# The Mitochondrial Influence on Nuclear Gene Expression in Cytoplasmic Male-Sterile *Brassica napus*

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**Cover:**
Flowering CMS-line 4:19
Photo Maria Abrahamsson

# Abstract

Phenotypic, genetic and molecular studies were made on *Brassica napus* (*Arabidopsis thaliana*) lines carrying mitochondria with DNA from both *B. napus* and *A. thaliana*. The lines were isogenic regarding the nuclear and chloroplast genomes, which consisted of *B. napus* DNA. Most lines contained large but variable portions of *A. thaliana* mitochondrial DNA detected by a dense set of *A. thaliana* specific markers. Several of the *A. thaliana* sequences gave rise to novel transcripts. The lines were divided into three classes; male-sterile, semi-sterile and fertile according to their stamen morphology. The flowers of the male-sterile lines were characterized by replacement of stamens with carpeloid organs, which resemble the *apetala3* (*ap3*) and *pistillata* (*pi*) mutants found in *A. thaliana*. The *AP3* and *PI* gene expression were lower in the male-sterile lines. The *AP3* expression was down-regulated in the stamens shortly before these organs developed carpeloid characteristics. Repression of *PI* succeeded that of *AP3* and might be a consequence of loss of *AP3* activity. Low levels of AP3 and PI proteins were found in a male-sterile line. These results suggest that *AP3* expression in stamens depends on proper mitochondrial function and correct nuclear–mitochondrial interactions. To study the nuclear gene expression profiles of flower buds in the lines, two different types of microarrays were used. Gene expression profiling revealed that a large number of genes differed in expression between the lines. These results show that the mitochondrial genome strongly influences nuclear gene expression and reveal the importance of retrograde signalling between the mitochondria and the nucleus. In addition, the mitochondria directly or indirectly influence several different aspects of plant development and metabolism, since not only flower morphology but also growth rate, flowering time and adenylate content were affected. An additional CMS-system, *B. napus* (Ogu-INRA) was also studied. The conclusion is that the two CMS-systems differ in expression at stage 8, likely due to timing. The nuclear gene expression of the *B. napus* (*A. thaliana*) CMS-line was altered already at stages 0-5 while the expression of the Ogu-INRA CMS-line was altered at stage 8. This was reflected in the phenotypes of the two different CMS-lines.

*Keywords*: *APETALA3*, flower development, homeotic genes, male-sterility, microarray, mitochondrial gene expression, *PISTILLATA*, restoration of fertility, retrograde signalling, somatic hybrids

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*Till min farfar, Gustav Adolf Carlsson i Hallstorp*

# Contents

# Appendix

## Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

**I.** Carlsson, J., Leino, M. & Glimelius, K. 2007. Mitochondrial genotypes with variable parts of *Arabidopsis thaliana* DNA affect development in *Brassica napus* lines. *Theoretical and Applied Genetics* DOI 10.1007/s00122-007-0593-2.

**II.** Carlsson, J., Lagercrantz, U., Sundström, J., Teixeira, R., Wellmer, F., Meyerowitz, E.M. & Glimelius, K. 2007. Microarray analysis reveals altered expression of a large number of nuclear genes in developing cytoplasmic male sterile *Brassica napus* flowers. *The Plant Journal 49,* 452-462.

**III.** Carlsson, J., Bitton, F., Renou, J-P., Budar, F. & Glimelius, K. The impact of novel mitochondrial genomes on nuclear gene expression in two *Brassica napus* CMS-systems. (manuscript).

**IV.** Sundström, J.F., Carlsson, J., & Glimelius, K. Mitochondrial regulation of the nuclear encoded transcription factors APETALA3 and PISTILLATA in a *Brassica napus* CMS-system. (manuscript).

Paper **I** and **II** are reproduced by permission of the journals concerned.

# Abbreviations

| | |
|---|---|
| ADP | adenosine diphosphate |
| *AG* | *AGAMOUS* |
| *At* | *Arabidopsis thaliana*, thale cress |
| *A. thaliana* | *Arabidopsis thaliana*, thale cress |
| At3 | *Arabidopsis thaliana* chromosome III |
| *AP1* | *APETALA1* |
| *AP2* | *APETALA2* |
| *AP3* | *APETALA3* |
| *ap3* | *apetala3* mutant |
| *AtAOX1a* | *A. thaliana* alternative oxidase 1a |
| ATP | adenosine triphosphate |
| *Bn* | *Brassica napus*, rapeseed |
| *B. napus* | *Brassica napus*, rapeseed |
| CATMA | Complete Arabidopsis Transcriptome MicroArray |
| cDNA | complementary DNA |
| *DcMADS2* | *Daucus carota APETALA3* |
| *DcMADS3* | *Daucus carota PISTILLATA* |
| CMS | cytoplasmic male-sterility |
| *DEF* | *DEFICIENS* |
| DNA | deoxyribonucleic acid |
| EU | European Union |
| *GLO* | *GLOBOSA* |
| INRA | l'Institut National de la Recherche Agronomique |
| *LFY* | *LEAFY* |
| n | the haploid chromosome number |
| nap | *napus* |
| Ogu | Ogura |
| *orf* | *open reding frame* |
| PCR | polymerase chain reaction |
| *PI* | *PISTILLATA* |
| *pi* | *pistillata* mutant |
| *PLE* | *PLENA* |
| pol | Polima |
| PPR | pentatricopeptide repeat |
| qRT-PCR | quantitative RT-PCR |
| RAPD | random amplified polymorphic DNA |
| *Rf* | *restorer-of-fertility* |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase PCR |
| SAM | shoot apical meristem |
| *STK* | *SEEDSTICK* |
| *SEP1-4* | *SEPALLATA1-4* |
| *SHP1-2* | *SHATTERPROOF1-2* |
| *SQUA* | *SQUAMOSA* |
| *UFO* | *UNUSUAL FLORAL ORGANS* |
| *WAP3* | *Triticum aestivum APETALA3* |
| *WPI1* | *Triticum aestivum PISTILLATA* |

# Introduction

It is of great importance to understand flower development and plant reproduction. Not only for the mere satisfaction of getting a deeper understanding of the mechanisms behind flower development, but also since plants are of major importance for human consumption. We utilise plants for a wide range of purposes, for example to build houses, produce fabrics and paper, as fuels and of course as food. The knowledge of flower development and plant reproduction may be used by breeders to generate novel hybrids and cultivars designed to suit the demands that producers and consumers may have.

Plants propagate sexually through seeds or vegetativelly via for example roots, tubers, stem or leaf cuttings, or by tissue culture. Sexual reproduction is of advantage since new and desired traits can be obtained for example when creating new hybrids. The breeders are able to combine desired traits through crossings between parental lines carrying the traits asked for. A practical problem in breeding, however, is that many plant species used for human food production are self-pollinating. That is when breeders wish to cross different parental lines and combine certain genotypes with desired traits they need to manipulate the hybridisation in one way or another. One way is to remove the stamens of the mother-line by emasculation and thus inhibit pollen production. This laborious procedure can be circumvented by using male-sterile lines as mother-lines. Male-sterility can either be regulated by nuclear genes or mitochondrial genes that is, cytoplasmic male sterility (CMS). CMS-lines do not produce viable pollen, and the female fertility is un-affected. Even though a lot of research has been carried out regarding flower development and CMS, the precise mechanisms behind CMS are yet to be understood.

# Flower development

Large portions of the genetic regulation of flower development is similar for all angiosperms (Soltis *et al.*, 2002; Jack, 2004; Kramer & Hall, 2005; Krizek & Fletcher, 2005). The typical angiosperm flower consists of four whorls of distinct organs (Figure 1). The two outermost whorls, the sterile perianth, are denoted first and second and consist of sepals and petals respectively. The third whorl harbours the male reproductive organs, the stamens, and the forth and innermost whorl bear the female reproductive organs, the carpels.

The genetic regulation of flower development have been elucidated mainly through studies of the two plant species *Arabidopsis thaliana* and *Antirrhinum majus* and is summarised in the ABC-model (Schwarz-Sommer *et al.*, 1990; Coen & Meyerowitz, 1991; Theißen, 2001). In the ABC-model (Figure 1) the floral organs are under control of the three gene functions, A, B and C, which act in a combinatorial manner. The A function alone specifies the sepals, while the A and B functions together produce the petals. B together with C defines stamens and the

C function alone specifies the carpels. The C function also prevents an indeterminate floral meristem growth. Furthermore, the A and C functions are antagonistic to each other.

In *A. thaliana* the A function is determined by *APETALA1* (*AP1*) (Irish & Sussex, 1990; Mandel *et al.*, 1992; Gustavson-Brown *et al.*, 1994) and *APETALA2* (*AP2*) (Bowman *et al.*, 1989; Kunst *et al.*, 1989; Bowman *et al.*, 1991). The B function is controlled by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Bowman*, et al.*, 1989; Bowman*, et al.*, 1991; Jack *et al.*, 1992; Goto & Meyerowitz, 1994). The C function is regulated by *AGAMOUS* (*AG*) (Bowman*, et al.*, 1989; Yanofsky *et al.*, 1990; Bowman*, et al.*, 1991; Mizukami & Ma, 1992). In addition, *AP2* and *AG* interact antagonistically (Bowman*, et al.*, 1991). Orthologous genes have been found in a wide range of species such as *A. majus*, *Daucus carota* and *Triticum aestivum* (Kramer *et al.*, 2004; Hernandez-Hernandez *et al.*, 2007), and the ABC-model has been described in a variety of species for example *Petunia hybrida* (Angenent *et al.*, 1992), *Oryza sativa* (Nagasawa *et al.*, 2003) and *Zea mays* (Whipple *et al.*, 2004). The ABC-model has been extended with an E-function. The four *SEPALLATA* genes (*SEP1*, *SEP2*, *SEP3* and *SEP4*) (Ma *et al.*, 1991; Purugganan *et al.*, 1995; Pelaz *et al.*, 2000; Malcomber & Kellogg, 2005) regulate the E-function. *SEP1-3* redundantly regulate the petal, stamen and carpel development and *SEP4* is required for the sepal development. There is also a regulation of the ovule-development that sometimes is referred to as the D-function. Ovule-identity is determined by *AG*, *SEEDSTICK* (*STK*) (Rounsley *et al.*, 1995) and *SHATTERPROOF1* and *2* (*SHP1* and *SHP2*) (Ma*, et al.*, 1991; Savidge *et al.*, 1995; Flanagan *et al.*, 1996).

If the A-function is removed the theoretical flower phenotype will develop carpels in whorls 1 and 4, and stamens in whorls 2 and 3 (Figure 1). The *ap2* mutant in *A. thaliana* has this phenotype, while the *ap1* mutant has additional alterations such as an extra set of miniature flowers within the original flower. A deletion of the B-function will give flowers with sepals in the two outermost whorls and carpels in the remaining two whorls. This phenotype is found in *ap3* and *pi* mutants. The removal of the C-function will give flowers with only sepals and petals, and this phenotype is found in the *ag* mutant. Finally, when the E-function is completely deleted, that is in the quadruple *sep* mutant, the flower only produces leaf-like structures. Intriguingly, CMS-lines sometimes obtain homeotic conversions of the stamens that are transformed into carpeloid or petaloid structures (Kofer *et al.*, 1991; Zubko *et al.*, 1996; Ogihara *et al.*, 1997; Linke *et al.*, 1999b; Murai *et al.*, 2002; Leino *et al.*, 2003; Linke *et al.*, 2003) that partly resemble the B- and C-mutants found in *A. thaliana*.

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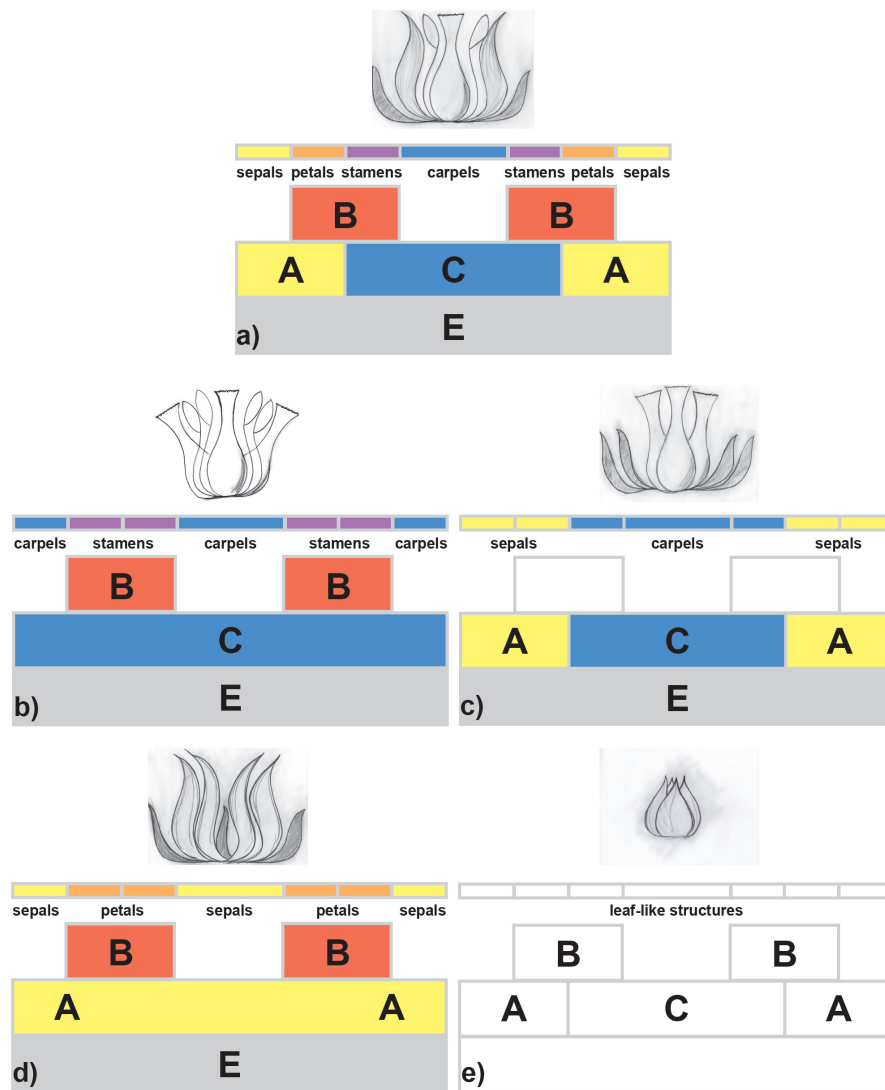
**Figure 1**. In the extended ABC-model the gene functions A (*AP1* and *AP2*), B (*AP3* and *PI*), C (*AG*) and E (*SEP1-4*) acts in a combinatorial manner to produce a proper flower with sepals in whorl 1, petals in whorl 2, stamens in whorl 3 and carpels in whorl 4 (a). In A-mutants (b) whorl 1 and 2 organs are homeotically converted into carpels and stamens. The B-mutants (c) carry sepals and carpels in whorl 2 and 3 instead of petals and stamens. The flower of the C-mutant (d) consists of only sepals and carpels, while the E-mutants (e) resemble a bud of leaf-like organs.

# Cytoplasmic male sterility

The CMS phenotype is caused by disturbed nuclear-mitochondrial interactions (Figure 2) and has been detected in more than 150 species (Kaul, 1988). It is often observed in hybrid lines obtained from intra- or inter-specific crosses, that is lines with the nucleus from one species and the cytoplasm from another (Figure 2). In general the stamens and the sporogenous tissues are affected which result in inhibited pollen production. Pollen formation is disrupted at the meiotic or postmeiotic stages in many of the studied and commercially utilized CMS-systems. For example, in *P. hybrida* (*Petunia parodii*) (Conley & Hanson, 1995), *Heliantus annuus* (PET1) (Smart *et al.*, 1994) and *Z. mays* (Texas) (Warmke & Lee, 1977) the tapetal layer in the anthers is degenerated or aborted. In *Phaseolus vulgaris* (*pvs*) (Abad *et al.*, 1995) and *Sorghum bicolor* (A3) (Tang *et al.*, 1998) microspores and pollen are formed and the development is arrested before the pollen is released.

In other CMS-systems the proper organ identity of stamens is disturbed and homeotic conversions are often observed. Moreover, petals can be modified as well as parts of the carpels. In these instances only the male fertility is affected leaving female fertility intact (Hanson & Bentolila, 2004; Chase, 2007; Pelletier & Budar, 2007). Homeotic conversions of the stamens are clearly observed in a number of CMS-lines. In for example, the *Brassica napus* CMS-lines analysed in this thesis stamens are replaced by carpeloid organs with ovule-like structures found at the internal margins of the unfused carpeloid structures (Leino*, et al.*, 2003; Teixeira *et al.*, 2005a). Similar homeotic modifications have also been found in other CMS-systems such as *D. carota* (Linke*, et al.*, 1999b; Linke*, et al.*, 2003), *T. aestivum* (Ogihara*, et al.*, 1997; Murai*, et al.*, 2002) and *Nicotiana tabacum* (Kofer*, et al.*, 1991; Zubko*, et al.*, 1996). These CMS-phenotypes resemble the homeotic conversions of stamens to carpels found in *A. thaliana ap3* and *pi* mutants (Bowman*, et al.*, 1989; Bowman*, et al.*, 1991). Several recent reports have shown that the CMS-inducing gene or genes indeed causes alterations in the expression of transcription factors regulating floral development (**II**) (Zubko *et al.*, 2001; Murai*, et al.*, 2002; Linke*, et al.*, 2003; Hama *et al.*, 2004; Geddy *et al.*, 2005; Teixeira*, et al.*, 2005a).

*D. carota* CMS-lines display a wide range of flower morphologies depending on which sub-species that is used as cytoplasmic donor, for example petaloid stamens, rudimentary stamens, no or rudimentary petals and stamens (Linke *et al.*, 1999a) and normal flowers with brown anthers (Nothnagel *et al.*, 2000) are found. Flowers with carpeloid organs in whorl three are also observed (Linke*, et al.*, 2003). In these flowers a reduction in expression of *DcMADS2* and *DcMADS3*, orthologous of *A. thaliana AP3* and *PI* respectively, were observed.

*T. aestivum* has also been combined with an extensive number of cytoplasms from a variety of related species, resulting in a corresponding range of flower phenotypes (Kaul, 1988), for example homeotic conversions of the stamens into carpeloid structures have been observed (Murai & Tsunewaki, 1993; Murai*, et al.*,

2002). It was shown that *T. aestivum AP3* (*WAP3*) and *T. aestivum PI* (*WPI1*) were expressed in the lodicules, but not in the primordia of the carpeloid stamens. Contrary, *WAP3* and *WPI1* in fully fertile *T. aestivum* were expressed in the lodicules and in the primordia of the stamens (Murai, *et al.*, 2002; Hama, *et al.*, 2004).
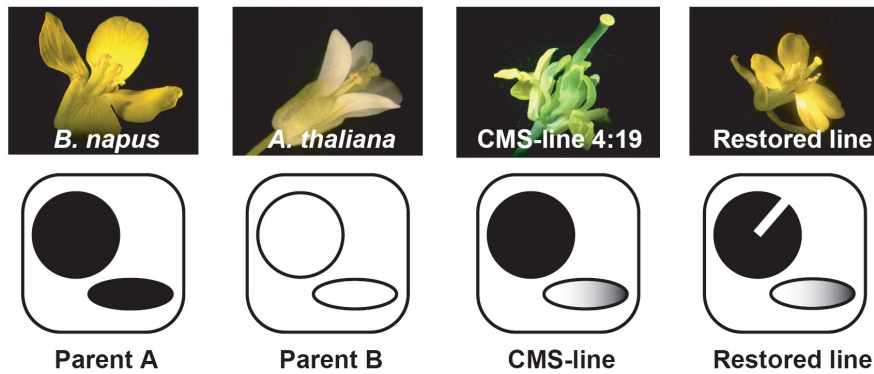


**Figure 2**. A schematic representation of a CMS-system exemplified by the *B. napus* (*A. thaliana*) system discussed in this thesis. The basis of CMS is that two parents are combined so that the offspring are male-sterile (the CMS-line). The CMS-line inherits the nucleus from parent A and the mitochondria from parent B. Incompatibilities between the mitochondrial and nuclear genomes give rise to the male-sterility that is maternally inherited. The restored line is isogenic to the CMS-line, but in addition it carries a piece of nuclear DNA (a *Restorer-of-fertility*, *Rf*, gene) from parent A. This *Rf*-gene can bring back the male-fertility in the CMS-line.

In the *B. napus* (*A. thaliana*) CMS-system parent A is represented by *B. napus* and parent B by *A. thaliana*. The CMS-line 4:19 was generated through protoplast fusions between the two parental lines. This gave a rearranged mitochondrial genome consisting of DNA from both parents. A pure *B. napus* nucleus was obtained after reoccurring back-crosses to *B. napus*. The restored line is isogenic to the CMS-line with the disomic addition of the *A. thaliana* chromosome III that harbours one or several *Rf*-genes. Circle = nucleus; ellipse = population of mitochondria; rectangle = *Rf*-gene; black = *B. napus* DNA; white = *A. thaliana* DNA; shaded black and white = mix of *B. napus* and *A. thaliana* DNA. (Photos M. Leino, S. Thyselius)

Alloplasmic lines as well as protoplast fusions have been produced between various species within the genus *Nicotiana*, both for breeding purposes as well as for genetic studies. The resulting CMS-lines display a large variation of flower phenotypes depending on which mitochondrial genome is combined with the *N. tabacum* nuclear genome. It is for example possible to find flowers with no stamens, stamens without anthers but with filaments, petaloid stamens and stigmatoid stamens (Bonnett *et al.*, 1991; Kofer, *et al.*, 1991). Another example is the combination of *N. tabacum* with two different genera within the *Solanaceae* family. The lines of the two resulting CMS-systems, *N. tabacum* (*Hyoscyamus niger*) and *N. tabacum* (*Scopolia carniolica*), were divided into two categories (Zubko, *et al.*, 1996). The first category developed "green flowers", flowers which are lacking true corolla and stamens and have three types of pistils; fasciated pistils, separated pistils and one pistil per flower. In fact, the phenotype perfectly

matches the phenotype expected from mutations in the genes responsible for the B-function. The second category have a more normal flower phenotype, but do not produce fertile pollen (Zubko, *et al.*, 1996). Using an *A. majus GLOBOSA* (*GLO*) cDNA probe it was shown that the *GLO* expression level was significantly lower in the "green flower"-lines than in the two parental lines (Zubko, *et al.*, 2001). *GLO* is the orthologous of *A. thaliana PI*.

When combining the mitochondria from *Nicotiana repanda* with the *N. tabacum* nucleus stigmatoid tissue on the stamens are observed and occasionally the stamens bear ovule-like structures (Bonnett, *et al.*, 1991). *In situ* hybridization experiments have revealed that the floral organ identity genes *NTDEF*, *NTGLO* and *NAG1*, the orthologous of *A. thaliana AP3*, *PI* and *AG*, do not differ in expression pattern compared to the fertile *N. tabacum* line (Farbos *et al.*, 2001). In the *N. tabacum* (*N. repanda*) CMS-line stamens are sometimes fused with the carpel resulting in mosaic organs. This phenotype in part mimic mutant phenotypes described for the *A. thaliana SUPERMAN* (*SUP*) gene, which in *A. thaliana* marks the boundary between third and fourth whorl organs. This indicates that the down-regulation of the *NtSUP* gene could be responsible for part of the CMS phenotype (Bereterbide *et al.*, 2001; Bereterbide *et al.*, 2002). In agreement with this notion, a transgenic *N. tabacum* (*N. repanda*) CMS-line expressing the *A. thaliana SUP* gene under the control of the constitutive 35S promoter is partially restored with respect to the fused organ phenotype (Bereterbide, *et al.*, 2002; Hernould *et al.*, 2002).

The two *B. napus* CMS-systems, *B. napus* (nap) and *B. napus* (pol) display modified flower phenotypes when the plants are cultivated at low temperatures (Fan & Stefansson, 1986). The male-sterile lines of *B. napus* (nap) and *B. napus* (pol) are characterised by short stamens with undeveloped anthers. Morphological studies of the *B. napus* (nap) and *B. napus* (pol) CMS-lines revealed that when the anther tissue developed into sporogenous cells the anthers have lost their symmetry and are lacking one to three locules (Geddy, *et al.*, 2005). The microspore mother cells are often clumped together and detached from the tapetum layer. Even though anther development is impaired a small amount of viable pollen is formed. Despite that no homeotic conversions of the stamens are observed the *AP3* promoter is differentially activated in the *B. napus* (pol) CMS-line in comparison to fertile *B. napus* (Geddy, *et al.*, 2005).

The nuclei and chloroplasts of the *B. napus* (*A. thaliana*) lines (Figure 2) described in this thesis contain only *B. napus* DNA (Forsberg *et al.*, 1998; Leino, *et al.*, 2003), while the mitochondrial genomes consists of rearranged *B. napus* and *A. thaliana* DNA (**I**; (Leino, *et al.*, 2003). The male-sterile lines display homeotically converted stamens that resemble carpels with stigmatoid tissues and ovule-like structures (Leino, *et al.*, 2003; Leino, 2005; Teixeira, 2005; Teixeira, *et al.*, 2005a). The petals are not homeotically converted but reduced in size. Several other observations have been made. For example, the cell divisions of the L1-L3 layers in two of the CMS-lines are disturbed (Teixeira, *et al.*, 2005a). Adenylate levels are reduced in some but not all male-sterile lines (**I**) (Teixeira *et al.*, 2005b), suggesting that the reduced adenylate-levels are pleiotropic effect and not causal

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with respect to the male-sterile phenotype. The novel mitochondrial background also affects the expression of nuclear genes (**I**, **II**, **III**). Many of the genes reflect the altered floral phenotype, for example stamen specific genes are expressed at a lower level, while carpel specific genes are expressed at higher levels in the CMS-line. The flowers of the male-sterile *B. napus* (*A. thaliana*) lines partly resemble the *A. thaliana* B-class mutants *ap3* and *pi*.

A well-known and exploited CMS inducing cytoplasm in the *Brassica* genus is the Ogura cytoplasm initially discovered in *Raphanus sativus* (Ogura, 1968). This cytoplasm has been transferred to *B. oleracea* and *B. napus* (Bannerot *et al.*, 1974) as well as to *B. juncea* (Kirti *et al.*, 1995). In the *B. oleracea* (Ogura) CMS-lines, petaloid and carpeloid stamens were occasionally observed (McCollum, 1979; McCollum, 1981). In *B. juncea* the Ogura cytoplasm induced floral abnormalities like petaloid anthers, and stamens with aborted microspores (Kirti*, et al.*, 1995; Meur *et al.*, 2006). In lines of *B. napus* cv. Westar with Ogura cytoplasm the flowers developed three types of stamens depending on the temperature. Under low temperature the stamens developed into carpeloid structures bearing stigmatoid surfaces and external ovules. Normal anthers were produced at high temperatures even though the microspore development was affected (Polowick & Sawhney, 1987). To correct for flower abnormalities somatic hybridisation between lines with the Ogura cytoplasm and normal *B. napus* cytoplasm was performed (Pelletier *et al.*, 1983; Vedel *et al.*, 1986). Several lines with rearranged mitochondrial genomes and varying phenotypes were obtained after the protoplast fusions. Some cybrids displayed feminised stamens whereas others had a normal stamen phenotype, but did not produce pollen (Gourret *et al.*, 1992). This latter type is known as Ogu-INRA. The mitochondrial protein ORF138 has been linked to the male-sterility and are thought to cause the Ogu-INRA CMS phenotype (Bonhomme *et al.*, 1991; Bonhomme *et al.*, 1992). In conclusion, the Ogura cytoplasm confers a range of flower phenotypes depending on the nuclear genome it is combined with as well as which other mitochondrial genes except ORF138 that are kept after somatic hybridisations.

All CMS-lines described here are affected in whorl three, but the stamens are disrupted in several ways. Most of the examples presented have homeotic alterations. The homeotic alterations partly resembling the B-function mutants will be discussed in this thesis. Besides the flower specific modifications found in the male-sterile lines other aberrations have been associated with CMS; for example reduced vegetative development (Malik *et al.*, 1999; Leino*, et al.*, 2003) and impaired ATP production (Bergman *et al.*, 2000; Sabar *et al.*, 2003; Teixeira*, et al.*, 2005b).

## CMS-associated genes

CMS is caused by disturbances in the nuclear-mitochondrial interaction, demonstrated by maternal inheritance of the male-sterile phenotype. Male-sterility can be obtained by combining different mitochondrial and nuclear genomes (Figure 2). Molecular studies of CMS plants have correlated the trait with the expression of novel chimeric genes. Although mitochondrial CMS-associated genes have been suggested in many systems the link to the male-sterile phenotype is often lacking. However, in some cases strong correlations between the gene and the CMS-phenotype have been found (Hanson & Bentolila, 2004; Pelletier & Budar, 2007). These CMS-associated loci share some similarities. For instance are *open reading frames* (*orf*s) and novel sequences of unknown origin combined with sequences of standard mitochondrial genes. A close physical association to, and in some cases co-transcription with, standard mitochondrial genes is another observation. Often, the CMS-associated loci are found close to ATP-synthase subunit genes. This is three common, but not compulsory properties, for CMS loci. It has been observed in the fully sequenced mitochondrial genomes of *A. thaliana* (Marienfeld *et al.*, 1997; Unseld *et al.*, 1997), *B. napus* (Handa, 2003), *Beta vulgaris* (Kubo *et al.*, 2000), *O. sativa* (Notsu *et al.*, 2002), *Z. mays* (Clifton *et al.*, 2004) and *N. tabacum* (Sugiyama *et al.*, 2005) that the mitochondrial genomes of fertile plants contain many more *orf*s with typical CMS properties than would occur by random. Possibly these genes can cause CMS, but are suppressed by nuclear regulators (*Rf*-genes). If such mitochondria are moved to a novel nuclear background the suppressors are removed and CMS is expressed.

## Restoration of fertility

CMS phenotypes can be restored resulting in male-fertile flowers producing pollen through the influence of nuclear genes (Figure 2). Nuclear genes called *restorers-of-fertility* (*Rf*-genes), that can suppress the CMS flower phenotype and restore pollen production have been identified (Hanson & Bentolila, 2004). CMS *Rf*-genes are often found in the nuclear genome of the cytoplasmic donor species (Figure 2) and restored lines can be produced by introgression of parts of nuclear DNA from this species to the CMS-line. This has been accomplished in for example *N. tabacum* CMS-lines (Burns *et al.*, 1978; Gerstel *et al.*, 1978), *T. aestivum* (*Triticum thimophevii*) cytoplasm (Livers, 1964) and numerous *B. juncea* CMS-lines (Prakash *et al.*, 2001; Banga *et al.*, 2003; Pathania *et al.*, 2003). The molecular identity of several *Rf*-genes has been described (Bentolila *et al.*, 2002; Brown *et al.*, 2003; Desloire *et al.*, 2003; Koizuka *et al.*, 2003; Klein *et al.*, 2005; Wang *et al.*, 2006). All but one (Cui *et al.*, 1996) has been shown to encode proteins belonging to the pentatricopeptide repeat (PPR) family.

The majority of PPR proteins in *A. thaliana* are predicted to localize to mitochondria or plastids (Lurin *et al.*, 2004). Several PPR proteins have been shown to bind to, and mediate, the processing of different RNAs (Kotera *et al.*, 2005; Schmitz-Linneweber *et al.*, 2005; Okuda *et al.*, 2006; Schmitz-Linneweber *et al.*, 2006; Wang*, et al.*, 2006; Hattori *et al.*, 2007). This function of PPR proteins agrees with the properties expected of *Rf*-genes, as alterations of CMS

associated transcripts commonly are observed in restored lines (Tang *et al.*, 1996; Dill *et al.*, 1997; Li *et al.*, 1998; Wen & Chase, 1999). An intriguing result from the comparison of the *Rf*-genes from several CMS-systems is that they share a high sequence similarity to certain clusters of *PPR*-genes in the *A. thaliana* genome, for example a 23Mb region of chromosome I (Lurin*, et al.*, 2004; Geddy & Brown, 2007). This might indicate a common mode of action of the *Rf*-genes and the *PPR*-genes found in this 23Mb region, or that this specific set of *A. thaliana PPR*-genes are putative *Rf*-genes. So far no single *Rf*-gene has been identified in a CMS-system restoring the homeotic transformations of floral organs. However, in the *B. napus* (*A. thaliana*) system, discussed in this thesis, it has been shown that the introgression of the entire *A. thaliana* chromosome III restores the homeotically transformed stamens. Fertile pollen is produced although the stamens are shorter than in the wild type (Leino *et al.*, 2004). *A. thaliana* chromosome III contains 96 annotated *PPR*-genes some of which are very similar to known *Rf*-genes. Thus, one or some of these might be responsible for the observed restoration. Interestingly, *AP3*, which is down-regulated transcriptionally in this CMS-system, is located on chromosome III and one possibility is that the introduction of the *A. thaliana AP3* gene is responsible for the observed restoration.

## Retrograde signalling

It is evident that the mitochondria have the ability, through the CMS-associated *orf*s or chimeric genes, to influence nuclear gene expression. Mitochondrial regulation of nuclear gene expression is called retrograde signalling, retrograde communication, retrograde stress signalling or retrograde regulation  (Liao & Butow, 1993; Patil & Walter, 2001; Rodermel, 2001; Butow & Avadhani, 2004; Rhoads & Vanlerberghe, 2004). The broad definition of retrograde signalling is "*cellular responses to changes in the functional state of mitochondria*" (Butow & Avadhani, 2004) or of plastids (Rodermel, 2001; Surpin *et al.*, 2002). The opposite signalling, from the nucleus to the organelles, is referred to as anterograde regulation (Scarpulla, 2006).

Mitochondrial signalling has mainly been studied in *Saccharomyces cervisiae* since it is able to survive regardless of its mitochondrial status; it even survives with mitochondria lacking DNA. Moreover several studies have been made in mammalian systems (Butow & Avadhani, 2004; Liu & Butow, 2006). Several retrograde regulatory pathways have been established. For example, a retrograde pathway has been found in *S. cervisiae* with deficient citric acid cycle. The pathway adopts the respiratory-deficient cells to the novel situation and the glutamate biosynthesis is secured through other pathways (Liu & Butow, 2006).

Molecular studies of for examples alterations in the mitochondrial genomes, inhibition of the electron transport chain and different stresses have shown that the nuclear gene expression is affected in such conditions. CMS is the most frequent example of retrograde signalling in plants. It was shown that the nuclear encoded *A. thaliana* alternative oxidase 1a (*AtAOX1a*) was induced due to inhibition of the

electron transport chain or the citric acid cycle (Zarkovic *et al.*, 2005). The authors suggested that the induction of *AtAOX1a* occurs via distinct but overlapping pathways that in addition, are tissue specific. In a microarray study it was shown that most nuclear encoded respiratory genes involved in different mitochondrial functions did not respond to the inhibition of the electron transport chain, except for *AtAOX1a* and cytochrome c (Yu *et al.*, 2001). It was also shown that the genes were regulated similarly under several conditions such as aluminium stress, cadmium stress, disease responses or under hydrogen peroxide treatment.

Alterations in the mitochondrial genome that result in retrograde regulation also lead to for example chlorotic leaves or embryo lethality (Newton *et al.*, 2004; Rhoads & Subbaiah, 2007). Chlorotic leaves have been found in *N. tabacum* (*P. hybrida*) (Bonnett *et al.*, 1993), *A. thaliana* (Sakamoto *et al.*, 1996), *T. aestivum* (*T. aegilops*) (Mukai & Tsunewaki, 1976) and *Solanum lycopersicum* (*Lycopersicon pennellii*) (Bonnema *et al.*, 1995). In *Z. mays* the so called non-chromosomal stripe (NCS) mutants have been characterized, and shown to carry specific mitochondrial DNA deletions (Newton & Coe, 1986). An altered mitochondrial background has also been demonstrated to affect the starch content in several *S. tuberosum* hybrids (Lössl *et al.*, 1994). Taken together, it is not only impairment of the mitochondrial electron transport chain or the citric acid cycle that affect the nuclear gene expression, but also abiotic and biotic stresses and other mitochondrial dysfunctions such as alterations in the mitochondrial genome (Newton*, et al.*, 2004; Rhoads & Subbaiah, 2007).

## *Arabidopsis thaliana* and *Brassica napus*

*Arabidopsis thaliana* (L.) Henh. (n=5) is a small weed that belongs to the family of *Brassicaceae* and it is closely related to crop species within this family (Cavell *et al.*, 1998). The species is native to Western Euroasia and Northern Africa, but has become naturalised throughout the world (Al-Shehbaz & O'Kane Jr, 2002; Hoffmann, 2002). Due to its small size, high level of selfing and short generation time it has become the main model plant in genetic studies (Meinke *et al.*, 1998; Somerville & Koornneef, 2002; Bevan & Walsh, 2005). Furthermore it is easy to grow, to cross and to transform *A. thaliana* (Bechtold *et al.*, 1993; Meinke*, et al.*, 1998; Desfeux *et al.*, 2000). Today large collections of mutants and ecotypes are available (Meinke*, et al.*, 1998; Alonso-Blanco & Koornneef, 2000). The nuclear (TheArabidopsisGenomeInitiative, 2000), mitochondrial (Unseld*, et al.*, 1997) and chloroplast (Sato *et al.*, 1999) genomes have been sequenced, which makes the *A. thaliana* even more useful for genetic studies. The sequencing efforts have given rise to a wide range of well-annotated *A. thaliana* microarrays, for example cDNA microarrays like the CATMA (Crowe *et al.*, 2003; Hilson *et al.*, 2004) and oligonucleotide microarrays such as the Affymetrix (Lockhart *et al.*, 1996; Lipshutz *et al.*, 1999). The first time *A. thaliana* was described in the literature in Sweden was 1745 (Nordstedt, 1920).

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*Brassica napus* L. (n=19) is an allotetraploid species derived from interspecific crosses between *B. oleracea* L. (n=9) and *Brassica rapa* L. (n=10) all in the *Brassicaceae* family (U, 1935; Röbbelen, 1960; Parkin *et al.*, 1995; Bohuon *et al.*, 1996). Wild forms have been found in Gothland, Sweden, (Nordstedt, 1920), in the Netherlands and in Great Britain, and it is believed that *B. napus* originates from Europe (Rakow, 2004). The first time *B. napus* was described in the literature in Sweden was during the Middle Ages (Nordstedt, 1920).

Both winter and summer annual forms of *B. napus* are grown as oilseed. Within the European Union (EU), Germany and France are the largest producers of *B. napus* and *B. rapa*. The EU produces 10,000-15,000 millions kg *B. napus* and *B. rapa* each year grown on approximately 4 million hectares. In Sweden the production of *B. napus* and *B. rapa* per year is 120-200 million kg, cultivated on about 74 thousand hectares (Jordbruksverket, 2006). There are also root-forming *B. napus* types (swede in English, kålrot in Swedish) grown as vegetables or fodder. *Brassica* oilseed production is one of the worlds most important vegetable oils after soybean and cotton seed (Rakow, 2004). The *B. napus* cultivars grown in the EU is of the zero erucic acid, low glucosinolate type (that is of canola-quality). The fatty-acid composition in canola-quality *B. napus* is considered to have high nutritional value for human consumption and regarded as the healthiest vegetable oil on the market (Rakow, 2004). For example more than 90% of the fatty acids are unsaturated, and of these are 10% Omega 3 fatty acids. That is 15 times more Omega 3 fatty acids than in olive oil (Svenskraps, 2007b). From the canola-type of *B. napus* oil fatty acid methyl esters (FAME) can be produced, which are utilised as bio-fuels in the transport industry and glycerol in the cosmetic industry (Rakow, 2004; Svenskraps, 2007a).

The mitochondrial genome of *B. napus* has been sequenced (Handa, 2003), but the nuclear or chloroplast genomes have not. However, several restriction fragment length polymorphism (RFLP) maps have been generated for *B. napus* (Ferreira *et al.*, 1994; Parkin*, et al.*, 1995; Sharpe *et al.*, 1995; Uzunova *et al.*, 1995). There exist at least six independent maps of the *B. napus* nuclear genome generated through different techniques, such as RFLP and random amplified polymorphic DNA (RAPD) (Quiros & Paterson, 2004). As mentioned above, *B. napus* is an allotetraploid species derived from interspecific crosses between *B. rapa* and *B. oleracea* with genomes denoted A and C respectively. *B. rapa*, *B. oleracea* and *B. nigra* (genome B) are related (U, 1935; Lagercrantz & Lydiate, 1995; Parkin*, et al.*, 1995; Lagercrantz & Lydiate, 1996), and from these species new allotetraploid hybrids have been obtained (Figure 3; (U, 1935).

The genera of *Brassica* and *Arabidopsis* are closely related (Scheffler *et al.*, 1997; Brunel *et al.*, 1999; Parkin *et al.*, 2002; Parkin *et al.*, 2005), and the estimated date of divergence between the two genera is 20 million years ago (Koch *et al.*, 2001). *A. thaliana* and *B. napus* share on average 87% exon sequence similarity (Cavell*, et al.*, 1998). This enables us to use *A. thaliana* as a model when studying *B. napus*. In addition it is possible to use the tools available for *A. thaliana*. Several studies have shown that *A. thaliana* microarrays can be successfully used for the analysis of gene expression in *Brassica* species (**II**, **III**)

(Girke *et al.*, 2000; Lee *et al.*, 2004), and it is assumed that the genes detected by the microarrays represent the *B. napus* homologues of the *A. thaliana* genes. Moreover, *A. thaliana* specific probes or primers have been shown to be useful when studying the *B. napus* genome with for example Southern blotting or PCR (Cavell*, et al.*, 1998; Brunel*, et al.*, 1999).
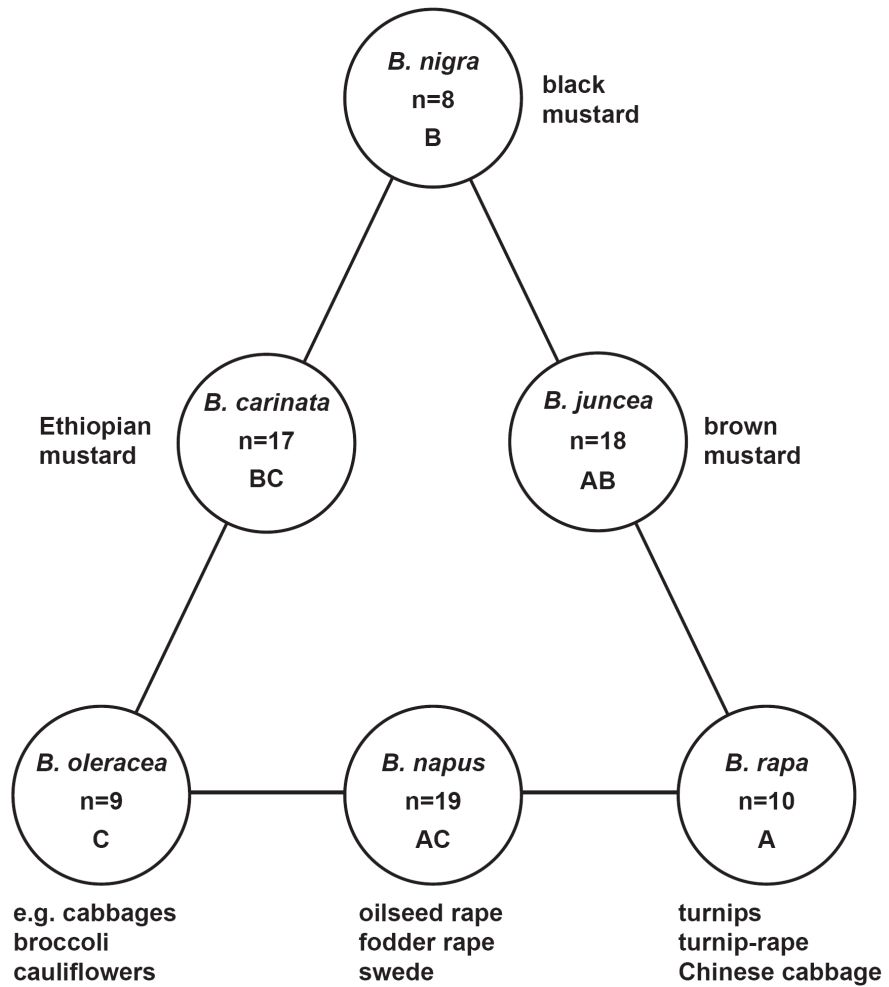


**Figure 3**. Representation of the genomic relation between six species in the genus *Brassica* based on U (1935).

# Measuring nuclear gene expression

There are many tools available for studying the different *A. thaliana* transcriptomes. Several studies of *A. thaliana* transcriptomes have been performed using microarrays, for example the *A. thaliana* pollen transcriptome (Honys & Twell, 2003; Honys & Twell, 2004; Pina *et al.*, 2005) and the floral development transcriptome (Schmid *et al.*, 2005; Wellmer *et al.*, 2006) have been studied. The results are easily available in many databases, for example in *Genevestigator* (https://www.genevestigator.ethz.ch/; (Zimmermann *et al.*, 2004), through the *Arabidopsis eFP Browser* (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and the *Arabidopsis information resource* web-page (http://www.arabidopsis.org/). Since *B. napus* and *A. thaliana* share a high nucleotide sequence identity in the coding regions of the genomes the possibilities exist to screen more or less the complete genome of *B. napus* by utilising the knowledge about *A. thaliana*.

*A. thaliana* microarray slides have been used successfully to study the *B. napus* nuclear gene expression profiles (**II**, **III**) (Girke*, et al.*, 2000). There are additional examples of heterologous systems where *A. thaliana* microarrays have been used effectively to study an alien species (Horvath *et al.*, 2003; Lee*, et al.*, 2004). It is assumed in this thesis that the genes that were detected by the microarrays represent the *B. napu*s homologues of the *A. thaliana* genes and for simplicity, the *A. thaliana* gene annotations are used throughout this thesis if nothing else is stated.

Genes of interest found in a microarray screens should be verified by an additional method, for example Northern analysis, qRT-PCR and *in situ* hybridisation. The first two measure the amount of RNA in a similar fashion as microarrays, while *in situ* hybridisation localise the expression to specific tissues. Northern analysis and qRT-PCR are regarded as being more sensitive than the microarray analysis. Low amounts of RNA and small differences in expression are more easily detected. In contrast, *in situ* hybridisation not only discriminate if the RNA is expressed or not, but also give the additional information of were the RNA is expressed.

# Objectives

The main objective was to study a set of fertile, semi-sterile and male-sterile *B. napus* (*A. thaliana*) lines, to get an insight of the effect of the mitochondria on the nuclear gene expression. The specific aims were to:

- describe the number of floral organs, the floral organ size and flower morphology for the *B. napus* (*A. thaliana*) lines
- determine flowering time, shoot dry-weight and adenylate content for the *B. napus* (*A. thaliana*) lines
- analyse the *A. thaliana* DNA content in the mitochondria of the *B. napus* (*A. thaliana*) lines
- study the expression pattern of a sub-set of mitochondrial markers in the *B. napus* (*A. thaliana*) lines
- examine the expression profiles of *AP1*, *AP2*, *AP3*, *PI*, *AG*, *LFY* and *UFO* in the *B. napus* (*A. thaliana*) lines
- identify and characterise the nuclear gene expression profiles using microarrays in two different CMS-systems
- verify and follow up a selection of nuclear genes picked out in the microarray studies
- scrutinise the nuclear encoded genes *AP3* and *PI*

# Results and discussions

The intention with the studies in the present thesis was to deepen the knowledge about the nuclear gene expression in a set of *B. napus* (*A. thaliana*) lines to elucidate the impact of the mitochondria on the nuclear genome and the CMS-phenotype. To do so, the flower morphology, flowering time, growth rate and adenylate content was determined for 22 lines and one fertile *B. napus* cultivar (**I**). For each line the mitochondrial DNA content was analysed and the expression of a set of mitochondrial *orf*s was established. Futhermore, the nuclear gene expression was determined for five nuclear encoded genes using qRT-PCR (**I**). The nuclear transcriptome of young flower buds of one of the male-sterile lines (CMS-line 4:19), a restored line, the *B. napus* cv. Hanna +At3 line and the fertile *B. napus* cultivar were described using *A. thaliana* microarrays (**II**, **III**). Moreover, the nuclear gene expression of the *B. napus* (Ogu-INRA) CMS-system was studied (**III**). The two nuclear encoded genes *AP3* and *PI* involved in stamen formation were studied in detail (**II**, **IV**).

## Plant material

To start with, populations of *A. thaliana* (+) *B. napus* somatic hybrids (Yamagishi *et al.*, 2002) and *B. napus* (+) *A. thaliana* somatic hybrids (Forsberg *et al.*, 1994; Forsberg*, et al.*, 1998) were screened for male-sterility, with the aim of finding male-sterile lines with a pure *A. thaliana* nucleus and mitochondria with

rearranged DNA mainly from *B. napus*. This would have made the following studies more straight-forward since it would have been possible to utilise the knowledge about the nuclear genome of the model plant *A. thaliana* more easily. However, no male-sterile *A. thaliana* (*B. napus*) progenies were found. The progenies found were either fully fertile or the studied lines did not set any seed. A reason to why no male-sterile plants were found could be that too few individuals were included in the screen. In larger populations of somatic hybrids it might have been possible to find male-sterile plants.

### The B. napus (A. thaliana) lines

Thus, the plant material studied in this thesis was derived from several unique somatic hybrids between *B. napus* cv. Hanna and *A. thaliana* ecotype Landsberg *erecta* (Forsberg*, et al.*, 1998). From the somatic hybrids, progenies were obtained by utilising *B. napus* cv. Hanna as the pollinator (Leino*, et al.*, 2003; Leino, 2005). From repeated back-crosses, progenies were obtained and of these, 21 *B. napus* (*A. thaliana*) lines were chosen (Figure 4). The *B. napus* (*A. thaliana*) lines were back-crossed a minimum of four times and several lines were back-crossed twelve times (for details see Table 1 in **I**). These 21 lines had pure *B. napus* nuclear (Bohman *et al.*, 1999; Leino*, et al.*, 2003) and chloroplast (Raats, 1999; Leino*, et al.*, 2003) genomes. The mitochondrial genomes were unique for most lines and contained *B. napus* mitochondrial DNA and *A. thaliana* mitochondrial DNA, partly recombined (**I**) (Leino*, et al.*, 2003; Leino, 2005).

The phenotypes of the obtained *B. napus* (*A. thaliana*) lines ranged from male-sterile lines combined with aberrant flower phenotypes to male-fertile lines with normal *B. napus* flowers (Figure 4; **I**). The most obvious phenotypic difference between the lines was the feature of the stamens (Leino*, et al.*, 2003; Teixeira, 2005; Teixeira*, et al.*, 2005a). Ten of the lines had homeotically converted stamens and were classified as male-sterile (**I**). The stamens of these ten lines resembled carpels with stigmatoid tissues and ovule-like structures (Figure 5). One of these male-sterile lines, line 4:19, was studied in detail in this thesis (**I-IV**). Line 4:19 was also named *the CMS-line* (**II**, **IV**), *the sterile line 4:19* (**I**) or *the CMS-line 4:19* (**III**). Eight lines had a phenotype similar to *B. napus* that is with normal stamens producing pollen (**I**). These lines were classified as fertile. In addition three lines were classified as semi-sterile (**I**). They had pollen-producing anthers. However, the stamens were too short to allow self-pollination.

The flowers of the 21 lines were analysed with *B. napus* as a reference line. The reference line was also named *the fertile B. napus* (**II**, **IV**), *cv. Hanna* (**I**) and *the maintainer line* (**III**, **IV**). Each of the 21 lines studied developed four distinct floral whorls and from each whorl, with a few exceptions, the expected number of floral organs developed, that is four sepals, four petals, four long stamens, two short stamens and one pistil (for details see Table 1 in **I**). While the number of organs was the same in most lines, petal size and stamen length varied between the classes (for details see Table 1 in **I**). All fertile lines grouped together and were, in general, significantly different from the male-sterile and semi-sterile lines

regarding petal and stamen size (**I**). The male-sterile and semi-sterile lines were divided into several sub-groups.

The semi-sterile lines had in general petals of intermediate size and the shortest stamens, compared to the fertile and male-sterile lines (**I**). The male-sterility was stable, maternally inherited and proven to result from the combination of the nuclear genome of *B. napus* with mitochondria displaying rearranged DNA from both *B. napus* and *A. thaliana* mitochondrial DNA (**I**) (Forsberg*, et al.*, 1998; Leino*, et al.*, 2003; Leino *et al.*, 2005).

*The B. napus (A. thaliana) restored line and the B. napus cv. Hanna +At3 line*

The *B. napus* (*A. thaliana*) restored line carried two copies of the *A. thaliana* chromosome III (At3), but was otherwise isogenic to the CMS-line 4:19 (Leino*, et al.*, 2004; Leino, 2005). The flowers of the restored line resembled the flowers found in the semi-sterile class described above (Figure 4; **I**). The petals were larger in the restored line compared to the CMS-line 4:19, but not as large as the *B. napus* petals. The stamens developed into proper filaments with anthers that produced viable pollen. The homeotically converted whorl three organs found in the CMS-line 4:19 were restored. However, the stamens formed were not as tall as in the ordinary fertile *B. napus* cultivar (**I**).

The restored line was used as the pollinator and crossed with *B. napus* cv. Hanna. Of the generated progenies an offspring that stably inherited the *A. thaliana* chromosome III was selected (personal communication, Dr. M. Leino, April 2007). This line was named *B. napus* cv. Hanna +At3 (Figure 4; **III**). The flower phenotype of the line resembled that of *B. napus* cv. Hanna (**III**). The line *B. napus* cv. Hanna +At3 had not only the capacity to restore male-fertility, but also a lower susceptibility to certain fungal pathogens compared to *B. napus* (personal communication, Msc. M. Kaliff and Dr. J. Staal, April 2007). It has previously been shown that the *A. thaliana* chromosome III harbours resistance genes specific to *A. thaliana* that can induce resistance against *Leptosphaeria maculans* in *B. napus* (Bohman *et al.*, 2002).

*The restored line restores a subset of male-sterile lines*

The *B. napus* (*A. thaliana*) restored line had the ability to partially restore male-fertility in four male-sterile lines (**I**). The offspring of the male-sterile line 4:19 had the expected restored flower phenotype that is, a phenotype similar to the restored line, even though less pollen was formed. The offspring of the male-sterile lines 9:13, 14:4 and 48:60 obtained a mix of flowers with either homeotically converted stamens or short stamens producing pollen. This indicates that the putative *Rf*-gene or -genes present on the *A. thaliana* chromosome III in the restored line had the capacity to restore the phenotype of a subset of the male-sterile lines. The remaining six male sterile lines and the semi-sterile lines were not restored.
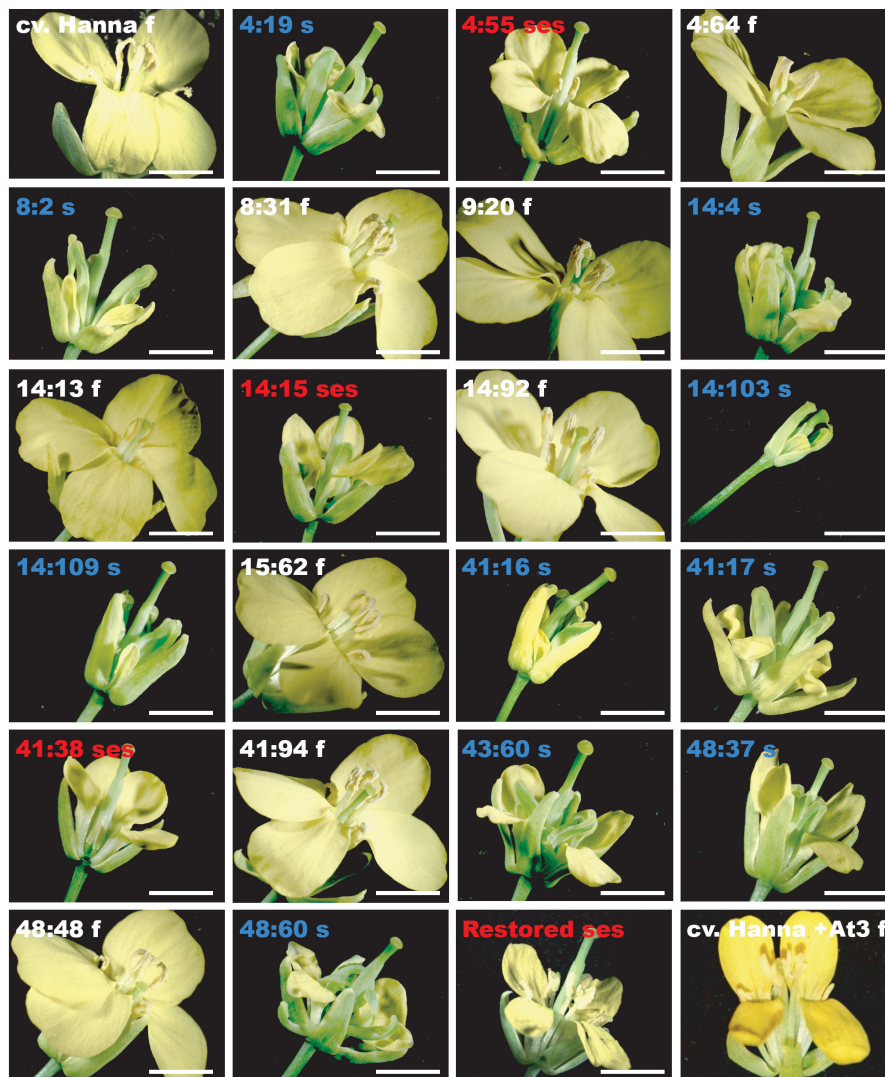
**Figure 4.** An illustration of the flower phenotypes observed in *B. napus* cv. Hanna, the *B. napus* (*A. thaliana*) lines, the restored line and the *B. napus* cv. Hanna +At3 line that are described in this thesis. The figure is a reprint from **I** with the addition of *B. napus* cv. Hanna +At3. s = male-sterile line; ses = semi-sterile line; f = fertile line. (Photos M. Leino, J. Carlsson)
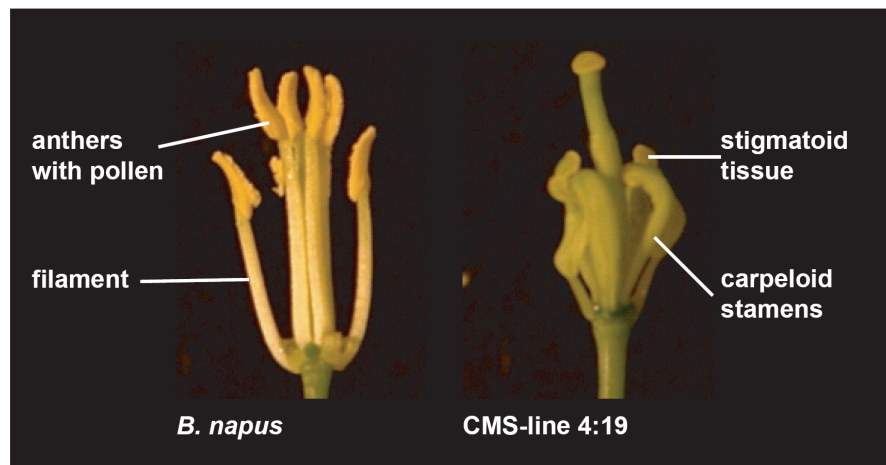
**Figure 5**. Stamen morphology of fertile *B. napus* cv. Hanna and the CMS-line 4:19. *B. napus* has normal stamens producing pollen, while the CMS-line has carpeloid stamens with stigmatoid tissue and ovule-like structures. The male-sterile *B. napus* (*A. thaliana*) lines have the same type of stamens as CMS-line 4:19. (Photos J. Carlsson)

On *A. thaliana* chromosome III, the "restorer-chromosome", several *PPR*-genes were found. As pointed out in the *Introduction* several nuclear *Rf*-genes have been found to be *PPR*-genes. One of these were chosen as a *Rf*-gene candidate and transformed into the male-sterile line 4:19 (unpublished results, Dr. J. Sohlberg and Dr. M. Leino, May 2007). The resulting transformants appeared to be partially restored but this has to be evaluated further. The results so far indicate that the *PPR*-gene restores the male-fertility to almost the same extent as in the restored line. Besides the *PPR*-genes the B- gene *AP3* is localized to chromosome III. Since *AP3* is involved in the formation of stamens it was chosen as an additional *Rf*-gene candidate. It has been transformed into the CMS-line 4:19. Progenies from the transgenic lines are under investigation (personal communication, Dr. M. Leino, May 2007). A conclusion based on the results so far is that the *PPR*-gene may have the ability to restore the levels of *AP3*-gene expression and thus, restore fertility.

### The B. napus (Ogura) CMS-system

In addition a French CMS-system, the *B. napus* (Ogu-INRA) CMS-system (Pelletier*, et al.*, 1983; Bonhomme*, et al.*, 1992; Desloire*, et al.*, 2003), was studied (**III**). It consisted of three lines; a maintainer line (*B. napus* cv. Pactol), a CMS-line (*B. napus* (Ogu-INRA)) and a restored line (the *PPRB*-line). Both cv. Pactol and the *PPRB*-line had a normal *B. napus* flower phenotype, while the CMS-line had normal stamens but no pollen.

The CMS-system *B. napus* (Ogu-INRA) is today effectively used in hybrid seed production. The CMS-line Ogu-INRA, were originally produced through protoplast fusions between a *B. napus* cultivar and a *B. napus* CMS-line with a *B.*

*napus* nuclear genome in a *R. sativus* cytoplasm (Pelletier*, et al.*, 1983). The resulting lines were back-crossed with fertile *B. napus* and the progenies carried *B. napus* nuclear and chloroplast genomes, morover the CMS-inducing mitochondrial genome was retained. The mitochondrial genome carries *orf138* that cause the CMS-phenotype (Bonhomme*, et al.*, 1991; Bonhomme*, et al.*, 1992). The restored line, the *PPRB*-line, is isogenic to the Ogu-INRA CMS-line but in addition carries the *Rf*-gene *PPRB* (Brown*, et al.*, 2003; Desloire*, et al.*, 2003; Koizuka*, et al.*, 2003), that has been transformed into the line (Uyttewaal *et al*. manuscript in prep.). The gene-product from *PPRB* reduces the amount of ORF138 proteins in the restored line by an unknown mechanism (Bellaoui *et al.*, 1999). Both the CMS-line Ogu-INRA and the *PPRB*-line have the nuclear background of the maintainer-line *B. napus* cv. Pactol.

## Flower-development in *B. napus* parallels that of *A. thaliana*

As a first step flower development was studied. The studies were made to relate the flower development of *B. napus* cv. Hanna to *A. thaliana* (Müller, 1961; Smyth *et al.*, 1990) and previously described *B. napus* flower development (**II**) (Polowick & Sawhney, 1986; Teixeira, 2005; Teixeira*, et al.*, 2005a). The description of the flowers and the defined stages were the bases for all lines studied in this thesis.

The shoot apical meristem (SAM) of *A. thaliana* seedlings is flat or slightly convex and consists of a biseriate tunica enclosing the corpus during vegetative growth. Prior to floral transition the SAM still has a shallow corpus and a biseriate tunica. At floral transition SAM changes from a slightly convex to a distinct dome-shape structure due to cell divisions (Vaughan, 1955; Miksche & Brown, 1965). The flower primordia develop on the flanks of the altered SAM (Polowick & Sawhney, 1986; Smyth*, et al.*, 1990).

Flower initiation of *B. napus* is similar to that of *A. thaliana*. It occurs continuously in a spiral at each floral apex, with the most recent developed bud at the tip and the oldest flower or bud at the base (Polowick & Sawhney, 1986; Smyth*, et al.*, 1990). Some minor differences in timing and order of appearance are obvious when the specific organs are established. However, since the differences are small and no definitions of flower stages for *B. napus* have been established the flower stages defined by Müller (1961) and Smyth *et al*. (1990) for *A. thaliana* have been used.

*B. napus* and *A. thaliana* show a parallel developmental pattern until stage 4 (Table 1). At stage 1 the flower primordia are visible as an outgrowth and at stage 2 the outgrowth becomes more like a sphere. The sepal primordia start to develop at stage 3. In *A. thaliana* the sepal primordia arise in the following order; the abaxial primordium, then the adaxial primordium and last the two lateral primordia (Smyth*, et al.*, 1990). In *B. napus* the sepals arise in a slightly different order, namely, the two lateral primordia arise directly after the abaxial primordium and thereafter the adaxial primordium is developed (Polowick & Sawhney, 1986).

When the abaxial sepal primordium overlies the flower primordium, stage 4 is reached. During the stages 3 and 4 the primordium becomes more and more stalked.

In *A. thaliana* the petal and stamen primordia develop during stage 5. It is difficult to tell if the petal primordia develop after the long stamens or at the same time. The four long stamens (or inner stamens) arise before the short (outer) stamens (Smyth*, et al.*, 1990), that is, the long stamen primordia and the petal primordia are present when the short stamen primordia arise. In *B. napus* the long stamen primordia develop before the short stamen primordia and the petal primordia arise after the stamen primordia (Polowick & Sawhney, 1986). That is, in *B. napus* the stamen primordia develop during stage 5 while the petal primordia develop during stage 6 and 7.

Stage 6 in the two species begins when the sepals fully cover the bud. Stamen and petal primordia develop further and a hollow tube that will become the gynoecium starts to appear. During stage 7 the primordia of the long stamens become stalked, the gynoecium is of the same height as the long stamens and the petal primordia are hemispherical. At stage 8 the locules appear as protrusions on the anthers. Petal primordia become stalked during stage 9 and a rapid lengthening of all organs occur. By the end of stage 9 the tip of the gynoecium is not yet differentiated. At stage 10 the petals reach the top of the short stamens. During stage 10 the opening in the gynoecium starts to diminish. In stage 11 the gynoecium which is closed develops stigmatic papillae. Stage 12 is reached when the petals are as tall as the long stamens (corresponds to stage B2 (Müller, 1961)). During stage 12 the organs elongate (Smyth*, et al.*, 1990). Except for the occurrence and development of the petal primordia the organ development is similar in *B. napus*. The anthers in *B. napus* are of the same length as the gynoecium at stage 12, while they are approximately half the gynoecium length in *A. thaliana*.

When the sepals open, stage 13 is reached (stage B3). Anthesis occurs at stage 13. At stage 14 (B4) the long anthers are taller than the gynoecium and at stage 15 (B5) the gynoecium extends over the long stamens. The petals and sepals are withering during stage 16 (B6) and during stage 17 (B7) all organs fall off from the green siliques. The siliques turn yellow during stage 18 (B8). The valves separate from dry siliques at stage 19 (B9) and finally at stage 20 (B10) the seeds fall (Müller, 1961; Polowick & Sawhney, 1986; Smyth*, et al.*, 1990). It appears as if *B. napus* releases its pollen slightly later than *A. thaliana*. Moreover, *B. napus* does not release its seeds as easily as *A. thaliana*. This is probably due to breeding, since it is important to keep the seeds in the siliques to facilitate harvest.

Flower development of the two male-sterile lines 4:19 and 41:17 and the two semi-sterile lines 4:55 and 41:38 have also been studied (Teixeira, 2005; Teixeira*, et al.*, 2005a), and is similar to that of *B. napus*. At stage 8 it is possible to distinguish the male-sterile lines from the semi-sterile lines and *B. napus*. The locules of the stamens in the semi-sterile lines and *B. napus* are clearly visible, but the locules are missing in the two male-sterile lines. From stage 8 and onwards the

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whorl three organs of the male-sterile lines develop into carpeloid organs with ovule-like structures and stigmatoid tissues. The differences regarding the stamens in the semi-sterile lines are not obvious until stage 13. At that stage the normal fertile *B. napus* has long stamens that are of the same height as the gynoecium, while the semi-sterile lines have long stamens that are about half the height of the gynoecium.

**Table 1**. A summary of the flower development in *A. thaliana* and *B. napus*.

| Floral stages | Development in *A. thaliana* according to Müller (1961) and Smyth *et al*. (1990) | Development in *B. napus* | Note |
|---|---|---|---|
| 1 | flower primordia arises | flower primordia arises | |
| 2 | flower primordia as spheres | flower primordia as spheres | |
| 3 | sepal primordia arise | sepal primordia arise | 1 |
| 4 | sepals overlie meristem | sepals overlie meristem | |
| 5 | petal and stamen primordia arise | stamen primordia arise | 2 |
| 6 | sepals enclose bud and gynoecium primordia arise | sepals enclose bud, petal and gynoecium primordia arise | 2 |
| 7 | long stamen primordia stalked at base | long stamen primordia stalked at base | 2 |
| 8 | locules appear in long stamens | locules appear in long stamens | |
| 9 | petal primordia stalked at base | petal primordia stalked at base | |
| 10 | petals level with short stamens | petals level with short stamens | |
| 11 | stigmatic papillae appear | stigmatic papillae appear | |
| 12 B2 | petals level with long stamens | petals level with long stamens | 3 |
| 13 B3 | bud opens, petals visible, anthesis | bud opens, petals visible, anthesis | |
| 14 B4 | long stamens extend above stigma | long stamens extend above stigma | |
| 15 B5 | stigma extends above long stamens | stigma extends above long stamens | |
| 16 B6 | petals and sepals withering | petals and sepals withering | |
| 17 B7 | all organs fall from green siliques | all organs fall from green siliques | |
| 18 B8 | siliques turn yellow | siliques turn yellow | |
| 19 B9 | valves separates from dry siliques | valves separates from dry siliques | 4 |
| 20 B10 | seeds fall | seeds fall | 4 |

1 In *A. thaliana* the sepal primordia arise in the following order: abaxial, adaxial and last the two lateral primordia, and in *B. napus* the order is slightly different: abaxial, the two lateral primordia and last the adaxial one.
2 Petal primordia arise later in *B. napus*, at stage 6-7.
3 The anthers of *B. napus* are more or less of the same length as the gynoecium at stage 12, while the *A. thaliana* anthers are half the gynoecium length.
4 The siliques of *B. napus* do not open as easily as in *A. thaliana*.

## The influence of novel mitochondrial genomes on flower development

The 21 *B. napus* (*A. thaliana*) lines were divided into three classes according to their stamen phenotype, which were assumed to be affected by the mitochondrial background. To elucidate the mitochondrial genome composition the *A. thaliana* mitochondrial DNA content was determined for each line using 36 *A. thaliana* specific markers. Six of the lines, all classified as fertile, were lacking all *A. thaliana* mitochondrial markers evaluated (For details see Table 3 in **I**). These six lines could completely lack *A. thaliana* mitochondrial DNA, but it is possible that they contain rearranged *B. napus* mitochondrial DNA and/or pieces of *A. thaliana* mitochondrial DNA for which no markers were available. The remaining 15 lines contained at least eight of the 36 *A. thaliana* mitochondrial DNA markers. Such lines were found in all three classes described above.

Fifteen *A. thaliana* mitochondrial markers were found in fertile lines (**I**) and these are supposed to represent mitochondrial DNA that does not influence the fertility in the *B. napus* (*A. thaliana*) lines. The conclusion is that the presence of *A. thaliana* mitochondrial DNA in a *B. napus* nuclear background does not lead to male-sterility, but rather a specific *A. thaliana* fragment or a specific combination of fragments.

Most lines had a unique combination of *A. thaliana* mitochondrial markers (**I**). The exceptions were the six fertile lines mentioned above and four male-sterile lines. The six fertile lines were lacking the *A. thaliana* markers screened for. The two male-sterile lines 14:103 and 48:37 shared the same set of markers, and the lines 43:60 and 48:60 shared another distinct set of markers. The male-sterile lines 43:60 and 48:60 were fairly similar regarding the flower phenotype, adenylate-content and the other characteristics measured, while 14:103 and 48:37 differed distinctly regarding at least the flower phenotype (**I**). This indicates that there are additional dissimilarities in the mitochondrial DNA-content, such as differences in the amount of *A. thaliana* DNA and/or in the amount of *B. napus* mitochondrial DNA and/or the rearrangements of the DNA.

The three markers VI, XXI and XXXV are present in all male-sterile and semi-sterile lines (**I**). However, none of the regions represented by these markers were expressed in any of the 21 lines and neither in *B. napus* nor in *A. thaliana*. The proximity of these markers was analysed with the purpose of finding genes or *orf*s. The region close to marker XXXV was not studied further since no gene or *orf* were found in the vicinity. Marker VI is close to *rpl5*. The expression of *rpl5* was not specific for any of the three classes described above. Marker XXI is close to *cox3*. A novel *cox3* transcript was found in all semi-sterile and male-sterile lines, except for the semi-sterile line 41:38. This expression was absent in the fertile lines (**I**). Thus, marker XXI, or rather *cox3*, is a gene associated with CMS and a putative CMS-inducing gene.

Additional three markers, representing three *orf*s, were of special interest. These three *orf*s have been studied previously and were thought to be associated to CMS (Leino*, et al.*, 2005). Marker V, *orf139*, and marker XX, *orf240a*, were found in all male-sterile and semi-sterile lines. However, *orf139* was also found in the fertile lines 9:20 and 14:92, while *orf240a* was found in the fertile line 14:92. Marker XXX, *orf294*, was found in all male-sterile and semi-sterile lines except the semi-sterile line 4:55 and the male-sterile line 41:17. These three *orf*s were expressed in the lines where they were detected, except for in line 9:20. The fertile line 9:20 hade no *orf139* transcripts (**I**). This gives, together with the results mentioned above, three regions denoted by marker V (*orf139*), markers XX-XXI (*orf240a* and *cox3*) and marker XXX (*orf294*), in the *A. thaliana* mitochondrial DNA that are CMS-associated and putative CMS-inducing genes. Thus, transcripts from either of these regions or a combination may cause the CMS-phenotype in the male-sterile lines.

Both fertile and male-sterile lines contained *A. thaliana* mitochondrial markers (**I**). Thus, *A. thaliana* mitochondrial DNA did not induce CMS as such. It was rather a specific fragment or a specific combination of fragments that was important. Likely candidates are the three areas around markers V-VI (*orf139* and *rpl5*), XX-XXI (*orf240a* and *cox3*) and XXX (*orf294*). Furthermore, these candidates give rise to novel transcripts that are not found in the *A. thaliana* mitochondrial transcriptome. It appears as if it is the presence of novel transcripts rather then the presence of *A. thaliana* DNA that give rise to CMS. One could speculate if there are novel transcripts originating form the *B. napus* mitochondrial genome that could be CMS-inducing.

Two mitochondrially encoded transcripts were picked up in the first microarray study (**II**). The two transcripts had a higher expression in the CMS-line 4:19 in comparison to *B. napus* cv. Hanna at all stages examined. The two transcripts were identified as AtMg01080 (*atp9*) and a sequence located up-stream in the 5'-UTR of *orf139* (here named GP001E3). GP001E3 matches a 500-nucleotide sequence expressed in *A. thaliana* (Holec *et al.*, 2006), and is co-transcribed with *orf139* (Leino*, et al.*, 2005). Additional mitochondrial encoded transcripts from the gene AtMg00220 (*cob*) was picked up in the second microarray study (**III**). The lower expression of AtMg01080 and AtMg02200 in the CMS-line reflects a changed expression in the mitochondria. Even though *atp9* and *cob* do not appear to be CMS-associated genes, in the sense of being CMS-inducing genes, they clearly are affected by the alterations in the mitochondria. These novel regulations of mitochondrial genes have previously been shown for other mitochondrial genes in the *B. napus* (*A. thaliana*) CMS-line (Leino*, et al.*, 2005).

## Nuclear gene expression

Nuclear gene expression in the two CMS-systems has been studied using *A. thaliana* microarrays. In publication **II** microarray slides harbouring 10,816 elements corresponding to approximately 5,000-6,000 unique genes were used (Wellmer *et al.*, 2004). These were floral specific cDNA arrays well suited to study the transcriptome of floral tissues. Full-genome CATMA-arrays (including most of the nuclear, mitochondrial and chloroplast genomes) printed in France were used in publication **III** (Crowe*, et al.*, 2003; Hilson*, et al.*, 2004). The aim of the CATMA (Complete Arabidopsis Transcriptome MicroArray) project was to design and produce gene-specific sequence tags (GSTs) covering most *A. thaliana* genes (Thareau *et al.*, 2003). The GST repertoire is used by numerous groups for the production of DNA arrays for transcript profiling experiments.

244 unique genes were differentially expressed between the CMS-line 4:19 and *B. napus* cv. Hanna at one or several stages (**II**). This showed that the *A. thaliana* recombined mitochondrial DNA in the CMS-line had a strong influence on nuclear gene expression in buds during early flower development, which also is supported by the second microarray-study (**III**). The number of differentially expressed genes increased with progressing developmental stages (**II**). Most genes

were altered at stage 8, a stage where clear phenotypic differences between the lines were found. Many of the genes were preferentially or presumably expressed in carpels or in stamens. In general the carpel specific genes had a higher expression while the stamen specific had a lower expression (**II**), which probably reflects the phenotype of the CMS-line. One group of genes were preferentially differentially expressed during stages 0-5. These genes were to a high extent stamen and/or pollen specific. Several of these genes encoded members of pectinesterases, multicopper oxidases and glycoside hydrolases families (**II**), thought to be involved in cell-wall modifications (Torki *et al.*, 1999; Micheli, 2001). The changed gene expression of these cell-wall modifying genes may cause the aberrations observed in the L1-L3 layers (Teixeira, 2005; Teixeira*, et al.*, 2005a).

Since the alterations in the *B. napus* (*A. thaliana*) CMS-system occur at an early flower developmental stage, that is the switch from a normal flower development to an abnormal one, occurs at an early stage (**II**, **III**), this system was studied at stages 0-5 and stage 8. While, *B. napus* (Ogu-INRA) was studied only at stage 8 since the modifications starts later (**III**). Of the nuclear encoded genes that were differentially expressed in one or several comparisons the differences were statistically significant for 72 genes in the *B. napus* (Ogu-INRA) CMS-system (Table 4 in **III**), and 665 genes in the *B. napus* (*A. thaliana*) CMS-system (Table 5 in **III**). Sixteen genes were found to be in common for both systems. Few similarities between the *B. napus* (*A. thaliana*) and *B. napus* (Ogu-INRA) CMS-systems were found. One possible explanation could be that the CMS-phenotypes develop at different stages in the two systems. The homeotic conversions of the stamens in *B. napus* (*A. thaliana*) occur earlier than the abortion of pollen in *B. napus* (Ogu-INRA). Still, some similarities were found. The conclusion is that each CMS-system is regulated in a fairly unique manner.

Of the 665 genes found in the *B. napus* (*A. thaliana*) CMS-system almost 80% were annotated to *A. thaliana* chromosome III, while the remaining 20% were evenly distributed on the remaining four chromosomes (Table 6 in **III**). On the contrary, the 72 genes found in the *B. napus* (Ogu-INRA) CMS-system were evenly distributed on the five *A. thaliana* chromosomes (Table 6 in **III**). In two of the *B. napus* (*A. thaliana*) lines, *A. thaliana* chromosome III was added to the nuclear genome, which explains why genes from this chromosome are over-represented in the *B. napus* (*A. thaliana*) CMS-system. The true *A. thaliana* transcripts will most probably have a higher affinity to the microarray probes relative to the *B. napus* homologous genes, and will thus contribute to higher signals. Another explanation could be that the addition of *A. thaliana* chromosome III causes an increased dosage of the genes found on the chromosome. Most likely it is a combination of both.

Of the genes found in the first study between the CMS-line and *B. napus* cv. Hanna, 243 genes were found to differ significantly at one or several stages (**II**). In the second study of the CMS-line and cv. Hanna, utilising the CATMA microarrays, 35 genes with a differential expression profile at one or two stages was observed (**III**). It is reasonable to expect that a similar number of genes would

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be found for the same comparison in both studies. One explanation to why this was not the case is that some genes are only present on one of the arrays. Of the 243 genes found on the cDNA-arrays 29 were absent on the CATMA microarrays. Of the 35 genes found on the CATMA microarrays, 26 genes were lacking on the cDNA-array.

Of the 214 genes present on both arrays, 189 genes had a significant different expression profile only on the cDNA-arrays (**II**, **III**). The reason to why these 189 genes were not found in the both studies could be explained by the differences between the two types of arrays. The CATMA microarrays include short probes that are highly specific for each *A. thaliana* gene to avoid cross-hybridisations within gene-families. This indicates that only genes which are highly conserved between the two species will hybridise well. The probes of the cDNA-arrays were relatively large and it was more likely for any given *B. napus* gene to hybridise well to the array. This has to be analysed further.

25 genes had a significant different expression profile in both studies. Eight of these 25 genes were present in comparison I. Seven of these eight genes display a similar expression profile while one of the genes (At5g20630) differs slightly (**III**). Moreover, 15 of the 25 genes were found in comparison II, seven genes were found in comparison III and finally 14 genes were found in comparison IV. The results from the two microarray-studies made are in agreement with each other even though the number of genes varied. Due to the high specificity of the CATMA microarrays only a narrow set of genes will be detected, while the cDNA-arrays gave a more complete list.

In comparison I, 35 genes had a different expression profile at one or both stages (**III**). The modified nuclear gene expression in the CMS-line in comparison to cv. Hanna is caused by the altered mitochondrial genome and is displayed as male-sterility and an altered flower phenotype. That is, the genes that were found to be differentially expressed in comparison I are indirectly or directly regulated by the mitochondrial genome and they are candidate genes likely to cause the CMS-phenotype. Genes that are involved in transcription were over-represented in comparison I. Apparently the modified mitochondria directly or indirectly have influenced several transcription factors or parts of the transcription machinery found in the nucleus. In comparison A, three genes had a significant different expression profile at one or both stages. Two genes (At1g44970 and At2g07727) had a lower expression and one gene (At2g40080) had a higher expression. The *B. napus* (*A. thaliana*) comparisons I and the *B. napus* (Ogu-INRA) comparison A were both comparisons between a CMS-line and a fertile maintainer-line. Genes that differed in expression in a similar manner in both comparisons were supposed to be a common CMS-response for the two CMS-systems. Two such genes were found, that is At1g44970 and At2g07727. The changed expression of At1g44970, a putative peroxidase, in both systems could reflect a mitochondrial induced stress-response, or that the CMS inducing pathway is interlinked with a stress response pathway. The differences in the amount of genes found in the two CMS-systems could be explained by that the CMS-trait is turned on later in *B. napus* (Ogu-INRA) compared to *B. napus* (*A. thaliana*).

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In comparison II, 412 genes had a different expression profile at one or both stages when comparing the restored line with the CMS-line (**III**). Except for the addition of *A. thaliana* chromosome III the two lines are isogenic regarding the mitochondrial and chloroplast genomes and the *B. napus* part of the nuclear genome. Genes that were observed in comparison II were probably differentially expressed due to that the two different nuclei responded differently on the mitochondrial influence. The lack of overlap between functional categories between comparison I and II are most likely due to the biased expression of *A. thaliana* chromosome III genes. In comparison B, 57 genes had an altered expression profile at one or both stages. Fifty of these genes had a higher expression in the *PPRB* restored line in comparison to the CMS-line, and seven had a lower expression. The *B. napus* (*A. thaliana*) comparisons II and the *B. napus* (Ogu-INRA) comparison B were both comparisons between a restored line and a CMS-line. Seven genes were shared between the two comparisons. As for comparisons I and A, the differences in the amount of genes found in the two CMS-systems in comparison II and B could be explained by the fact that the CMS trait is turned on later in *B. napus* (Ogu-INRA) compared to *B. napus* (*A. thaliana*). The seven genes are believed to be related to several stress-responses, for example pathogen responses or responses to salt-stress. The genes could be part of stress-response pathways regulate by or via the mitochondria. These pathways or part of them might also be involved in the CMS-regulation, since CMS is caused by dysfunctional mitochondria, which thus creates a stress situation for the cells.

In comparison III, 87 genes had a different expression profile at one or both stages (**III**). Comparison III parallels comparison I. That is, the modified nuclear gene expression in the restored line in comparison to cv. Hanna+At3 is caused by the altered mitochondrial genome. However, it is not manifested as an altered flower phenotype. The genes that had a changed expression in comparison III were differentially expressed due to the altered mitochondrial background, since the nuclear genomes are isogenic between the two lines. This indicates that the modified mitochondria and dysfunctions may evoke for example several stress-responses. It also indicates that the mitochondrial effects on the nucleus depend on the nuclear background. In comparison I the altered mitochondria acts on a pure *B. napus* nucleus while in comparison III the *B. napus* nucleus has the addition of chromosome III from *A. thaliana*. In both comparisons the novel mitochondria can induce a modified nuclear gene expression. However, it is not the same set of nuclear genes that are affected. This may partly be explained by the biased expression of *A. thaliana* chromosome III genes discussed above.

Comparisons I and III could also be further analysed to get a broader understanding on retrograde signalling in plants, since it is possible to compare the action of the same type of mitochondria in two different nuclear backgrounds. Three sets of genes were found when comparing comparisons I and III (**III**). One group of 21 genes were specific to comparison I that is, these genes directly or indirectly respond to retrograde signalling and are causing the CMS-phenotype. A second group containing 73 genes are specific to comparison III, and are assumed

to respond to retrograde signalling and cause changes specific to the restored line. The third group included 14 genes that are shared between comparison I and III. These genes respond to retrograde signalling but are not affecting the phenotype.

In comparison IV, 577 genes had a different expression profile at one or both stages (**III**). Except for the addition of *A. thaliana* chromosome III the two lines are isogenic regarding the mitochondrial and chloroplast genomes and the *B. napus* part of the nuclear genome. Genes that were observed in comparison IV were probably differentially expressed due to that the two types of nuclei responded differently on the mitochondrial influence, or that the extra chromosome itself affected, or contributed, to an altered nuclear expression. The addition of *A. thaliana* chromosome III influenced the nuclear gene expression, but no apparent phenotype was observed.

In comparison C, 48 genes had a different expression profile at one or both stages (**III**). Thirty-nine of these genes had a higher expression in the *PPRB* restored line in comparison to the cv. Pactol, and nine had a lower expression. No similar comparisons were made in the *B. napus* (*A. thaliana*) CMS-system.

Several nuclear encoded genes had a modified expression profile in the CMS-line 4:19 in comparison to *Brassica napus* cv. Hanna (**II**, **III**), and in the restored line in comparison to *B. napus* cv. Hanna +At3 (**III**). In the comparisons two pairs of lines were compared, both pairs are isogenic regarding the nuclear genome but have different mitochondrial genomes. The conclusion is that the altered mitochondria have influenced nuclear gene expression, and that this was done through retrograde signalling. The retrograde signalling appeared to mainly affect genes involved in transcription and stress responses.

## The adenylate-content is not correlated to CMS

It has previously been shown that the male-sterile lines 4:19 and 41:17 had a lower adenylate-content than *B. napus* cv. Hanna (Teixeira, 2005; Teixeira*, et al.*, 2005b). This was in agreement with the proposed energy-hypothesis discussed in the *Introduction*. The hypothesis that has been put forward try to explain the floral phenotypes of CMS-lines based on the idea that an increased demand for respiratory function and energy equivalents during flower development cannot be provided for by the abnormal mitochondria of a CMS-line (Tadege & Kuhlemeier, 1997; Hanson & Bentolila, 2004; Linke & Börner, 2005). A similar result was obtained in this thesis (**I**). Genes that had a lower expression in the CMS-line 4:19 in comparison with *B. napus* cv. Hanna were overrepresented in the functional category *Energy*, for example genes encoding proteins involved in glycolysis, in the citric acid cycle or components of the electron transport chain (**II**). Taken together, these results indicate that altered adenylate-content and/or energy-production may cause the male-sterility phenotype for the two male-sterile lines. In publication **I** however, the adenylate-content was measured in all 21 *B. napus* (*A. thaliana*) lines. This study showed that there was no or a low correlation between adenylate-content and male-sterility. Therefore, the new interpretation is

that the altered mitochondria influence the adenylate-content in the *B. napus* (*A. thaliana*) lines, at least in the 15 lines that have an altered adenylate-content in comparison to *B. napus*. This influence could be a retrograde signalling pathway that parallels the pathway influencing male-sterility rather than causing CMS. Moreover, the lines displayed aberrations in flowering time and growth rate (**I**). The conclusion is that the mitochondria directly or indirectly influence several regulatory pathways of plant development and metabolism. The flowering time, growth rate and adenylate-content had a low correlation with male-sterility (**I**). The assumption is that the three characteristics had no or a low influence on male-sterility.

## The importance of *APETALA3* and *PISTILLATA* in the CMS flower phenotype

The expression levels of the five ABC-genes (*AP1*, *AP2*, *AP3*, *PI*, *AG*), *LFY* and *UFO* was estimated for all 21 *B. napus* (*A. thaliana*) lines, the restored line and fertile *B. napus* using qRT-PCR (**I**). During stages 0-5 were no significant differences between the three classes, fertile, male-sterile and semi-sterile, found. At stage 8 were no significant differences found between classes regarding *AG* and *UFO*. However, for *AP3* and *PI* the male-sterile and semi-sterile lines had a significantly lower expression than the fertile lines and *AP1*, *AP2* and *LFY* had a significantly higher expression than the fertile lines. It has been shown that the *AP1* transcripts are elevated in *ap3* mutants in *A. thaliana* (Sundström *et al.*, 2006). The relatively high levels of *AP1* transcripts in the male-sterile lines could be due to the low levels of *AP3*, a parallel to what is observed in the *ap3* mutant. When comparing each gene line by line it was only *AP3* and *PI* that had a clear correlation to male-sterility. Most male-sterile and semi-sterile lines had a significantly lower expression of these two genes in comparison to the fertile cv. Hanna at both stages (**I**). *AP3*, *PI*, *AG*, *LFY* and *UFO* had similar expression profiles in **I**, **II** and in Teixeira *et al.* (2005a). In conclusion, only *AP3* and *PI* are correlated to the CMS-phenotype, while the remaining five genes have no or a low correlation indicating that they not are affected by the modified mitochondria.

No phenotypic differences were observed between the CMS-line 4:19 and *B. napus* cv. Hanna during the early flower stages 0-5, while homeotically transformed third whorl organs were easily recognised at stage 8 in the CMS-line (**I**) (Teixeira*, et al.*, 2005a; Teixeira*, et al.*, 2005b). The fully open flowers of the male-sterile lines showed a phenotype that partly resembled that of the *A. thaliana* B-class mutants *ap3* and *pi*. *AP3* and *PI* showed reduced expression levels in the CMS-line 4:19 in comparison to *B. napus*, according to the two microarray-studies and the qRT-PCR analyses performed in this thesis (**I**, **II**, **III**, **IV**). In stage 3 floral buds in the CMS-line as well as in *B. napus AP3* and *PI* were expressed in both whorls two and three. At stage 5 the *AP3* expression in whorl three of the CMS-line disappeared. The *PI* hybridization signal in whorl three of the CMS-line disappeared gradually from stage 7 and was completely abolished in stage 10 (**II**). The decreased expression levels of *AP3* and *PI* were detected for all CMS-lines studied (**I**). Clearly, the mitochondria directly or indirectly down-regulates the

gene expression of *AP3* and *PI* specifically in whorl three, and it is likely since both *AP3* and *PI* encode the homeotic transcription factors, that the male-sterility phenotype is caused by the down-regulation of those two genes.

The spatial expression of *AP3* and *PI* as well as the expression levels in the restored line was similar to those of cv. Hanna, indicating the possibility of *A. thaliana* chromosome III to restore the expression of *AP3* and *PI*, both spatially and the amount of transcripts (**I**, **III**, **IV**). The restored line contains *AP3* alleles from both *B. napus* and *A. thaliana*, and both are expressed (**IV**). This indicates that the addition of an *A. thaliana* copy of *AP3* do not mask the lack of *BnAP3* expression, rather is the expression restored. The expression might instead be restored by one of the *PPR*-genes present on *A. thaliana* chromosome III. The AP3 and PI proteins were present in all three lines, although to a lesser extent in the CMS-line. In addition, the expression of *AP3*, *PI*, *LFY* and *UFO* in the restored line in comparison to the male-sterile line 4:19 was closer to the expression in *B. napus* cv. Hanna (**I**). The presence of low levels of *AP3* and *PI* transcripts and proteins in the CMS-line are likely due to a normal expression in the petals.

Thus, the conclusion is that down-regulation of *AP3* and *PI* in whorl three organs in the CMS-lines caused the CMS-phenotype. The down-regulation of these two genes was a direct or indirect response of retrograde signalling. First a down-regulation of the *AP3* expression was obtained followed by a down-regulation of *PI* expression, which likely is a consequence of the lowered *AP3* levels.

# Conclusions

An altered composition of the mitochondrial DNA, or rather the presence of novel mitochondrial transcripts, has a large effect on nuclear gene expression. The mitochondrial influence on the nuclear gene expression is due to expression of novel *orf*s rather than to incompatibilities between species. The phenotypic modifications that occur as a consequence of the altered nuclear gene expression are preferentially found in whorl three flower organs. Not only the B-genes *AP3* and *PI* are targets, but also other genes acting in whorl three. Besides this the mitochondria directly or indirectly influence other regulatory pathways of plant development and metabolism.

# Future perspectives

The future perspectives can be divided into three categories. The first category includes concrete experiments that could be followed up in the near future. In the second category concrete but time consuming experiments are proposed. In the

last and third category ideas that would be intriguing but perhaps a bit far fetched to follow are suggested.

A first step would be to follow up the analyses of the presence or absence of APETALA3 (AP3) and PISTILLATA (PI) proteins in the CMS-line 4:19 (**IV**), by using the antibodies for immunolocalisation and study the localisation of these two proteins in the floral buds. Such studies may elucidate if the two genes are regulated at a transcriptional or translational level.

In order to study the importance of *AP3* and *PI* in the CMS-phenotype it would be appealing to transform the CMS-line 4:19 with *AtAP3* and/or *AtPI* to observe if the *A. thaliana* copies of these two genes are expressed in the presence of the CMS-inducing mitochondria and thus could restore the flower phenotype. In addition the CMS-line 4:19 transformed with *BnAP3* or *BnPI* driven by the 35S-promoter have to be evaluated.

The putative *PPR Rf*-gene (At3g22470) was introduced in the CMS-line 4:19 through crosses with a transgenic *B. napus* line carrying the *PPR*-gene. It appears as if the gene is expressed and partially restores the male-fertility (personal communication, Dr. J. Sohlberg and Dr. M. Leino, May 2007). To gain more insights about its function *A. thaliana* mutants could be studied.

The *A. thaliana* DNA contribution to the mitochondrial genome of the somatic hybrid lines has been mapped (**I**). A rather large and time-consuming but interesting project would be to do a corresponding *B. napus* marker map or to sequence the complete mitochondrial genomes of the lines. The sequencing of the genomes could provide information about the rearrangements in the mitochondrial genomes. In addition, this mapping-project could include fine-mapping of the *A. thaliana* markers associated with the male-sterile and semi-sterile lines (that is markers V (*orf139*), XX-XXI (*orf240a* and *cox3*) and XXX (*orf294*)) to detect putative CMS-inducing *open reading frames*. It would also be intriguing to fine-map and characterise marker XIII, the marker that is lacking in all hybrids (**I**). Finally, the addition of other fertile lines (and perhaps male-sterile and semi-sterile lines) in the mapping populations could help to reduce the number of markers associated with sterility, and/or to increase the ability to do correlations between mitochondrial markers and other characteristics. These extra lines could also be of interest in studies regarding retrograde signalling. Moreover the mapping of the two species could tell us more about the mitochondrial recombination. The fine-mapped genes would be candidates for mitochondrial CMS-inducing genes, from which gene expression, protein expression and function, cellular localisation, *etc.* could be studied.

Another project would be to choose and to study other nuclear encoded genes found in the two microarray studies (**II**, **III**). For example, characterise the differentially expressed genes that had a significant difference in expression during the early stages 0-5 (that is genes found in for example cluster I in publication **I**), and to elucidate whether they may be involved in the initiation of the CMS-trait or not.

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The complete set of *B. napus* (*A. thaliana*) lines (**I**) could be used for studying different aspects of retrograde signalling not only connected to CMS. These lines could be further characterized, regarding different metabolites, such as sugar and starch, as well as other developmental aspects for example shoot height and leaf size. These characteristics could be correlated to the mitochondrial markers described in publication **II**. Retrograde signalling could also be studied using the CMS-line 4:19 in comparison to *B. napus* cv. Hanna or the *B. napus* cv. Hanna +At3 in comparison to the restored line.

The mitochondrial genomes of the *B. napus* (*A. thaliana*) lines studied in this thesis harbour pieces of DNA from both *A. thaliana* and *B. napus* (**II**). It would be interesting to test what happens with the flower phenotype if pure *A. thaliana* mitochondria were introduced to a *B. napus* nuclear background. Will mitochondria with a combined genome between *B. napus* and *A. thaliana* give the same effect on a *B. napus* nucleus as pure *A. thaliana* mitochondria, that is, does the phenotype arise due to a conflict between species or due to alterations in the mitochondrial genome?

# Sammanfattning på svenska

Varje dag äter vi människor livsmedel som innehåller stärkelse, protein och oljor från olika grödor. Dessa livsnödvändiga näringsämnen utvinns ur frön som fås från blommans pistill efter att den har befruktats av pollen från ståndarna. Därför är det av stor vikt att förstå de mekanismer som styr blomutvecklingen, speciellt utvecklingen av ståndare och pistill.

## Blommor är bisexuella

Vanligen består en blomma av fyra olika organ, ordnade i fyra kransar (whorls på engelska). I den yttersta, nummer ett, finns foderbladen som täcker blommans knopp. Kronbladen som hos många arter ofta är mycket uppseendeväckande vad gäller färg och form, återfinns i krans två. Krans tre omfattar ståndarna, blommans hanorgan. I den innersta kransen, nummer fyra, finns pistillen, blommans honorgan, där bildas frön efter befruktningen. Blommor är med andra ord bisexuella, men det finns undantag.

Det finns ett flertal gener i cellkärnan som styr bildandet av blomman. Den så kallade ABC-modellen (Figur 1) ger en översikt av hur en blomma bildas. A-generna (*APETALA1* och *APETALA2*) ger upphov till foderbladen, och bildar tillsammans med B-generna (*APETALA3, AP3,* och *PISTILLATA, PI*) kronbladen. B-generna tillsammans med C-genen (AGAMOUS) bildar i sin tur ståndarna. Enbart C-genen ger upphov till pistillen. Om någon av generna i ABC-modellen är avstängd kommer blomman att sakna ett eller flera organ. Om till exempel en av B-generna är avstängd utvecklas en blomma som saknar ståndare men som har många foderblad och pistiller. Många av de fyllda blommorna som vi till exempel ser i blomsteraffärer och trädgårdar har fått C-genen avstängd.

Denna avhandling handlar om hur cellens energifabrik, mitokondrien, kan påverka arvsmassan, generna, i cellkärnan hos växter. Genom att mitokondrien påverkar vissa gener i cellkärnan kan blommorna hos en växt utvecklas på andra sätt än det normala, förväntade mönstret. För en växtförädlare kan kunskapen om vilka gener som är inblandade i cellkärnans och mitokondriernas interaktioner användas för att producera hansterila blommor vilket kan underlätta framtida förädlingsstrategier. Som modell-system har raps använts, en gröda vars frön har stor betydelse för produktionen av hälsosammare matoljor men även för produktionen av biodisel.

## Hansterila blommor ger möjlighet till korsbefruktning

Många växter är inte bara bisexuella, de är självbefruktare också. Det vill säga en och samma invid är både pappa och mamma. För att få in nya egenskaper behöver en självbefruktare korsa sig med en annan individ, korsbefruktning. Detta är speciellt viktigt i växförädling, då man ofta vill kombinera egenskaper från olika individer. Det har visat sig att avkomman från två olika individer är bättre än

föräldrarna med avseende på vissa egenskaper. Det är sådana avkommor som hittas i fröpåsarna som är märkta F1-hybrider. När växtförädlare vill korsbefrukta två individer kan till exempel ståndarna tas bort från den förälder som ska bli moderplantan. I naturen kan två typer av hansterilitet uppkomma. Den ena sorten orsakas av mutationer i cellkärnan. Den andra, som kallas cytoplasmisk hansterilitet, orsakas av förändringar i mitokondrien.

Cytoplasmisk hansterilitet (CMS), beror på att mitokondrien och cellkärnan inte kan kommunicera på ett korrekt sätt (Figur 2). CMS ärvs från generation till generation genom att mitokondrierna överförs via äggcellen, alltså på mödernet. Hansterilitet genom CMS kan se ut på många olika sätt, till exempel kan ståndarna i en blomma helt sakna pollen eller så kan ståndarna vara pistillika (Figur 5). Fördelen med hansterila blommor är att de på ett enkelt sätt möjliggör korsbefruktning.

## Backtravs DNA i mitokondrien ger hansteril raps

Raps (*Brassica napus* L. ssp. *napus*) odlas som oljegröda i stora delar av världens tempererade områden, bland annat i Sverige. I Sverige odlas 120-200 tusen ton raps och rybs per år, i EU är motsvarande siffra 10-15 miljoner ton. Omförestrad rapsolja så kallad RME används som biodisel. En biprodukt vid omförestringen är glycerol som bland annat används av kosmetikaindustrin i till exempel hudkrämer. Rapsolja brukar kallas Nordens olivolja och är mycket hälsosam, rent av nyttigare än olivolja. Till exempel innehåller rapsolja en hög andel enkel- och fleromättade fettsyror samt 15 gånger mer Omega 3 än olivolja. Raps är nära släkt till flera andra arter som används som livsmedel, t. ex. kål (Figur 3). Även om de kan tyckas olika så tillhör kålrot (*B. napus* L. ssp. *rapifera*) samma art som raps. På Gotland, i Nederländerna och i Storbritannien har vilda bestånd av raps hittats.

Backtrav (*Arabidopsis thaliana* (L.) Heynh.) är en växt som är spridd över större delen av världen. Den växer på torra och öppna marker som till exempel i klippskrevor och vid vägkanter. Backtrav är välbeskriven och används som modellväxt för att förstå hur växter fungerar. Fördelarna med att använda backtrav som modellväxt är att den är liten, är lätt att odla, har en liten arvsmassa samt att dess arvsmassor i cellkärnan, mitokondrien och kloroplasten är kartlagda.

Växterna som studerats i denna avhandling har en cellkärna från raps och mitokondrier vars arvsmassa kommer från både backtrav och raps (Figur 4). Växt-linjerna bildades genom att celler från raps respektive backtrav fick smälta samman till nya celler. Från dessa celler bildades det nya plantor. Varje planta är modern till en unik linje. Det gemensamma namnet för alla linjerna är *B. napus* (*A. thaliana*). Bland dessa linjer finns hansteril raps med ståndare som ser ut som pistiller (Figur 4).

## Växternas mitokondrier har ett stort inflytande på blommans utseende

Det övergripande syftet med denna avhandling är att uppnå en fördjupad förståelse av interaktionen mellan cellkärna och mitokondrierna, samt hur denna interaktion påverkar blomutvecklingen. Avhandlingen fokuserar främst på det som sker i cellkärnan. Studier av fertila, semisterila och hansterila *B. napus* (*A. thaliana*) linjer har gjorts för att få en överblick av mitokondriernas effekt på cellkärnan.

De 21 *B. napus* (*A. thaliana*) linjerna kan delas in i tre klasser; fertila, semisterila och hansterila (Figur 4). De 8 fertila linjerna har blommor som liknar rapsblommor, det vill säga normala blommor med pollen. Dessutom kan de få frön. De 10 hansterila linjerna har feminiserade ståndare, alltså ståndare som ser ut som pistiller. Dessa blommor får inget pollen men kan fortfarande få frön. De tre semisterila linjerna har miniatyr rapsblommor. Ståndarna är små, men producerar pollen. Precis som de andra linjerna kan även de semisterila linjerna få frön. Dessutom växer linjerna olika mycket, de innehåller olika mycket adenylater och blommar vid olika tidpunkter. Slutsaten är att mitokondrien kan påverka generna i cellkärnan och därmed reglera växtens utveckling.

Att identifiera och beskriva de gener som är inblandade i interaktionen mellan cellkärna och mitokondrierna och som dessutom kontrollerar utvecklingen av blomman har varit ett delprojekt. Ett urval av gener följdes upp med detaljerade analyser av genernas och proteinernas uttryck. Om generna och proteinerna uttrycks förväntar vi oss en normal blomma, om de däremot är avstängda förväntar vi oss att se det som blommor med avvikande utseende. Flera kärnkodade gener uppvisar en förändrad uttrycksprofil i bland annat den hansterila linjen 4:19 jämfört med raps, vilket beror på att mitokondrien påverkar hur generna uttrycks. Förändrade uttrycksprofiler observerades även i andra linjer. Generna *AP3* och *PI*, som bland annat behövs för att det ska bli ståndare, har en lägre nivå i de hansterila linjerna jämfört med raps. Det innebär att B-genfunktionen är avstängd och att det inte bildas ståndare i krans tre i de hansterila linjerna. Sammantaget innebär detta att mitokondrierna har ett stort inflytande på hur ett flertal generna i cellkärnan regleras. För blomknoppar innebär det att utvecklingen av blomorganen påverkas. Denna reglering tycks i huvudsak påverka gener i krans tre, vilket ger feminiserade ståndare.

Med hjälp av mitokondrien kan växtförädlarna stänga av de gener som behövs i krans tre för att ståndare med pollen ska bildas, på så sätt skapas plantor utan fertila ståndare som fungerar som moderlinjer. Detta underlättar möjligheterna för korsbefruktning, och nya, bättre plantor fås. Vad det gäller raps så skulle detta kunna underlätta för att få nya rapssorter som innehåller till exempel en än större andel nyttiga oljor.

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