primers, dNTPs, RNAse H and DNA polymerase I the cDNA was synthesized. After adaptor ligation, the library can be completed via PCR by using Illumina sequencing (Novogene).

The RNA sequencing of the *B. napus* microspores was conducted with the Illumina High-Seq 50 bp system generating single-ends while using the Ovation SoLO RNA-Seq System from NuGen (SanCarlos; USA), according to the manufacturer's instructions. For the microspore RNA, fragmentation was not necessary, due to the already short sized RNA. Furthermore, instead of a mRNA enrichment, the rRNA was extracted.

For the computer analysis, the raw reads went through a process of quality control, with trimming and removal of adapters and low quality reads by using OmicsBox (Biobam, Valencia, Spain). The trimmed reads were assembled to the *B. napus* reference genome (http://www.genoscope.cns.fr/brassicanapus/) using HISAT (Kim *et al*., 2015). For the assembly of the alignments into possible transcripts, StringTie (Pertea *et al*., 2015) was used. Salmon (Patro *et al*., 2017) was used for generating counts and the transcript file. The normalization of the data, the illustration of the principle component analysis (PCA) plot as well as the analysis of differentially expressed genes (DEGs) was performed by using 3D RNA seq (Guo W., *et al*., 2019). To this point, the RNA seq computer analysis was performed by Dr. Chris Carrie.

The program Blast2Go/Omnibox was used for gene ontology (GO) enrichment analysis of the DEGs by using the false discovery rate (FDR) cutoff 0.5 and performing Fisher’s exact

test enrichment analysis. The graphical presentation of GO terms was also done with 3D RNA-seq. Furthermore, Mapman BINs (Thimm *et al*., 2004) were used for functional annotation of all the DEGs.

2.2.6 Software

The sequences were either received from Tair (https://www.arabidopsis.org/), Aramemnon (http://aramemnon.uni-koeln.de/; Schwacke *et al*., 2003) or NCBI (https://www.ncbi.nlm.nih.gov/). The tools used for obtaining plasmid maps were: VectorNTI (Invitrogen) and CLC workbench (Quiagen). The target peptide of chloroplastic located proteins was determined with the program ChloroP (http://www.cbs.dtu.dk/services/ChloroP/; Emanuelsson *et al.,* 1999). The determination of the transmembrane domain for the YTH was done using TOPCONS (http://topcons.cbr.su.se/; Tsirigos *et al.,* 2015). Analysis of images was done with ImageJ. For obtaining the alignment, MAFFT alignment version 7 (https://mafft.cbrc.jp/alignment/server/index.html) in combination with Multiple Align show (https://www.bioinformatics.org/sms/multi_align.html) was used.

3 Results

3.1 The male sterility gene

One of the major aims of this project was to identify the male sterility (ms) gene of the MSL system. In a map-based cloning approach performed by the NPZ, three different genes were identified as possible ms gene candidates: *XTH16* (xyloglucan endotransglucosylase-hydrolase) (At3g23730), BAK1-Associating Receptor-Like Kinase 1 (*Bark1*) (At3g23750) and a potential *DNA-Methyl-Transferase* (At3g23740). For further analysis the closest *A. thaliana* homologs were selected. *A. thaliana* is closely related to *B. napus* with an exon similarity of approximately 87% (Cavell *et al*., 1998). This relation in combination with a shorter life cycle and the fact that less space is needed for cultivation results in a preferential model organism for analyzing ms traits from *B. napus*. Sequencing analysis of the *XTH16* gene in *B. napus* performed in the laboratory of Prof. Jürgen Soll demonstrated that no significant mutations were identified between the C545-line and Monty. So, XTH16 was not considered a likely candidate as the ms gene. The proposed DNA-Methyl-Transferase and Bark1 were still possible ms gene candidates because of the variation of mutations in their sequence in the C545-line compared to the Monty line and were therefore studied in more detail.

3.1.1 Characterization of the proposed DNA-Methyl-Transferase

For analysing the possible function for the suggested DNA-Methyl-transferase, T-DNA insertion mutants were utilised to observe a possible difference in the phenotype compared to wildtype (WT) in *A. thaliana*. Two T_DNA insertion lines (SALK_076646 and SALK_042862) were used to study the proposed DNA-Methyl-Transferase. In addition to the analysis of single knockouts, double mutants were generated by crossing with a *tic40* knockout (ko) line (SALK_057111) in order to either eliminate or find indications of an AtTic40 involvement in male sterility (Figure 4). The single ko plants of the *dna-methyl-transferase* showed a similar phenotype to Col-0 while the phenotype of the double knockout mutants resembled the tic40 single knockout (Figure 4 A-D). The lines were genotyped for the correct T-DNA insertions using the left and right primer (LP+RP) for the WT gene and the left border (LB) primer for the T-DNA insertion (Figure 4 F-G). All plants shown are homozygous knockouts for either the *dna-methyl-transferase*, *tic40* or homozygous double mutants. However, loss of the *DNA-Methyl-Transferase* gene in *A. thaliana* did not lead to a sterile phenotype (Figure 4 E), neither in the single knockout, nor in the double knockouts with *tic40*. The anthers displayed normal pollen development and the plants developed siliques comparable to the Col-0 control (Figure 4 B, D and E).

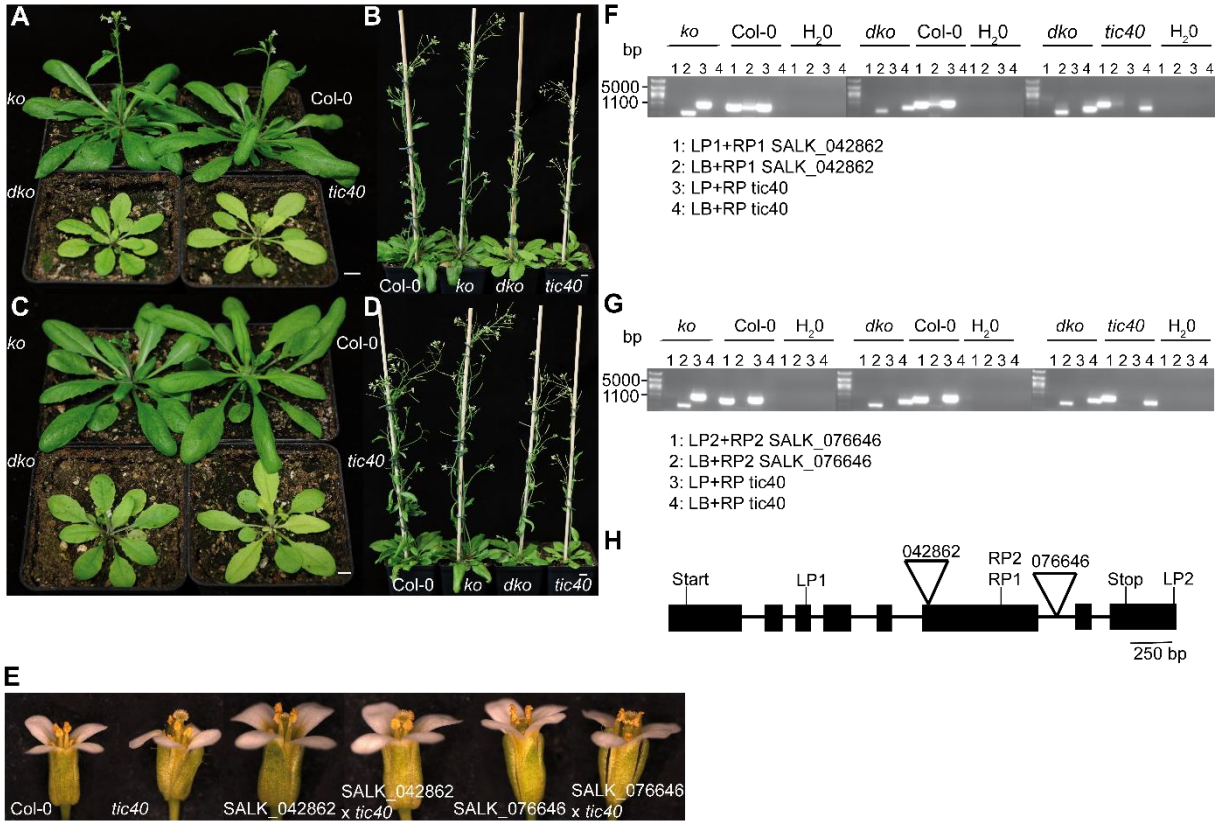

Figure 4: Phenotyping and Genotyping of two SALK lines of the predicted DNA-Methyl-Transferase. A: Three weeks old plants of SALK_042862 (ko), Col-0, SALK_042862 x *tic40* (dko) and *tic40*. **B**: Three weeks old plants of SALK_076646 (ko), Col-0, SALK_076646 x *tic40* (dko) and *tic40*. **C**: Five weeks old plants of SALK_042862 (ko), Col-0, SALK_042862 x *tic40* (dko) and *tic40*. **D**: Five weeks old plants of SALK_076646 (ko), Col-0, SALK_076646 x *tic40* (dko) and *tic40*. **E**: Pictures, taken with the stereo microscope (magnification: 1.6x), of fully opened *A. thaliana* flowers for the indicated genotype. **F**: Genotyping results of SALK_042862 lines. **G:** Genotyping results of SALK_076646 lines. **H**: Location of the t-DNA insertions of the two SALK lines in the model gDNA of the predicted DNA-Methyl-Transferase. The black boxes illustrate the exons.

To determine the localisation of the DNA-Methyl-Transferase, the translation product was used for *in vitro* protein imports into isolated chloroplasts and mitochondria. Beside the translated precursor of the DNA-Methyl-Transferase, the alternative oxidase (AOX) and Glyceraldehyde-3-phosphate dehydrogenase B (GAP B) were used for import controls into mitochondria and chloroplasts, respectively. The control pre-proteins (AOX and GAP B) both imported successfully into their respective organelle (Figure 5). This is displayed by the smaller mature protein, which is protease protected by the membrane (Figure 5 A/B). Proteinase K is an enzyme which was used to digest non-imported preproteins binding to the outside of mitochondria. For import of pre-proteins across the inner mitochondrial membrane, the membrane potential Δψ is necessary (Geissler *et al*., 2000). The use of valinomycin disturbs the proton-motive force and therefore inhibits import into mitochondria. Thermolysin is a thermostable protease which digests proteins binding to the outer chloroplast membrane.

(Cline *et al*., 1984). Hence, only the imported mature protein (m) should be visible after successful import. There was no import observed for the DNA-Methyl-Transferase neither in mitochondria nor in chloroplasts, because no processed mature version of the DNA-Methyl-Transferase was obvious after PK or thermolysin treatment. These experiments gave first insights, that the proposed DNA-Methyl-Transferase might not be localised in chloroplasts.

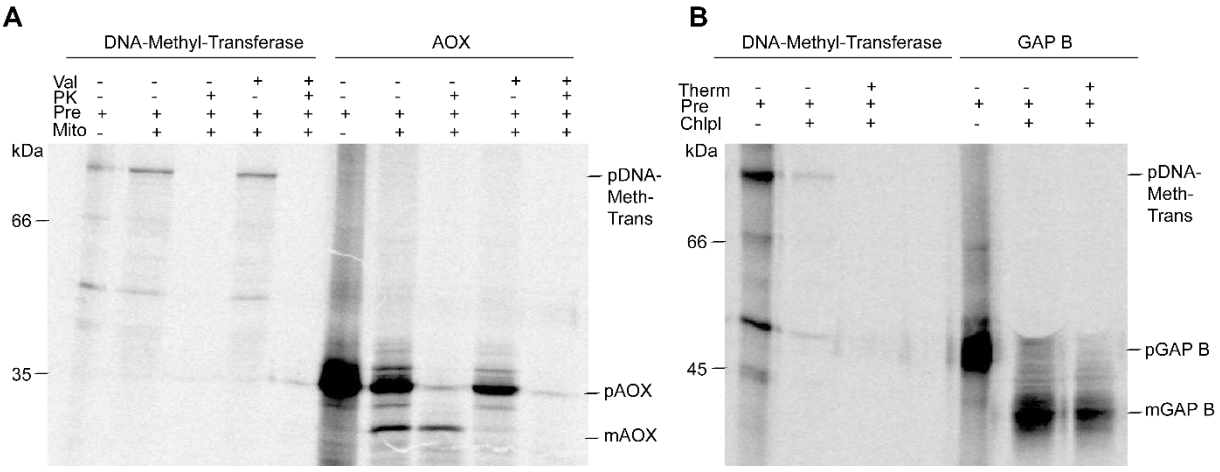

Figure 5: *In vitro* protein import of the DNA-Methyl-Transferase translation product in isolated mitochondria and chloroplasts. p: precursor protein. m: mature protein **A**: In vitro import in isolated mitochondria (Mito) from *A. thaliana* with *in-vitro* translated precursors (Pre) which were labelled with ^{35}S methionine. As positive import control in mitochondria, the alternative oxidase (AOX) was used. The samples were treated with Valinomycin (Val) and/or Proteinase K (PK). **B**: In vitro import in isolated chloroplast (Chlpl) from peas with *in-vitro* translated precursors (Pre) which were labelled with ^{35}S methionine. Glyceraldehyde-3-phosphate dehydrogenase B (GAP B) was used as positive import control. The samples were partially treated with Thermolysin (Therm).

The fertile phenotype of the knockout plants and the unlikely localisation in chloroplasts render the proposed *DNA-Methyl-Transferase* to be an unlikely candidate as the male sterility gene.

3.1.2 Characterization of Bark1 regarding male sterility

A second possible ms candidate gene was *Bark1*. Bark1 was shown to interact with BAK1/SERK3, which is a member of a receptor family localised in the plasma membrane (Kim *et al.,* 2013).

First of all, a RNAi line of *Bark1* was generated due to an absence of a suitable T-DNA-insertion line. After transformation of the RNAi plasmid (pK7GWIWG2(I)) into Col-0 ecotype, the seeds were collected and selected on kanamycin plates. Several lines of the T2 generation were chosen for qRT-PCR to determine the *Bark1* expression level. In Figure 6 C only *bark1* RNAi line #14 showed a reduced *Bark1* expression. Looking closer at the

phenotype, there are no obvious differences to the Col-0 control plants (Figure 6 A) and all RNAi plants were observed to be fertile (Figure 6 B). Hence, since it is unlikely that Bark1 is localised in the chloroplast because it interacts with a plasma membrane protein (Kim *et al.,* 2013) and the lack of sterility in *bark1* RNAi lines, all indicate that Bark1 is also most likely not the ms gene.

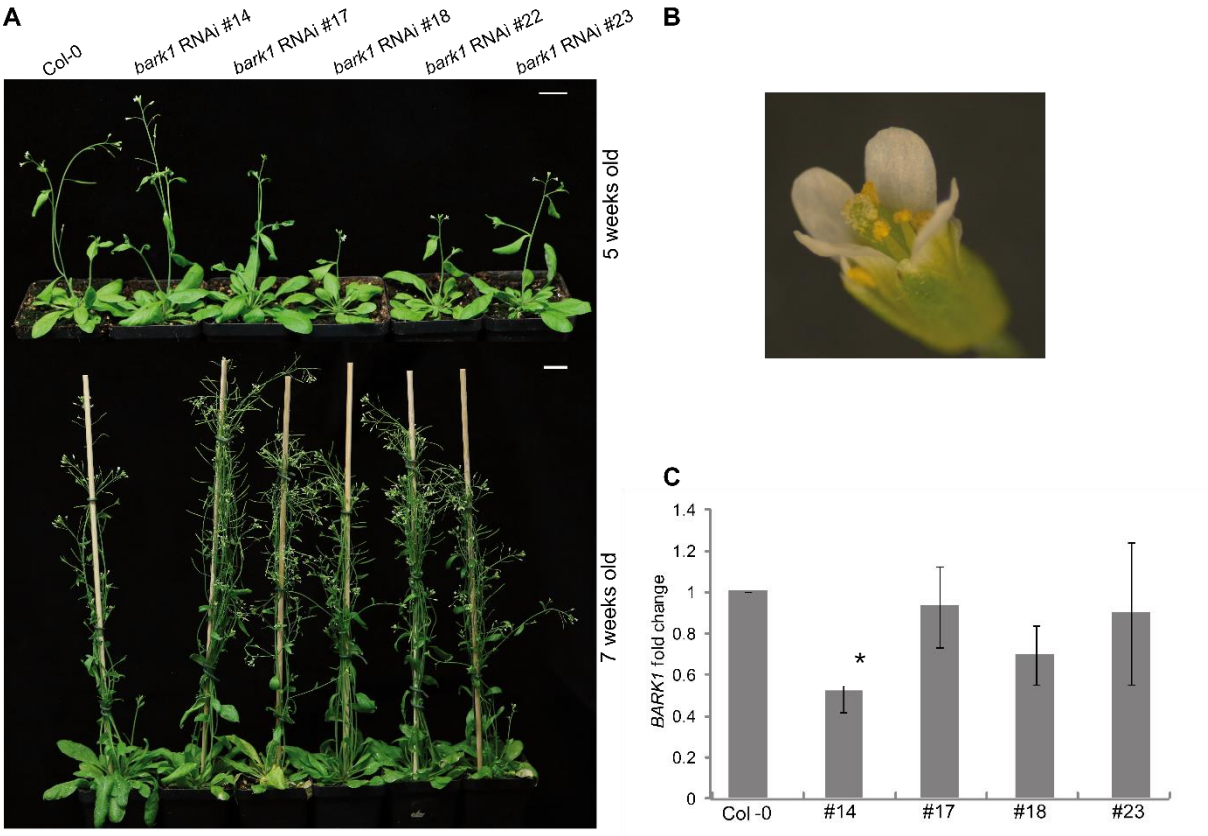

Figure 6: Phenotypes of the *BARK1* RNAi lines and qRT_PCR analysis of the T2 generation. A: The analysed *bark1* RNAi lines at an age of 5 weeks and 7 weeks after sowing. The scale bar indicates 2 cm. **B:** Open *A. thaliana* flower of *bark1* RNAi #14 line. Magnification: 1.6x **C:** qRT-PCR analysis of the *bark1* RNAi lines. Three individual samples and three replicates of each line were quantified. *AtActin*2 was used as reference gene. The data was evaluated using the $2^{-\Delta\Delta\text{-ct}}$ method. With students t-test, the significance between Col-0 and the RNAi lines was determined (p-value: * = $p<0.05$).

3.1.3 Mass spectrometry analysis after the pulldown of *B. napus* proteins with BnTic40 C9

With the assumption, that the restorer gene might interact with the ms gene (Dun *et al.,* 2014), the purified BnTic40C9 protein was used for detection of different interaction partners in *B. napus*. This biochemical approach was tried, because mapping failed to identify an obvious ms candidate.

In the alignment (Figure 7 A) it can be seen that a serine is present in position 408 of BnTic40C9 protein sequence, while all other BnTic40s as well as the *A. thanliana* Tic40 contain an alanine at this position. The amino acid alanine cannot be phosphorylated, while for serine phosphorylation is possible. For this experiment two soluble versions of BnTic40C9 were purified with a GST-tag. Version 1 was the WT BnTic40C9 containing a serine. In the second version the serine was mutated by site directed mutagenesis to aspartate (D). The aspartate shows a similar structure to the phosphorylated serine with the negative charge and can be used as a phosphor-mimicking control (Figure 7 B).

Therefore, the conducted pulldown experiment should also show if phosphorylation has an effect on the Tic40 behaviour. Phosphorylation is a major post-translational protein modification (Waegemann and Soll, 1996). In combination with dephosphorylation it is important for regulation of protein import (Lamberti *et al.*, 2011; Nickel *et al.*, 2015). Being phosphorylated via kinases, the protein can change its conformation especially when interacting with other proteins. The aim of this pulldown experiment was to identify possible interaction partners of the BnTic40 C9 as well as the BnTic40 $C9_{S\text{-}D}$. Both proteins were expressed with a glutathione S-transferase (GST) tag. After heterologous overexpression of the two proteins in *E. coli*, they were purified using glutathione sepharose.

After incubation of the BnTic40C9 and BnTic40 $C9_{S\text{-}D}$ with the protein extract isolated from Monty cotyledons, possible interaction partners of the Tic40 proteins were pulled down and the GST tag was cleaved off the Tic40 protein with the according protease. The eluate was expected to only contain proteins interacting with Tic40, Tic40 and the protease. This protein suspension was collected and TCA precipitated before loading it on an 8% SDS-gel (Figure 7 C). The black arrow heads indicate the proteins of interest, containing possible BnTic40 interaction partners. These lanes were cut out from the gel after colloidal coomassie staining, and then sent for the mass spectrometry based analysis. Mass spectrometry results were analysed according to their sequence coverage, amount of matching peptides and size.

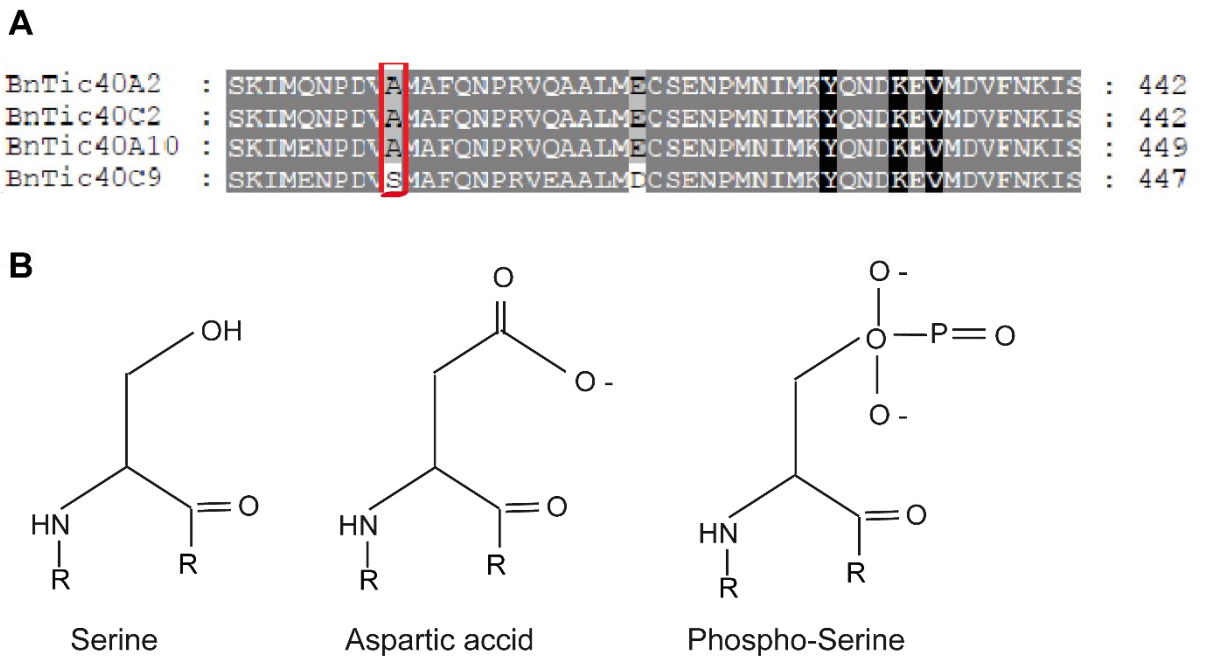

Figure 7: Pulldown *of B. napus* proteins isolated from Monty with BnTic40 C9 and BnTic40 C9-D A: Section of the alignment of the different Tic40s. The position of the investigated amino acid of the BnTic40 C9 is labelled in red. **B:** Structure of serine, aspartic acid and phospho-serine for visualization of the phosphor-mimicry. **C:** Colloidal coomassie stained gel of the pulldown experiment. The eluate represents the interacting partners if BnTic40, BnTic40 and the protease after release from the sepharose. The black arrowheads indicate the bands, which were extracted and sent for mass spectrometry analysis. The red arrowheads indicate the Tic40 proteins.

In Table 3 the possible Tic40 interaction candidates identified by mass spectrometry are listed. Only matches with a sequence coverage of at least 10% are listed in the table below. The comparison was done with the *Brassica rapa* genome.

Table 3: Results of the mass spectrometry analysis after the pulldown of *Brassica* proteins with the BnTic40C9 and its mutated form.

	ID	Name	Molecular Weight [kDa]	Sequence coverage	localisation
BnTic40 C9~70 kDa lane	Bra006027	mtHsp70	72.859	21%	mitochondria
	Bra031301	Isocitrate lyase	63.493	16%	glyoxysome
	Bra016676	Thioglucoside glucohydrolase	62.262	20%	secretory pathway
	Bra011792	Phosphoenol pyruvate carboxikinase	73.853	20%	cytosol
	Bra015491	Acyl-CoA oxidase	76.294	10%	mitochondria peroxisome
BnTic40 C9 ~ 30 kDa lane	Bra008608	Tic40	49.725	14%	chloroplast
	Bra001049	putative 14-33 protein	29.549	10%	cytoplasm/ nucleus
	Bra040592	putative 14-33 protein	29.545	10%	unknown
	Bra037818	putative 14-33 protein	28.241	13%	unknown
BnTic40 $C9_{S-D}$~70 kDa lane	Bra006027	mtHsp70	72.859	26%	mitochondria
	Bra031301	Isocitrate lyase	63.493	17%	glyoxysome
	Bra001843	Isocitrate lyase	60.859	17%	glyoxysome
	Bra016676	Thioglucoside gucohydrolase	62.262	18%	secretory pathway
	Bra011794	mtHsp70	73.251	10%	mitochondria
	Bra015491	Acyl-COA oxidase	76.294	10%	mitochondria, peroxisome
BnTic40 $C9_{S-D}$~30 kDa lane	Bra008608	Tic40	49.725	15%	chloroplast
	Bra008355	putative 14-33 protein	28.169	33%	unknown
	Bra006203	AAC/2	41.006	15%	mitochondria
	Bra016811	Glyoxalase I homolog	31.924	19%	unknown
	Bra001049	putative 14-33 protein	29.594	29%	unknown
	Bra018266	40S ribosomal protein S3-2 like	27.481	11%	unknown

The data of Table 3 indicated that there is little difference between the interaction partners of BnTic40C9 WT and the mutated form. The appearance of Hsp70 is not surprising, because it

has been previously suggested that Tic40 interacts with Hsp70 (Su and Li, 2010). The Hsp70s, which were found as interaction partners of the BnTic40C9 WT and BnTic40C9$_{S\text{-}D}$ proteins, are only mitochondrial Hsp70s, although Tic40 is a chloroplastic protein and therefore also chloroplastic Hsp70 would have been expected. However, this approach did not reveal any potential candidate ms genes, as it is believed that only chloroplastic proteins should be contemplated.

3.1.4 Discovery of a chimeric gene as possible ms gene

In 2016, two different groups claimed to have found the male sterility gene (Xia *et al.* 2016, Deng *et al*. 2016) in their described Chinese male sterility systems. Given that the BnTic40C9 is also the restorer gene for these male sterility systems (Dun *et al.,* 2011; Dun *et al.,* 2014), it was reasonable to contemplate it as the ms gene for the MSL system. Both publications describe a gene on chromosome A7 which encodes for a chimeric protein. The described gene shows high sequence similarities to three different *A. thaliana* genes, a ribonuclease III family protein (RNC1) (AT4G37510), a pre-mRNA processing splicing factor (AT1G80070), while the C-terminus covers most of the mitochondrial Hsp70 (mtHsp70) (AT4G37910). The Middle fragment of this gene shows no similarities to any known gene (Figure 8).

The location of this gene on the A07 chromosome, places it very close to the region identified by mapping by the NPZ. Interestingly while Xia *et al.*, 2016 found it to be localised in the chloroplast, Deng *et al.*, 2016 claimed a nuclear localisation.

First of all, for this work it was necessary to clone the possible ms gene. Because all basic cloning approaches failed to obtain the proposed ms gene from any lines, a 5' RACE-PCR was conducted. One clone was found which covered the chimera coding sequence from start to the end of the Middle part (see Figure 8). This product was used for cloning the N-terminus and the Middle part of the Chimera, while the C-terminus was synthetically synthesized by a company. In the end, it was possible to assemble the whole chimeric gene using the GoldenGate system. Due to its chimeric structure, it will be referred to as the Chimera gene or ms gene for simplicity.

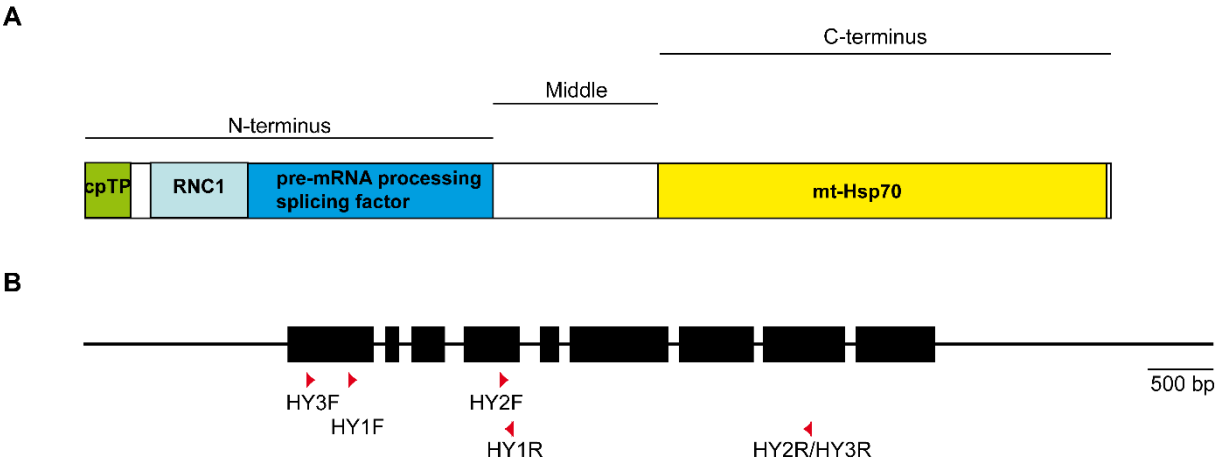

Figure 8: The chimeric ms gene. The size of the model structure (A) cannot be compared with the length of the gDNA (B). **A:** Proposed model of the ms gene. The N-terminus contains the proposed chloroplastic TP (cpTP) and resembles a pre-mRNA processing splicing factor. The C-terminus of the chimera shows an 88% sequence identity with the *A. thaliana* mitochondrial Hsp70.1 sequence (NCBI blast). **B:** Schematic illustration of the gDNA with all the exons (black boxes). The binding sites of oligonucleotides which were used for the PCR (Figure 9 C) are indicated with red arrow heads.

3.1.4.1 First analyses of the possible ms gene

To determine in which lines of the MSL system the ms gene is present and expressed, qRT-PCR was conducted as well as PCR on *B. napus* genomic DNA (gDNA.) Furthermore it was analyzed, if the expression of the Chimera changes during heat treatment at 37 °C in the C545 and WT line (Monty). It was hypothesized, that the ms gene should only be found in the sterile lines (C545 and A-line). The B-line is fertile, but only has the mutated version of the restorer gene, therefore should lack the ms gene. The Monty line is also fertile and possesses a functional restorer gene. Accordingly there might be a chance for the ms gene to also be expressed in the Monty line.

RNA was isolated from flowerbuds with a size of 2 mm in which the pollen development should have reached the uninuclear microspore stage (Zhou *et al.,* 2012) and converted into cDNA. Then qRT-PCR was conducted on the C545 and Monty flower buds both untreated and heat treated at 37 °C. Although there is no indication for a change in the expression after heat treatment (Figure 9 A), this experiment also gave the first indication, that the ms gene is not verifiable on a transcriptomic level in Monty, because there was no PCR result visible after qRT-PCR (Figure 9 B). A second approach was done by conducting PCR on isolated gDNA out of leaves from all four lines of the MSL system to determine if the gene is present. Therefore three specific primer pairs were used (Figure 8 B). The oligonucleotide sequences specific for the chimeric gene were obtained from (Xia *et al.*, 2016). Interestingly, the marker fragments of the ms gene can only be detected in the two male sterile lines, the C545 and

the A-line, but not in Monty and the B-line (Figure 9 C). These results show, that the ms gene seems to be present in the C545 and A-line, but cannot be detected in Monty or the B-line.

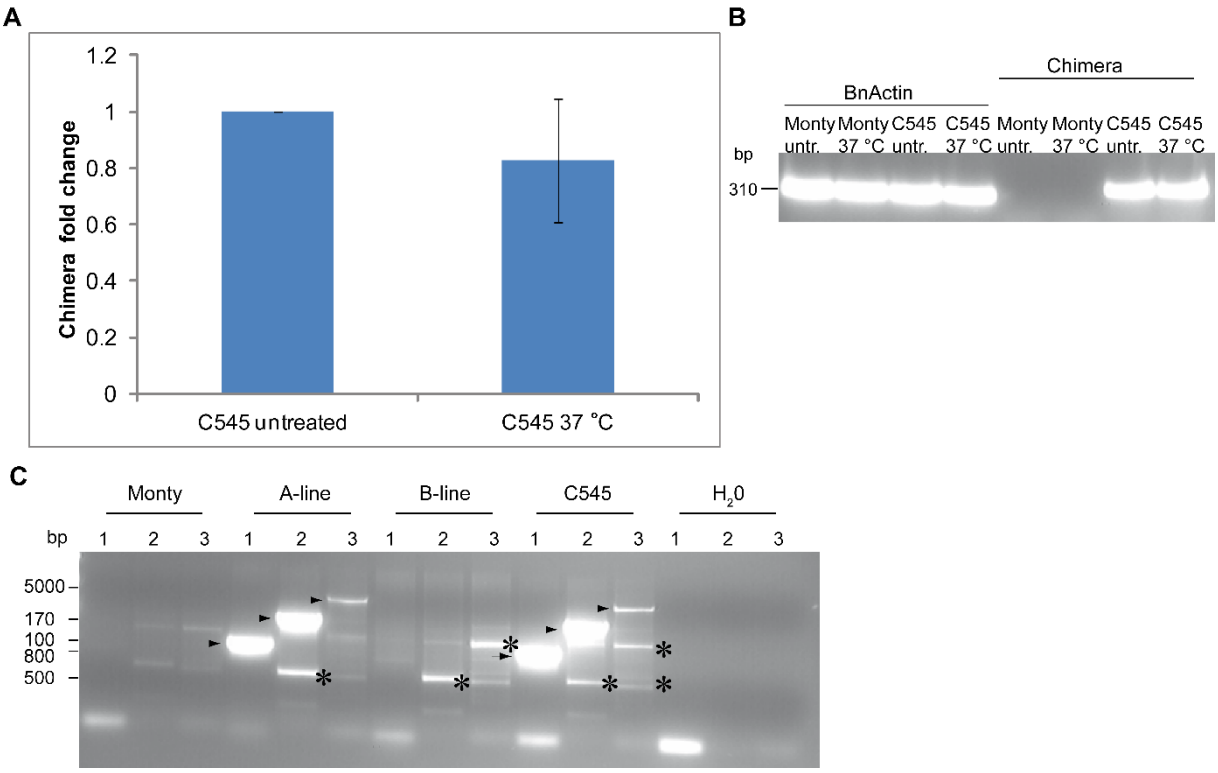

Figure 9: Analysis of the *chimeric* ms gene on a genomic and transcriptomic level. A: qRT-PCR results of extracted cDNA from flower buds (2 mm) from the C545 line untreated and treated at 37 °C under high humidity for three days. The qRT-PCR was also performed with isolated cDNA from Monty flower buds. *BnActin* was used as reference gene. **B:** The results of the qRT-PCR were analysed on an agarose gel (1%). **C:** PCR was done with the gDNA from the four different *B. napus* lines. The gDNA was extracted out of leaf material. The different primer pairs with the estimated size of the PCP product are: 1: HY1, ~1200 bp; 2: HY2, ~2500 bp; 3: HY3, ~3860 bp. The correct PCR products are labelled with arrow heads. The asterisks indicate PCR lanes which display unspecific PCR products.

After cloning the proposed ms gene, it was assembled with its native promoter, the nos terminator and the BASTA resistance marker into a vector which is suitable for stable plant transformation (Figure 10 E). This construct was stably transformed into *A. thaliana* ecotype Col-0. In the T1 generation transformed plants expressing the chimera ms gene displayed a sterile phenotype in contrast to Col-0 plants (WT) (Figure 10 A and B). Furthermore, pollen staining with Alexander staining solution was conducted with the anthers of the mutant as well as Col-0 as a control (Figure 10 A and B). The Alexander staining method stains viable pollen purple (Figure 10 A), while nonviable pollen stay green (Figure 10 B). All things considered, the plants transformed with the full-length chimeric gene construct showed a sterile phenotype with small anthers, no viable pollen or no pollen formation at all and short siliques compared to Col-0 (Figure 10 C). To confirm the plants contained the correct

construct, they were screened for the ms gene with the cloning primers for the N-terminus, Middle and C-terminus of the gene (Figure 10 D). Surprisingly, the same full-length Chimera construct under the Cauliflower Mosaic Virus (CaMV) 35S promoter led to no positively transformed plants, therefore it was impossible to analyse the influence of the full length ms gene while being overexpressed in *A. thaliana*.

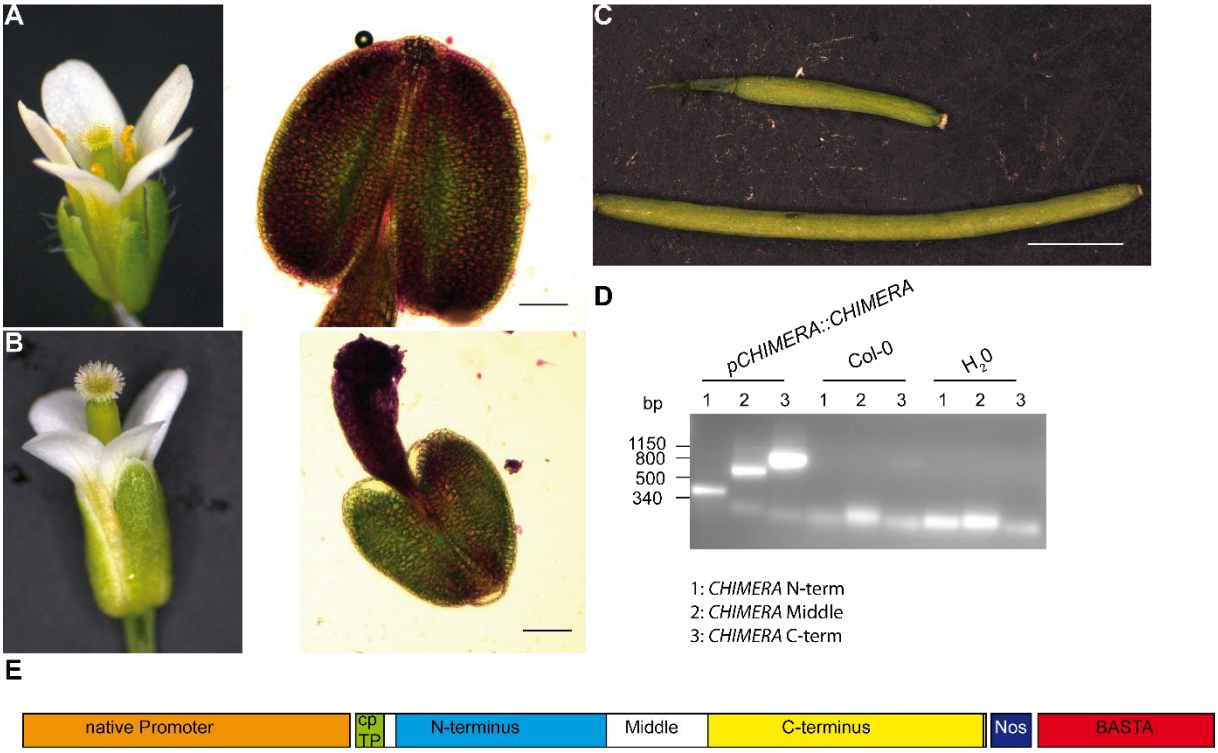

Figure 10: Full length Chimera construct under the native promoter after transformation in *A. thaliana.* A: Stereo microscope picture of the flower and Alexander staining (light microscope) of the anther (Col-0). **B:** Stereo microscope pictures of the flower and Alexander staining (light microscope) of the anther (*pCHIMERA::CHIMERA*). The scale bar indicates 100 µM. **C:** Comparison of Col-0 (lower) and *pCHIMERA::CHIMERA* (upper) silique. The scale bar indicates 3 mm. **D:** Genotyping results. The oligonucleotides used were the same as used for the cloning. **E:** Schematic representation of the *pCHIMERA::CHIMERA* construct.

To examine the localisation of the Chimera protein, two GFP fusion constructs were designed. The FL Chimera construct as well, as just the N-terminus (Figure 11), were fused to the 5' end of the coding sequence of GFP under the 35S promoter. After transient transformation of tobacco with the Chimera FL-GFP and Chimera N-terminus-GFP fusion constructs, intact protoplasts were isolated and analysed under a laser scanning microscope. Theoretically the constructs should contain the chloroplast targeting peptide to guide the proteins to chloroplasts. For both constructs the GFP signal and the autofluorescence of the chloroplasts overlay in the merged picture (Figure 11 A). This indicates a chloroplastic localisation of the chimeric protein. To confirm the expression of the GFP-fused proteins in

tobacco, western blot analysis of extracted tobacco proteins was conducted (Figure 11 B). The male sterile phenotype of the *pCHIMERA::CHIMERA* transformed *A. thaliana* plants and the chloroplastic localisation of the chimeric gene are strong indications for its possible function as the ms gene in the MSL system.

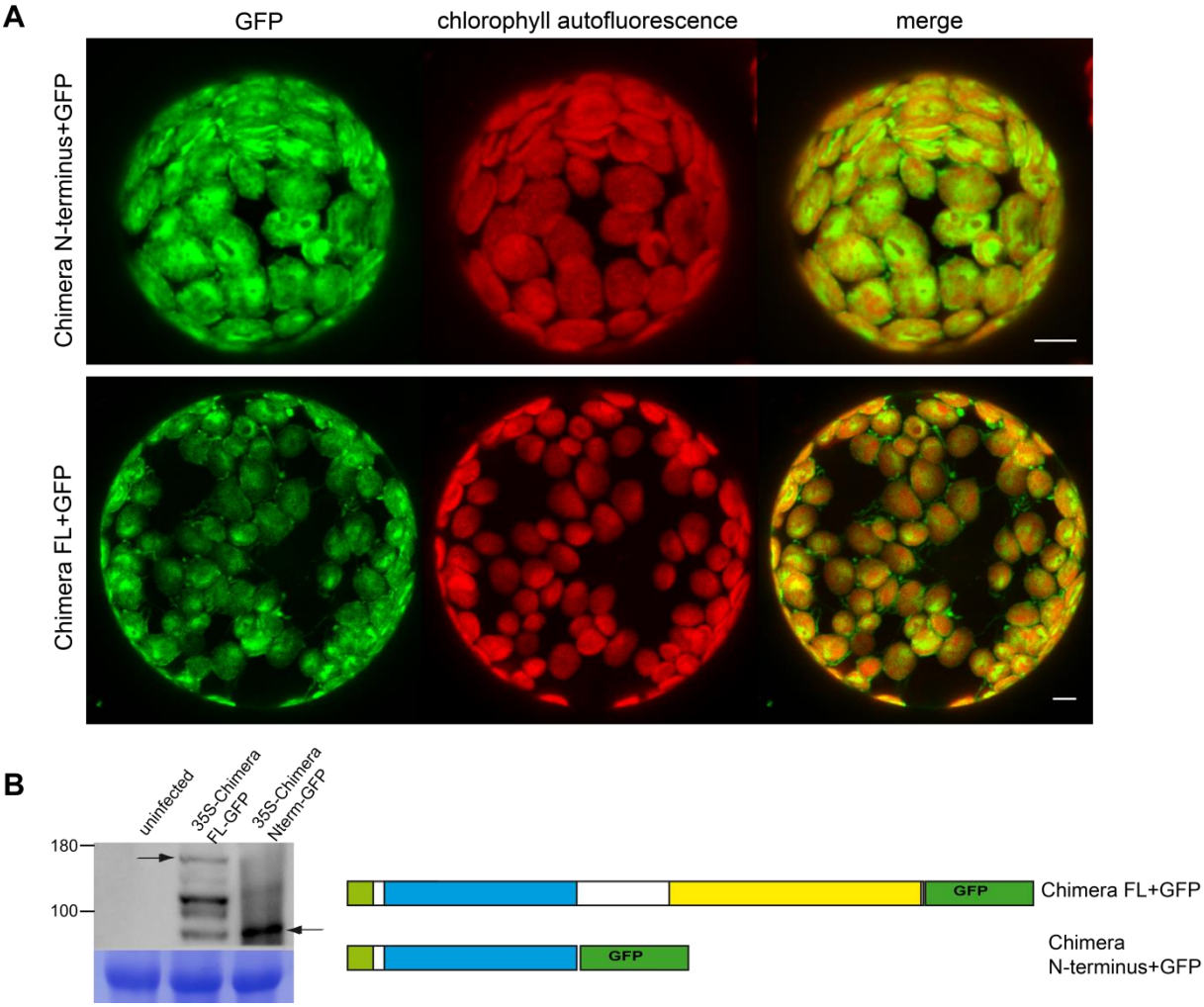

Figure 11: Localisation studies of the N-terminus and the full length (FL) Chimera protein. A: GFP fluorescence and chloroplast autofluorescence detected in isolated protoplasts from transient transformed tobacco. The scale bar indicates 5 µM. **B:** Immunoblot detection of GFP fused proteins after protein isolation from tobacco leaves. Expression was confirmed via confocal fluorescence microscopy. For each sample 25 µg of protein were loaded and detected with antisera against GFP. Equal loading was confirmed with coomassie staining. The estimated size of the Chimera FL+GFP construct after processing is ~175 kDa and for the Chimera N-terminus+GFP ~82 kDa, which is indicated by the black arrows. The stated sizes in the picture are in kDa. **C:** Schematic overview of the GFP fusion proteins.

3.1.4.2 Further analyses of the ms gene candidate

With the discovery that the chimeric gene is the most likely ms gene candidate, further questions arose concerning this project. Since the structure of the chimeric gene had an

estimated protein size of around 150 kDa, and because it consists of Ribonuclease III/ pre-mRNA processing splicing factor, the Middle part and the mtHsp70 C-terminus (Figure 8), the gene was segmented into three fragments. The intention was to analyse which part is responsible for causing male sterility. Therefore, different constructs were cloned and assembled using the GoldenGate system (Table 4). After transformation in *agrobacteria* and stable transformation of Col-0 ecotype of *A. thaliana*, the phenotypes of the T1 generation were analysed (Figures 12 and 13).

In the following sections of this thesis, the *A. thaliana* mutants were named after the gene part of the chimeric gene (Table 4). In contrast to the full-length construct of the chimeric ms gene, no uniform signs for male sterility could be detected for any of the fragmented constructs which were used (Figure 12).

Table 4: Composition of the constructs transformed in *A. thaliana* which can be seen in Figure 12 and 13. The cloning and assembly was done via the GoldenGate system. Numbers indicate the base pairs starting with the start codon.

#	Name	Promoter	Other	Gene part	Terminator	Plant Resistance
1	p*CHIMERA:: CHIMERA*	Chimera native		Chimera FL	Nos	BASTA
2	*pCHIMERA:: CHIMERA* 1-1637	Chimera native		Chimera N-terminus	Nos	BASTA
3	*pCHIMERA:: CHIMERA* 2295-4161	Chimera native	SSUtp	Chimera C-terminus	Nos	BASTA
4	*p35S:: CHIMERA* 1-1637	CamV 35S		Chimera N-terminus	35S	BASTA
5	*p35S:: CHIMERA* 2295-4161	CamV 35S	SSUtp	Chimera C-terminus	35S	BASTA
6	p*CHIMERA:: CHIMERA* 1-2296	Chimera native		Chimera N-terminus+ Middle	Nos	BASTA
7	p*CHIMERA:: CHIMERA* 1638-4161	Chimera native	SSUtp	Chimera Middle+ C-terminus	Nos	BASTA
8	*p35S:: CHIMERA* 1638-4161	CamV 35S	SSUtp	Chimera Middle+ C-terminus	35S	BASTA
9	p*CHIMERA:: CHIMERA* 1638-2296	Chimera native	SSUtp	Chimera Middle	Nos	BASTA
10	*p35S:: CHIMERA* 1638-2296	CamV 35S	SSUtp	Chimera Middle	35S	BASTA

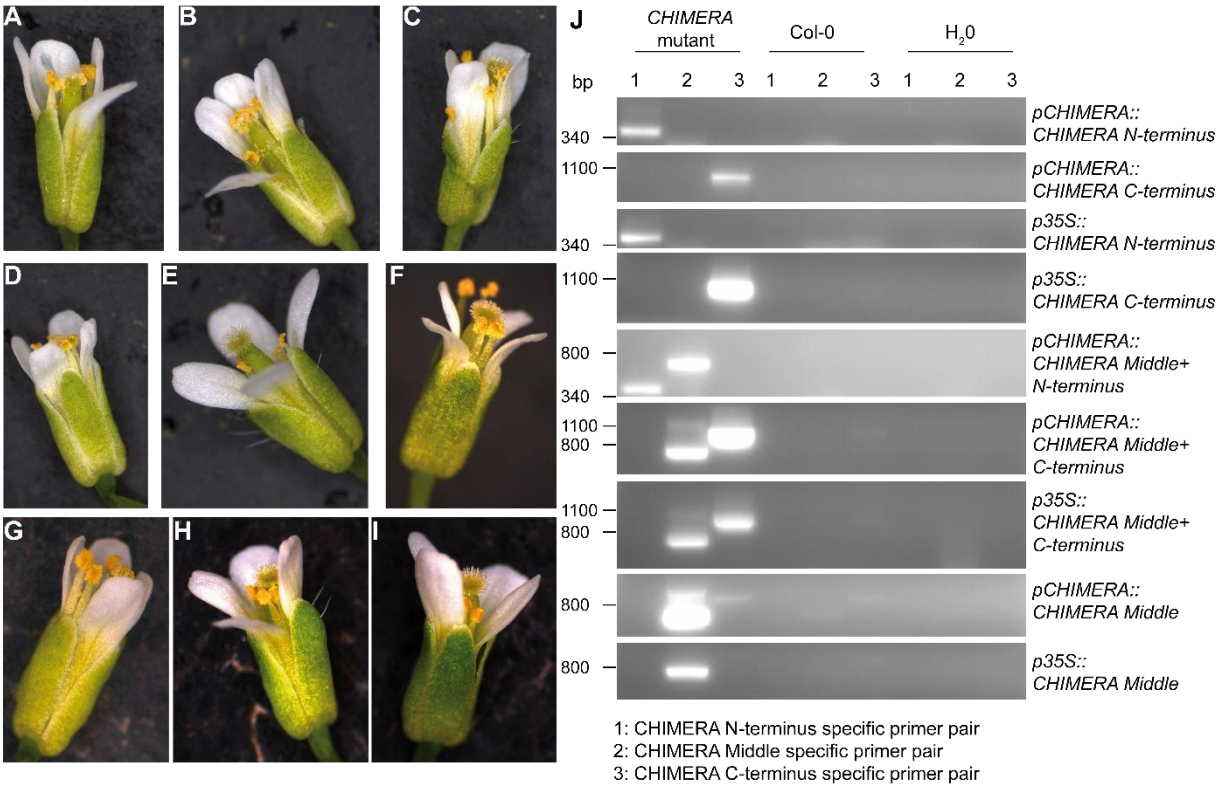

Figure 12: Stable transformed A. thaliana Col-0 plants with different constructs containing parts of the ms gene. A: *pCHIMERA::CHIMERA* 1-1637 (N-terminus) **B:** *pCHIMERA::CHIMERA* 2295-4161 (C-terminus) **C:** *pCHIMERA::CHIMERA* 1638-2296 (Middle) **D:** p35S*::CHIMERA* 1-1637 (N-terminus) **E:** p35S*::CHIMERA* 2295-4161 (C-terminus) **F:** p35S*::CHIMERA* 1638-2296 (Middle) **G:** *pCHIMERA::CHIMERA* 1-2296 (N-terminus+Middle) **H:** *pCHIMERA::CHIMERA* 1638-4161 (Middle+C-terminus) **I:** p35S*::CHIMERA* 1638-4161 (Middle+C-terminus) **J:** Genotyping results using the gene specific primers which were also used for cloning the different Chimera fragments.

The flowers of transformed *A. thaliana* plants showed normal anthers with visible pollen on the surface (Figure 12). The plants were also screened for confirmation of the successful transformation with the different constructs (Figure 12 J), using the molecular cloning primers for the N-terminus, Middle and C-terminus of the chimeric gene to verify the correct construct. Furthermore, measuring of the silique length also showed a similar length like Col-0 except for the full-length construct under the native promoter (Figure 13).

Due to low transformation rates the construct p35S*::CHIMERA N-terminus+Middle* was omitted from the analysis. For the construct *pCHIMERA::CHIMERA N-terminus+Middle*, around 20% of the plants produced less seeds compared to the rest. A similar result was obtained for the p35S*::CHIMERA Middle+C-terminus* plants. However, for these transgenic plants even 40-50% showed little to no seed production. The reason for this is not obvious in Figure 13 is most likely, because three plants were randomly picked for measuring the siliques. The silique length of all the chimera constructs in *A. thaliana* were measured and displayed in Figure 13 B.

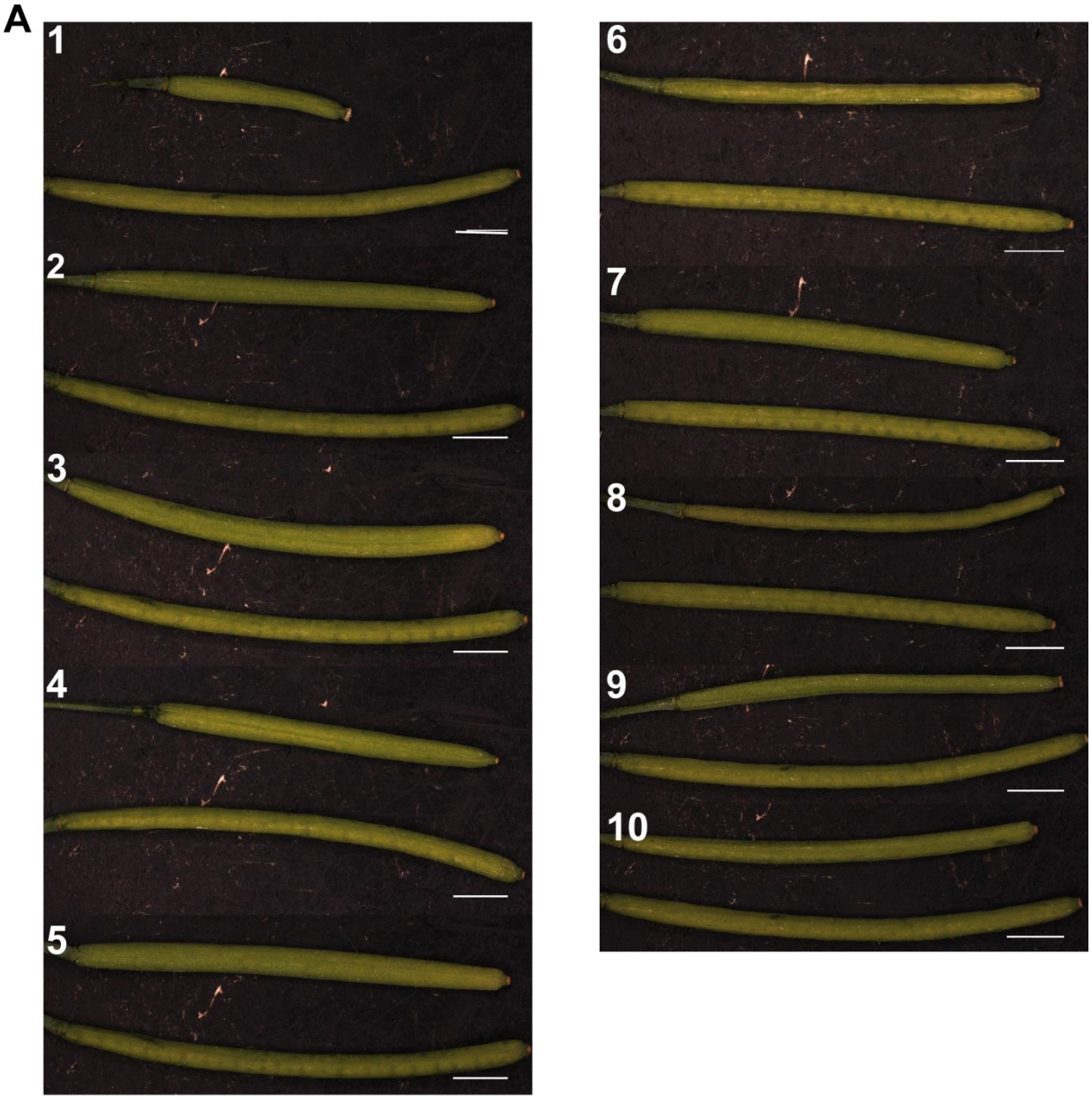
A
1
2
3
4
5
6
7
8
9
10
B
Silique length [cm]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
Col-0
1
2
3
4
5
6
7
8
9
10

Figure 13: Siliques of the different chimera constructs. A: The lower silique is always a Col-0 sample. The scale bar indicates for 0.2 cm. **1:** *pCHIMERA::CHIMERA* FL. **2:** *pCHIMERA::CHIMERA* N-terminus. **3:** *pCHIMERA::CHIMERA* C-terminus. **4:** *p35S::CHIMERA N-terminus.* **5:** *p35S::CHIMERA C-terminus.* **6:** *pCHIMERA::CHIMERA N-terminus+Middle.* **7:** *pCHIMERA::CHIMERA Middle+C-terminus.* **8:** *p35S::CHIMERA Middle+C-terminus.* **9:** *pCHIMERA::CHIMERA Middle.* **10:** *p35S::CHIMERA Middle.* **B:** Measuring the silique length of all chimera mutants in the Col-0 background in cm. For each construct, 50 siliques from three plants (150 siliques in total) were measured. The numbers indicate the constructs according to Table 4. With students t-test, the significance between Col-0 and the mutant lines was determined (p-value: *** = p<0.001).

Interestingly, only the p35S*::Chimera Middle* plants showed an obvious growth phenotype with pale green leaves (Figure 14 A). After BASTA selection, approximately 70-80% of the resistant plants showed a pale green phenotype and partially slower growth. This can be seen in Figure 14 A with four plants of the p35S*::Chimera Middle* transformants, which show different variations of this pale green phenotype. In comparison, the other Chimera mutants under the 35S promoter (Figure 14 B) show a normal dark green phenotype comparable to the Col-0 ecotype. Some of the p35S*::CHIMERA Middle* plants even died before they were able to make seeds. Only the T1 generation of the *A. thaliana* CHIMERA mutants were analysed. Due to the growth phenotype observed the following generations of the p35S*::CHIMERA Middle* plants warrant further analysis in the future.

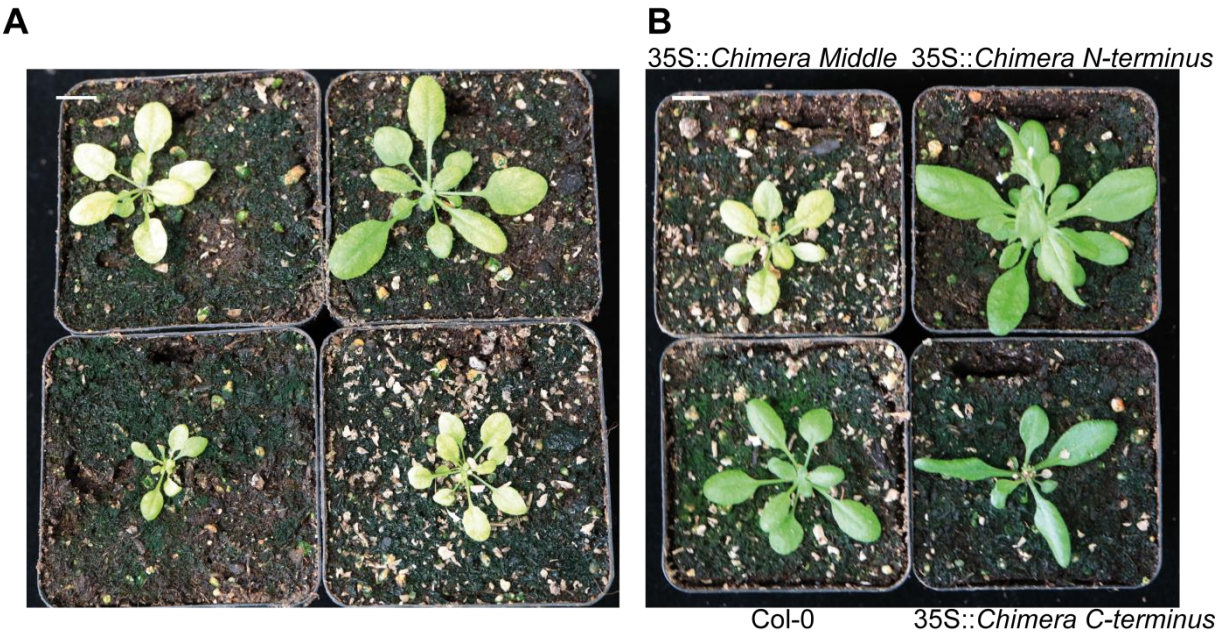

Figure 14: Phenotype of the *p35S::CHIMERA Middle* construct at 5 weeks. The scale indicates for 1 cm. **A:** Four individual plants *of p35S::CHIMERA Middle*. **B:** The p35S*::CHIMERA Middle* in comparison to Col-0, p35S*::CHIMERA N-terminus* and p35S*::CHIMERA C-terminus*.

In conclusion, the data presented so far indicate that only the full-length chimeric gene functions as the ms gene and not one specific part is responsible for causing sterility in *A. thaliana*.

3.2 Detailed analysis of the four BnTic40s in the MSL system including the restorer gene

As previously mentioned, there are four Tic40 proteins in *B. napus*, yet only one of them (BnTic40C9) was identified as a restorer gene for the genic male sterility systems (NPZ, Dun *et al.*, 2011, Dun *et al.,* 2014). However, the four Tic40s as well as the Tic40 from *A. thaliana* show a high sequence similarity in their protein coding sequences (Figure 16). In the alignment the *Bntic40c9* from the A-, B- and C545-lines is also displayed. The sequences were obtained by sequence analysis of the four MSL lines. Due to its 2 bp mutation the sequence ends with a premature stop codon and results in a non-functional protein. The WT form of the BnTic40C9 is only present in Monty (Figure 15).

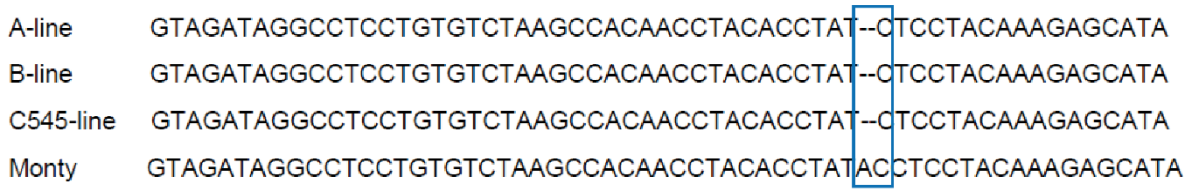

Figure 15: Alignment of the BnTic40C9 sequence in the four MSL-lines: A-line, B-line, C545-line and Monty. The two bp mutation is marked in blue.

Interestingly, the BnTic40 A2 and C2, as well as C9 and A10 display a close resemblance in their sequence. Therefore it was important to analyse which amino acids are responsible for the function of BnTic40C9 as the restorer gene. The significant amino acid differences between the BnTic40C9 in comparison to the other Tic40s were investigated in more detail and are marked green (Figure 16).

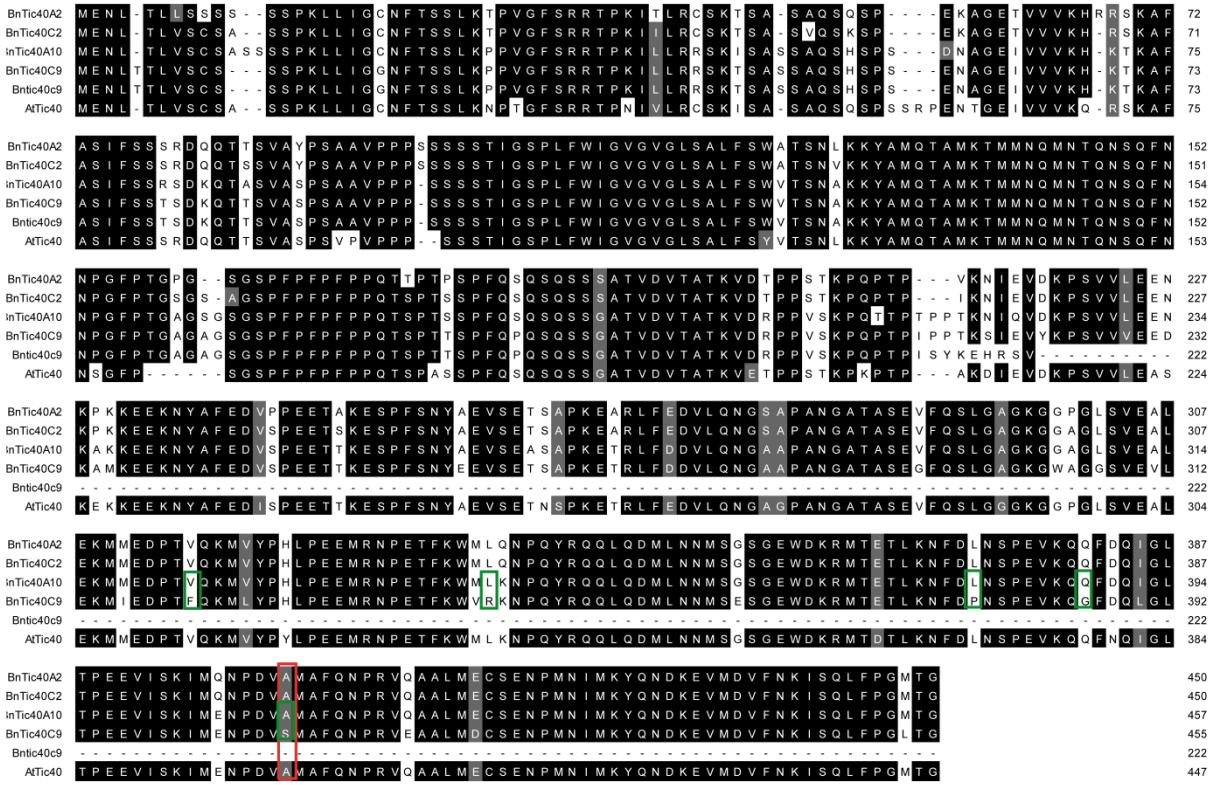

Figure 16: Alignment of the *B. napus* Tic40 protein sequences with the Tic40 from *A. thaliana*. The sequence for the BnTic40 A2 is the sequence obtained after cloning the gene from the MSL system. All other BnTic40 sequences were obtained from (https://www.genoscope.cns.fr/brassicanapus/). The *A. thaliana* sequence was retrieved from Tair. The exchanged amino acids for the YTH experiment, are labelled in green (section 3.2.3/3.2.4). The exchange for the pulldown (section 3.1.3) is tagged in red. Black colour indicates identical amino acids in all sequences, while grey boxes indicate similar amino acids.

3.2.1 Complementation of the *A. thaliana tic40* ko line (SALK_057111)

It is hypothesized that BnTic40C9 gained a novel function as a restorer gene for the male sterility system (Dun *et al.*, 2014). Given that in *B. napus* four Tic40 genes exist, the question arises, if it is necessary for the BnTic40 C9 to keep its function as an import component. Especially in the MSL system, the loss of function of the BnTic40C9 in combination with the expression of the ms gene results in a sterile phenotype but no obvious paleness compared to the WT line. In *A. thaliana* the loss of function of the one Tic40 gene results in a pale phenotype, indicating the import capacity of chloroplasts is impaired. With the complementation of the *tic40* ko line (SALK_05777) with the four *BnTic40* genes individually, the effect of all four *Brassica* Tic40 proteins on the *attic40* pale green phenotype was analysed. Furthermore, this experiment could indicate if BnTic40C9 lost its function as an import component by gaining its function as a restorer gene.

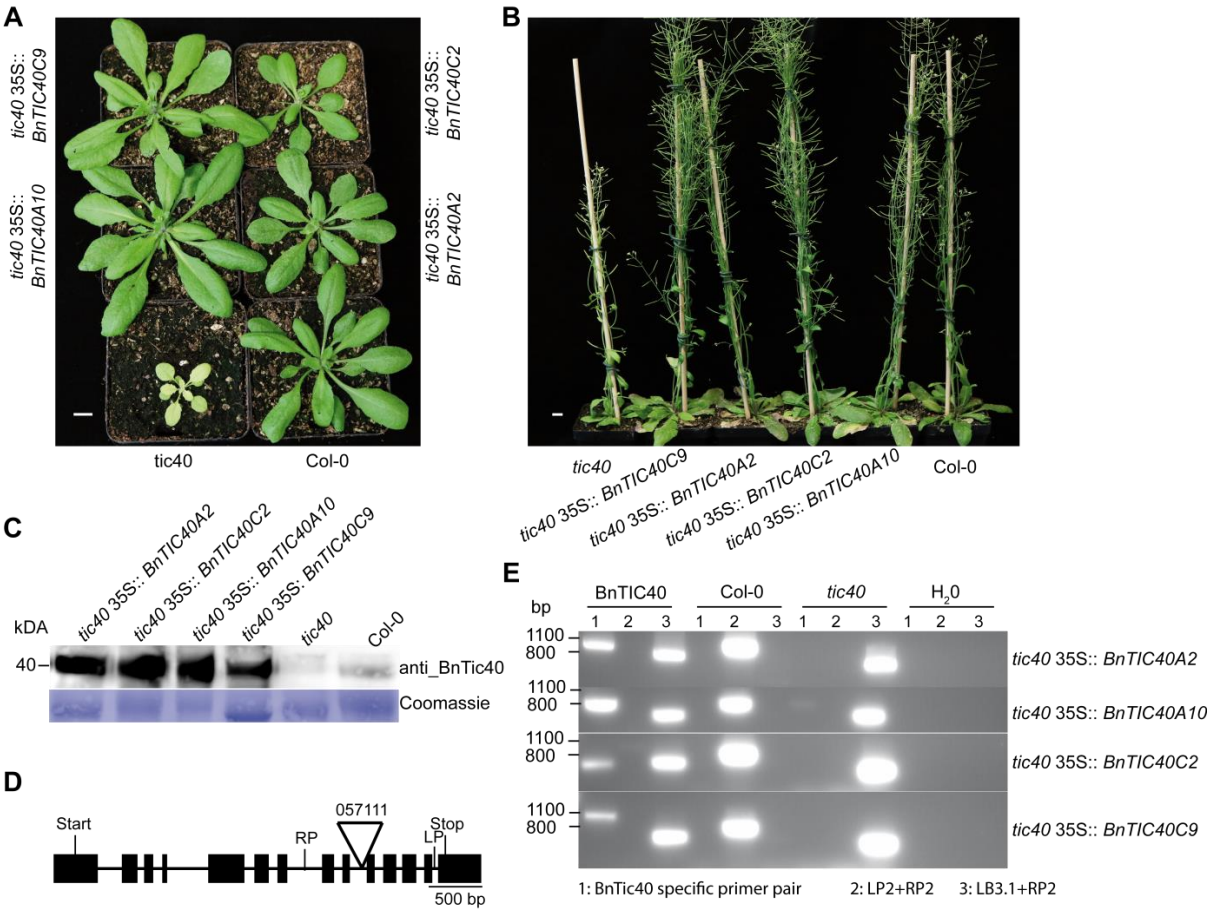

Figure 17: *A. thaliana* tic40 ko complementation with the four *B. napus Tic40* genes C9, C2, A10 and A2. A: Phenotypes of 4 week old plants of the T3 generation. The scale bar indicates 1 cm. **B:** Phenotypes of 7 week old plants of the T3 generation. The scale bar indicates 1 cm. **C:** Western blot for protein analysis of Tic40 in the plant tissue. 20 µg of total plant protein was loaded each time. The coomassie stained blot was used as loading control. **D:** Illustration of the tic40 genomic T-DNA insertion. The correct BnTic40 in each complementation was confirmed via sequencing. Exons are represented by the black boxes. **E:** Genotyping results by using the stated oligonucleotide combinations.

After transformation of the *BnTic40* genes into the *A. thaliana tic40* plants and following hygromycin selection, the plants were analysed. All four complementation lines (*tic40* 35S*::BnTIC40C9*; *tic40* 35S*::BnTIC40C2*; *tic40* 35S*::BnTIC40A2*; *tic40* 35S*::BnTIC40A10*) in their T3 generation showed a dark green phenotype (Figure 17 A and B) similar to the Col-0 line and a comparable wildtype growth rate in contrast to the obvious slow growth of the *tic40* ko line. The results were confirmed via western blots on the total protein content from leaves, showing a clear overexpression of the BnTic40s compared to the AtTic40 in Col-0 Figure (17 C). PCR screening of the plants was performed with BnTic40 specific oligonucleotides and the left primer (LP), right primer (RP) and left border primer (LB) to confirm the T-DNA insertion and the correct complementation construct (Figure 17 D-E). All plants were homozygous for a*ttic40* T-DNA insertion confirming that the complementation was

successful. In conclusion, all BnTic40s can individually complement the *tic40* phenotype, which was also shown by (Dun *et al.,* 2014). This is an indication, that the *BnTic40C9* gene with its new role as restorer gene still retained its function as import component.

3.2.2 qRT-PCR analysis of the four BnTic40 in Monty and the C545 line

The sterility of the C545 line in the MSL system can be reverted by heat treatment at 37 °C for three days. With qRT-PCR it was analysed if the expression levels of the four Tic40s change in the C545 line and Monty and if heat treatment leads to a difference in gene expression.

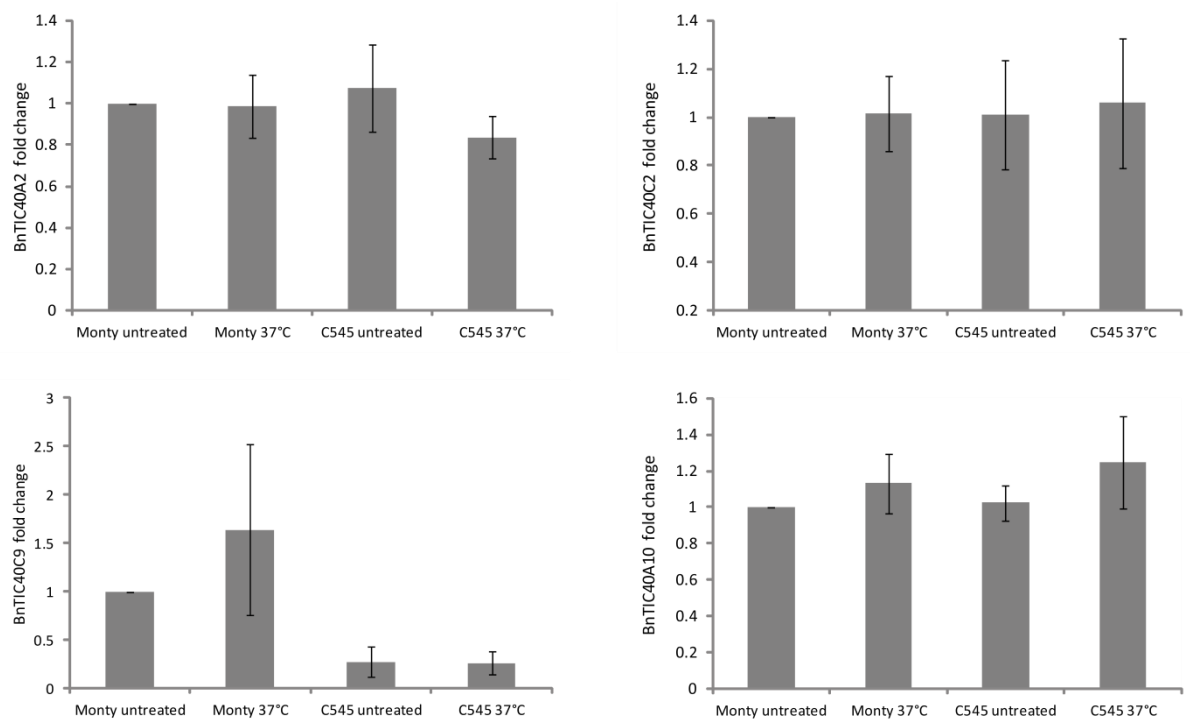

Figure 18: qRT-PCR analysis of the four *BnTic40* genes in Monty and the C545 line untreated and treated for three days at 37 °C. RNA was isolated from flower buds with a size of 2 mm. The PCR was done with three individual samples and three replicates. *BnActin3* was used as reference gene. The data was evaluated using the $2^{-\Delta\Delta\text{-ct}}$ method.

Due to the mutation of the *Bntic40C9mut*, resulting in an early stop codon in the sequence, the expression of *BnTic40C9* in the C545-line is reduced compared to Monty (Figure 18). Nevertheless, qRT-PCR analysis did not indicate any evidence that one of the other three BnTic40s adopts the function of the BnTic40C9 as an import component as there is no obvious change in the expression in the C545-line for *BnTic40 A2*, *C2* or *A10*. These results provide further evidence for the BnTic40C9 to keep its function during protein import in spite of its new gained function as restorer gene.

3.2.3 Analysis of possible interaction between the four *BnTic40* genes and the ms gene

Since the function of the *BnTic40C9* as restorer gene was suspected to evolve around the same time frame as the chimeric male sterility gene (Xia *et al*., 2016) as well as their similar expression patterns (Dun *et al.*, 2011; Xia *et al*., 2016), it is likely to assume that these two genes might interact directly with each other. Furthermore, the Chimera and Tic40 proteins are both located within chloroplasts. To determine if the Chimera interacts with the Tic40 proteins, yeast two hybrid (YTH) assays were performed. For the YTH interaction studies, the four *B. napus* Tic40s were cloned again with the GoldenGate system into the prey plasmids. The chimeric gene was used as a FL construct as well as the Middle part, N-terminus and C-terminus, which were cloned as bait constructs. All sequences were cloned without their target peptide (Chimera) and transmembrane domain (Tic40), as these are known to be incompatible with YTH assays. The YTH system is based on the procedure of bringing the attached Gal4 DNA binding domain and the Gal4 activation domain in close proximity if interaction between the prey and bait proteins occurs. Thereby the transcription of the reporter genes is triggered (Luban and Goff, 1995).

After activation of the reporter genes AURI1-C, ADE2, HIS3 and MEL1, proteins interacting with each other should be able to grow as white colonies on plates lacking adenine and histidine. The mating experiment was plated out on SD plates without Leucine and Tryptophan (-2) to control for positive mating of bait and prey containing yeast, but also on SD plates without Leucine, Tryptophan, Adenine and Histidine (-4) to test for positive interactions. As a positive control, an interaction between murine tumour suppressor p53 protein and SV40 large T antigen was tested. The positive interaction control of SV40 and p53 growth on SD (-2) as well as on SD (-4) plates and is white in colour. The negative control on the other hand (Lam and SV40), only growth on the SD (-2) plates with a red colour which indicates for no positive interaction. Interestingly, the same positive growth was observed with the restorer gene (BnTic40 C9) as well as the Middle fragment of the ms gene on the SD (-2) and (-4) plates (Figure 19 A and B). Why there was no interaction observed between the restorer gene and the full length ms gene, was answered after western blot analysis of the yeast proteins. Therefore, the different proteins with their interaction partner were grown, proteins were extracted and then loaded on a SDS-gel. After blotting the c-myc antibody was used for the bait constructs and the BnTic40 specific antibody detected the prey proteins (Figure 19 C and D). Apparently, the Chimera FL could not be detected on the western blot, which leads to the conclusion that this protein is not being expressed in the yeast system. Hence, no interaction with the Chimera FL protein could take place.

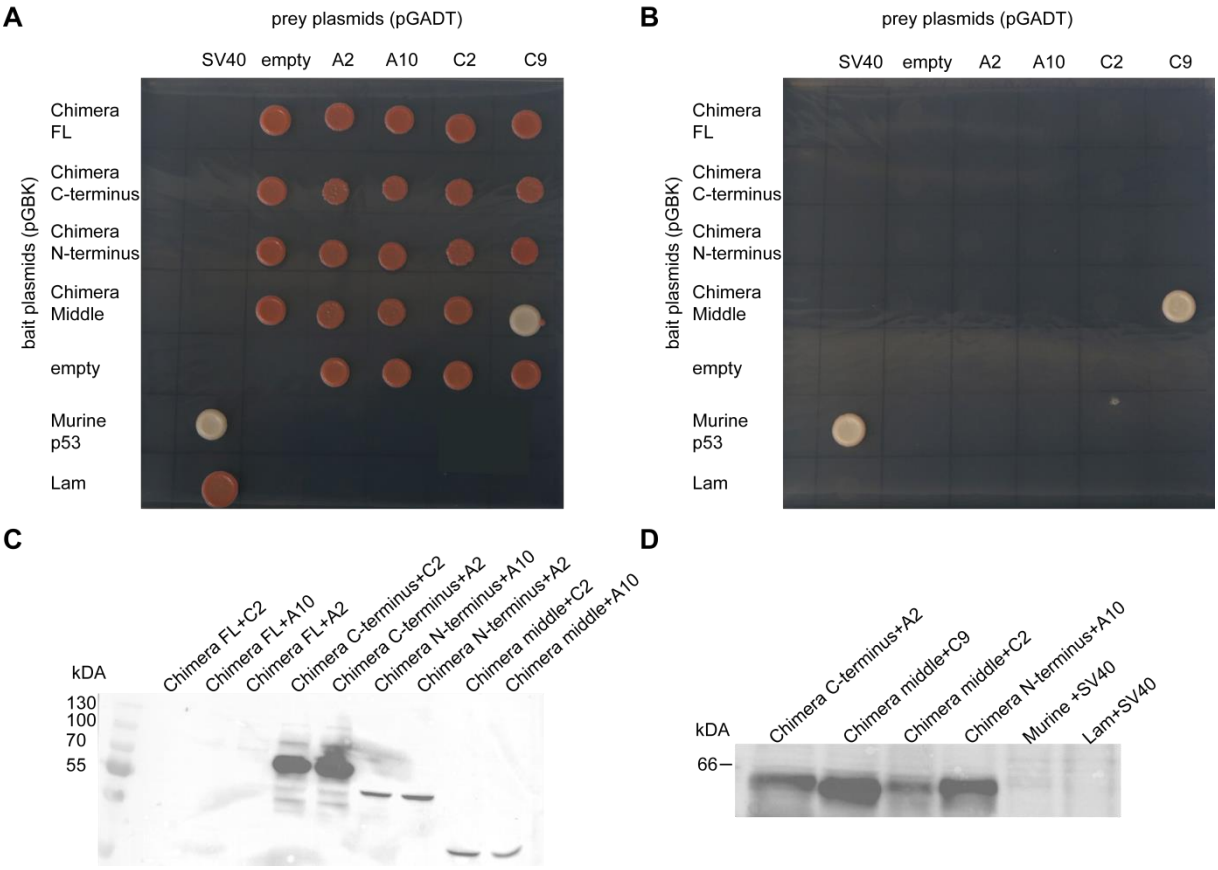

Figure 19: Yeast two hybrid interaction experiment with the *B. napus* Tic40s as prey proteins and Chimera, also in fragmented versions, as bait proteins. A: Mating control plate: SD-Leu/Trp **B**: Interaction plate: SD-Leu/Trp/His/Ade **C**: Western blot was conducted with anti-cmyc antiserum for detection of the bait proteins. **D**: Western blot with anti-BnTic40sol antibody for detecting the prey proteins. The YTH interaction experiment was performed by Carina Engstler.

3.2.4 The different amino acid exchanges between the restorer gene and the BnTic40 A10

Between the different Tic40s in *B. napus*, the Tic40A10 exhibits the closest sequence similarity (95.5%) to the restorer gene. Another question which was relevant to the overall thesis needed to be asked: why does the BnTic40 A10 not function as a restorer gene, even though it has this close similarity to BnTic40C9? To answer this question, different mutated constructs were cloned. In each one, a different amino acid of the C9 protein was exchanged with the corresponding amino acid of the A10 protein. Furthermore, one construct was designed with the first part of C9 and the second part of the A10 protein. These C9 mutations were cloned into the prey plasmid of the YTH system. With the YTH assay, we wanted to determine, if these amino acid exchanges are enough to alter the behaviour of the BnTic40 C9 protein. The amino acids at position 321 (phenylalanine (F) → valine (V)), 343 (arginine (R) → leucine (L)), 378 (proline (P) → L), 386 (glycine (G) → glutamine (Q)) and 408 (serine (S) → alanine (A)) were exchanged.

Figure 20 shows, that the exchange of the amino acids on position 321 (F → V) and separately on 343 (G → Q) lead to the result that Tic40C9 does no longer interact with the Middle part of the chimera protein anymore. Apparently, either changing from a phenylalanine (F) to a valine (V) and an arginine (R) to a leucine (L) seems to make a difference for the interaction. Interestingly, a point mutation in position 386 leads to a new interaction of the C9 protein with the C-terminus of the chimeric protein, which resembles the mtHsp70. A possible interaction with BnTic40C9 and mtHsp70 was already observed earlier (Figure 7; Table 3).

Again, the expression of the proteins in the yeast system was confirmed by conducting western blots (Figure 20 C and D).

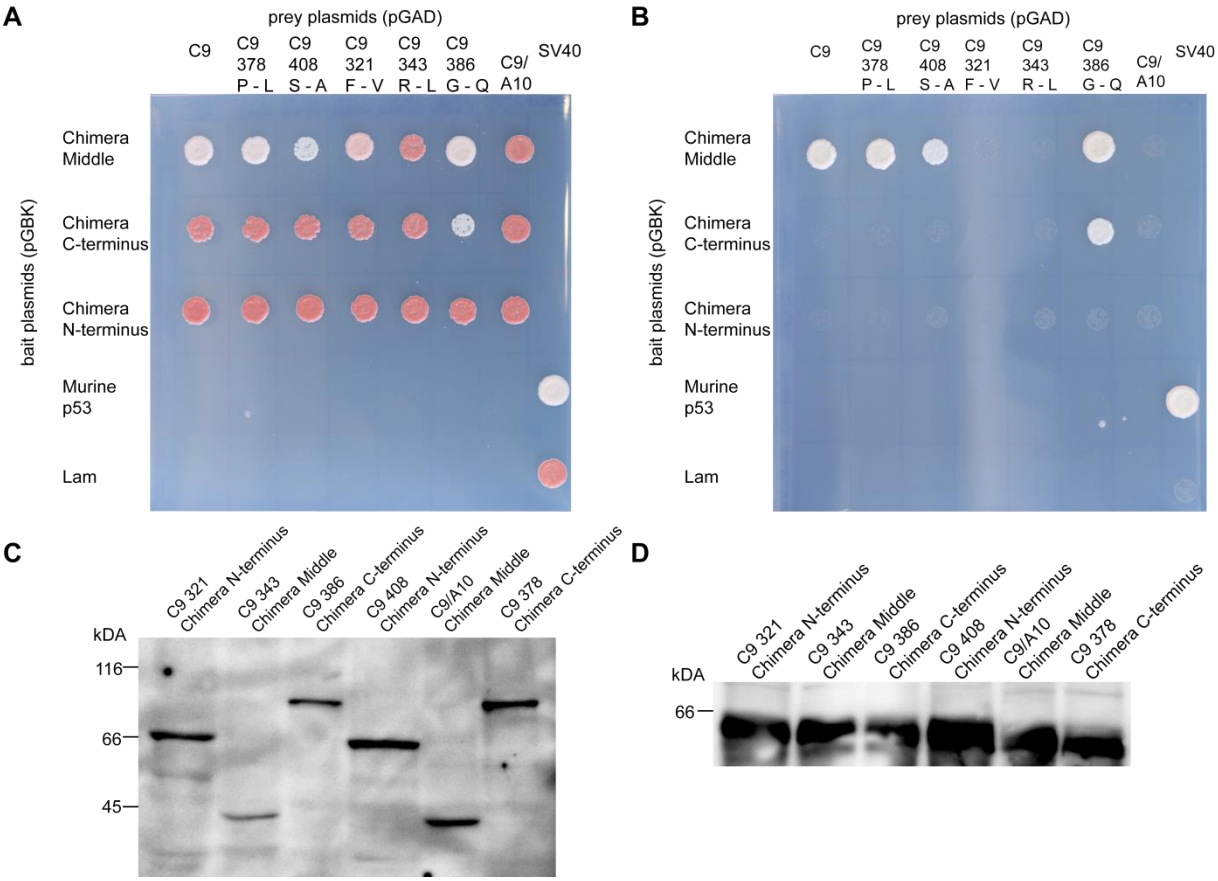

Figure 20: Yeast two hybrid interaction experiment with the *Brassica napus* Tic40C9 mutations as prey proteins and Chimera in fragmented versions, as bait protein. A: Control plate: SD-Leu/Trp. **B**: Interaction plate: SD-Leu/Trp/His/Ade. **C**: Western blot with anti-cmyc antiserum, detecting the bait proteins. **D**: Western blot with anti-BnTic40sol antibody for detecting the prey proteins. 25 µg of protein was loaded for each interaction. The YTH interaction experiment was performed by Carina Engstler.

3.2.5 Stable transformation of Col-0 with the ms gene in combination with the BnTic40s

To test the restorer function of the BnTic40C9 and the inhibition of this function with a simple exchange of one amino acid, *A. thaliana* Col-0 plants were stably transformed. Therefore constructs containing the native promoter of the ms gene as well as the ms gene in combination with either BnTic40 C9, C2 and A10 in full length under the control of the native C9 and A10 promoter were designed (Table 5). The BnTic40A2 was not used, because the assembly of the full length construct for the GoldenGate system failed. All the following results were received from the T1 generation.

Table 5: Constitution of the *A. thaliana* mutants shown in figure 21

		Construct 1		Construct 2			
#	**vector**	**promoter**	**gene**	**promoter**	**gene**	**resistance**	**background**
102	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40A10*	*Tic40A10*	BASTA	Col-0
103	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40A10*	*Tic40C2*	BASTA	Col-0
104	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40A10*	*Tic40C9*	BASTA	Col-0
106	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40C9*	*Tic40A10*	BASTA	Col-0
107	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40C9*	*Tic40C2*	BASTA	Col-0
108	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40C9*	*Tic40C9*	BASTA	Col-0
113	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40C9*	$Tic40C9_{378}$	BASTA	Col-0
114	pCAMBIA	*Chimera*	*CHIMERA*	*Tic40C9*	$Tic40C9_{408}$	BASTA	Col-0

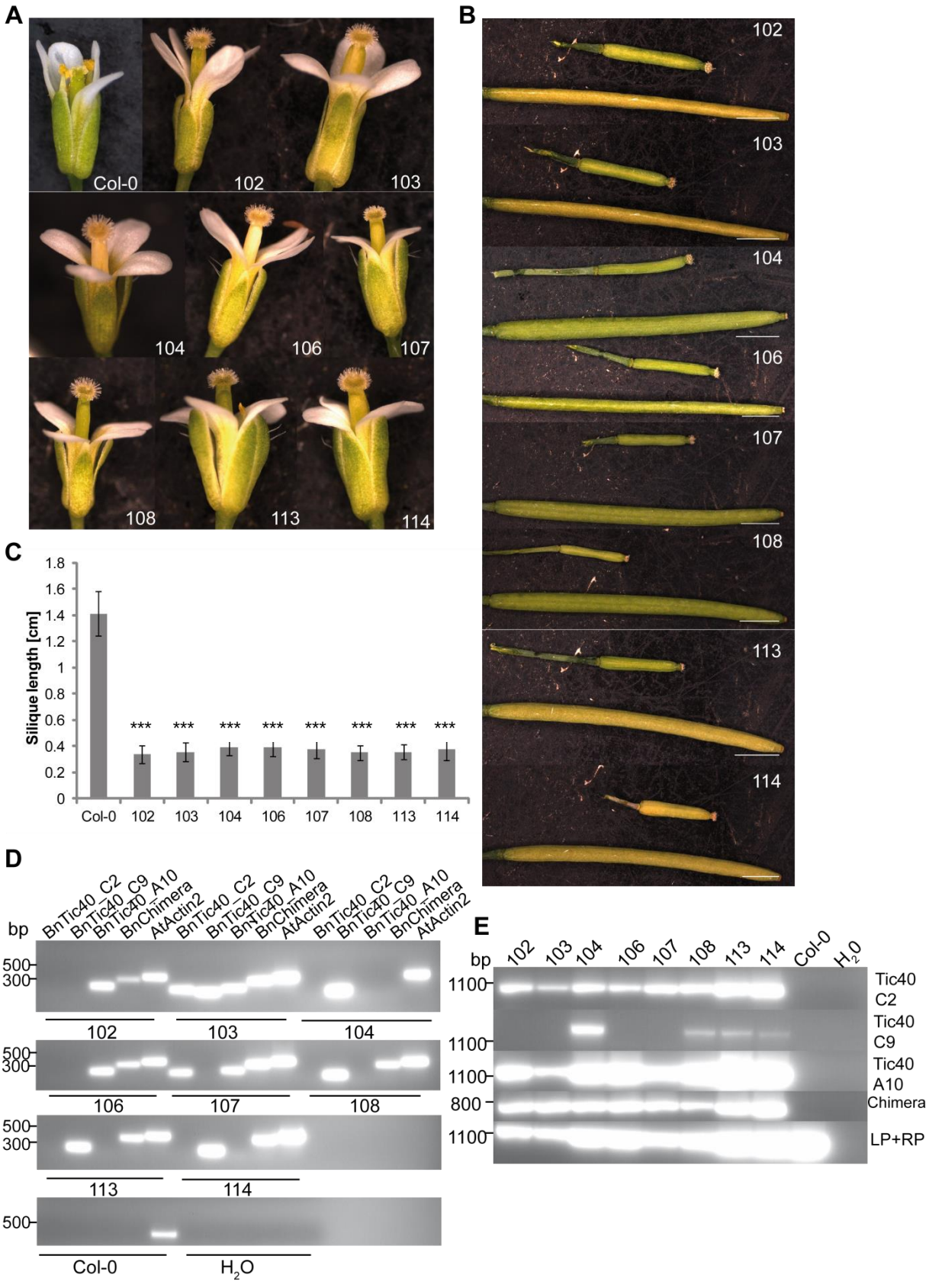
A
Col-0
102
103
104
106
107
108
113
114
B
102
103
104
106
107
108
113
114
C
Silique length [cm]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
Col-0
102
103
104
106
107
108
113
114
D
bp
BnTic40_C2
BnTic40_C9
BnTic40_A10
BnChimera
AtActin2
500
300
102
103
104
106
107
108
113
114
Col-0
H2O
E
bp
102
103
104
106
107
108
113
114
Col-0
H2O
1100
800
Tic40 C2
Tic40 C9
Tic40 A10
Chimera
LP+RP

Figure 21: Analysis of the Col-0 mutants after transformation with different constructs containing BnTic40 in combination with the ms gene. The composition of the different constructs is listed in Table 5. **A:** Pictures taken with the stereo microscope of the opened *A. thaliana* flowers in contrast to Col-0. **B:** Images of the *A. thaliana* siliques always in combination with Col-0 (silique below). The scale bar indicates 2 mm. **C:** Graph of the silique length in cm. For three plants each, 50 siliques were measured per construct. The graph shows the mean value and the standard deviation. With students t-test, the significance between Col-0 and the mutant lines was determined (p-value: *** = $p<0.001$). **D:** Expression analysis of the four BnTic40s and the ms gene. The qRT-PCR results were run on an agarose gel. To confirm that actually the C9 was expressed, PCR results for line 104, 108, 113 and 114 were send for sequencing. The qRT-PCR was done with three biological and three technical replicates. **E:** Genotyping results for the different mutant plants. The primers for BnTic40 C2, C9 and A10 were used for cloning in the Golden Gate system. For detection of the ms gene, the Chimera Middle primers (Golden Gate cloning) were used. As positive control the LP/RP primers for SALK_057111 were used to assure the intactness of the Col-0 gDNA. Because of the close sequence similarity of the BnTic40s, the primers for C2 and A10 are apparently also amplifying the other three BnTic40s, while the oligonucleotide combination for the C9 is very specific.

The *A. thaliana* plants with the constructs of Table 5 all displayed a sterile phenotype (Figure 21 A-C). These plants do not produce pollen, barely make any visible anthers and the siliques are substantially shorter compared to the Col-0 ecotype similar to the results with the Chimera construct (Figure 10). In the next step to confirm the expression of the Tic40s especially of the BnTic40C9 qRT-PCR was confirmed (Figure 21 D). The fact that sometimes even the A10 or C9 primer combinations lead to a positive result is most likely due to the close sequence similarity of the Tic40s. It was confirmed via sequencing that the PCR product with the BnTic40A10 primer of the line #103 actually is BnTic40C2. The PCR products for the BnTic40C9 (line 104 and 108) were also sequenced and confirmed the transformation of the restorer gene. Although the Chimera is so low expressed that it sometimes does not even show a product on the gel (Figure 21 D) and was barely detectable during the qRT-PCR analysis, the expression level is sufficient for the *A. thaliana* plants to become sterile. The plants were screened using BnTic40 specific primer and primers for the Middle fragment of the Chimera. As a positive control for Col-0, LP and RP oligonucleotides for *tic40* were used (Figure 21 E). The expectation was that plants containing the restorer gene (BnTic40C9) should be fertile despite the presence of the ms gene meaning the restorer function of BnTic40C9 could not be confirmed in *A. thaliana*. One explanation might be, despite the high exon similarity, the system regulating pollen development in *A. thaliana* is different to the *B. napus* male sterility system.

3.3. Possible restoration of fertility in the sterile C545 line

The MSL system used in this work consists of four lines, although it is a genic male sterility system. Two of these lines (A- and C545 line) are sterile while the maintainer (B) and restorer (Monty/WT) lines are fertile. For the analysis of the phenotype, the RNA sequencing analysis and the treatment with different conditions only the C545 and restorer line was used. For a breeding company, it can be difficult to obtain seeds for their sterile lines. For the C545 line, it is known, that the sterility can be partially reversed by heat treatment at 37 °C for at least three days. Admittedly, that is a rather energy consuming way to produce seeds for this line. In this part of the thesis, the phenotype of the C545 line treated under different conditions was analysed, while in section [3.5], the corresponding RNA-sequencing results for the different treatments are shown.

All the following flower pictures are representative for three independent treatments.

As expected, there is an obvious difference in the phenotype between the sterile C545 line and the restorer line (Monty) (Figure 22). The different flower sizes express different developmental stages. For Monty a complete development of the anthers with pollen production can be seen. The anthers of the C545 line on the other hand are strongly reduced in their growth and are unable to produce pollen (Figure 22 A and B).

For the heat treatment at 37 °C in our laboratory, a Percival growing chamber was used. The high humidity level was reached by adding a large vessel with water into the plant growing chamber. Figure 22 C shows the different developmental stages of heat treated flowers from the C545-line. Compared to Monty flowers (Figure 22 A) there are barely any differences visible. The image of the whole flower clearly shows a strong pollen production and release from the anthers.

Figure 22: Different stages of anther development. These are shown in flower sizes from 0.3 cm up to 1 cm in 0.1 cm steps as well as an example of a fully opened flower. The scale bar indicates 2 mm. Pictures were taken with the stereo microscope (magnification: 0.8x) **A**: Monty untreated. **B**: C545 untreated. **C:** 37 °C heat treatment for 3 days in the C545 line.

Another approach, which was first conducted by the NPZ and then performed under various conditions in our laboratory, was treating the plants with auxin in combination with an ethylene donor (10 µM 1-NAA and 100 µM ethephon). After the treatment, the plants were allowed to keep growing in a climate chamber at 28 °C with a humidity level of 70% and a day/night rhythm of 20 h/4 h. For simplicity these conditions will be referred to as auxin

treatment only. The first attempts which involved only one auxin spraying did not lead to fertile flowers. The *B. napus* plants were then treated four times with auxin during their 14 days in the climate chamber.

For the phenotyping results, the flower buds of the C545-line were documented 1-3 days after the plants were transferred from the climate chamber back to the greenhouse. Figure 23 shows the auxin treated flowers and also the control flowers. There is not a huge difference visible between only mild heat treatment of the plants and simultaneously spraying the flowers and unopened buds. Both samples show an anther development which differs to the untreated C545-line (Figure 22). Nevertheless the pollen development seemed to be more evident with the auxin treatment compared to mild heat treatment alone. The anthers without auxin treatment appeared to be rather smooth compared to the control plants exposed to 28 °C only (Figure 23 A and B). Smooth anthers are usually a sign for the lack of pollen release. These results were also used for RNA sequencing to achieve more insights into the transcriptome changes during the treatments.

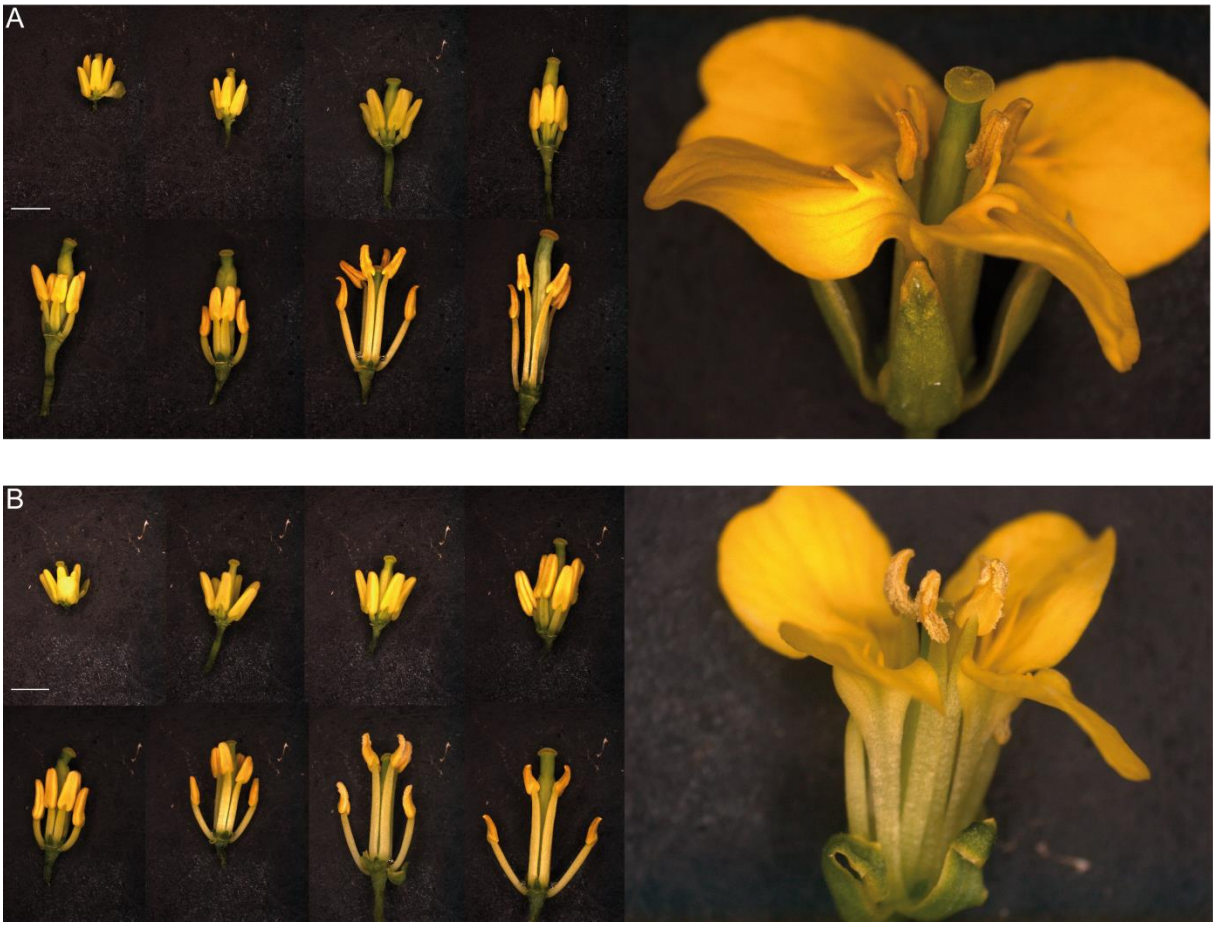

Figure 23: Anther development of flower sizes from 0.3 to 1 cm in 0.1 cm steps as well as an example of a fully opened flower. The scale bar indicates 2 mm. A: C545 treated at 28 °C and 70% humidity for two weeks. **B**: C545 sprayed with auxin and treated at 28 °C and 70% humidity for two weeks. The pictures were taken one to three days after the plants were moved from the climate chamber back to the greenhouse and four to six days after the last auxin treatment.

Since the treated flowers did not always have many siliques after treatment, it had to be determined if the pollen were actually viable. A different staining solution was applied to *B. napus* pollen than the one applied to the *A. thaliana* mutants, because the *B. napus* pollen did not stain well with the Alexander staining. A 1% acetocarmine staining solution was manufactured and applied to free pollen only instead of the whole anthers. This change of method was necessary to get a clearer picture of the pollen viability due to the larger size of *B. napus* anthers compared to *A. thaliana*.

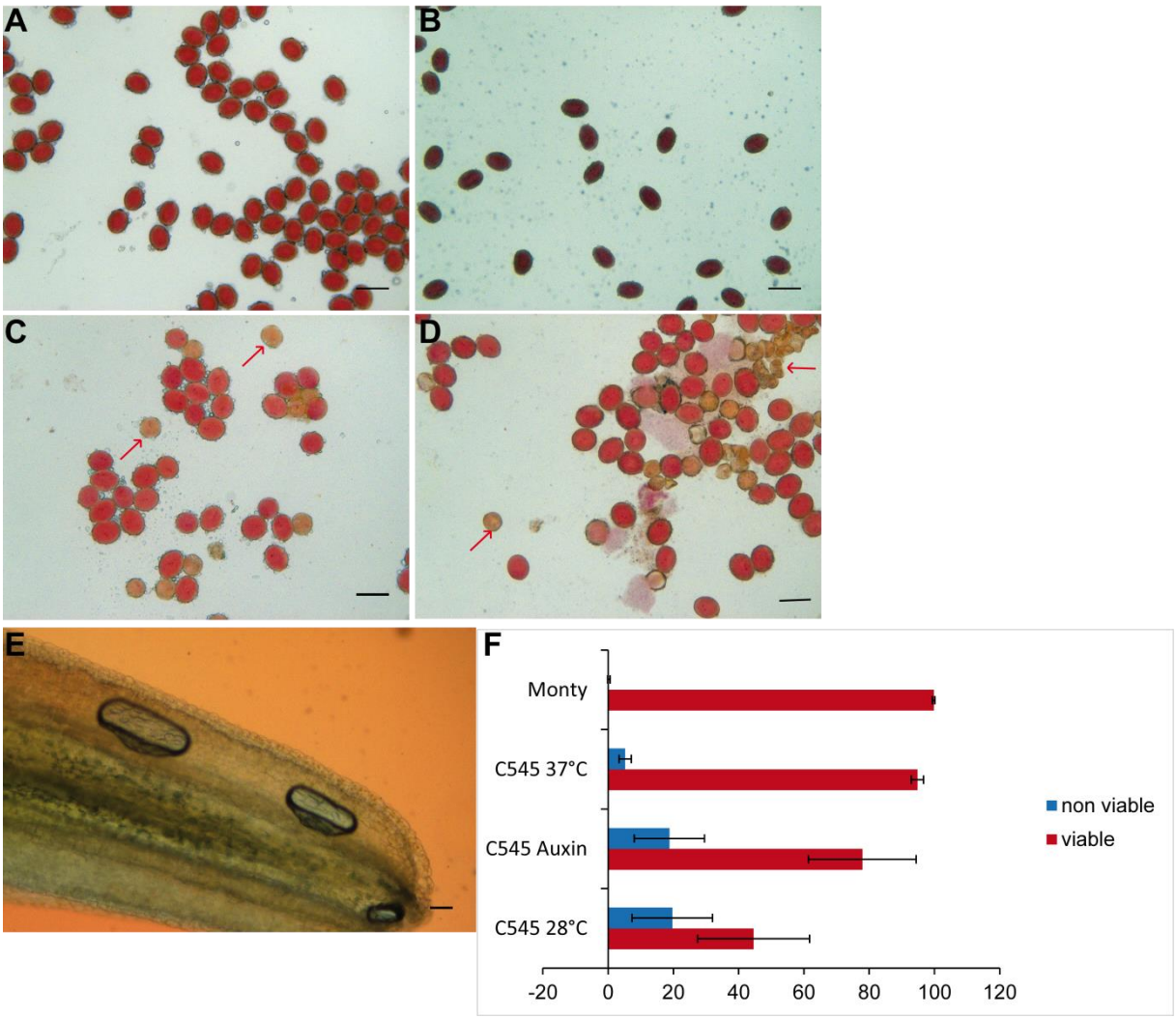

Figure 24: Acetocarmine staining of *B. napus* pollen and anther. The scale bar indicates for 50 µm. **A**: Monty untreated. **B**: C545 treated with 37 °C and 80-90% humidity for 3 days. **C**: C545 sprayed with auxin and treated with 28 °C and 70% humidity for two weeks. **D**: C545 treated with 28 °C and 70% humidity for two weeks. **E**: Anther of C545 untreated **F**: Amount of viable pollen among the different treatments. There exist no pollen for C545 untreated; the experiment was done three times with three flowers each, counting 100 pollen: n=900 for Monty, n=870 for C545 auxin, n=578 for C545 28 °C and n= 900 for C545 37 °C.

While fertile or rather viable pollen displayed an intense red colour, the inviable pollen have a beige to brown colour (compare Figure 24 A-D). For the C545 untreated line, only an anther

in the staining solution is shown (Figure 24 E) because there was obviously no pollen to extract. Interestingly the sterile C545-line after heat treatment at 37 °C exhibited similar pollen viability to Monty (Figure 24 A and B). Treatment at 28 °C with and without auxin surprisingly resulted only in some unviable pollen (Figure 24 F). This was also confirmed by counting viable and unviable pollen after staining (Figure 24 F). Apparently, the heat treatment at 37 °C seems to be the most effective option for regaining fertility in the sterile C545-line.

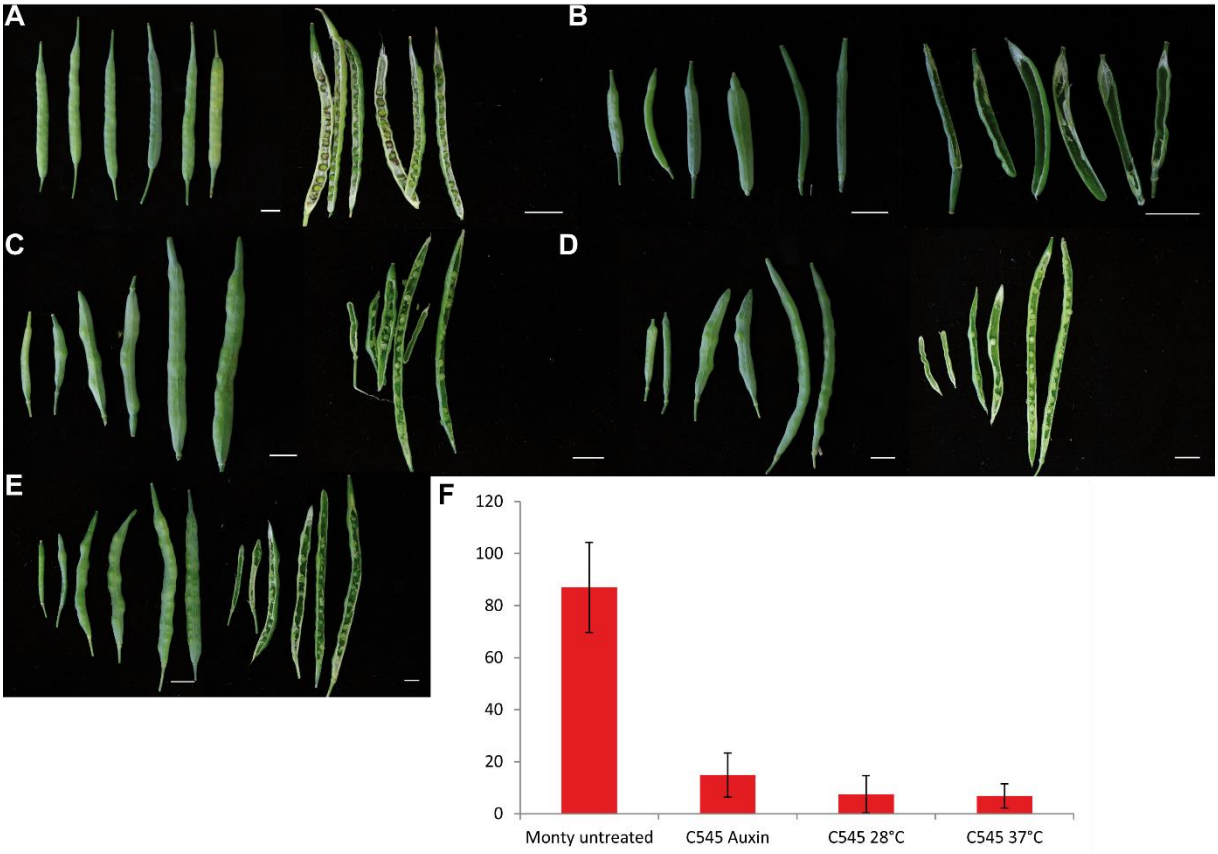

Figure 25: Representative *B. napus* siliques after different treatments in a closed and opened up arrangement. The scale bar indicates 1 cm. A: Monty untreated. **B:** C545 untreated**. C:** C545 treated with 28 °C and 70% humidity for two weeks. **D:** C545 sprayed with auxin and treated with 28 °C and 70% humidity for two weeks. **E:** C545 treated with 37 °C and 80-90% humidity for three days. **F:** Amount of siliques containing > 5 seeds, as the majority of the siliques in the fertile Monty line, on a whole plant (mean value). Monty n=7, C545 auxin n=17, C545 28 °C, n=16, C545 37 °C, n=30 (n corresponds to the number of plants).

To complete the phenotypic analysis and to investigate whether the different treatments can actually reverse the sterile line to fertile and to estimate the effectiveness, the formed siliques were investigated. After at least three different treatments the long siliques were counted, which are representative for Monty used as control. Figure 25 shows the siliques which were found on Monty (Figure 25 A), the C545-line untreated (Figure 25 B) and after the different treatments (Figure 25 C-E). It also displays an illustration of the data which were obtained

after counting the siliques (Figure 25 F). Interestingly, the result after counting the siliques proposed, that the treatment with auxin at 28 °C is more efficient to transform the sterile C545 line to fertile, while the pollen staining demonstrated a higher pollen viability after the 37 °C treatment. It is important to keep in mind, that the plants were only treated at 37 °C for three days, while the auxin treatment took place over two weeks. Hence, more flowers in different developmental stages could be reached during the longer time frame compared to three days of treatment at 37 °C.

We had already created a sterile *A. thaliana* line containing the possible ms gene. To identify the possible mechanism in the plants, which must be happening during the heat treatment, the *A. thaliana* mutant plants were treated the same way as the *B. napus*. One possible explanation for the restoration of fertility after heat treatment might be, that the ms gene breaks down due to heat or stress to the plant system. However, our *A. thaliana* plants did not survive 3 days at 37 °C, even with slow adaption to the increasing temperature. The auxin treatment and heat exposure to 28 °C with 70% humidity and 20 h of light, could be performed with the *A. thaliana* mutants containing the chimeric gene under the native promoter. Just like the *B. napus* plants, the *A. thaliana* plants were first treated with auxin when they started flowering and then moved into the 28 °C climate chamber. In a period of two weeks, the plants were treated four times in total with auxin. Moreover, mutant plants were also kept at 28 °C for two weeks without any auxin treatment. The sterile *A. thaliana* plants containing the ms gene did not develop anthers after mild heat and auxin treatment in contrast to the *B. napus* flowers (Figure 26). This might rule out a possible breakdown of the protein as a result of heat treatment. It is more likely that the restoration of the fertility after heat treatment in *B. napus* is a chain reaction of different processes, which cannot be found in *A. thaliana* despite the close genetic relationship. It cannot be excluded after all, that the Chimera protein might get damaged at higher temperatures.

Figure 26: Col-0 transformed with *Chimera:CHIMERA FL* after different treatments. The scale bar represents 1 mm. A: native Promoter: CHIMERA FL treated for 2 weeks at 28 °C. **B:** native Promoter: CHIMERA FL treated for 2 weeks at 28 °C and were sprayed four times with auxin (10 µM 1-NAA and 100 µM ethephon). The pictures were taken with the stereo microscope and show an open flower, as well as the corresponding siliques.

3.4 Transcriptome analysis via RNA sequencing

3.4.1 Transcriptome analysis of whole *B. napus* flower buds

The phenotyping results of the *B. napus* plants in section 3.3 gave the first insights about the physiological effect of different treatments to the flowers of the plant.

Moreover, one of the major questions for this project is still open. Which mechanisms occur in the plants that lead to fertility after heat treatment? To fully understand the C545 response to high temperatures a RNA sequencing analysis approach was conducted. With the phenotyping results it indicated that auxin/ethylene treatment, as well as moderate heat treatment over a longer period of time, also might partially restore fertility in the sterile C545-line. For the treatment at 37 °C for three days it was already known that it leads to seed production for the C545 line. Therefore, flower buds of five different sample types were collected, RNA isolated and sent for sequencing. The conditions are exactly the same as shown before in section 3.3. Table 6 shows an overview of the sample types, the replicates and the treatments which were used for RNA sequencing analysis. In total 15 samples were analysed.

Table 6: *B. napus* flower bud samples used for RNA sequencing analysis. The replicates as well as the different treatments are stated in the table.

Line	Bud size	Sample size/replicates	Treatment
Monty	2 mm	3 samples	Untreated
C545	2 mm	3 samples	37°C for 3 days
C545	2 mm	3 samples	28 °C for 14 days
C545	2 mm	3 samples	Auxin/ ethylene and 28°C for 14 days
C545	2 mm	3 samples	Untreated

After a preliminary quality control of the RNA, the cDNA library was constructed. Sequencing was conducted using the Illumina platform.

After trimming of the reads of the sequencing adapters and removing low quality reads, the clean reads were mapped against the *B. napus* genome. Overall between 77 and 79% of the reads were mapped to the genome (Table 7).

Henceforth differential expression (DE) analysis was conducted. The mapped reads of all the libraries were run through the online available 3D RNA sequencing program. The cut off was an adjusted p-value < 0.01 and (log_2 fold change) $log_2FC \geq 1$. For normalization of the read

counts into log_2-CPM (count per million reads), the TMM (trimmed mean of M-values) method was used. Read quality control and read count were generated by Dr. Chris Carrie.

Table 7: Number of clean and mapped reads after RNA sequencing of the flower buds data.

Sample	Clean reads	Mapped reads	Mapped reads (%)
C545 untreated 1	90018054	70426936	78.24
C545 untreated 2	68794798	53920616	78.38
C545 untreated 3	78338043	61846537	78.95
Monty untreated 1	70997821	55624679	78.35
Monty untreated 2	72416351	56866795	78.53
Monty untreated 3	80232006	62281912	77.63
C545 37 °C 1	70442030	55368524	78.60
C545 37 °C 2	77239906	60513022	78.34
C545 37 °C 3	78445149	61422527	78.30
C545 28 °C 1	84138544	66367753	78.88
C545 28 °C 2	85447631	67299805	78.76
C545 28 °C 3	85082647	67077499	78.84
C545 Auxin 1	85858779	67410288	78.51
C545 Auxin 2	77016499	60887832	79.06
C545 Auxin 3	80521487	63576795	78.96

The results of the principle component analysis (PCA) are usually presented in a two-dimensional plot. PCA recognizes not only the variation between the replicates but also of the whole data set. PCA1 pictures the most variation of the data and PC2 respectively the second most variation (Koch *et al.,* 2018). Accordingly, the PCA plot in Figure 27 A already gives the first impression that the most variation occurs between the Monty and the C545 line after treatment at 37 °C. The three different sample types C545 untreated, C545 28 °C and C545 Auxin, which cluster more closely together, indicated a low variation between the data sets for these treatments. Moreover, there is barely any variation between the single replicates. Figure 28 B shows the amount of differentially expressed genes (DEG). Up regulated genes are visualized in red and down regulated genes in blue. The most differentially expressed genes (16109 genes: 8146 upregulated and 7963 downregulated genes) were obtained by comparing the C545 37 °C heat treatment to C545 untreated. The least amount of DEGs can be found between C545 untreated and C545 28 °C (2636 genes).

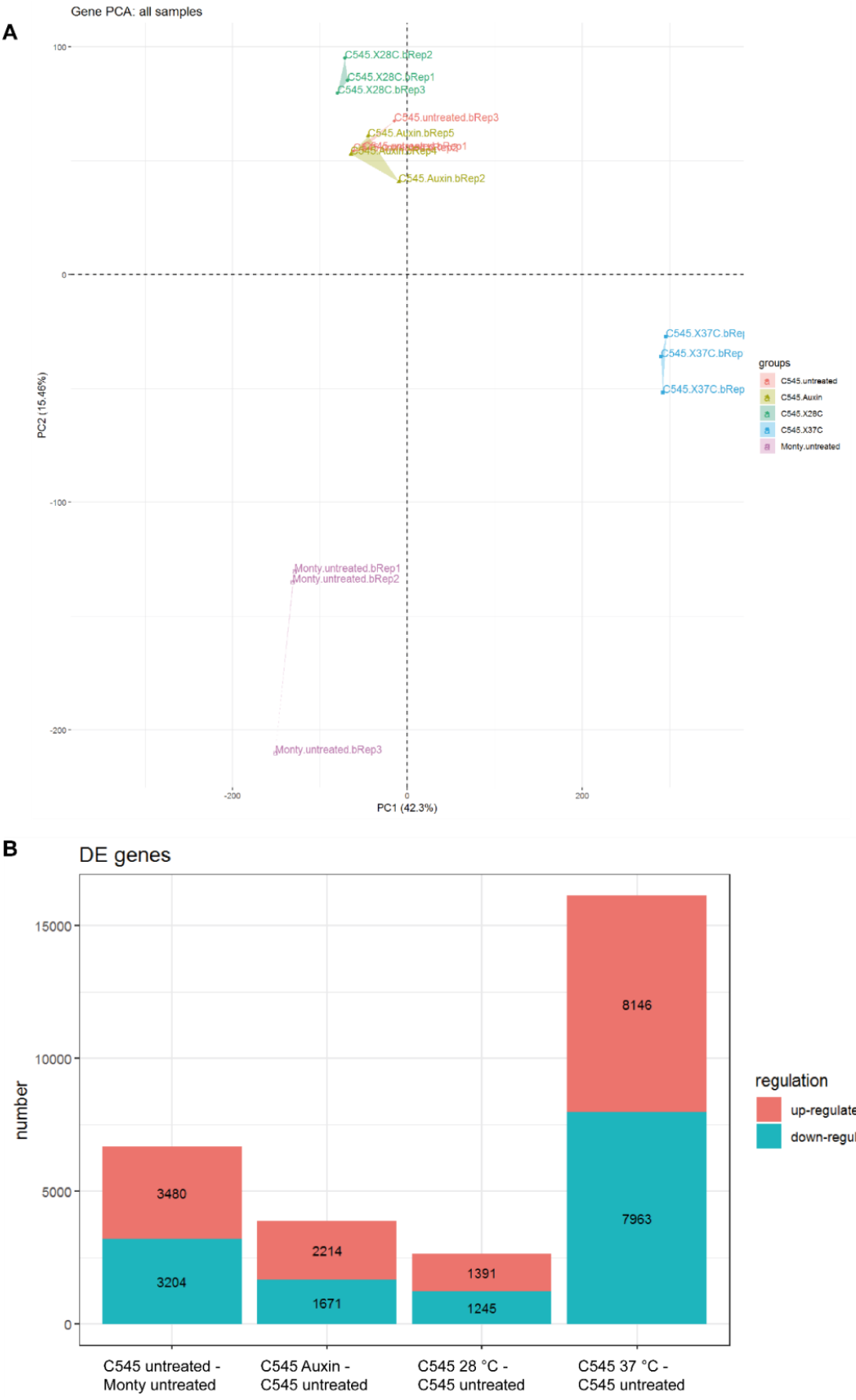

Figure 27: Differential expression analysis of whole flower buds. **A:** PCA plot of all RNA sequencing samples. **B:** Overview of the amount of differentially expressed genes.

The Venn diagrams (Figure 28) gave insights in the amount of DEGs which overlap in the different samples and treatments. For example, the same 329 up regulated and 775 down regulated DEGs could be found between the comparison of C545 untreated vs Monty and C545 37 °C vs C545 untreated. These up and down regulated genes were further investigated by using gene ontology (GO) term analysis. For conducting functional analysis with GO classification, the genes needed to be mapped to the *B. napus* accession numbers first. Then the program Blast2Go/Omnicsbox was used. The used cut off was a false discovery rate (FDR) value of 0.05 and the annotations were always "reduced to most specific", which resulted in the most specific GO terms. The GO terms were distributed by the program between biological process (BP), cellular component (CC) and molecular function (MF). From all emerging GO terms, the top 30 according to their -log10(FDR) value were selected for illustration.

The GO term analysis for these genes revealed stress related GO terms for up regulated genes (Figure 28 C) like “response to chitin”, “response to wounding”, “response to oxidative stress” or “response to antibiotic” For the 775 down regulated genes the GO terms “fatty acid biosynthetic process” and “pollen exine formation” are enriched. Interestingly, these GO terms clearly identify processes involved in pollen development, are down regulated in the C545 untreated and then become further down regulated after heat treatment. However, based on the phenotyping results, the 197 upregulated DEGs and 594 downregulated genes between C545 37 °C, C545 28 °C and C454 Auxin treatment might also be interesting for further analysis.

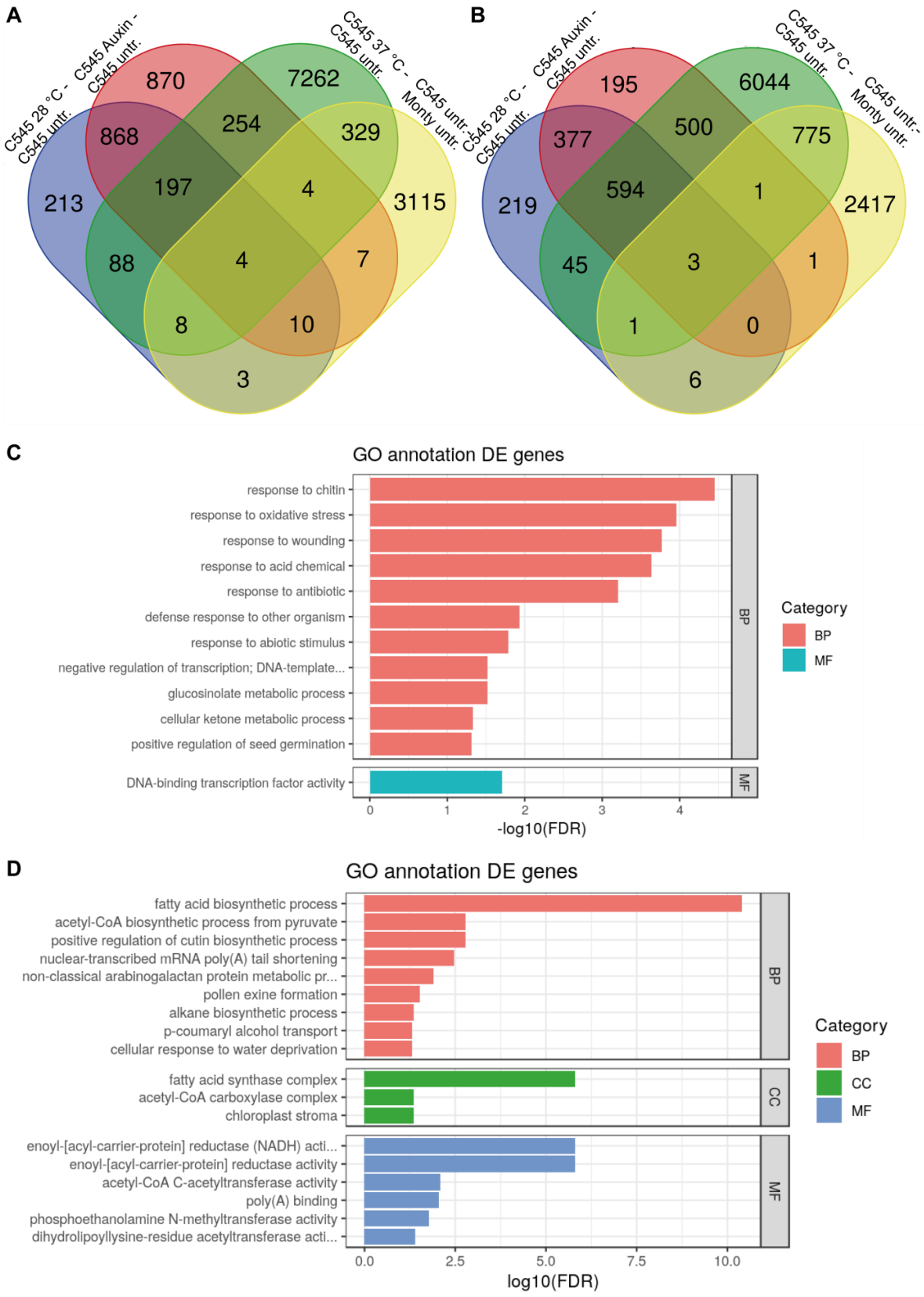

Figure 28: Venn diagram of all DEGs in flower buds. **A:** upregulated genes. **B:** downregulated genes. **C:** Overlapping 329 up regulated genes between C545 37 °C in contrast to C545 untreated and C545 compared to Monty with the according GO term annotations. **D:** Overlapping 775 down regulated genes C545 37 °C in contrast to C545 untreated and C545 compared to Monty with the according GO term annotations. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function

Another approach was clustering the DEGs of all different sample types into to several groups by generating a heatmap (Figure 29 A). Therefore the DEGs were clustered into 10 groups according to their expression patterns. In cluster 1 the majority of genes display a higher expression in the untreated C545 buds but are expressed at a lower level in Monty. Interestingly in cluster 6, the genes after the C545 37 °C heat treatment show a higher expression level, while for the C545 untreated, the expression level for the genes is lower. For further analysis, Clusters 1, 4, 6, and 7 were investigated by applying GO term classifications. The genes in cluster 1 show lower expression level after heat treatment (37 °C) compared to the untreated C545 line. Annotation to GO terms revealed (Figure 29 B) the involvement of “peptidyl-cysteine oxidation”, “polyketide and phenylpropanoid synthesis” (BP). But also the genes annotated to the GO terms “cell wall” (CC) and “AT DNA binding” as well as “fatty acid binding” (MF) were down regulated after heat treatment. In Cluster 4, the gene expression level in C545 untreated and C545 28 °C is higher compared to C545 37 °C. The accompanying GO terms (Figure 29 C) are DNA related (BP), involve microtubule (CC and MF) and also ubiquitin ligases (MF).

Cluster 6 shows a higher gene expression level in C545 37 °C. The GO term analysis revealed “Response to chitin, water deprivation and wounding” (BP), which are probably all responses to the heat stress. Cluster 7 displays a similar pattern to cluster 6 and in the GO term analysis there were also indications for stress found (“response to fungus, karikin and drug”) as well as hormonal influences ("response to abscisic acid", "regulation of jasmonic acid mediated signalling pathway").

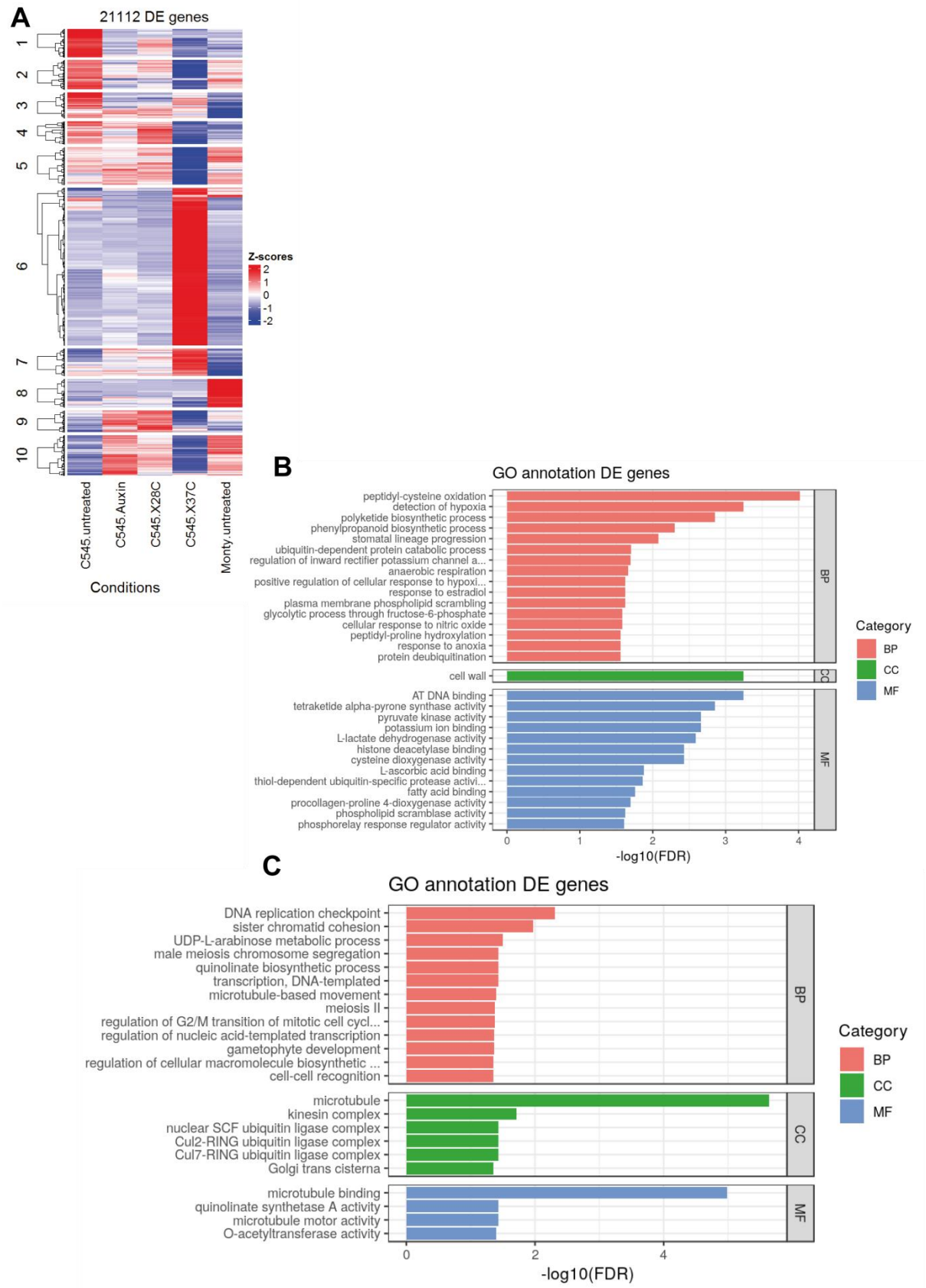

Figure 29: Clustering of all DEGS from all conditions into 10 groups. The colour scale displays higher expression of genes in red and low expressed genes in blue. **A:** Heatmap of DEGs between the sample types. **B:** GO term analysis of Cluster 1. **C:** GO term analysis of Cluster 4. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function.

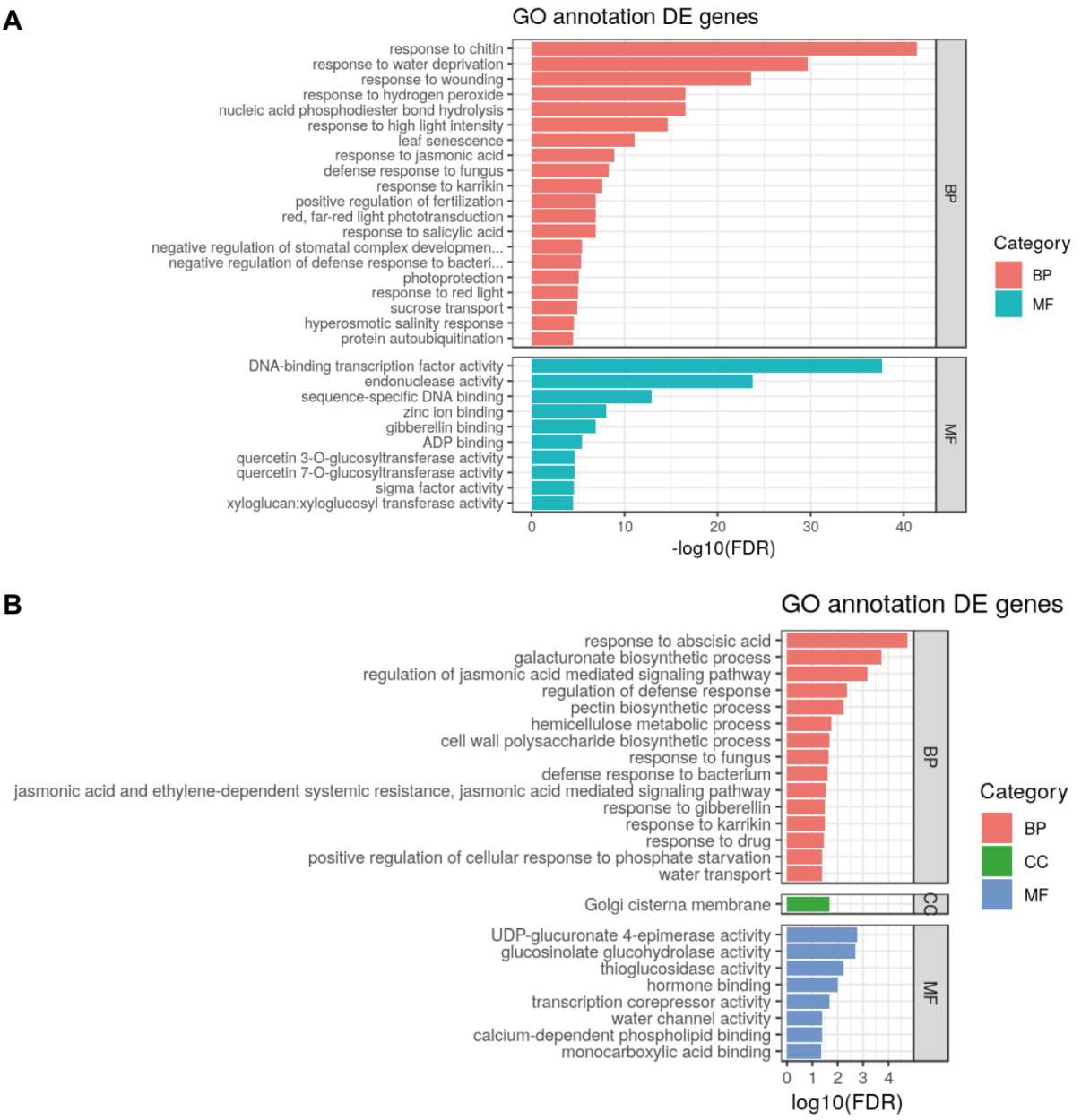

Figure 30: GO term analysis of selected heatmap clusters. A: GO term analysis of Cluster 6. **B:** GO term analysis of Cluster 7. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function

In the next step, all of the DEGs between C545 37°C vs C545 untreated and C545 untreated vs Monty were analysed using GO term annotations. These comparisons resulted in the most amount of DEGs (see Figure 27 B). Interestingly, the upregulated genes (Figure 31 A) mainly seemed to be responses to stress. The down regulated (Figure 31 B) genes were related to “spermidine/ fatty acid/ amide biosynthetic process” (BP) but also “fatty acid synthase” (CC) and “arginine decarboxylase” (MF). It appeared, that the untreated C545 line itself indicates a higher stress level compared to the Monty line. The results of the GO term analysis for C545 37 °C vs C545 untreated showed a similar pattern (Figure 32). For the up

regulated genes (Figure 32 A) in the BP category most GO terms seem to be related to stress responses (“response to wounding”, “Response to water deprivation”, “response to fungus”). For the down regulated GO terms (Figure 32 B), “sporopollenin biosynthetic process” (BP), but also fatty acid binding” were enriched, which are indications for down regulation of genes involved in pollen development.

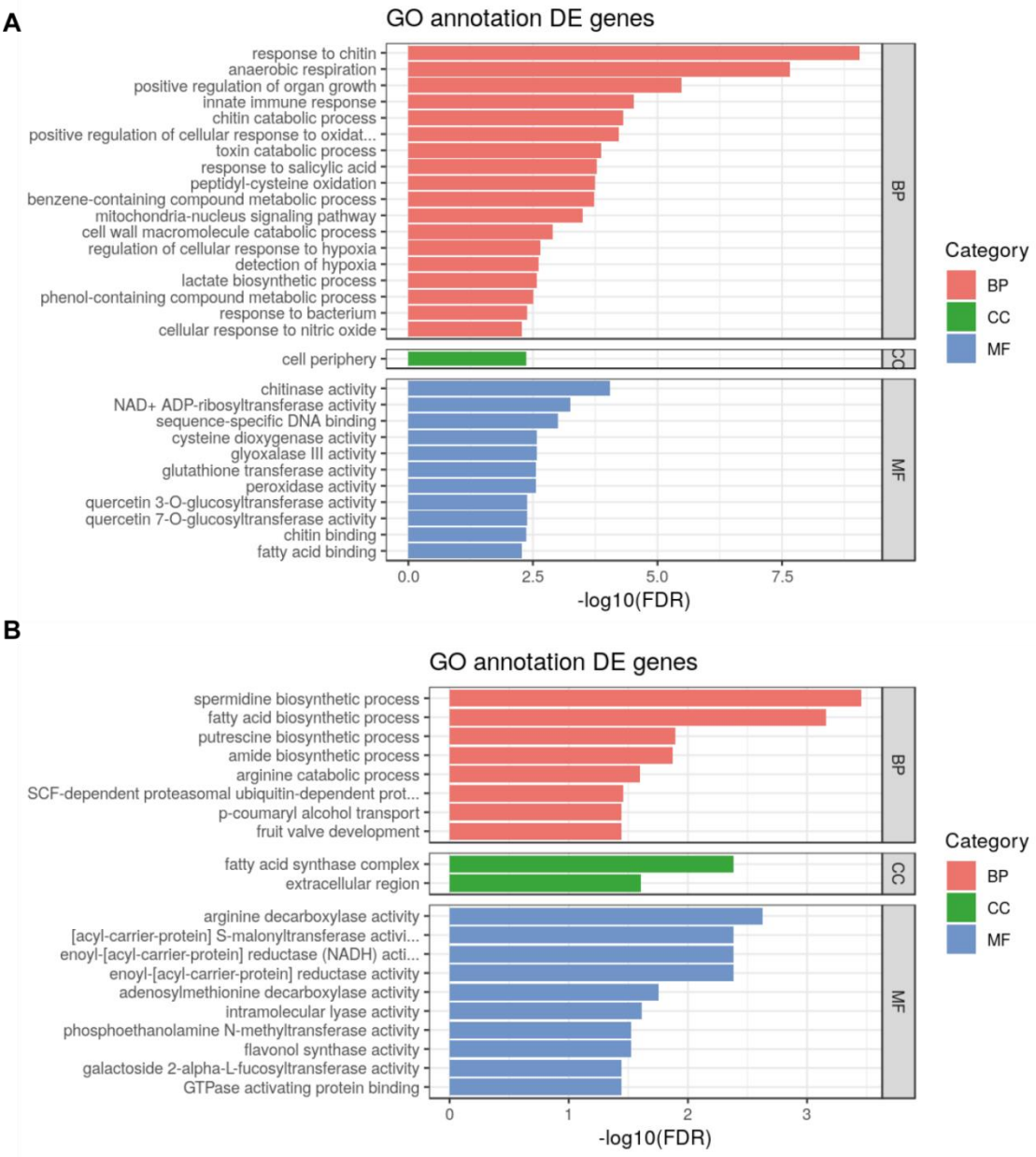

Figure 31: GO term analysis between C545 untreated and Monty untreated. A: GO term analysis of up regulated DEGs **B:** GO term analysis of down regulated DEGs. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function.

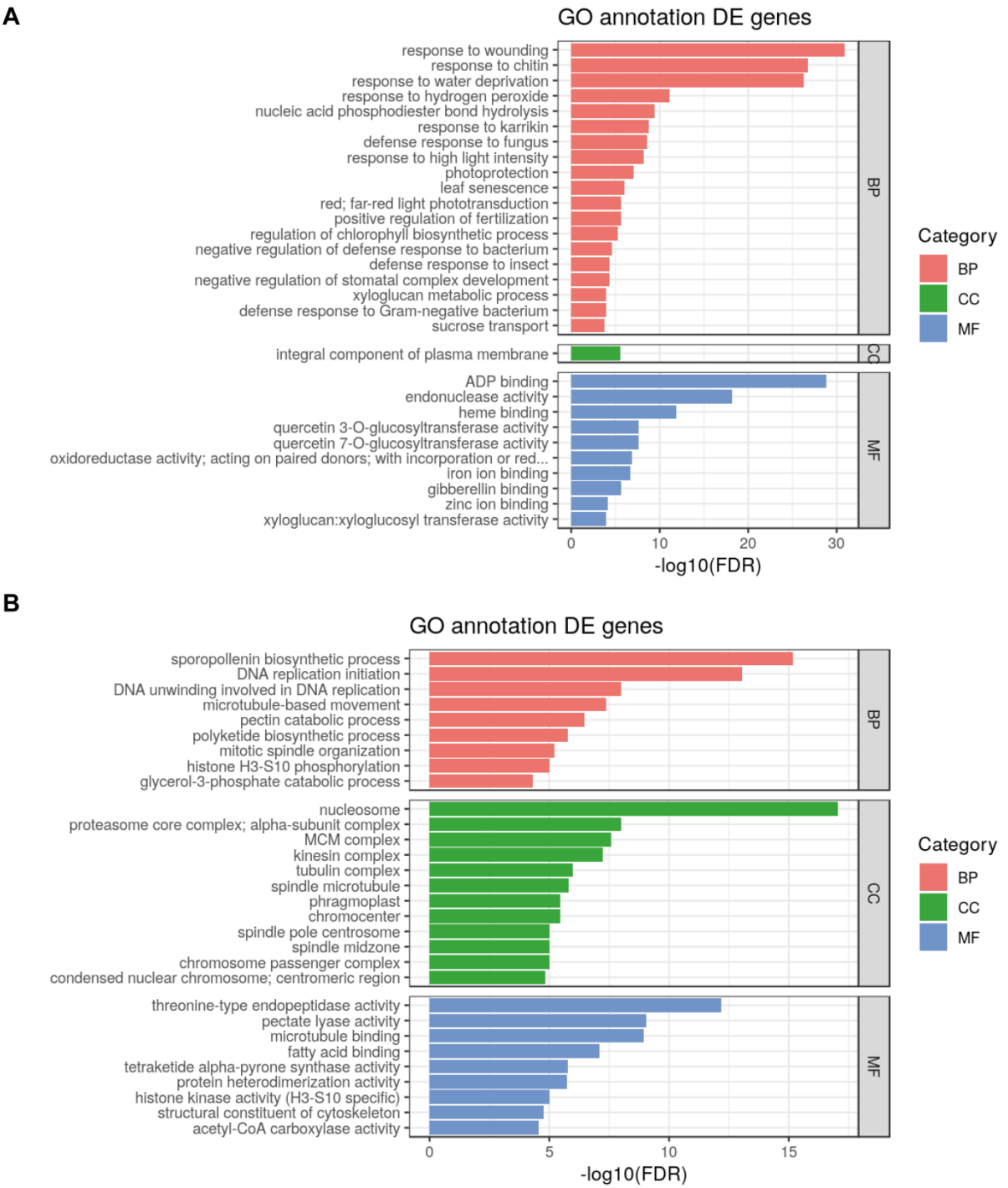

Figure 32: GO term analysis between C545 untreated and C545 37°C. A: GO term analysis of up regulated DEGs **B:** GO term analysis of down regulated DEGs. The top 30 GO terms according to their -log10(FDR) value are plotted BP: biological process; CC: cellular component; MF: molecular function.

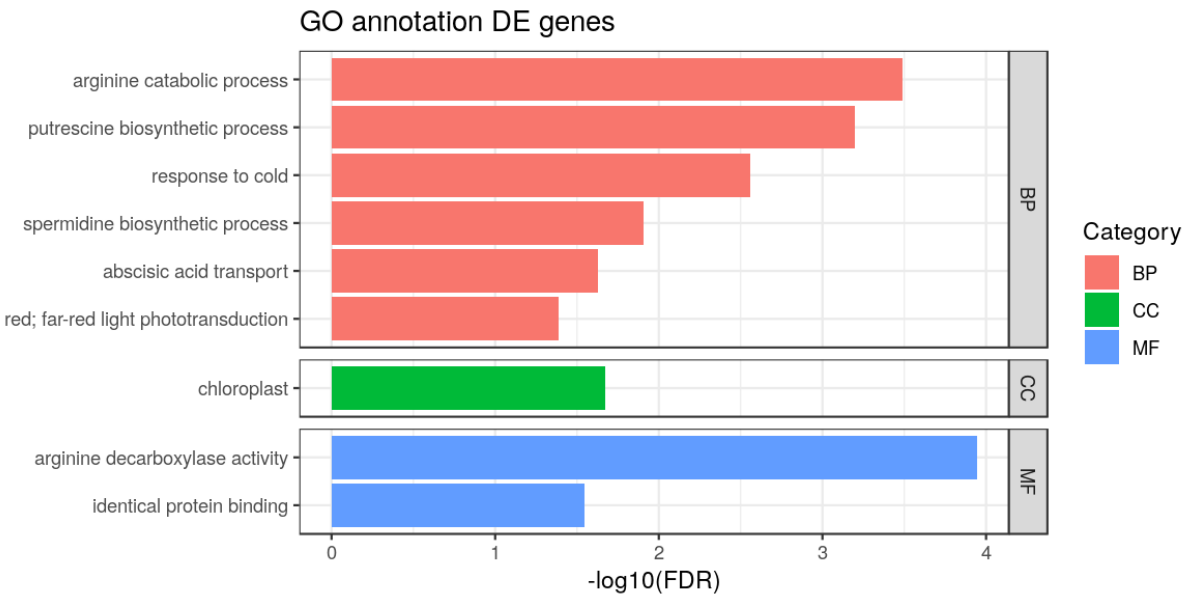

Figure 33: GO term analysis of DEGs only existing in C545 vs Monty down and C545 37°C vs C545 untreated up. BP: biological process; CC: cellular component; MF: molecular function.

Additionally, all DEGs which are down regulated in the comparison of C545 untreated to Monty but also exist in the list for up regulated genes in C545 37 °C vs C545 untreated were selected and annotated to the *B. napus* GO terms (Figure 33). The aim was to find the relevant genes, which are down regulated in the C545 line when compared to the fertile Monty line, but then become up regulated after intense heat treatment (37 °C). In this process, the GO terms "arginine catabolic process", "Spermidine biosynthetic process" and "Arginine decarboxylase activity" were accumulated. These annotations play a role in the synthesis of polyamines. Polyamines play different roles in the plant metabolism. It is known, that they are important for tress tolerance. However, they might also be involved in pollen development (Aloisi *et al.,* 2016). The GO term analysis also revealed, that chloroplast genes seem to be enriched (CC). The ms gene is localised in the chloroplast and can only be detected in the sterile lines. Therefore it can be assumed, that chloroplastic pathways in the sterile line are affected.

The DEGs between the different sample types were also run through the Mapman program. The according figures (Figure 34 and Figure 35) show an overview of up (blue boxes) and down regulated (red boxes) genes for metabolism. The most changes were seen when comparing the C545 treated at 37 °C to the C545 line untreated (Figure 34). Especially the mechanisms concerning the cell wall, lipids and the secondary metabolism seem to be affected. In contrast, the comparison of the C545 line 28 °C (Figure 35 B) treated vs untreated and C545 Auxin treated vs untreated (Figure 35 A) showed only a few changes in the metabolism overview as expected for the lower amount of DEGs. The most DEGs play a

role for the secondary metabolism and cell walls. These results somehow resemble the phenotyping results for the mentioned treatments. With heat treatment at 28 °C, the flowers made normal sized anthers, but compared to the fertile restorer line, regained the least amount of viable pollen. Furthermore the comparison of C545 28 °C to C545 untreated resulted in the least amount of DEGs. Heat treatment at 37 °C however, resulted in the development of viable pollen in the C545 line that was comparable to the Monty line. For the RNA sequencing results, the most DEGS were found when comparing the C545 37 °C to the C545 untreated.

Because of the reappearing Ubiquitin related GO terms during analysis of this data (Figure 31 B/ 32 B), the DEG data was run through the Mapman pathway for proteasome and ubiquitin degradation related genes (Figure 36). Most DEGs appeared to be concerning the proteasome, the F-Box and RING finger domain. Interestingly, it appears that the DEGs for the F-Box in C545 37 °C vs C545 untreated and C545 untreated vs Monty are mostly down regulated (Figure 36 A and B). For C545 Auxin vs C545 untreated (Figure 36 C) and C545 28 °C vs C545 untreated on the other hand (Figure 36 D), more genes seemed to be up regulated, which is indicated by the higher amount of blue coloured boxes for F-Box related genes. Ubiquination describes the transfer of one or more ubiquitin proteins to the target proteins. With this post-translational modification, the ubiquinated proteins are recognized by the proteasome and get degraded. The ubiquitin-dependant regulation during protein import into chloroplasts might help with the plants stress tolerance.

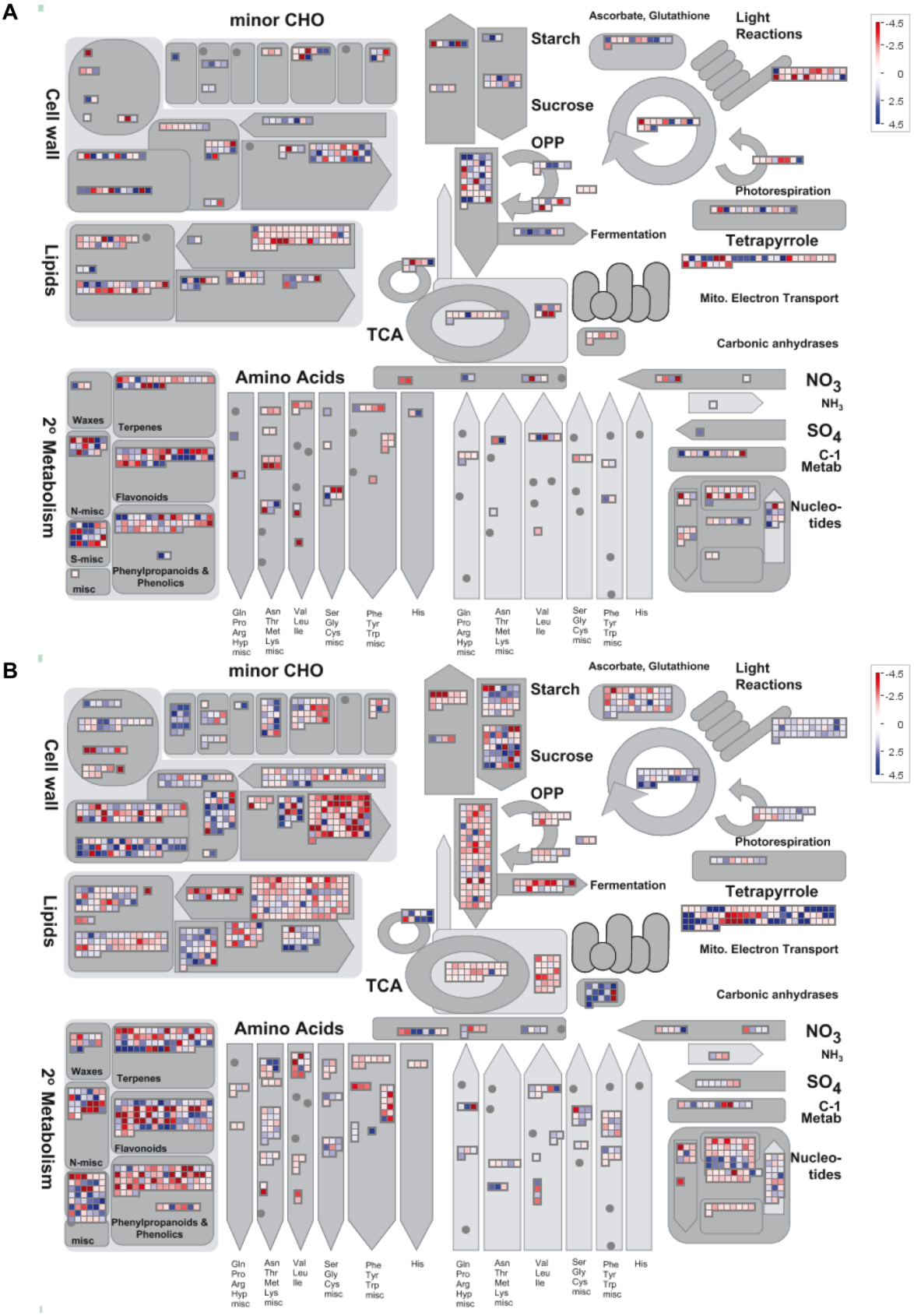

Figure 34: Mapman illustration of the up- and down regulated DEGs. **A:** C545 untreated compared to Monty. **B:** C545 treated at 37° C compared to C545 untreated. Red boxes indicate down regulated genes, while blue boxes represent up regulated genes.

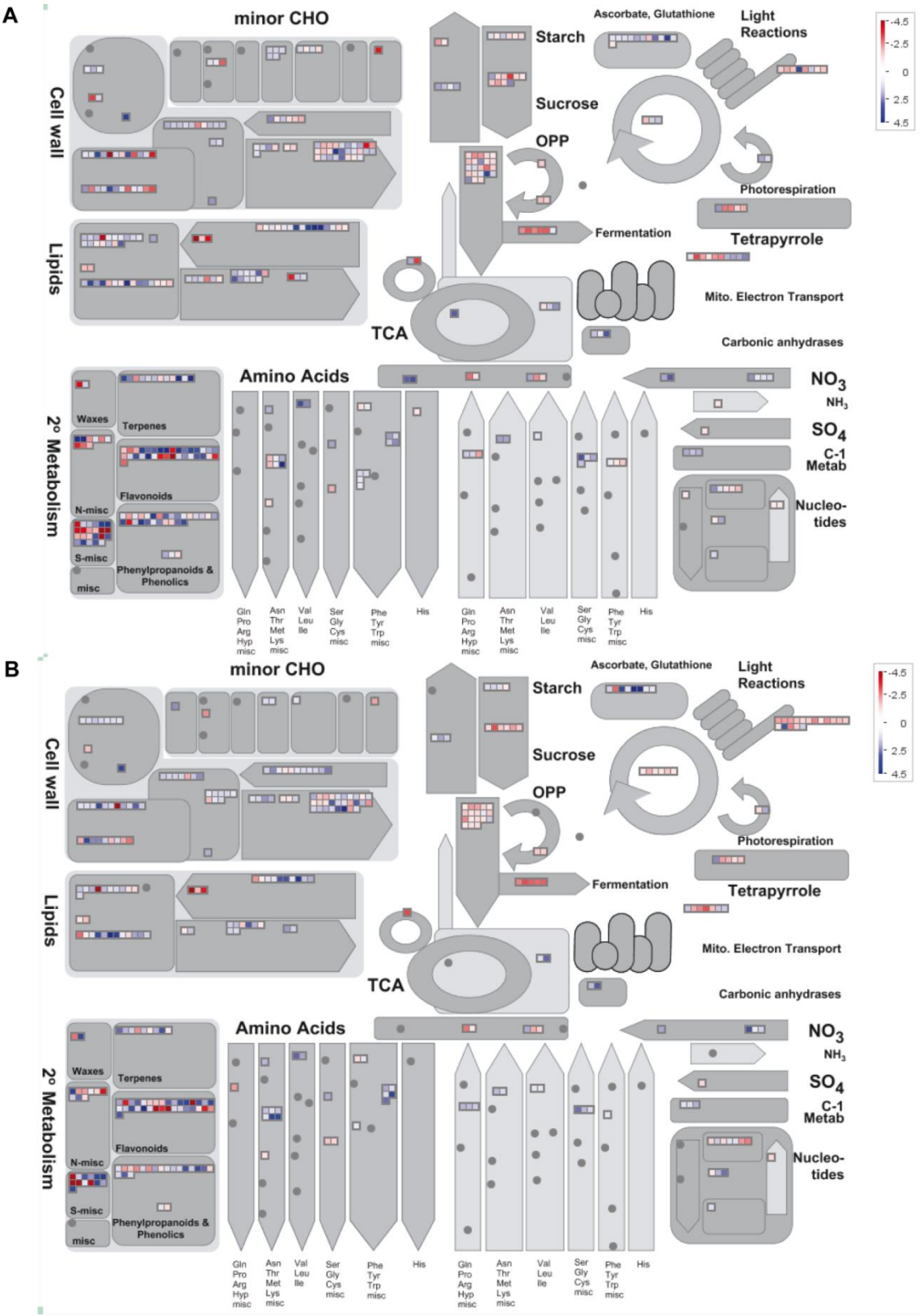

Figure 35: Mapman illustration of the up- and down regulated DEGs. **A:** C545 Auxin treated compared to C545 untreated. **B:** C545 treated at 28° C compared to C545 untreated. Red boxes indicate down regulated genes, while blue boxes represent up regulated genes.

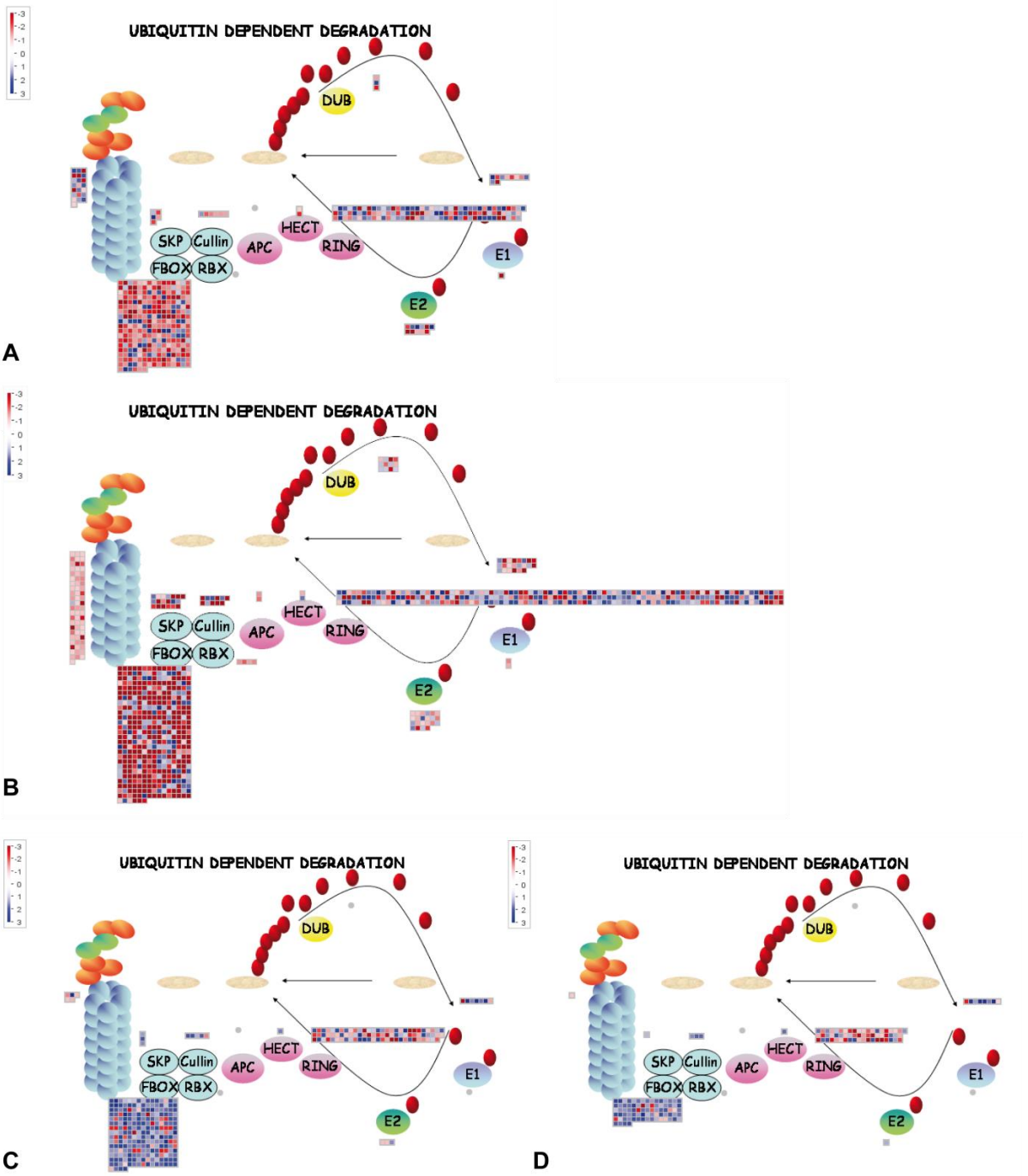

Figure 36: Mapman BINs of Ubiquitin dependent degradation. A: Monty vs C545 untreated. **B:** C545 37 °C vs C545 untreated. **C:** C545 Auxin vs C545 untreated. **D:** C545 28 °C vs C545 untreated. Red coloured boxes indicate for down regulated genes and blue boxes for up regulated genes.

All things considered, for the RNA sequencing data of the *B. napus* flower buds, for the C545 line compared to Monty and after heat treatment with 37 °C the up regulated DEGs seemed to be mostly related to stress responses. The down regulated DEGs annotated to polyamines, fatty acid bisynthesis (C545 vs Monty) and sporopollenin biosynthesis (C545

untreated vs 37 °C). For both comparisons, also ubiquitin-proteasome related DEGs were donw regulated, but interestingly in the majority up regulated (F-Box related) for C545 Auxin treated and C545 28 °C compared to C545 untreated.

3.4.2 Transcriptome analysis of *B. napus* microspores

Studying gene expression, the analysis of single cell types proves to be difficult. However, using laser microdissection (LMD) in combination with RNA sequencing analysis is a useful method to isolate and analyse specific tissues or cell types. LMD possesses different advantages for tissue analysis in combination with RNA sequencing, especially for transcriptome analysis of a specific cell types. By using LMD, the cells of interest can be separated from the surrounding tissue, without the necessity of cell type specific markers (Schmid *et al*., 2012). To investigate which specific genes for pollen development play a role, microspores were analysed. Anthers, constituting one section of the male reproductive part in plants, consist of different cell layers and are the location where pollen development takes place. Microspores are released after the tetrad stage during pollen development. The workflow of obtaining microspore RNA before the actual sequencing process is visualized in Figure 37.

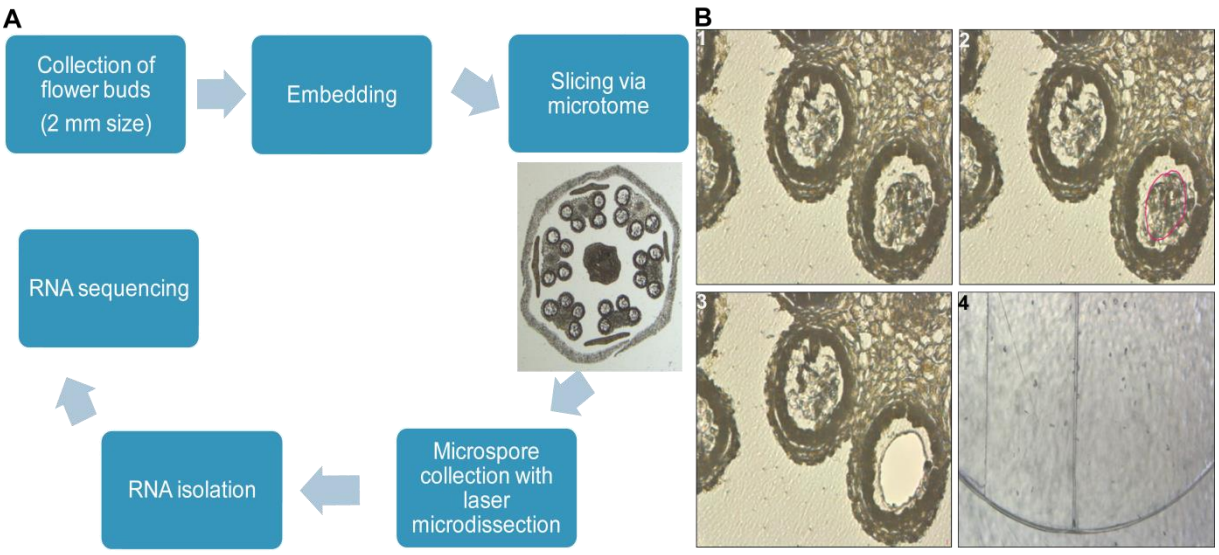

Figure 37: Workflow for the collection of microspores. A: *B. napus* flowers were all harvested at the same developmental stage, embedded and then sectioned via a microtome. The embedding media was removed before the next step. The microspores were collected by using laser microdissection from all the sections from each individual flower. After RNA isolation, the samples were sent for sequencing. **B:** Laser microdissection of microspores**.** 1: Flower bud section with focus on the anther. 2: Selection of the region of interest via the computer program. 3: Anther after dissection with the laser. 4: Image of the lid of the 500 µl tube with the already dissected samples.

The flower buds were collected at an equal developmental stage, characterized by their size. After embedding the flowers for the sectioning process, the embedding media had to be removed again for LMD. During the LMD process, the region of interest was removed with a laser and collected in a small sample tube (Figure 37 B). The RNA was isolated from the microspores and then sent for sequencing. In this sequencing analysis, only four sample types were used. The sterile line C545 untreated and treated with 37 °C for three days, as well as Monty untreated and heat treated (37 °C for three days).

The RNA sequencing analysis was performed as described in section 3.4.1. An alternative library preparation was used in comparison to the whole buds, because of the low amounts of RNA which was obtained after LMD. Therefore the RNA was only sequenced as 50 bp single end while a pair end approach was conducted for the whole flower buds. In Table 8, the number of mapped reads compared to the number of clean reads are listed. Compared to the data of the whole flower buds, the mapping rate for the clean reads fluctuates between 44 and 83%, depending on the individual sample. This is most likely due to the lower quality of the RNA obtained after the longer handling of the samples.

Table 8: Number of clean and mapped reads after RNA sequencing of microspore data.

Sample	Clean reads	Mapped reads	Mapped reads (%)
C545 heat 1	92703839	41417343	44.68
C545 heat 2	86285310	55856028	64.73
C545 heat 3	84985972	38764202	45.61
C545 heat 4	83675805	50381957	60.21
C545 untreated 1	86259286	56608119	65.63
C545 untreated 2	82966048	44036217	53.08
C545 untreated 3	89747062	71886319	80.10
C545 untreated 4	86790287	54065698	62.29
Monty heat 1	101761645	72349300	71.10
Monty heat 2	101356673	68572476	67.65
Monty heat 3	90244030	64285893	71.24
Monty heat 4	95146991	71595551	75.25
Monty untreated 1	99031988	76849911	77.60
Monty untreated 2	92426240	72634128	78.59
Monty untreated 3	91269908	74751039	81.90
Monty untreated 4	96332209	79220863	82.24

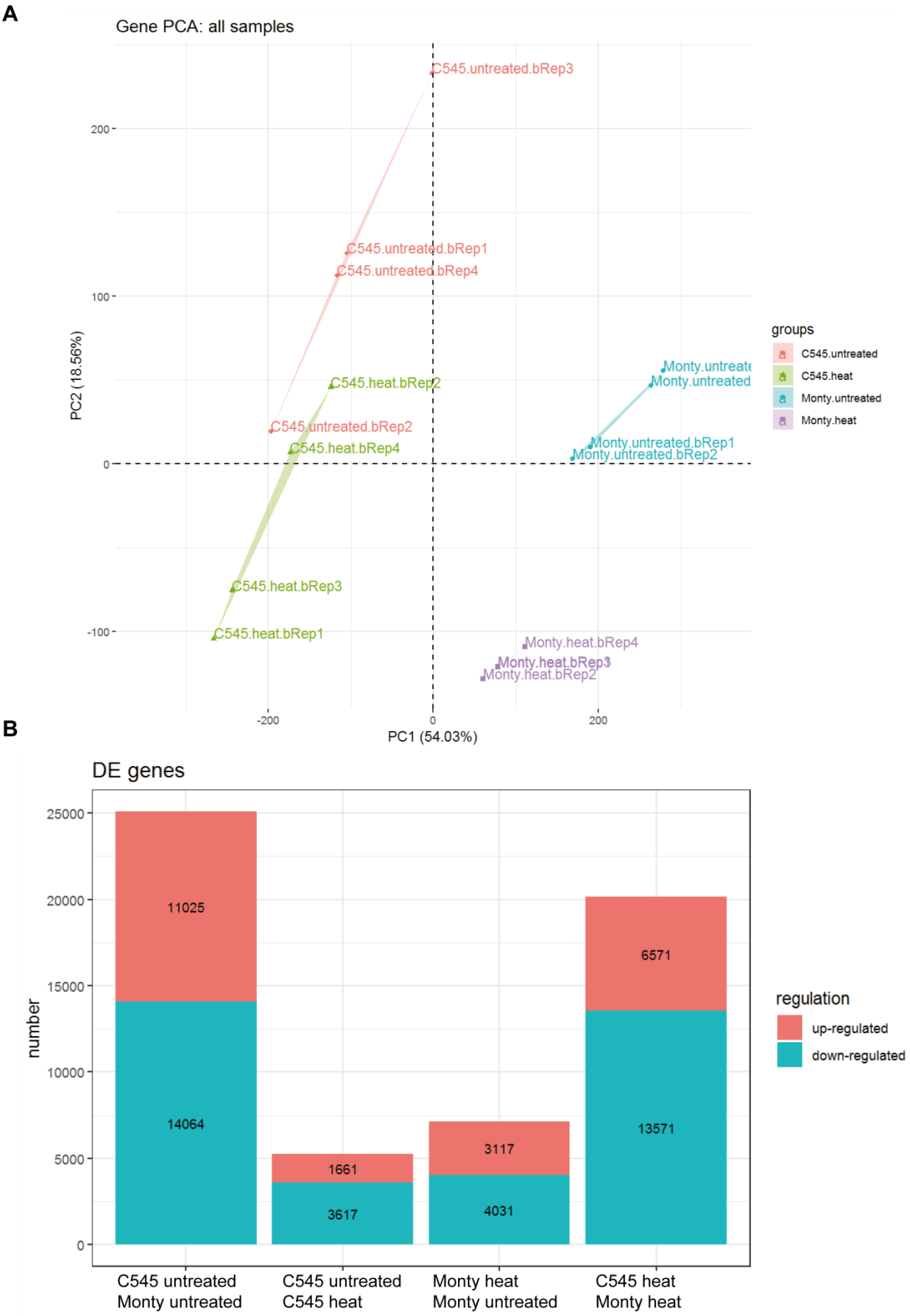

Figure 38: Differentially expression analysis of microspores. **A:** PCA plot of all RNA sequencing samples. **B:** Overview of the amount of differentially expressed genes.

The comparison of the RNA sequencing results from section 3.4.1 with the clustering in the PCA blots from the microspores data (Figure 38 A) shows a higher variance between each replicate. This can probably be explained by the different handling of the set ups. For the data for the whole flower buds, the RNA was directly isolated after the harvest. For the data of section 3.4.2 on the other hand, the flower buds had to be sectioned with the microtome first, then the microspores were collected via LMD before the RNA was isolated. Figure 38 A also pictures, that the most variation occurred between Monty and the C545 line (with or without heat treatment). After DEG analysis (Figure 38 B) of all clean reads, the comparison of C545 untreated to Monty untreated resulted in 11025 up- regulated genes and 14064 down regulated genes. The least amount of DEGs was observed when comparing C545 untreated to heat treated (1661 up regulated genes and 3617 down regulated genes). The amount of DEGs in the comparisons between the lines and the treatments is completely different to the DEGs analysis of the flower buds (Figure 27). For the flower buds the comparison of the C545 line untreated to the C545-line heat treated (37 °C) resulted in the most amount of DEGs (16109), while in the microspore data the least amount of DEGs was observed. Furthermore only 6684 genes were differentially expressed between the C545-line and Monty in whole flower buds, while in microspores the comparison resulted 25089 DEGs.

The DEGs were also clustered via a heatmap (Figure 39 A) into 10 groups. Cluster 1 showed, that the DEGs are less up regulated after heat treatment in the C545-line compared to the untreated sterile line. For Monty, the genes already had a mostly lower expression value when untreated while for the C545 line the expression level of the genes reduces after heat treatment. GO term analysis of Cluster 1 (Figure 39 B) though only reveals general processes from the plant cell, like “Golgi membrane” (CC), “microtubule based process/ binding” (MF; BP). Cluster 5, which shows a low expression of the genes after heat treatment in the C545 line indicates amongst others, for ubiquitin and proteasome dependent processes (CC) (Figure 439 C).

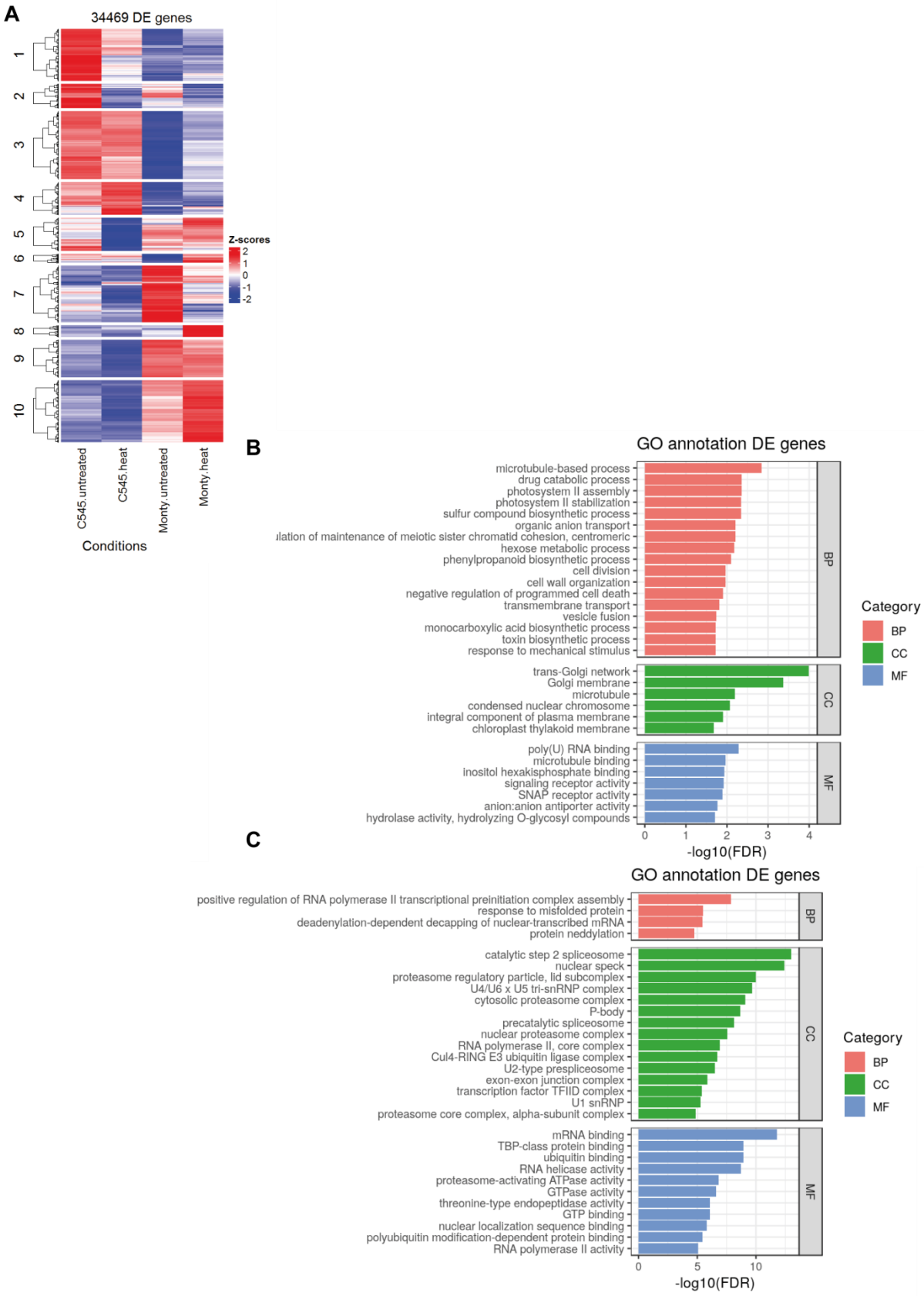

Figure: 39: Cluster analysis of DEGs. **A:** Heatmap of DEGs between the sample types. **B:** GO term analysis of Cluster 1. **C:** GO term analysis of Cluster 5. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function.

The comparison between the sample types was also investigated by annotating the data to GO terms. For the microspore data, only the C545 untreated to Monty untreated and the C545 heat treated compared to C545 untreated were investigated.

The GO term analysis of C545 untreated versus Monty untreated (Figure 40) revealed that up regulation for genes were involved in “protein phosphorylation” (BP), plasma membrane related (CC) or “heme and iron ion binding” (MF) (Figure 40 A). The down regulated genes annotated to GO terms related to protein translation processes (Figure 40 B).

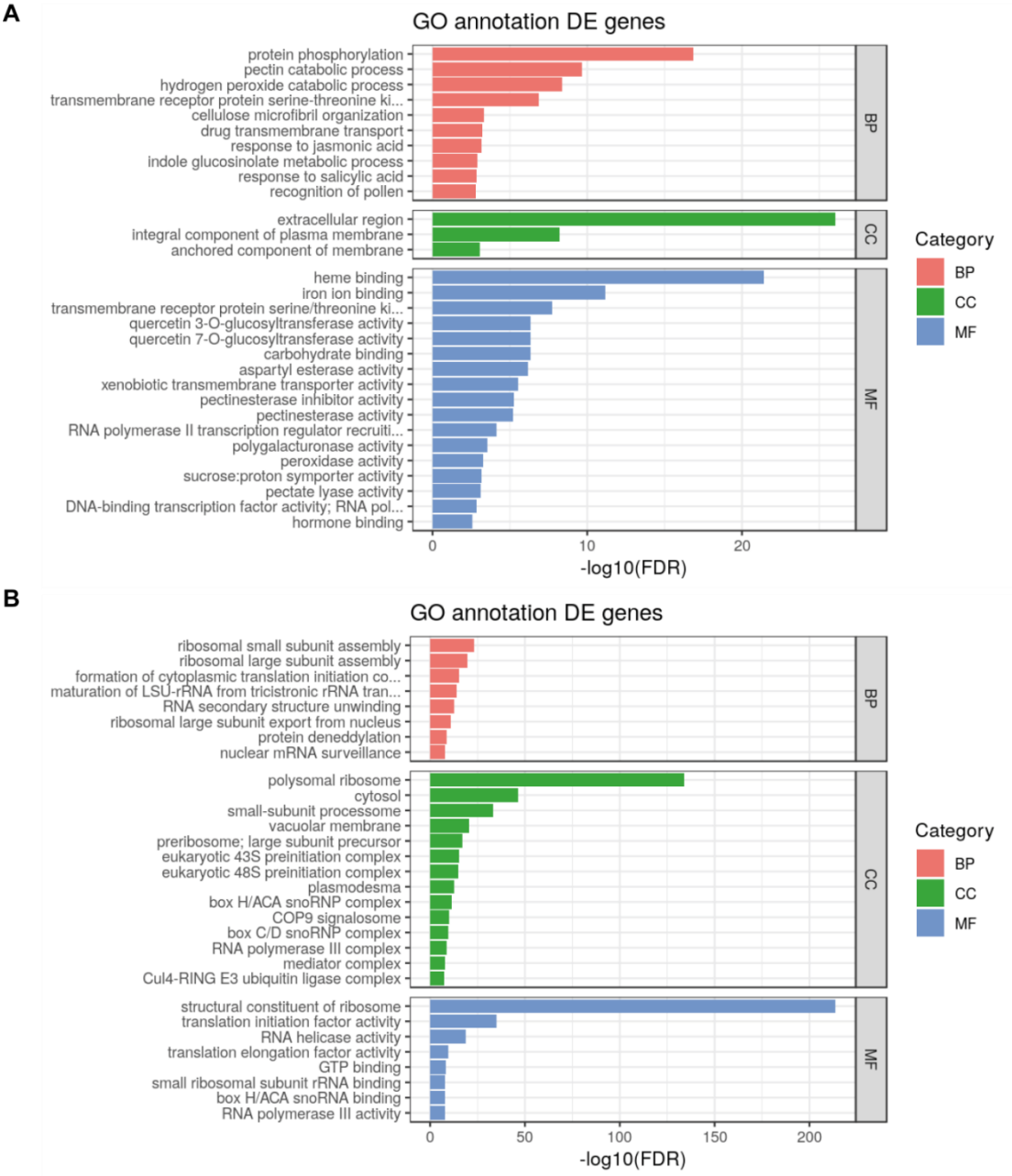

Figure 40: GO term analysis of C545 untreated in contrast to Monty untreated. A: Clustering of upregulated DEGs. **B:** Clustering of downregulated DEGs. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function.

The comparison of the C545-line heat treated to the C545-line untreated resulted in only transcription related GO terms for up regulated genes (Figure 41 A). The down regulated genes (Figure 41 B) were involved in “sporopollenin/polyketide biosynthetic process” (BP) and ribosome related genes (CC/MF) amongst others. Sporopollenin is an important component of the outer pollen layer (exine) (Piffanelli *et al.*, 1998; Wilson and Zhang, 2009). Interestingly, the GO term "Sporopollenin biosynthetic process" was also down regulated in the comparison between C545 37 °C vs C545 untreated for the whole flower buds. In male sterile plants, pollen development is obviously affected. It can be speculated, that heat treatment might influence sporopollenin biosynthesis, which is important for exine formation of the pollen wall.

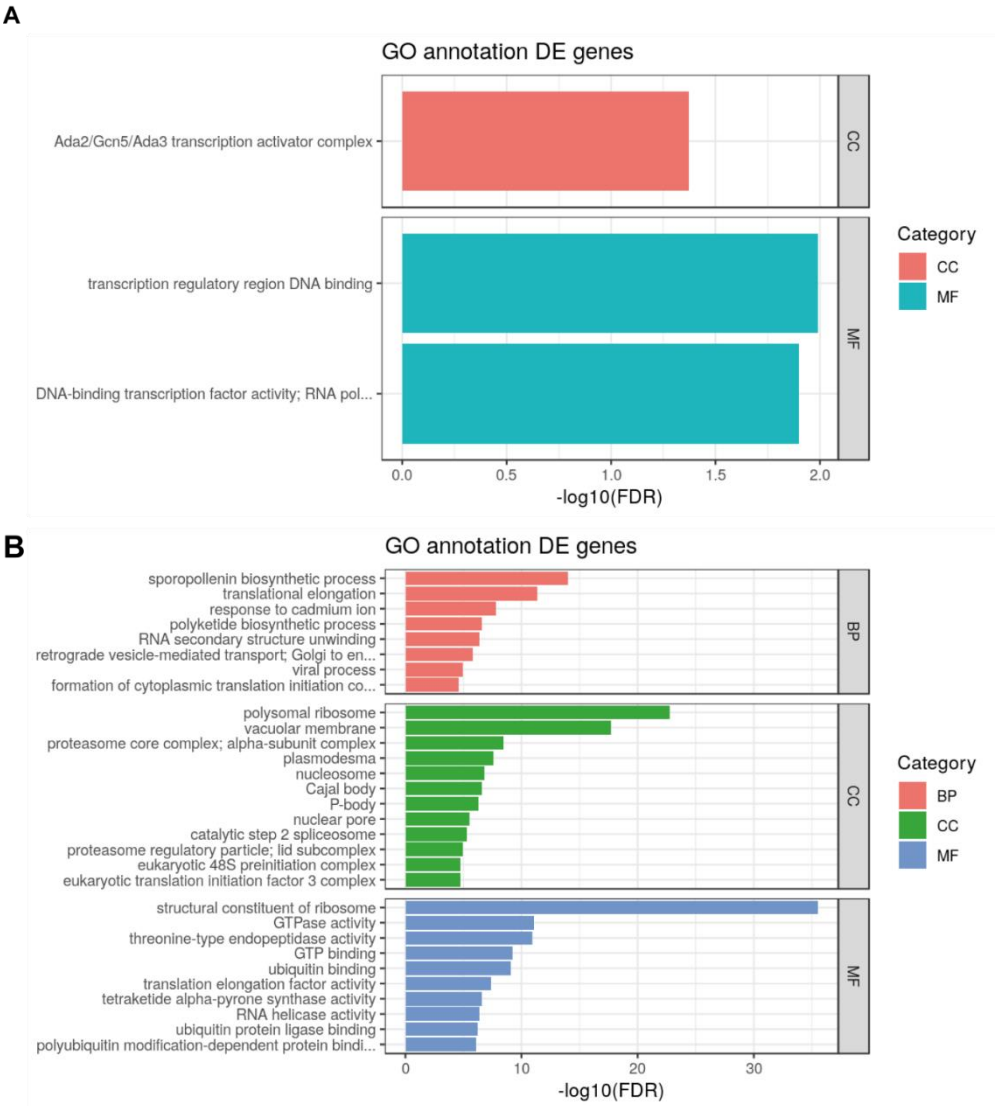

Figure 41: GO term analysis of C545 heat treated in contrast to C545 untreated. A: Clustering of up regulated DEGs. **B:** Clustering of down regulated DEGs. The top 30 GO terms according to their -log10(FDR) value are plotted. BP: biological process; CC: cellular component; MF: molecular function.

With the shown RNA sequencing results, it is important to keep in mind that these datasets derive from two different approaches. For section 3.4.1, RNA was isolated from whole flower buds, while for section 3.4.2, the analysed RNA was tissue specific and only collected from microspores. Accordingly, a comparison between the two generated RNA sequencing results is difficult to achieve.

With all the generated RNA sequencing data, there is still scope for more detailed analysis in order for the understanding of the mechanism behind male sterility and its reversion after different treatments. So far, the data only provides an overview and therefore only a general idea about what might be happening. To this point of the analysis, many plant responses after heat treatment are related to stress, especially in up regulated DEGs. Sporopollenin biosynthesis might be affected in the untreated sterile line (C545), which would explain the sterile phenotype.

4 Discussion

With the growing world population and limited food resources, more research for improving crop production is essential. The proposed increase in temperature will have an impact on the crop production. Therefore it is important to understand the defense strategies and processes which lead to heat tolerance in the plant when exposed to higher temperature, especially with the purpose of improving yield as predictions hypothesize a negative impact with temperature increase for the yield of wheat, rice maize and soybean (Zhao *et al.*, 2017). This is particularly interesting for the MSL system, where the sterile C545 line produces fertile flowers after heat treatment at relatively high temperatures (37 °C). Different ms systems in a variety of crops have already been analysed and a number of ms and restorer genes have already been identified. In recent years, RNA sequencing is a major approach in gaining first insights in the molecular mechanism which is happening during male sterility in different plant species, for example broccoli, rape seed or radish (Mei *et al*., 2015; Pei *et al*., 2017; Tang *et al*., 2019).

4.1 The male sterility gene

In general, ms systems (either CMS or GMS) are controlled by two genes, the so called ms gene and the restorer gene. The ms genes were mostly discovered by a mapping approach. For CMS genes, distinctions in the mitochondrial transcriptome or proteome in the CMS line were mainly analysed (Chen and Liu, 2014). For the MSL system the NPZ narrowed down the chromosomal region to three genes. After ruling out one candidate immediately, two genes which are known in *A. thaliana* as Bark1 (At3g23750) and a proposed DNA-methyl-transferase (At3g23740) remained. The possible candidates were examined further in the progress of this work and analyzed according to possible male sterility traits. As already mentioned, it was assumed that the ms gene should have the same subcellular localization as the restorer gene to make an interaction possible. In any case, both ms candidates showed neither signs for a chloroplastic localization, nor for male sterility in *A. thaliana*. *A. thaliana* is closely related to *B. napus* with an exon similarity of approximately 87% (Cavell *et al*., 1998). This relation in combination with a shorter growing period, the fact that it is easier to cultivate and its diploid genome results in a preferential model organism for analyzing ms traits from *B. napus*. Unfortunately, no further probable ms candidates could be considered after the pulldown with the *B. napus* restorer gene (*Bn*Tic40 C9) (Table 3). With the knowledge gained over the course of this project, this is easily explainable. While using rape seed protein extract from the restorer line (Monty), no ms gene is present. Therefore, it would be more efficient to use protein extract from either one of the two male sterile lines (A- or

C545-line). However, with the low expression of the ms gene in the C545-line, which was barely detectable when using qRT-PCR, it might be unlikely to pull down the ms gene. Furthermore, it did not seem that phosphorylation, imitated by the phospho-mimicry mutant, made a difference for the binding of interaction partners of the BnTic40C9. However it should be noticed, that with the pulldown only mtHsp70 was found interacting with BnTic40C9 but no cpHsp70.

The ms gene was discovered for the GMS systems 7-7365ABC and 9012AB (Xia *et al.,* 2016; Deng *et al.,* 2016). Both groups describe the same gene. This gene consists of several fragments from known genes. The N-terminus consists of RCN1, which is a protein that comprises two ribonuclease II domains, and a pre-mRNA processing splicing factor. The Middle part of this chimeric gene does not resemble any known gene and the C-terminus shows a close homology to the mtHsp70 sequence. This is interesting because Tic40 is known to have a Hop/Hip (Hop: Hsp70 and Hsp90 organizing protein; Hip: Hsp70 interacting protein) domain (Chou *et al.,* 2003). The localization of this chimeric gene was either described as nuclear (Deng *et al.,* 2016) or chloroplastic (Xia *et al*., 2016). These findings allowed us to examine a new candidate as ms gene for the MSL system. Why was it logical to contemplate this gene for the European ms system used in this thesis? The GMS system 7-7365ABC consists of three lines of which the 7-7365A line is the sterile line, while the B- and C545-line both show fertility. The 9012AB system is a two line system. Comparable to the sterile lines we used in this work from the MSL system, the 7-7365A line can regain fertility after heat-shock treatment (Zhu *et al*., 2010). Furthermore, the restorer gene (BnMS3) for these systems was identified as *BnTic40 C9* (Dun *et al.*, 2011; Li *et al*., 2012; Dun *et al*., 2014), which is essentially the same gene as the restorer gene in the MSL system (confirmed by the NPZ). In the next step, the chimeric gene was cloned from the sterile lines by using RACE-PCR. Stable transformation of this proposed ms gene into *A. thaliana* led to a sterile phenotype, which was the first indication for its function as ms gene. The same result was achieved by (Xia *et al*., 2016; Deng *et al.,* 2016). For determination of the cellular localization of the chimeric gene, a GFP fusion protein was constructed. After transient expression in tobacco leaves it was clear, that the CHIMERA-GFP signal displayed a clear localization in chloroplasts for the full length construct as well as the N-terminal sequence. This result also confirms that the N-terminus alone, which contains the proposed chloroplastic transit peptide (according to ChloroP), is responsible for the chloroplast targeting. We thus confirmed the chloroplast localisation (Xia *et al.,* 2016) which contradicts the expression in the nucleus (Deng *et al*., 2016). Unfortunately, a more detailed analysis of the localization of the ms gene was not possible. After isolation of chloroplasts from Monty and the C545 line and separation into stroma and thylakoids, the antibody raised against the Middle segment of the Chimera could not detect the protein in the plant fractions (data not

shown). This might be explained by a low expression of the Chimera protein. Though its interaction with the BnTic40C9 protein and the lack of prediction of a transmembrane domain (using TOPCONS) leads to the assumption, that the chimeric protein might be a soluble protein localised in the stroma or the intermembrane space.

For further analysis of the chimeric gene in the MSL lines qRT-PCR was conducted. The expression levels of the *Chimera* did not change after 37 °C in the C545 line and no expression was detected in the Monty line. Although Monty is fertile, because it contains the fully functional restorer gene, it might also have the ms gene. This assumption was ruled out after the qRT-PCR results and the PCR with specific primer combinations done on the gDNA of all four lines. Hence, the ms gene can only be detected in the sterile A- and C545 lines.
The ms gene was fragmented accordingly to its chimeric structure and then transformed again into *A. thaliana*. Interestingly, only the full length chimeric gene always caused sterility in *A. thaliana.* The pale phenotype, which can be observed with the Middle (1638-2296 bp) fragment is probably caused by the overexpression with the 35S promoter, because there was no phenotype detectable by using the native promoter. A comparable experiment was done with 7-7365ABC ms system, where the different fragments of the ms gene were analysed in *A. thaliana* (Zhang *et al.,* 2020). This group describes that for the longer constructs (full length and N-terminus in combination with the Middle part (1-2296 bp)) some plants show a chlorotic phenotype, while for the N-term and Middle construct, all plants were described as chlorotic and sterile. This is interesting, because with the 35S promoter we did not get any transformation results for the full length ms gene and the N-terminus in combination with the Middle part in *A. thaliana*. Furthermore, some *A. thaliana* mutants transformed with the ms gene N-terminus and Middle (1-2296 bp) produced less to no seeds under the native promoter, compared to the WT. Yet, not all of them did. In general, by using the native promoter, we could also detect this chlorotic phenotype on some mutant plants, which can be seen in Figure 4b (Zhang *et al.,* 2020), but assumed it was connected to the BASTA treatment.

To this point all the results strongly indicate, that the chimeric gene also functions as ms gene in the MSL system. It was demonstrated that only the full length Chimera under the native promoter leads to sterility in *A. thaliana* plants and that the ms gene only exists in the sterile A- and C545-lines of the MSL system. The chloroplastic localisation hints for a chloroplastic cause of male sterility.

4.2 Analysis of the restorer gene

Although the BnTic40C9 was identified as the restorer gene for the MSL system via mapping done by the NPZ there were still unanswered questions about this gene. *BnTic04C9* was also identified as restorer gene for the Chinese 7-7365ABC male sterility system (Dun *et al.*, 2011, Dun *et al.*, 2014).

The restorer gene is a homologue to the *A. thaliana* Tic40, whose role in protein import into chloroplasts is still not fully understood (Chou *et al*., 2006; Chiu *et al*., 2008; Kikuchi *et al*., 2013; Bédard *et al*., 2017; Lee and Hwang, 2019). The *attic40* mutant shows a pale but fertile phenotype. We demonstrated that all four *B. napus* Tic40 proteins can complement the phenotype of the *attic40* mutant. This was also shown for the Tic40s of the 7-7365ABC male sterility system (Dun *et al*., 2014). The restorer function of the BnTic40C9 was described as a "neofunctionalization" event, leading to this new function in male sterility (Dun *et al.,* 2014). With the existence of three further Tic40s in *B. napus*, the question arose, if the BnTic40C9 might have lost its function during protein import into chloroplasts. The qRT-PCR results in Monty and the C545 line showed no difference in the expression level for BnTic40 A2, C2 and A10. Furthermore, there is no change in expression after heat treatment (37 °C) similar to the ms gene expression. Accordingly, there is no indication that one of these proteins has to compensate for the restorer function of BnTic40C9. Obviously, it can be argued, that the BnTic40 A2, A10 and C2 also did not show a change of expression levels in the sterile lines containing the mutated version of the restorer gene. After all, with the complementation of the *attic*40 ko mutant, it can be assumed that BnTic40C9 still functions as a regular Tic40 protein.

With the determination of the same localisation of the ms and the restorer gene in chloroplasts, the possibility of an interaction between the ms gene and the restorer gene was analysed using the YTH experiment. The possibility of an actual interaction between these two genes was proposed because of the assumption, that the restorer gene obtained its restorer function around the same time as the ms gene developed (Dun *et al*., 2014). Nevertheless, the same group could not prove an interaction between the expressed proteins of the restorer gene and ms gene for the 7-7365ABC system (Xia *et al*., 2016). Interestingly, in this thesis, the restorer gene interacted only with the Middle part of the chimeric gene, which is the only segment of unknown function. It was suspected, that the BnTic40 C9 might interact with the C-terminus of the ms gene, because of its close homology the mtHsp70-1. The results also showed that only the BnTic40C9 interacted with the ms gene out of the four *B. napus* Tic40s. Accordingly, the YTH experiment revealed, that the Middle section of the ms gene seems to play the most important part in the ms function.

The four BnTic40s have a close sequence similarity (see Figure 12) but only the BnTic40C9 was determined as restorer gene by the NPZ. With the exchange of specific amino acids, which distinguish the C9 protein from the remaining three Tic40 proteins, the YTH experiment was repeated. It became obvious during the exchange of phenylalanine to valine at position 321 and arginine to leucine at position 343, that the Tic40C9 protein lost its ability to interact with the ms gene. Furthermore it was shown, that the exchange of glycine to glutamine (aa 386) lead to an interaction of the mutated version of the C9 protein with the C-terminus of the ms protein, as well as the Middle part. Surprisingly, only the exchange in position 321 had an influence on the restorer function in *A. thaliana* (Zhang *et al.*, 2020). This group also showed in an interaction experiment while using YTH between BnTic40C9 and BnToc33 that no interaction occurred after the mutation at position 321, but could not see the same result with the amino acid 343. Therefore, they claim that amino acid 321 is essential for the restoring function (Zhang *et al.,* 2020). However, the outer envelope protein BnToc33 is a completely different interaction partner and with Tic40 being a known inner membrane protein, the occurrence if this interaction might need further evaluation. In this thesis, we also tried to apply the male sterility system into *A. thaliana* to confirm the importance of the amino acids 321 and 343 for the restoring function of Tic40C9. WT *A. thaliana* was transformed simultaneously with the ms gene and the restorer gene. Unfortunately, no restorer function was observed. All plants showed a sterile phenotype, although the expression of BnTic40C9 was confirmed via qRT-PCR. The expression of the chimeric gene was also confirmed by using qRT-PCR and was obvious because of the sterile phenotype. The possible reasons why this experiment did not work are many. The obvious one would be that despite their close relation, this male sterility system from *B. napus* does not function in *A. thaliana*. However, it was shown by crossing of the *A. thaliana* mutant containing the ms gene with the mutant of the restorer gene that the BnTicC9 can also restore fertility in *A. thaliana* (Xia *et la.,* 2016; Zhang *et al.,* 2020). It might be, that crossing is more efficient compared to the transformation of one plasmid containing two foreign genes into *A. thaliana*. Nevertheless, because of the mapping approach, done by the NPZ the mutation of the BnTic40 C9 in the sterile lines and the fact that it interacts with the ms protein *in vitro* suggests its function as restorer gene.

From the results received via qRT-PCR and PCR on gDNA it can be assumed, that the MS gene in the MSL system is only present in the two sterile lines (A- and C545 line). The functional restorer gene (BnTic40C9) on the other hand, can only be found and restorer/WT line (Monty), while the sterile lines and the maintainer line contain a loss of function mutation (qRT-PCR and sequencing results).

4.3 Analyzation of the plant mechanism after different treatments via phenotyping and RNA sequencing

The phenotyping of the flowers and pollen from the C545 line and Monty showed the obvious difference between these two lines. Monty was fertile and therefore grew flowers with fully developed anthers and produced viable pollen as was shown using pollen staining. The sterile C545-line on the other hand developed only crudely formed and very small anthers. These anthers did not produce pollen. For maintaining the sterile line to be used in hybrid-breeding, the production of seeds is mandatory. For EGMS lines, this can either be achieved by crossing techniques of the according GMS lines (see Figure 3, Introduction) or through a change of environmental conditions. For the C545 line, heat treatment at 37 °C in combination with a high humidity (~80%) leads to the development of functional anthers. This was proven by phenotyping of the flowers as well as pollen staining. Still, heat treated C545 line produced less siliques containing pollen than the untreated Monty line. The flower buds of the sterile line probably need to be exposed to heat at a specific developmental stage to produce functional pollen. A time period of three days is not enough to affect all flowers, because of their different development stages. Increasing the time period under heat treatment though, would likely result in a higher stress level and probably dying of the plants. Interestingly, high temperature can cause male sterility for other plant species (Kim *et al.*, 2001). In their study, heat shock induced at 42 °C for 4 h with 85% humidity resulted in aborted seed production for some of the analysed *A. thaliana* flowers (Kim *et al*., 2001).

Using high temperatures of 37°C in combination with high humidity for gaining *B. napus* seeds is rather costly and elaborate for breeding purposes, especially when a large amount of seeds needs to be obtained. Another option to receive anthers in the C545 line was treatment of the flowers and buds with Auxin and ethephon, which was first tested by the NPZ. Auxin is a phytohormone, known for its function in development and growth. It plays a role in anther and pollen development by prohibiting premature pollen development, but also inducing anther dehiscence and filament growth (Cecchetti *et al*., 2008). The most abundant form of auxin in plants is indole-3-acetic acid (IAA) (Lavy and Estelle, 2016). Ethylene is mostly known for its function in fruit ripening and flower senescence. We observed what seemed like fully developed anthers after auxin treatment, but the pollen viability was reduced compared to the fertile Monty line or the heat treatment at 37 °C. Growing the sterile plants at just 28 °C also seemed to make a difference for anther development. Although the anthers were more developed compared to the untreated sterile flowers, the pollen were either not always viable or not even released from the anthers.

Interestingly, heat treatment of *A. thaliana* mutants transformed with the full length ms gene could not confirm the results which were obtained in *B. napus*. First of all, even after slow

adaption to higher temperatures, the *A. thaliana* plants did not survive treatment at 37 °C. However, a partial transformation for some *A. thaliana* plants from sterility to fertility with heat treatment in a range of 33-36 °C and high humidity (75-85%) could be shown (Zhang *et al*., 2020). We were able to keep the *A. thaliana* mutants containing the ms gene at the same conditions as the *B. napus* plants (heat treatment at 28 °C ± spraying with auxin, a humidity of 70% and 20 h of light) for two weeks. Contrary to the sterile *B. napus* line, no indication for a restoration of fertility was observed. This result speaks against the presumption that the ms protein might degrade at higher temperatures and that is how the C545 plants become fertile. In a different study (Sakata *et al*., 2010) auxin was applied to barley and *A. thaliana* during heat stress (30° C for barley/ 31 °C or 33 °C for Arabidopsis). It was demonstrated that the male sterile phenotype which was caused by the heat treatment could be abolished with auxin treatment of the plants. For barley, application of auxin resulted in normal length anthers and the production of mature pollen. In *A. thaliana*, the applied heat stress led to reduced stamen length, which could also be influenced with auxin treatment during the heat treatment, depending on the auxin concentration (Sakata *et al*., 2010). In this thesis, no difference in the anther length after auxin treatment at higher temperatures (28 °C) was detected. However, the *A. thaliana* mutant plants we used were sterile because they contained the ms gene from the MSL system but not due to heat. This result might indicate that the auxin hormone does not affect the male sterile phenotype which is induced by the ms gene.

With a transcriptome analysis approach, the molecular mechanisms behind treatments leading to fertility were analysed. For the experimental set up, two separate RNA sequencing analysis were conducted. First of all, flower buds from *B. napus* at the same developmental stage were collected from Monty untreated and the C545 line untreated, C545 37 °C, C545 Auxin/28 °C and C545 at 28 °C. The second approach involved genes only from microspores of the C545 and Monty line untreated and treated at 37 °C for three days.

For the RNA sequencing results of the whole *B. napus* flower buds, the most DEGs were calculated between the C545 line untreated and treated at 37 °C (16109 DEGs). The comparison of the C545 line untreated to the C545 line treated at 28°C for two weeks resulted in the least amount of DEGs (2636). Interestingly, after GO term analysis which clusters the DEGs according to their function, between the C545-line and Monty and the comparison between C545 37 °C and the untreated C545-line it seems that the upregulated genes mostly seem to be involved in plant stress responses, like “response to chitin” or “response to wounding”. The down regulated genes on the other hand, might give insights into the plant mechanism related to sterility. GO terms, which repeatedly appear were proteasome or ubiquitin related. This was highlighted by using Mapman BINs, showing several DEGs belong to the proteasome and the according components. The so-called

ubiquitin-proteasome system (UPS) has a proteolytic function and is involved in the degradation of proteins targeted to mitochondria and chloroplasts (Ling and Jarvis, 2013). Ubiquination describes the process of adding one to several ubiquitin molecules to target proteins of the proteasome. This process usually takes place post-translationally. The ubiquinated proteins are then recognized by the 26S proteasome in the cytosol and become degraded (Vierstra, 2009). The enzymes E1 ubiquitin activase, E2 ubiquitin conjugase and E3 ubiquitin ligase catalyse the reactions leading to the ubiquination of the target proteins. HECT, really interesting new gene (RING), U-box and cullin-RING ligase (CRL) belong to the four classes of E3 ligases in plants. The F-Box proteins belong to the S phase kinase-associated protein 1 (SKP1)–cullin 1 (CUL1)–F-box (SCF complex), which is an E3 ubiquitin ligase complex and belongs to the family of CLRs (Vierstra, 2009).

With the discovery of the SP1, which is a RING E3 ligase localised in the outer chloroplast membrane, it was shown, that protein degradation via the UPS also occurs to TOC components (Ling *et al*., 2012). The TOC components play an essential role in importing pre-proteins into the chloroplast (see introduction section 1.2). Later on, the function of SP1 in the regulation of abiotic stress was discussed, because the *A. thaliana* overexpressing SP1 showed a higher stress tolerance than the control wild type plants. The difference was only detectable under salinity and osmotic stress conditions while temperature stress did not seem to have an effect. An accumulation of ROS in *sp1* mutants in contrast of the SP1 overexpression line was also shown (Ling and Jarvis, 2015). Chloroplast are responsible for several functions. The photosynthesis, which takes place in the thylakoid membrane, is not only essential for the production of oxygen, but also central for the creation of reactive oxygen species (ROS). The authors propose for the SP1 function, by adding abiotic stress, the SP1 targets the TOC components by ubiquination, which leads to a reduced import of proteins. In addition this also affects proteins involved in photosynthesis which results in a reduction of ROS production in chloroplasts and therefore an increase in stress tolerance (Ling and Jarvis, 2015; Broad *et al*., 2016).

This is interesting, because in the RNA sequencing data for the flower buds in this thesis, the most DEGs in the Ubiquitin dependant degradation (using Mapman BINs) can be seen for the F-BOX and RING genes, which belong to the E3 ubiquitin ligases (Vierstra, 2009) like SP1. It might be speculated, that another E3 ligase might be responsible for the stress response in the *B. napus* plants, because no correlation for SP1 and heat could be displayed (Ling and Jarvis, 2015). In a transcriptome analysis conducted on *A. thaliana* siliques of mutant plants containing the chimeric ms gene in comparison to plants containing the ms gene and the BnTic40C9 restorer gene as well as WT, it was shown that up-regulated genes related to the ubiquitin system were enriched (Zhang *et al*., 2020). The authors propose that the chimeric ms gene might affect the ubiquination process, resulting in a disturbance of the

TOC components enriched. The transcriptome analysis was not conducted after heat treatment on these *A. thaliana* plants. However, it was also shown that the Chimera protein interacts with the E3 ligase BRUTUS (Zhang *et al*., 2020). This outcome is highly unlikely, because BRUTUS is localised to the nucleus (Kobayashi *et al.*, 2013), while the chimeric ms gene has a chloroplastic localisation (this work, Xia *et al.*, 2016).

In our data the ubiquitin related genes are down regulated after treatment at 37 °C. All things considered, it seems that the ubiquitin related GO terms seem to be a stress related response of the *B. napus* plant, at least after auxin treatment and treatment with 28 °C compared to the C545 untreated. For these data sets, the Mapman BINs appear mostly up regulated for F-Box related genes. In *B. napus* the E3 ligase thermal resistance gene 1 (BnTR1) was detected, which seems to be induced during heat stress and is localised in the plasma membrane (Liu *et al.,* 2014). The authors showed in their study, that overexpression of *BnTR1* in *B. napus* and *O. sativa* not only resulted in a higher resistance of the plants to heat stress (35° C) but also increased the pollen viability in *Oryza sativa* (*O. sativa)* compared to the control plants (Liu *et al.,* 2014). However, it needs to be kept in mind that the ubiquitin-proteasome related GO terms or Mapman BINs are mostly down regulated after heat treatment (37 °C) in the C545 line compared to the untreated sterile line.

The down regulated genes in the comparison between the C545-line untreated and heat treated, as well as C545 compared to Monty seemed to be the most interesting in relation to male sterility related genes. That is why, the DEGs which were both, down regulated in the C545 line untreated when compared to Monty and up regulated after heat treatment in the C545 line compared to the untreated sterile line, were selected. After GO term analysis, it seemed that most of these genes were clustered to GO terms related to polyamines. Polyamines can be found in all living organisms. The three most important polyamines in plants are putrescine, spermidine and spermine. The synthesis of putrescine in plants starts with l-arginine. It can either be synthesized directly from ornithine or from l-arginine involving three steps. Therefore the arginine decarboxylase (ADC) or the ornithine decarboxylase (ODC) are involved (Liu *et al.,* 2015). In *A. thaliana* the ODC gene does not exist (Hanfrey *et al.,* 2001). The ADC catalyzes the formation of agmatine and after two further steps, putrescine is produced. Putrescine can be converted into spermidine and spermidine into spermine (Sagor *et al*., 2013). Polyamines have several functions and seem to play a role in the plants stress response but also in cell division and cellular growth. In plants, abiotic stress leads to accumulation of polyamines as a response mechanism (Liu *et al*., 2015). Heat-shock treatment (42° C) of *A. thaliana* with the application of spermine led to reduced heat shock damage of the plants (Sagor *et al*., 2013). In *A. thaliana* the most amounts of polyamines resides in flowers, which suggests a function in flowering (Applewhite *et al*.,

2000). It was also reviewed, that polyamines are involved in every step from pollen development to pollen tube growth and fertilization (Aloisi *et al*., 2016).
Interestingly, the GO terms for polyamines on the data for the flower buds are down regulated in the C545 line compared to Monty and then up regulated after heat treatment (37 °C) compared to the C545-line untreated. This up regulation of polyamines after treatment at 37 °C therefore can either be a stress response of the plant or the necessary step for pollen development, which might explain the partial fertile phenotype of the C545-line.

The second data set of RNA sequencing data comprises only microspores which were isolated by using laser microdissection. The longer handling of the plant samples was necessary for the process including harvest of flower buds and the following embedding of the buds in paraffin for sectioning with the microtome. Only after the microspores were dissected, the RNA could be isolated. This resulted in a lower quality of the RNA compared to the data set of the RNA isolated from complete flower buds, which can be seen in the lower amount of mapped reads. For the microspores, only heat treatment (37° C for three days) on the C545 line and Monty was used. After DEG analysis, 25089 DEGs were observed between the C545-line untreated and Monty untreated, while the comparison between the heat treated C545 to the untreated sterile line displayed the least amount of DEGs (5278 DEGs). For the GO term analysis in the microspore data between C545 untreated and Monty untreated, protein phosphorylation is upregulated and ribosomal related GO terms are down regulated, which hints a down regulation of the genes involved in protein translation. When the heat treated C545 is compared to the untreated C545 the up regulated GO terms showed involvement in transcription, while the down regulated GO terms resulted amongst others in sporopollenin biosynthetic processes for biological processes. Male sterility is mostly related to defects in proper development of mature pollen. The formation of the pollen wall is essential in obtaining mature pollen. Sporopollenin is known to be the major component of the exine layer of the pollen wall (Wilson and Zhang, 2009). The biosynthesis of sporopollenin is still not completely understood. It probably starts with the synthesis of fatty acids. De novo fatty acid synthesis in plants occurs in plastids (Li-Beisson *et al*., 2010). It is assumed, that the composition of the sporopollenin precursors takes place in the tapetum. For their destination in the anther locule for exine formation, the transport via an ABC transporter is proposed (Quilichini *et al*., 2010; Quilichini *et al*., 2015).

Although, because of the different experimental setup of the RNA sequencing data of the microspores and the whole buds, the comparison between the two datasets should be treated with caution. The appearance of sporopollenin biosynthesis in the GO term analysis seems to be the mechanism in the plant which is not directly related to stress and important for pollen development. The biosynthetical process of sporopollenin development starts with

the synthesis of fatty acids in the chloroplasts, which is also where the ms gene and the restorer gene are localized. Therefore fatty acid synthesis might be a good starting point for a more detailed analysis of the RNA sequencing data, To this point it can only be speculated which mechanisms are actually occurring in the plant that result in some fertile flowers after heat treatment at 37 °C. The fact, that the mentioned GO terms are down regulated in the RNA sequencing analysis is difficult to explain. Especially the fatty acid biosynthesis and pollen exine formation which are both down regulated in the C545 line compared to Monty and in the C545 line 37 °C when compared to the untreated C545-line (Figure 25 B Venn diagram). Because of their importance in pollen development and maturation and the clear pollen development in some of the C545 flowers after heat treatment would lead to the expectation that the involved genes need to be up regulated. It seems, that the untreated sterile line already shows stress responses, presumably because of its inability to develop functional anthers and pollen which would result in the production of seeds. One explanation might be that the genes required for sporopollenin biosynthesis are up regulated in the C545 line in its desperate attempt to produce pollen, but do not emerge in the GO term analysis. Accordingly, after heat treatment these processes need to be down regulated first to be on a level to function normally.

In this analysis, the transcriptome data set of the whole flower buds seemed to give more insights into possible mechanism in the *B. napus* plants about why the C545 line is sterile compared to Monty and the question of how heat treatment (37 °) can result in some fertile flowers. To give an overview of the GO term analysis of the flower buds (Figure 43 A), in the comparison of C545 and Monty the up regulated genes seem to be plant stress responses. This leads to the assumption, that the C545 line is stressed in general compared to the fertile Monty line. The down regulation of genes related to polyamine biosynthesis, fatty acid biosynthesis, and ubiquitin related genes, might explain, while the C545 line is sterile. With the down regulation of fatty acid biosynthesis, pollen development might not be possible. After heat treatment at 37 °C, the up regulated GO terms are also related to stress, which is probably a heat stress response of the plants. Moreover, genes related to sporopollenin and to the ubiquitin-proteasome complex are down regulated. The further down regulation of the ubiquitin-proteasome system in the C545 line after heat treatment is difficult to explain. The down regulation of the sporopollenin biosynthesis might be necessary to reach the level for the involved genes to function normally and therefore allow production of pollen.

For the microspore data, it seems, that the translation processes are down regulated and after heat treatment (37 °C) the GO terms describing transcription are up regulated. Interestingly, the GO term “sporopollenin biosynthetic process” was enriched for down regulated genes in both RNA sequencing data sets (Figure 43).

For further analysis, specificallly selected DEGs could be verified by conducting qRT-PCR. The results would give an indication about the reliability of the RNA sequencing data and the expression levels of the DEGs. With the proposed chloroplastic regulation of male sterility in the MSL system, it also might be interesting to analyse the chloroplastic structure using TEM while comparing the sterile lines with the fertile lines. The pictures of the chloroplast structure could help defining the starting point of the male sterility induced by the male sterility gene in the chloroplast.

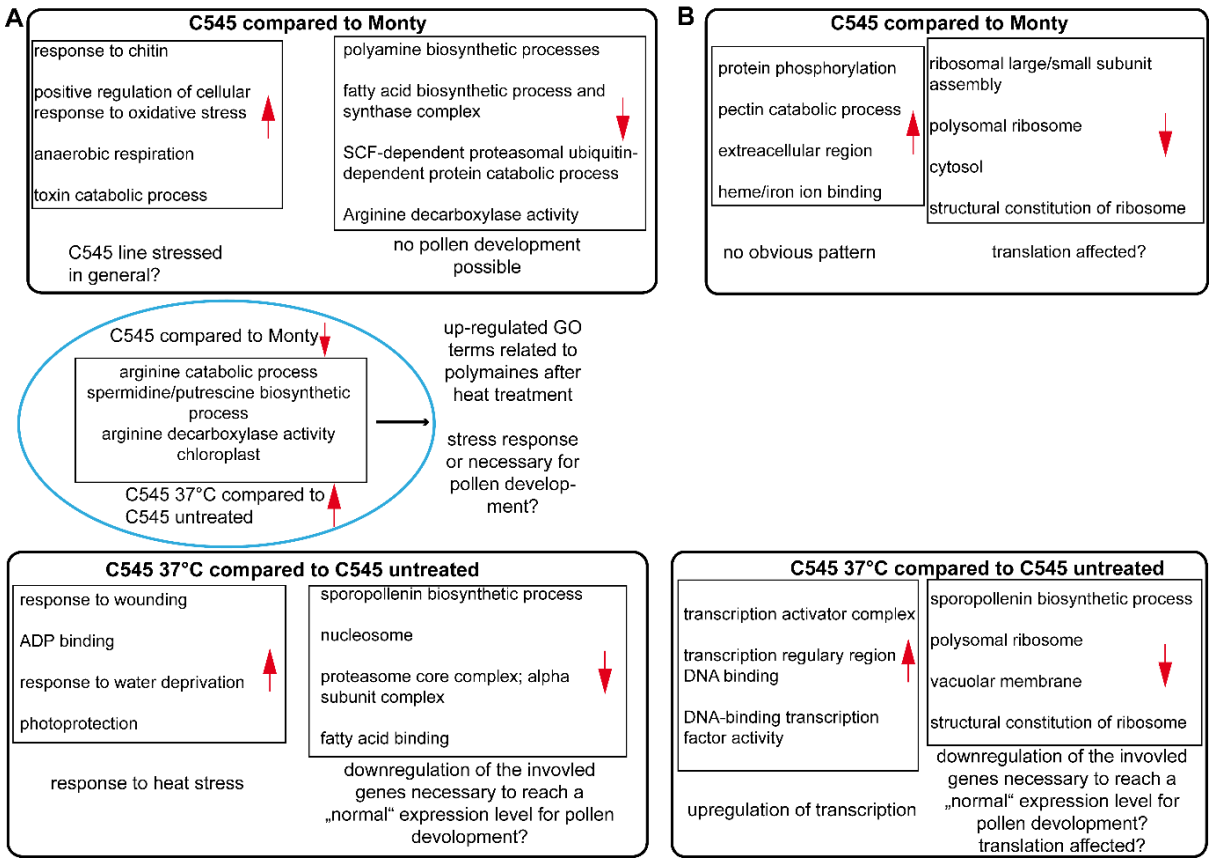

Figure 43: Overview of the GO term analysis between C545 and Monty as well as C545 37 °C compared to C545 untreated. The red arrows indicate if the GO terms are up regulated (↑) or down regulated (↓). The GO terms of the DEGs which exist in both data sets of the down regulated genes of C545 compared to Monty and are up regulated after 37 °C treatment in contrast to C545 untreated are circled in blue. **A:** Whole flower buds data set. **B:** Microspores data set.

4.4 Conclusion

Male sterility in crops and understanding the processes behind it is a complex topic. This study gives insights into the molecular mechanisms of the MSL system. We determined the ms gene and its localisation in the chloroplast as shown by (Xia *et al*., 2016) in the 7-7365ABC ms system. Because of the observed interaction between the restorer and ms gene, which can only take place in the chloroplast, it can be assumed that the regulation of the male sterility either takes place or is initiated in the chloroplasts. For conformation of this theory, a more detailed analysis of the obtained RNA sequencing data needs to be conducted. It might also be interesting to look specifically into transcription factors involved in pollen and anther development (mentioned in the introduction), especially which are the relevant DEGs clustering to the GO term sporopollenin biosynthesis. To this point, the question why heat treatment leads to fertility in the MSL system still cannot be answered satisfactorily. Furthermore it is still unclear, how the chimeric ms gene induces male sterility.
With the upcoming climate change and the associated increase in temperature, the answer to this question might be essential to prevent yield loss due to possible heat stress in crops.

5 References

Aarts, M.G.M., Hodge, R., Kalantidis, K., Florack, D., Wilson, Z.A., Mulligan, B.J., Stiekema, W.J., Scott, R., and Pereira, A. (1997). The *Arabidopsis MALE STERILITY 2* protein shares similarity with reductases in elongation/condensation complexes. The Plant Journal *12*, 615–623.

Akagi, H., Nakamura, A., Yokozeki-Misono, Y., Inagaki, A., Takahashi, H., Mori, K., and Fujimura, T. (2004). Positional cloning of the rice Rf-1 gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. Theor Appl Genet *108*, 1449–1457.

Akter, S., Huq, Md.A., Jung, Y.-J., Cho, Y.-G., and Kang, K.-K. (2016). Application of Single Nucleotide Polymorphism Markers for Selection of Male Sterility in Crop Plants. Plant Breed. Biotech. *4*, 379–386.

Albiniak, A.M., Baglieri, J., and Robinson, C. (2012). Targeting of lumenal proteins across the thylakoid membrane. Journal of Experimental Botany *63*, 1689–1698.

Alexander, M.P. (1969). Differential Staining of Aborted and Nonaborted Pollen. Stain Technology *44*, 117–122.

Aloisi, I., Cai, G., Serafini-Fracassini, D., and Del Duca, S. (2016). Polyamines in Pollen: From Microsporogenesis to Fertilization. Front. Plant Sci. *7*, 827.

Applewhite, P.B., Kaur-Sawhney, R., and Galston, A.W. (2000). A role for spermidine in the bolting and flowering of Arabidopsis. Physiol Plant *108*, 314–320.

Arnon, D.I. (1949). copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. Plant Physiol *24*, 1–15.

Baldwin, A., Wardle, A., Patel, R., Dudley, P., Park, S.K., Twell, D., Inoue, K., and Jarvis, P. (2005). A Molecular-Genetic Study of the Arabidopsis Toc75 Gene Family. Plant Physiol. *138*, 715–733.

Balsera, M., Soll, J., and Buchanan, B.B. (2009a). Chapter 10 Protein Import in Chloroplasts. In Advances in Botanical Research, (Elsevier), pp. 277–332.

Balsera, M., Goetze, T.A., Kovács-Bogdán, E., Schürmann, P., Wagner, R., Buchanan, B.B., Soll, J., and Bölter, B. (2009b). Characterization of Tic110, a Channel-forming Protein at the Inner Envelope Membrane of Chloroplasts, Unveils a Response to Ca^{2+} and a Stromal Regulatory Disulfide Bridge. J. Biol. Chem. *284*, 2603–2616.

Becker, T., Jelic, M., Vojta, A., Radunz, A., Soll, J., and Schleiff, E. (2004). Preprotein recognition by the Toc complex. EMBO J *23*, 520–530.

Bédard, J., Kubis, S., Bimanadham, S., and Jarvis, P. (2007). Functional Similarity between the Chloroplast Translocon Component, Tic40, and the Human Co-chaperone, Hsp70-interacting Protein (Hip). J. Biol. Chem. *282*, 21404–21414.

Bédard, J., Trösch, R., Wu, F., Ling, Q., Flores-Pérez, Ú., Töpel, M., Nawaz, F., and Jarvis, P. (2017). Suppressors of the Chloroplast Protein Import Mutant *tic40* Reveal a Genetic Link between Protein Import and Thylakoid Biogenesis. Plant Cell *29*, 1726–1747.

Benz, M., Bals, T., Gügel, I.L., Piotrowski, M., Kuhn, A., Schünemann, D., Soll, J., and Ankele, E. (2009). Alb4 of Arabidopsis Promotes Assembly and Stabilization of a Non Chlorophyll-Binding Photosynthetic Complex, the CF1CF0–ATP Synthase. Molecular Plant *2*, 1410–1424.

Binder, A., Lambert, J., Morbitzer, R., Popp, C., Ott, T., Lahaye, T., and Parniske, M. (2014). A Modular Plasmid Assembly Kit for Multigene Expression, Gene Silencing and Silencing Rescue in Plants. PLoS ONE *9*, e88218.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry *72*, 248–254.

Broad, W., Ling, Q., and Jarvis, P. (2016). New Insights Into Roles of Ubiquitin Modification in Regulating Plastids and Other Endosymbiotic Organelles. In International Review of Cell and Molecular Biology, (Elsevier), pp. 1–33.

Browne, R.G., Iacuone, S., Li, S.F., Dolferus, R., and Parish, R.W. (2018). Anther Morphological Development and Stage Determination in Triticum aestivum. Front. Plant Sci. *9*, 228.

Caliebe, A. (1997). The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. The EMBO Journal *16*, 7342–7350.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., and Righetti, P.G. (2004). Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis *25*, 1327–1333.

Cavell, A.C., Lydiate, D.J., Parkin, I., Dean, C., and Trick, M. (1998). Collinearity between a 30-centimorgan segment of *Arabidopsis thaliana* chromosome 4 and duplicated regions within the *Brassica napus* genome. Genome *41*, 62–69.

Cecchetti, V., Altamura, M.M., Falasca, G., Costantino, P., and Cardarelli, M. (2008). Auxin Regulates *Arabidopsis* Anther Dehiscence, Pollen Maturation, and Filament Elongation. Plant Cell *20*, 1760–1774.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., *et al*. (2014). Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science *345*, 950–953.

Chaudhury, A.M. (1993). Nuclear Genes Controlling Male Fertility. Plant Cell 1277–1283.

Chen, L., and Liu, Y.-G. (2014). Male Sterility and Fertility Restoration in Crops. Annu. Rev. Plant Biol. *65*, 579–606.

Chen, Y.-L., Chen, L.-J., Chu, C.-C., Huang, P.-K., Wen, J.-R., and Li, H. (2018). TIC236 links the outer and inner membrane translocons of the chloroplast. Nature *564*, 125–129.

Chiu, C.-C., and Li, H.-M. (2008). Tic40 is important for reinsertion of proteins from the chloroplast stroma into the inner membrane. The Plant Journal *56*, 793–801.

Chou, M.-L. (2003). Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. The EMBO Journal *22*, 2970–2980.

Chou, M.-L., Chu, C.-C., Chen, L.-J., Akita, M., and Li, H. (2006). Stimulation of transit-peptide release and ATP hydrolysis by a cochaperone during protein import into chloroplasts. The Journal of Cell Biology *175*, 893–900.

Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. (1984). Thermolysin Is a Suitable Protease for Probing the Surface of Intact Pea Chloroplasts. Plant Physiol. *75*, 675–678.

Deng, Z., Li, X., Wang, Z., Jiang, Y., Wan, L., Dong, F., Chen, F., Hong, D., and Yang, G. (2016). Map-based cloning reveals the complex organization of the BnRf locus and leads to the identification of BnRf b, a male sterility gene, in Brassica napus. Theor Appl Genet *129*, 53–64.

Dewey, R.E., Timothy, D.H., and Levings, C.S. (1987). A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proceedings of the National Academy of Sciences *84*, 5374–5378.

Ducos, E., Touzet, P., and Boutry, M. (2001). The male sterile G cytoplasm of wild beet displays modified mitochondrial respiratory complexes: The G cytoplasm of wild beet. The Plant Journal *26*, 171–180.

Dun, X., Zhou, Z., Xia, S., Wen, J., Yi, B., Shen, J., Ma, C., Tu, J., and Fu, T. (2011). BnaC.Tic40, a plastid inner membrane translocon originating from Brassica oleracea, is essential for tapetal function and microspore development in Brassica napus: Bnac.Tic40 affects tapetal development in Brassica napus. The Plant Journal *68*, 532–545.

Dun, X., Shen, W., Hu, K., Zhou, Z., Xia, S., Wen, J., Yi, B., Shen, J., Ma, C., Tu, J., et al. (2014). Neofunctionalization of Duplicated Tic40 Genes Caused a Gain-of-Function Variation Related to Male Fertility in Brassica oleracea Lineages. PLANT PHYSIOLOGY *166*, 1403–1419.

Emanuelsson, O., Nielsen, H., and Heijne, G.V. (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. *8*, 978–984.

Flores-Pérez, Ú., Bédard, J., Tanabe, N., Lymperopoulos, P., Clarke, A.K., and Jarvis, P. (2016). Functional Analysis of the Hsp93/ClpC Chaperone at the Chloroplast Envelope. Plant Physiol. *170*, 147–162.

Frauen, M., Noack, J., Paulmann, W., and Grosse, F. (2003). Development and perspectives of MSL-hybrids in winter oilseed rape in Europe. P. 316–318. In Proc. 11th Int. Rapeseed Confr., 6–10 July 2003. Copenhagen 3.

Frauen, M., Noack, J., Girke, A., and Paulmann, W. (2007). Ten years experience of development and cultivation of winter oilseed rape hybrids in Europe based on the MSL system. Proceedings of the 12th International Rapeseed Congress, Vol I: Genet Breeding, Wuhan, China, 26–30 March. Science Press USA Inc, Pp 39–41 3.

Frauen, M. and Paulmann, W (1999). Breeding of hybrid varieties of winter oilseed rape based on the MSL-system. Proc. 10th GCIRC Rapeseed Congr., Saskatoon, Canada.

Geissler, A., Krimmer, T., Bömer, U., Guiard, B., Rassow, J., and Pfanner, N. (2000). Membrane Potential-Driven Protein Import into Mitochondria: The Sorting Sequence of Cytochrome b_2 Modulates the Δψ-Dependence of Translocation of the Matrix-targeting Sequence. MBoC *11*, 3977–3991.

Goldberg, R.B., Beals, T.P., and Sanders, P.M. (1993). Anther development: basic principles and practical applications. Plant Cell *5*, 1217–1229.

Gómez, J.F., Talle, B., and Wilson, Z.A. (2015). Anther and pollen development: A conserved developmental pathway: Conservation of anther and pollen development. J. Integr. Plant Biol. *57*, 876–891.

Gould, S.B., Waller, R.F., and McFadden, G.I. (2008). Plastid Evolution. Annu. Rev. Plant Biol. *59*, 491–517.

Guo, W., Tzioutziou, N., Stephen, G., Milne, I., Calixto, C., Waugh, R., Brown, J.W.S., and Zhang, R. (2019). 3D RNA-seq - a powerful and flexible tool for rapid and accurate differential expression and alternative splicing analysis of RNA-seq data for biologists (Bioinformatics).

Hanahan, D., Jessee, J., and Bloom, F.R. (1991). [4] Plasmid transformation of Escherichia coli and other bacteria. In Methods in Enzymology, (Elsevier), pp. 63–113.

Hanfrey, C., Sommer, S., Mayer, M.J., Burtin, D., and Michael, A.J. (2001). Arabidopsis polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity: Arabidopsis polyamine biosynthetic decarboxylases. The Plant Journal *27*, 551–560.

Heijne, G., Steppuhn, J., and Herrmann, R.G. (1989). Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem *180*, 535–545.

Hinnah, S.C., Wagner, R., Sveshnikova, N., Harrer, R., and Soll, J. (2002). The Chloroplast Protein Import Channel Toc75: Pore Properties and Interaction with Transit Peptides. Biophysical Journal *83*, 899–911.

Honys, D., and Re, D. Male Gametophyte Development and Function. 13.

Hörmann, F., Küchler, M., Sveshnikov, D., Oppermann, U., Li, Y., and Soll, J. (2004). Tic32, an Essential Component in Chloroplast Biogenesis. J. Biol. Chem. *279*, 34756–34762.

Inaba, T., Alvarez-Huerta, M., Li, M., Bauer, J., Ewers, C., Kessler, F., and Schnell, D.J. (2005). Arabidopsis Tic110 Is Essential for the Assembly and Function of the Protein Import Machinery of Plastids. Plant Cell *17*, 1482–1496.

Inoue, H., Li, M., and Schnell, D.J. (2013). An essential role for chloroplast heat shock protein 90 (Hsp90C) in protein import into chloroplasts. Proceedings of the National Academy of Sciences *110*, 3173–3178.

Jiang, J., Zhang, Z., and Cao, J. (2013). Pollen wall development: the associated enzymes and metabolic pathways. Plant Biology *15*, 249–263.

Kawanabe, T., Ariizumi, T., Kawai-Yamada, M., Uchimiya, H., and Toriyama, K. (2006). Abolition of the Tapetum Suicide Program Ruins Microsporogenesis. Plant and Cell Physiology *47*, 784–787.

Kikuchi, S., Bédard, J., Hirano, M., Hirabayashi, Y., Oishi, M., Imai, M., Takase, M., Ide, T., and Nakai, M. (2013). Uncovering the Protein Translocon at the Chloroplast Inner Envelope Membrane. Science *339*, 571–574.

Kim, D., Langmead, B., and Salzberg, S.L. (2015). HISAT: a fast spliced aligner with low memory requirements. Nat Methods *12*, 357–360.

Kim, M.H., Kim, Y., Kim, J.W., Lee, H.-S., Lee, W.S., Kim, S.-K., Wang, Z.-Y., and Kim, S.-H. (2013). Identification of Arabidopsis BAK1-Associating Receptor-Like Kinase 1 (BARK1) and Characterization of its Gene Expression and Brassinosteroid-Regulated Root Phenotypes. Plant and Cell Physiology *54*, 1620–1634.

Kim, S.Y., Hong, C.B., and Lee, I. (2001). Heat Shock Stress Causes Stage-specific Male Sterility in Arabidopsis thaliana. J Plant Res *114*, 301–307.

Kobayashi, T., Nagasaka, S., Senoura, T., Itai, R.N., Nakanishi, H., and Nishizawa, N.K. (2013). Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. Nat Commun *4*, 2792.

Koch, C.M., Chiu, S.F., Akbarpour, M., Bharat, A., Ridge, K.M., Bartom, E.T., and Winter, D.R. (2018). A Beginner's Guide to Analysis of RNA Sequencing Data. Am J Respir Cell Mol Biol *59*, 145–157.

Kouranov, A., Chen, X., Fuks, B., and Schnell, D.J. (1998). Tic20 and Tic22 Are New Components of the Protein Import Apparatus at the Chloroplast Inner Envelope Membrane. The Journal of Cell Biology *143*, 991–1002.

Kovacheva, S., Bédard, J., Patel, R., Dudley, P., Twell, D., Ríos, G., Koncz, C., and Jarvis, P. (2004). In vivo studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import: Arabidopsis Tic knockout mutants. The Plant Journal *41*, 412–428.

Kovács-Bogdán, E., Benz, J.P., Soll, J., and Bölter, B. (2011). Tic20 forms a channel independent of Tic110 in chloroplasts. BMC Plant Biol *11*, 133.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature *227*, 680–685.

Lamberti, G., Drurey, C., Soll, J., and Schwenkert, S. (2011). The phosphorylation state of chloroplast transit peptides regulates preprotein import. Plant Signaling & Behavior *6*, 1918–1920.

Lavy, M., and Estelle, M. (2016). Mechanisms of auxin signaling. Development *143*, 3226–3229.

Lee, D.W., and Hwang, I. (2019). Protein import into chloroplasts via the Tic40-dependent and -independent pathways depends on the amino acid composition of the transit peptide. Biochemical and Biophysical Research Communications *518*, 66–71.

Li, M., and Schnell, D.J. (2006). Reconstitution of protein targeting to the inner envelope membrane of chloroplasts. The Journal of Cell Biology *175*, 249–259.

Li, J., Hong, D., He, J., Ma, L., Wan, L., Liu, P., and Yang, G. (2012). Map-based cloning of a recessive genic male sterility locus in Brassica napus L. and development of its functional marker. Theor Appl Genet *125*, 223–234.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S., Bird, D., DeBono, A., Durrett, T.P., et al. (2010). Acyl-Lipid Metabolism. Arabidopsis Book *8*.

Ling, Q., and Jarvis, P. (2013). Dynamic regulation of endosymbiotic organelles by ubiquitination. Trends in Cell Biology *23*, 399–408.

Ling, Q., and Jarvis, P. (2015). Regulation of Chloroplast Protein Import by the Ubiquitin E3 Ligase SP1 Is Important for Stress Tolerance in Plants. Current Biology *25*, 2527–2534.

Ling, Q., Huang, W., Baldwin, A., and Jarvis, P. (2012). Chloroplast Biogenesis Is Regulated by Direct Action of the Ubiquitin-Proteasome System. Science *338*, 655–659.

Liu, J.-H., Wang, W., Wu, H., Gong, X., and Moriguchi, T. (2015). Polyamines function in stress tolerance: from synthesis to regulation. Front. Plant Sci. *6*.

Liu, Z., Dong, F., Wang, X., Wang, T., Su, R., Hong, D., and Yang, G. (2017). A pentatricopeptide repeat protein restores nap cytoplasmic male sterility in Brassica napus. Journal of Experimental Botany *68*, 4115–4123.

Liu, Z.-B., Wang, J.-M., Yang, F.-X., Yang, L., Yue, Y.-F., Xiang, J.-B., Gao, M., Xiong, F.-J., Lv, D., Wu, X.-J., et al. (2014). A novel membrane-bound E3 ubiquitin ligase enhances the thermal resistance in plants. Plant Biotechnol J *12*, 93–104.

Luban, J., and Goff, S.P. (1995). The yeast two-hybrid system for studying protein—protein interactions. Current Opinion in Biotechnology *6*, 59–64.

Luo, C., Sun, Y., Zhang, Y., Guo, Y., Klima, M., and Hu, S. (2018). Genetic investigation and cytological comparison of two genic male sterile lines 9012A and MSL in Brassica napus L. Euphytica *214*, 124.

Lurin, C., Andrés, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyère, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., et al. (2004). Genome-Wide Analysis of Arabidopsis Pentatricopeptide Repeat Proteins Reveals Their Essential Role in Organelle Biogenesis. Plant Cell *16*, 2089–2103.

Lyska, D., Engelmann, K., Meierhoff, K., and Westhoff, P. (2013). pAUL: A Gateway-Based Vector System for Adaptive Expression and Flexible Tagging of Proteins in Arabidopsis. PLoS ONE *8*, e53787.

Marienfeld, J.R., Unseld, M., Brandt, P., and Brennicke, A. (1997). Mosaic Open Reading Frames in the Arabidopsis thaliana Mitochondrial Genome. Biological Chemistry *378*, 859–862.

May, T., and Soll, J. (2000). 14-3-3 Proteins Form a Guidance Complex with Chloroplast Precursor Proteins in Plants. Plant Cell *12*, 53–63.

McVetty, P.B.E., Lukow, O.M., Hall, L.M., Rajcan, I., and Rahman, H. (2016). Oilseeds in North America. In Reference Module in Food Science, (Elsevier), pp. 1–8.

Mei, S., Liu, T., and Wang, Z. (2016). Comparative Transcriptome Profile of the Cytoplasmic Male Sterile and Fertile Floral Buds of Radish (Raphanus sativus L.). IJMS *17*, 42.

Meuter-Gerhards, A., Riegert, S., and Wiermann, R. (1999). Studies on Sporopollenin Biosynthesis in Cucurbita maxima (DUCH.) — II. The Involvement of Aliphatic Metabolism. Journal of Plant Physiology *154*, 431–436.

Nickel, C., Soll, J., and Schwenkert, S. (2015). Phosphomimicking within the transit peptide of pHCF136 leads to reduced photosystem II accumulation in vivo. FEBS Letters *589*, 1301–1307.

Parkin, I.A.P., Sharpe, A.G., Keith, D.J., and Lydiate, D.J. (1995). Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). Genome *38*, 1122–1131.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods *14*, 417–419.

Pei, X., Jing, Z., Tang, Z., and Zhu, Y. (2017). Comparative transcriptome analysis provides insight into differentially expressed genes related to cytoplasmic male sterility in broccoli (Brassica oleracea var. italica). Scientia Horticulturae *217*, 234–242.

Pelletier, G., and Budar, F. (2007). The molecular biology of cytoplasmically inherited male sterility and prospects for its engineering. Current Opinion in Biotechnology *18*, 121–125.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.-C., Mendell, J.T., and Salzberg, S.L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol *33*, 290–295.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research *29*, 45e–445.

Piffanelli, P., Ross, J.H.E., and Murphy, D.J. (1998). Biogenesis and function of the lipidic structures of pollen grains. Sexual Plant Reproduction *11*, 65–80.

Qbadou, S., Becker, T., Mirus, O., Tews, I., Soll, J., and Schleiff, E. (2006). The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. EMBO J *25*, 1836–1847.

Quilichini, T.D., Friedmann, M.C., Samuels, A.L., and Douglas, C.J. (2010). ATP-Binding Cassette Transporter G26 Is Required for Male Fertility and Pollen Exine Formation in Arabidopsis. Plant Physiol. *154*, 678–690.

Quilichini, T.D., Grienenberger, E., and Douglas, C.J. (2015). The biosynthesis, composition and assembly of the outer pollen wall: A tough case to crack. Phytochemistry *113*, 170–182.

Richter, S., and Lamppa, G.K. (1998). A chloroplast processing enzyme functions as the general stromal processing peptidase. Proceedings of the National Academy of Sciences *95*, 7463–7468.

Rieseberg, L.H., Van Fossen, C., Arias, D., and Carter, R.L. (1994). Cytoplasmic Male Sterility in Sunflower: Origin, Inheritance, and Frequency in Natural Populations. Journal of Heredity *85*, 233–238.

Rudolf, M., Machettira, A.B., Groß, L.E., Weber, K.L., Bolte, K., Bionda, T., Sommer, M.S., Maier, U.G., Weber, A.P.M., Schleiff, E., et al. (2013). In Vivo Function of Tic22, a Protein Import Component of the Intermembrane Space of Chloroplasts. Molecular Plant *6*, 817–829.

Sagor, G.H.M., Berberich, T., Takahashi, Y., Niitsu, M., and Kusano, T. (2013). The polyamine spermine protects Arabidopsis from heat stress-induced damage by increasing expression of heat shock-related genes. Transgenic Res *22*, 595–605.

Sakata, T., Oshino, T., Miura, S., Tomabechi, M., Tsunaga, Y., Higashitani, N., Miyazawa, Y., Takahashi, H., Watanabe, M., and Higashitani, A. (2010). Auxins reverse plant male sterility caused by high temperatures. Proceedings of the National Academy of Sciences *107*, 8569–8574.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular cloning: A laboratory manual: Vol. 2 (S.l.: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Schmid, M.W., Schmidt, A., Klostermeier, U.C., Barann, M., Rosenstiel, P., and Grossniklaus, U. (2012). A Powerful Method for Transcriptional Profiling of Specific Cell Types in Eukaryotes: Laser-Assisted Microdissection and RNA Sequencing. PLoS ONE *7*, e29685.

Schnell, D.J. (2019). The TOC GTPase Receptors: Regulators of the Fidelity, Specificity and Substrate Profiles of the General Protein Import Machinery of Chloroplasts. Protein J *38*, 343–350.

Schnell, D., Kessler, F., and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science *266*, 1007–1012.

Schünemann, D. (2007). Mechanisms of protein import into thylakoids of chloroplasts. Biological Chemistry *388*, 907–915.

Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W.B., Flügge, U.-I., and Kunze, R. (2003). ARAMEMNON, a Novel Database for Arabidopsis Integral Membrane Proteins. Plant Physiol. *131*, 16–26.

Scott, R.J. (2004). Stamen Structure and Function. THE PLANT CELL ONLINE *16*, S46–S60.

Shi, L.-X., and Theg, S.M. (2010). A Stromal Heat Shock Protein 70 System Functions in Protein Import into Chloroplasts in the Moss *Physcomitrella patens*. Plant Cell *22*, 205–220.

Shi, L.-X., and Theg, S.M. (2013). The chloroplast protein import system: From algae to trees. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1833*, 314–331.

Singh, S., Dey, S.S., Bhatia, R., Kumar, R., and Behera, T.K. (2019). Current understanding of male sterility systems in vegetable Brassicas and their exploitation in hybrid breeding. Plant Reprod *32*, 231–256.

Sjuts, I., Soll, J., and Bölter, B. (2017). Import of Soluble Proteins into Chloroplasts and Potential Regulatory Mechanisms. Front. Plant Sci. *8*. 168

Sorensen, A.-M., Kröber, S., Unte, U.S., Huijser, P., Dekker, K., and Saedler, H. (2003). The *Arabidopsis ABORTED MICROSPORES* (*AMS*) gene encodes a MYC class transcription factor. The Plant Journal *33*, 413–423.

Stahl, T., Glockmann, C., Soll, J., and Heins, L. (1999). Tic40, a New “Old” Subunit of the Chloroplast Protein Import Translocon. J. Biol. Chem. *274*, 37467–37472.

Stengel, A., Benz, P., Balsera, M., Soll, J., and Bölter, B. (2008). TIC62 Redox-regulated Translocon Composition and Dynamics. J. Biol. Chem. *283*, 6656–6667.

Stengel, A., Benz, J.P., Buchanan, B.B., Soll, J., and Bölter, B. (2009). Preprotein Import into Chloroplasts via the Toc and Tic Complexes Is Regulated by Redox Signals in Pisum sativum. Molecular Plant *2*, 1181–1197.

Su, P.-H., and Li, H. (2010). Stromal Hsp70 Is Important for Protein Translocation into Pea and *Arabidopsis* Chloroplasts. Plant Cell *22*, 1516–1531.

Tang, X., Hao, Y.-J., Lu, J.-X., Lu, G., and Zhang, T. (2019). Transcriptomic analysis reveals the mechanism of thermosensitive genic male sterility (TGMS) of Brassica napus under the high temperature inducement. BMC Genomics *20*, 644.

Teixeira, P.F., and Glaser, E. (2013). Processing peptidases in mitochondria and chloroplasts. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1833*, 360–370.

Tester, M., and Langridge, P. (2010). Breeding Technologies to Increase Crop Production in a Changing World. Science *327*, 818–822.

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y., and Stitt, M. (2004). mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. The Plant Journal *37*, 914–939.

Timmis, J.N., Ayliffe, M.A., Huang, C.Y., and Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nat Rev Genet *5*, 123–135.

Tripp, J., Inoue, K., Keegstra, K., and Froehlich, J.E. (2007). A novel serine/proline-rich domain in combination with a transmembrane domain is required for the insertion of AtTic40 into the inner envelope membrane of chloroplasts. The Plant Journal *52*, 824–838.

Trösch, R., Töpel, M., Flores-Pérez, Ú., and Jarvis, P. (2015). Genetic and Physical Interaction Studies Reveal Functional Similarities between ALBINO3 and ALBINO4 in Arabidopsis. Plant Physiol. *169*, 1292–1306.

Tsirigos, K.D., Peters, C., Shu, N., Käll, L., and Elofsson, A. (2015). The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic Acids Res *43*, W401–W407.

Vierstra, R.D. (2009). The ubiquitin–26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol *10*, 385–397.

Vizcay-Barrena, G., and Wilson, Z.A. (2006). Altered tapetal PCD and pollen wall development in the Arabidopsis ms1 mutant. Journal of Experimental Botany *57*, 2709–2717.

Waegemann, K., and Soll, J. (1996). Phosphorylation of the Transit Sequence of Chloroplast Precursor Proteins. J. Biol. Chem. *271*, 6545–6554.

Wang, A., Xia, Q., Xie, W., Datla, R., and Selvaraj, G. (2003). The classical Ubisch bodies carry a sporophytically produced structural protein (RAFTIN) that is essential for pollen development. Proceedings of the National Academy of Sciences *100*, 14487–14492.

Wang, K., Guo, Z.-L., Zhou, W.-T., Zhang, C., Zhang, Z.-Y., Lou, Y., Xiong, S.-X., Yao, X., Fan, J.-J., Zhu, J., et al. (2018). The Regulation of Sporopollenin Biosynthesis Genes for Rapid Pollen Wall Formation. Plant Physiol. *178*, 283–294.

Wilson, Z.A., and Zhang, D.-B. (2009). From Arabidopsis to rice: pathways in pollen development. Journal of Experimental Botany *60*, 1479–1492.

Wilson, Z.A., Morroll, S.M., Dawson, J., Swarup, R., and Tighe, P.J. (2001). The Arabidopsis MALE STERILITY1 (MS1) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors: MS1 a transcriptional regulator of male gametogenesis. The Plant Journal *28*, 27–39.

Wu, C., Seibert, F.S., and Ko, K. (1994). Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. J. Biol. Chem. *269*, 32264–32271.

Xia, S., Wang, Z., Zhang, H., Hu, K., Zhang, Z., Qin, M., Dun, X., Yi, B., Wen, J., Ma, C., et al. (2016). Altered Transcription and Neofunctionalization of Duplicated Genes Rescue the Harmful Effects of a Chimeric Gene in *Brassica napus*. Plant Cell *28*, 2060–2078.

Yang, J., Tian, L., Sun, M.-X., Huang, X.-Y., Zhu, J., Guan, Y.-F., Jia, Q.-S., and Yang, Z.-N. (2013). AUXIN RESPONSE FACTOR17 Is Essential for Pollen Wall Pattern Formation in Arabidopsis. Plant Physiol. *162*, 720–731.

Zhang, W. (2006). Regulation of Arabidopsis tapetum development and function by DYSFUNCTIONAL TAPETUM1 (DYT1) encoding a putative bHLH transcription factor. Development *133*, 3085–3095.

Zhang, D., Luo, X., and Zhu, L. (2011). Cytological analysis and genetic control of rice anther development. Journal of Genetics and Genomics *38*, 379–390.

Zhang, Z., Fan, Y., Xiong, J., Guo, X., Hu, K., Wang, Z., Gao, J., Wen, J., Yi, B., Shen, J., et al. (2020). Two young genes reshape a novel interaction network in *Brassica napus*. New Phytol *225*, 530–545.

Zhang, Z.-B., Zhu, J., Gao, J.-F., Wang, C., Li, H., Li, H., Zhang, H.-Q., Zhang, S., Wang, D.-M., Wang, Q.-X., et al. (2007). Transcription factor AtMYB103 is required for anther development by regulating tapetum development, callose dissolution and exine formation in Arabidopsis: Molecular cloning and functional analysis of AtMYB103. The Plant Journal *52*, 528–538.

Zhao, C., Liu, B., Piao, S., Wang, X., Lobell, D.B., Huang, Y., Huang, M., Yao, Y., Bassu, S., Ciais, P., et al. (2017). Temperature increase reduces global yields of major crops in four independent estimates. Proc Natl Acad Sci USA *114*, 9326–9331.

Zheng, Y.-Y., Lin, X.-J., Liang, H.-M., Wang, F.-F., and Chen, L.-Y. (2018). The Long Journey of Pollen Tube in the Pistil. IJMS *19*, 3529.

Zhou, Z., Dun, X., Xia, S., Shi, D., Qin, M., Yi, B., Wen, J., Shen, J., Ma, C., Tu, J., et al. (2012). BnMs3 is required for tapetal differentiation and degradation, microspore separation, and pollen-wall biosynthesis in Brassica napus. Journal of Experimental Botany *63*, 2041–2058.

Zhu, J., Chen, H., Li, H., Gao, J.-F., Jiang, H., Wang, C., Guan, Y.-F., and Yang, Z.-N. (2008). *Defective in Tapetal Development and Function 1* is essential for anther development and tapetal function for microspore maturation in Arabidopsis. The Plant Journal *55*, 266–277.

Zhu, Y., Dun, X., Zhou, Z., Xia, S., Yi, B., Wen, J., Shen, J., Ma, C., Tu, J., and Fu, T. (2010). A separation defect of tapetum cells and microspore mother cells results in male sterility in Brassica napus: the role of abscisic acid in early anther development. Plant Mol Biol *72*, 111–123.

6 Appendix

Table 9: Oligonucleotides used in this thesis.

name	sequence (5'-3')	application
Bark1RNAiF	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT TGG ATC ACC	GATEWAY cloning
Bark1RNAiR	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TTA ACC TCC	GATEWAY cloning
HY1F	AGT CST TCC CTT TAC TCA GCT C	Xia et al., 2016
HY1R	GAT GYG GCT CMR TGA CAA CAG A	Xia et al., 2016
HY2F	TTC TGA TCC TWC GTG CGC T	Xia et al., 2016
HY2R	ACT TTG GGA ACA CGK GTC AT	Xia et al., 2016
HY3F	GCA ATG GAG CTT TGT TCT TC	Xia et al., 2016
HY3R	ACT TTG GGA ACA CGK GTC AT	Xia et al., 2016
BnChimera AB F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAA CCC CAT TAT TGA CAA T	antibody
Bn Chimera AB R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCC ACC TCC GGA TCA CCT CGA GCT GGA TTC ACG	antibody
BnTic40A2 F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GGA GAA CCT AAC CCT A	GATEWAY cloning
BnTic40C2 F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GGA GAA CCT AAC CCT A	GATEWAY cloning
BnTic40A10 F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GGA GAA TCT AAC CCT G	GATEWAY cloning
BnTic40C9 F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GGA GAA TCT AAC AAC CCT GGT T	GATEWAY cloning
BnTic40 A2/A10 at R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCC ACC TCC GGA TCA ACC CGT CAT TCC TGG GAA	GATEWAY cloning
BnTic40 C2 at R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCC ACC TCC GGA TCA ACC CGT CAT TCC AGG GAA	GATEWAY cloning
BnTic40 C9 at R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCC ACC TCC GGA TCA ACC CGT CAA TCC TGG GAA	GATEWAY cloning
5 RACE R1	GAT TAC GCC AAG CTT GTT CAC TTC CCT TGA TAA CAA TGT TGG	Xia et al., 2016
5 RACE R2	GAT TAC GCC AAG CTT CCT TCA AAA GTC TTG CGA ATT GGG ACA C	Xia et al., 2016
3 RACE F1	GAT TAC GCC AAG CTT GAT TTT GAA GGC CAC AGA ACC GCA GAT G	Xia et al., 2016
3 RACE F2	GAT TAC GCC AAG CTT CTC ATT CTG ATC CTT CGT GCG CTT TAC G	Xia et al., 2016
5 RACE R1 CC	GAT TAC GCC AAG CTT TTC CCG AGG TAA GCT TCA GCG GTC TCC	RACE cloning
5 RACE R2 CC	GAT TAC GCC AAG CTT AAC CGT GCC TGT TGG GTT GGT AAC GC	RACE cloning
5 RACE R3 CC	GAT TAC GCC AAG CTT CGA GCT GGA TTC ACG GAT CCT C	RACE cloning
5 RACE R4 CC	GAT TAC GCC AAG CTT GAG TTC ACG AAT CCT CTT CTT CTC	RACE cloning
3 RACE R1 CC	GAT TAC GCC AAG CTT AGG AGA ACA GAG AGA AGA AAC GAG	RACE cloning

3 RACE R2 CC	GAT TAC GCC AAG CTT AGA GGA TTC GTG AAC TCC AAA AGG	RACE cloning
BnChimera prom A-B F	ATGAAGACTTTACGGGTCTCAGCGGGTCCAGTCAAA CATTTACACAG	GG cloning
BnChimera prom A-B R	ATGAAGACTTCAGAGGTCTCACAGATCCTTTAGCAAA CTTCAGATTCG AGAAAGAAACAGAACCCTAGTGTTTTCTGCATT	
Chimera C-D F1	ATGAAGACTTTACGGGTCTCACACCATGCTCGCTCCCCTT AAGGT	GG cloning; genotyping
Chimera C-D R1	TAGAAGACAAAGTTTTCTCGGATGCGAGGGAG	GG cloning; genotyping
Chimera C-D F2	TAGAAGACAAAACTCAAGGCCTCCCACG	GG cloning
Chimera C-D R2	TAGAAGACAAAGtTTTCATGCCGTGCTGTATA	GG cloning
Chimera C-D F3	TAGAAGACAAAACTGCAGTGGCCATAGCG	GG cloning
Chimera C-D R3	TAGAAGACAAAATGCCAGATTGGATTTCCAGCGA	GG cloning
Chimera C-D F4	TAGAAGACAACATTGTGTATTAAAAACAAGATCA CAAAGGAGAACAGAGAGAAGAAACGAG	GG cloning
Chimera C-D R4	ATGAAGACTTCAGAGGTCTCACCTTGGTGTCTGCCCCGC TTGGAC	GG cloning; genotyping
Chimera C-terminus c-d F5	ATGAAGACTTTACGGGTCTCACACC ATGCCTATTGGGAATGATGTGATTG	GG cloning
Chimera C-terminus c-d R5	ATGAAGACTTCAGAGGTCTCACCTTGGTGTCTGCCCCGC TTG	GG cloning
Chimera N-terminus C-D F6	ATGAAGACTTTACGGGTCTCACACCATGCTCGCTCCCCTT AAGGT	GG cloning
Chimera N-terminus C-D R6	TAGAAGACAAAGTTTTCTCGGATGCGAGGGAG	GG cloning
Chimera N-terminus C-D F7	TAGAAGACAAAACTCAAGGCCTCCCACG	GG cloning
Chimera N-terminus C-D R7	ATGAAGACTTCAGAGGTCTCACCTTCTTGTTTGATTTTTC GTGCTGTTT	GG cloning
cpTP F11	ATGAAGACTTTACGGGTCTCATCTGaacaATGGCTTCCTC TATGCTCTC	GG cloning
cpTP R11	ATGAAGACTTCAGAGGTCTCAGGTGccCACCTGCATGCA GTTAACTC	GG cloning
Chimera middle C-D F8	ATGAAGACTTTACGGGTCTCACACC ATGAACCCCATTATTGACCATA	GG cloning; genotyping
Chimera middle C-D F8	ATGAAGACTTCAGAGGTCTCACCTTGCCTCGAGCTGGA TTCACGG	GG cloning; genotyping
Chim F1 Seq	ACCCCACCCTCTCAACAGCT	sequencing; genotyping
Chim F2 Seq	GAGCTTTCTTCCACATCTCA	sequencing
LIIß F1-2 49 Seq For	CCTGTATCGAGTGGTGATTTTG	sequencing
LIIß F1-2 49 Seq Rev	CGGATAAACCTTTTCACGCCC	sequencing
p35S For LII	TGATATCTCCACTGACGTAAGG	
T35S rev	5' TAT GCT CAA CAC ATG AGC GAA AC 3'	sequencing
BnTic40C9 C-D aF	ATGAAGACTTTACGGGTCTCACACC ATGGAGAATCTAACAACCCTGGT	GG cloning
BnTic40C9 C-D aR	TAGAAGACAATGTTTTCATAGCTGTTTGCATTGC	GG cloning

BnTic40C9 C-D bF	TAGAAGACAAAACAATGATGAACCAGATGAATAC	GG cloning
BnTic40C9 C-D 1F	ATGAAGACTTTACGGGTCTCACACCATGAGCCAG TTTAATAATCCTGGATTC	GG cloning; genotyping
BnTic40C9 C-D 1R	TAGAAGACAATGATGACTGGGATTGAGGCTGG	GG cloning
BnTic40C9 C-D 2F	TAGAAGACAAATCAGGTGCTACTGTTGA	GG cloning
BnTic40C9 C-D 2R	TAGAAGACAATTTATCTTCCTCTACGACAACAC	GG cloning
BnTic40C9 C-D 3F	TAGAAGACAATAAAGCGATGAAAGAAGAAAAGAACTA CGCCTTT GAAGATGTTTCCCCT	GG cloning
BnTic40C9 C-D 3R	TAGAAGACAATAATGTCTCCGTCATTCTCTTGT	GG cloning ; genotyping
BnTic40C9 C-D 4F	TAGAAGACAAATTAAAGAATTTTGACCCGAATAG	GG cloning
BnTic40C9 C-D 4R	ATGAAGACTTCAGAGGTCTCACCTTACCCGTCAATCCT GGGAAGA	GG cloning
BnTic40A2 C-D aF	ATGAAGACTTTACGGGTCTCACACC ATGGAGAACCTAACCCTACTTTC	GG cloning
BnTic40A2 C-D aR	TAGAAGACAAGGACGGTGGCGGCACTG	GG cloning
BnTic40A2 C-D bF	TAGAAGACAActcATCATCATCAACCATAGGATC	GG cloning
BnTic40A2 C-D bR	TAGAAGACAATGTTTTCATAGCTGTTTGCATTGC	GG cloning
BnTic40A2 C-D cF	TAGAAGACAAAACAATGATGAACCAGATGAACAC	GG cloning
BnTic40A2 C-D 1F	ATGAAGACTTTACGGGTCTCACACCATGAGCCAGTTTAAC AACCCTGG	GG cloning
BnTic40A2 C-D 1R	TAGAAGACAATGACGACTGGGATTGAGATTGG	GG cloning
BnTic40A2 C-D 2F	TAGAAGACAAGTCAAGTGCTACTGTTGAT	GG cloning
BnTic40A2 C-D 2R	ATGAAGACTTCAGAGGTCTCACCTTACCCGTCATTCCTG GGAAGA	GG cloning
BnTic40A10 C-D aF	ATGAAGACTTTACGGGTCTCACACC ATGGAGAATCTAACCTTGGTTTC	GG cloning; genotyping
BnTic40A10 C-D aR	TAGAAGACAATGTTTTCATAGCTGTTTGCATC	GG cloning
BnTic40A10 C-D bF	TAGAAGACAAAACAATGATGAACCAGATGA	GG cloning
BnTic40A10 C-D 1F	ATGAAGACTTTACGGGTCTCACACCatgACGCAAAA CAGCCAGTTTAATAAC	GG cloning
BnTic40A10 C-D 1R	TAGAAGACAATGACGACTGGGACTGAGATTGA	GG cloning
BnTic40A10 C-D 2F	TAGAAGACAAGTCAGGGGCTACTGTTGA	GG cloning
BnTic40A10 C-D 2R	TAGAAGACAAAACATCTTCAAAGGCGTAGTTCT	GG cloning
BnTic40A10 C-D 3F	TAGAAGACAATGTTTCCCCTGAGGAAAC	GG cloning
BnTic40A10 C-D 3R	TAGAAGACAATAATGTCTCCGTCATTCTCTTGT	GG cloning
BnTic40A10 C-D 4F	TAGAAGACAAATTAAAGAATTTTGACCTGAATAG	GG cloning; genotyping
BnTic40A10 C-D 4R2	ATGAAGACTTCAGAGGTCTCACCTT ACCCGTCATTCCTGGGAAGA	GG cloning

BnTic40C2 C-D aF	ATGAAGACTTTACGGGTCTCACACC ATGGAGAACCTAACCCTAGTTTC	GG cloning
BnTic40C2 C-D aR	TAGAAGACAACGATGACGGTGGCGGCACTGCT	GG cloning
BnTic40C2 C-D bF	TAGAAGACAAATCGTCATCATCAACCATA	GG cloning
BnTic40C2 C-D bR	TAGAAGACAATGTTTTCATAGCTGTTTGCATTGCAT	GG cloning
BnTic40C2 C-D cF	TAGAAGACAAAACAATGATGAACCAGATGAAC	GG cloning
BnTic40C2 C-D 1F	ATGAAGACTTTACGGGTCTCACACCatgACTCAAAA CAGCCAGTTTAATAATC	GG cloning; genotyping
BnTic40C2 C-D 1R	TAGAAGACAATGACGACTGGGATTGAGATTGG	GG cloning
BnTic40C2 C-D 2F	TAGAAGACAAGTCAAGTGCCACTGTTGA	GG cloning
BnTic40C2 C-D 2R	TAGAAGACAAACTTGTCTCAGAGACTTCTGCAT	GG cloning
BnTic40C2 C-D 3F	TAGAAGACAAAAGTGCTCCCAAGGAAGC	GG cloning
BnTic40C2 C-D 3R	TAGAAGACAATAATGTCTCCGTCATTCTCTTGT	GG cloning
BnTic40C2 C-D 4F	TAGAAGACAAATTAAAGAACTTCGACCTGAAT	GG cloning
BnTic40C2 C-D 4R2	ATGAAGACTTCAGAGGTCTCACCTT ACCCGTCATTCCAGGGAAGA	GG cloning; genotyping
BnTic40C09_Prom A-B F1	ATGAAGACTTTACGGGTCTCAGCGGGGATGGTCGGGTTA TAAGAATTA	GG cloning
BnTic40C09_Prom A-B R1	ATGAAGACTTCAGAGGTCTCACAGACTTTGGTTTAAGCTG TCCTGT	GG cloning
BnTic40A10_Prom A-B F	ATGAAGACTTTACGGGTCTCAGCGG TCTGCATTGTTTGATGACAC	GG cloning
BnTic40A10_Prom A-B R	ATGAAGACTTCAGAGGTCTCACAGA CTTTGTTGTCTCTTTGCTTT	GG cloning
C9 SDM 321 F/V for	AGATCCCACAgttCAGAAGATGC	side directed mutagenesis
C9 SDM 321 F/V rev	TCTATCATTTTCTCTAAAACTTCTACTG	side directed mutagenesis
C9 SDM 343 R/L for	CAAATGGGTGCTTAAGAATCCTC	side directed mutagenesis
C9 SDM 343 R/L rev	AAAGTTTCTGGGTTCCTC	side directed mutagenesis
C9 SDM 378 P/L for	GAATTTTGACCTGAATAGCCCCG	side directed mutagenesis
C9 SDM 378 P/L rev	TTTAAGGTCTCCGTCATTCTC	side directed mutagenesis
C9 SDM 386 G/Q for	AGTTAAGCAACAATTCGATCAATTAGGACTGACTCCAG	side directed mutagenesis
C9 SDM 386 G/Q rev	TCGGGGCTATTCGGGTCA	side directed mutagenesis
C9 SDM 408 S/A for	CCCTGATGTTGCAATGGCATTCC	side directed mutagenesis
C9 SDM 408 S/A rev	TTCTCCATTATCTTAGAGATGACTTC	side directed mutagenesis
C9 GG 391 rev	TAGAAGACAAAGTCCTAATTGATCGAATCC	GG cloning
A10 GG 394 for	TAGAAGACAAGACTGACTCCAGAAGAAGTC	GG cloning
Tic40C9SDM#33 for	GTTTCCCCTGAGGAAACC	side directed mutagenesis

Tic40C9SDM#33 rev	GTCTTCAAAGGCGTAGTTC	side directed mutagenesis
SALK_076646C LP	AAGAGATCACAGGAGGTTTTGC	genotyping
SALK_076646C RP	ACTCCTTCCTCCTTTTCTCCC	genotyping
SALK_042862C LP	GCTTCCATACTTGATGCAAGC	genotyping
SALK_042862C RP	AGGGAGAAAAGGAGGAAGGAG	genotyping
Salk LB1.3	ATTTTGCCGATTTCGGAAC	genotyping
SALK LP2	CTTTTTGGGCAATGGAGAAGTG	genotyping
SALK RP2	GGTGATAAAGAGGAATGAGTTGG	genotyping
BnChimera F RT	GAG GGT GTG AAT GCT GTC TT	qRT-PCR
BnChimera R RT	CTC TCC AGA ATC ATC TGT GG	qRT-PCR
Bnactin3 F RT	TCC ATC CAT CGT CCA CAG	qRT-PCR
Bnactin3 R RT	GCA TCA TCA CAA GCA TCC TT	qRT-PCR
BnTic40A2 F RT	GGA CTC CGA AAA TCA CC T	qRT-PCR
BnTic40A2 R RT	TCC TCC TGT GCT TCA CAA CC	qRT-PCR
BnTic40C2 F RT	GCC GAA CTC CTA AAA TCA TC	qRT-PCR
BnTic40C2 R RT	TCT GCT GAT CAC GGC TCG	qRT-PCR
BnTic40A10 F RT	AAG GGC TGG TTT GTC AGT AG	qRT-PCR
BnTic40A10 R RT	TTC ACC ACT CCC ACT CAT GT	qRT-PCR
BnTic40C9 F RT	GAA GTT AAG CAA GGA TTC GAT C	qRT-PCR
BnTic40C9 R RT	CAG TCC ATT AAC GCT GCT TC	qRT-PCR
BARK1 RT F	CCTTCTCGTCCTCCTCACAACCATA	qRT-PCR
BARK1 RT R	ACGGCGGAGGATTGAATGATTTAGC	qRT-PCR
Atactin2 F RT	AAT TAC CCG ATG GGC A	qRT-PCR
Atactin2 R RT	TCA TAC TCG GCC TTG GA	qRT-PCR
BnaC S-D for	CCC TGA TGT TGA CAT GGC ATT CCA GAA TCC TAG	side directed mutagenesis
BnaC S-D rev	TTC TCC ATT ATC TTA GAG ATG AC	side directed mutagenesis
3 RACE F1	GAT TAC GCC AAG CTT GAT TTT GAA GGC CAC AGA ACC GCA GAT G	Xia et al., 2016
3 RACE F2	GAT TAC GCC AAG CTT CTC ATT CTG ATC CTT CGT GCG CTT TAC G	Xia et al., 2016
5 RACE R3 CC	GAT TAC GCC AAG CTT CGA GCT GGA TTC ACG GAT CCT C	RACE cloning
5 RACE R4 CC	GAT TAC GCC AAG CTT GAG TTC ACG AAT CCT CTT CTT CTC	RACE cloning
DNA-Methyl-Transferase F GW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGA GATAGAACCATGACGGAATCTCTTCAG	GATEWAY cloning
DNA meth trans R GW	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCT CCGGATCAGGGGTCAAGTTGGGATGC	GATEWAY cloning

Table 10: Constructs used for this thesis.

construct	vector	application	source
BnaCTic40 C9 S-D #4	pGEX6p1	SDM; pulldown	this work
BnaCTic40 C9 WT#20	pGEX6p1	pulldown	B. Bölter
BARK1	pDONOR207	GATEWAY entry clone	this work
BARK1 RNAi	pK7GWIWG2 (I)	GATEWAYcloning; RNAi	this work
BnTic40 A2	pDONR207	GATEWAY entry clone	this work
BnTic40 C2	pDONR207	GATEWAY entry clone	this work
BnTic40 A10	pDONR207	GATEWAY entry clone	this work
BnTic40 C9	pDONR207	GATEWAY entry clone	this work
BnTic40 A2	pH2GW7	GATEWAY*; A. thaliana* tic40 complementation	this work
BnTic40 C2	pH2GW7	GATEWAY*; A. thaliana* tic40 complementation	this work
BnTic40 A10	pH2GW7	GATEWAY*; A. thaliana* tic40 complementation	this work
BnTic40 C9	pH2GW7	GATEWAY*; A. thaliana* tic40 complementation	this work
AtGAP B	pSP65	*In vitro* import	B. Bölter
AOX	pDest14	*In vitro* import	C. Carrie
BnDNA-Methyl-Transferase	pDONR207	GATEWAY entry clone	this work
BnDNA-Methyl-Transferase	pDest14	GATEWAY import clone	this work
35S::Chimera-GFP_BASTA	pCAMBIA	LII plasmid; loclisation studies	this work
35S::Chimera-1-1627-GFP_BASTA	pCAMBIA	LII plasmid; loclisation studies	this work
ChimeraProm::Chimera__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera_1-1637 __BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::SSUtp_Chimera _2295-4161__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::SSUtp_Chimera_ 1638-2296__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
35S::Chimera_1-1637__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
35S::SSUtp_Chimera_ 2295-4161__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
35S::SSUtp_Chimera_ 1638-2296__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera_ 1-2296__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera_ 1638-4161__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
35S::Chimera_1638-4161__BASTA	pCAMBIA	LII plasmid; *A.thaliana* stable transformation	this work
BnTic40A10Prom::BnTic40A10	pCAMBIA	LII Plasmid	this work
BnTic40A10Prom::BnTic40C9	pCAMBIA	LII Plasmid	this work
BnTic40A10Prom::BnTic40C2	pCAMBIA	LII Plasmid	this work
BnTic40C9Prom::BnTic40A10	pCAMBIA	LII Plasmid	this work

BnTic40C9Prom::BnTic40C9	pCAMBIA	LII Plasmid	this work
BnTic40C9Prom::BnTic40C2	pCAMBIA	LII Plasmid	this work
BnTic40C9Prom::BnTic40C9SDM378	pCAMBIA	LII Plasmid	this work
BnTic40C9Prom::BnTic40C9SDM408	pCAMBIA	LII Plasmid	this work
ChimeraProm::Chimera__ BnTic40A10Prom::BnTic40A10_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40A10Prom::BnTic40C9_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40A10Prom::BnTic40C2_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40C9Prom::BnTic40A10_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40C9Prom::BnTic40C9_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40C9Prom::BnTic40C2_ BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40C9Prom:: BnTic40C9SDM378_BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ChimeraProm::Chimera__ BnTic40C9Prom:: BnTic40C9SDM408_BASTA	pCAMBIA	LIII Plasmid; *A.thaliana* stable transformation	this work
ADH1Prom::BnTic40C9_445-1365	pGAD	LII Plasmid; YTH	this work
ADH1Prom::BnTic40C2_433-1350	pGAD	LII Plasmid; YTH	this work
ADH1Prom::BnTic40A10_480-1350	pGAD	LII Plasmid; YTH	this work
ADH1Prom::BnTic40A2_407-1371	pGAD	LII Plasmid; YTH	this work
ADH1Prom700::Chimera	pGBK	LII Plasmid; YTH	this work
ADH1Prom700::Chimera_1-1637	pGBK	LII Plasmid; YTH	this work
ADH1Prom700::Chimera_2295-4161	pGBK	LII Plasmid; YTH	this work
ADH1Prom700::Chimera_1638-2296	pGBK	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_445-1365_SDM321	pGAD	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_445-1365_SDM343	pGAD	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_445-1365_SDM378	pGAD	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_445-1365_SDM386	pGAD	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_445-1365_SDM408	pGAD	LII Plasmid; YTH	this work
ADH1Prom:: BnTic40C9_1-391/A10_394-457	pGAD	LII Plasmid; YTH	this work
BnTic40C9Promoter	pUC57	LI	this work

BnTic40A10Promoter	pUC57	LI	this work
BnChimeraPromoter	pUC57	LI	this work
BnTic40C9_445-1365	pUC57	LI; no TMD	this work
BnTic40A2_407-1353	pUC57	LI; no TMD	this work
BnTic40A10_480-1371	pUC57	LI; no TMD	this work
BnTic40C2_433-1350	pUC57	LI; no TMD	this work
BnTic40C9_445-1365_SDM321	pUC57	LI; no TMD	this work
BnTic40C9_445-1365_SDM343	pUC57	LI; no TMD	this work
BnTic40C9_445-1365_SDM378	pUC57	LI; no TMD	this work
BnTic40C9_445-1365_SDM386	pUC57	LI; no TMD	this work
BnTic40C9_445-1365_SDM408	pUC57	LI; no TMD	this work
BnTic40C9	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40C9_SDM378	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40C9_SDM408	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40A2	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40A10	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40C2	pUC57	LI; *A.thaliana* stable transformation	this work
BnTic40A2_407-560	pUC57	L0; YTH	this work
BnTic40A2_561-1350	pUC57	L0; YTH	this work
BnTic40C9_1-417	pUC57	L0; *A.thaliana* stable transformation	this work
BnTic40C9_418-566	pUC57	L0; *A.thaliana* stable transformation	this work
BnTic40C9_454-566	pUC57	L0; YTH	this work
BnTic40C9_567-698	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C9_699-1119	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C9_1120-1365	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_1-294	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_294-412	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_413-561	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_433-561	pUC57	L0; YTH	this work
BnTic40C2_562-784	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_785-1100	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40C2_1101-1350	pUC57	L0; *A.thaliana* stable transformation;YTH	this work

BnTic40A10_1-419	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40A10_420-572	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40A10_442-572	pUC57	L0; YTH	this work
BnTic40A10_573-740	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40A10_741-1121	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnTic40A10_1122-1371	pUC57	L0; *A.thaliana* stable transformation;YTH	this work
BnChimera_N-term and middle	pUC19	InFusionClone/ RACE PCR (ms gen)	this work
Chimera_2296-4161	pUC57	LI; C-terminus	this work
Chimera_1-293	pUC57	L0	this work
Chimera_294-1715	pUC57	L0	this work
Chimera_1716-2172	pUC57	L0	this work
Chimera_2173-4161	pUC57	L0	this work
Chimera_294-1637	pUC57	L0	this work
Chimera 1-1637	pUC57	LI	this work
Chimera FL	pUC57	LI	this work
Chimera_1636-2296	pUC57	LI	this work
tabacco_ssuTP	pUC57	LI; cTP	this work
dummy B-C	pUC57	LI	Binder *et al.*, 2014
dummy D-E	pUC57	LI	Binder *et al.*, 2014
dummy F-G	pUC57	LI	Binder *et al.*, 2014
BASTA F-G	pUC57	LI	Binder *et al.*, 2014
35S Promoter A-B	pUC57	LI	Binder *et al.*, 2014
35S Terminator E-F	pUC57	LI	Binder *et al.*, 2014
Nos Terminator E-F	pUC57	LI	Binder *et al.*, 2014
ADH1 Prom FL A-B	pUC57	LI	Binder *et al.*, 2014
ADH1 Prom 700 A-B	pUC57	LI	Binder *et al.*, 2014
Gal4AD pGAD B-C	pUC57	LI	Binder *et al.*, 2014
PGAL4BD pGBK B-C	pUC57	LI	Binder *et al.*, 2014
ScADH1 Term E-F	pUC57	LI	Binder *et al.*, 2014
Chimera C-term	pEX-K4	cloning for FL construct	Eurofins

Curriculum Vitae

Personal data

Family name: Schuhmann

First name: Petra

Date of birth: 07.06.1987

Education

Since 07/2014	PhD student Ludwig-Maximilians-Universität, Munich, Germany PhD thesis: Characterization of a thermosensitive male sterility system in *Brassica napus*
10/2010-03/2013	Master in Biosciences Technische Universität Kaiserslautern, Germany Master thesis: Detailed characterization of the adenine-nucleotide transporter pANT1 from *Arabidopsis thaliana* in the liposomal system
10/2007-09/2010	Bachelor in Biosciences Technische Universität Kaiserslautern, Germany Bachelor thesis: Localisation studies of carbonic anhydrase IV mutants in *xenopus* oocytes
09/2006-07/2007	Voluntary year in the forest kindergarten in Kaiserslautern
03/2006	Abitur (university entrance level) Albert-Schweitzer Gymnasium, Kaiserslautern, Germany

Conference

09/2017: Botanikertagung in Kiel, Germany

Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt wurde.

München, den 11.03.2020 _____

Petra Schuhmann

Erklärung

Hiermit erkläre ich, dass ich zuvor nicht versucht habe, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen. Die vorliegende Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

München, den 11.03.2020 _____

Petra Schuhmann

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