Developmental studies of cytoplasmic male-sterile *Brassica napus* lines

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2005

Acta Universitatis Agriculturae Sueciae
Agraria 2005:8

ISSN 1652-6880
ISBN 91-576-7007-2

Tryck: SLU Service/Repro, Uppsala 2005

Que me quereis, perpétuas saudades?

Que me quereis, perpétuas saudades?
Com que esperança inda me enganais?
Que o tempo que se vai não torna mais,
E se torna, não tornam as idades.

Razão é já, ó anos, que vos vades,
Porque estes tão ligeiros que passais,
Nem todos para um gosto são iguais,
Nem sempre são conformes as vontades.

Aquilo a que já quis é tão mudado,
Que quase é outra cousa, porque os dias
Têm o primeiro gosto danado.

Esperanças de novas alegrias
Não mas deixa a Fortuna e o Tempo errado,
Que do contentamento são espias.

Luís de Camões
(1524-1579)

To my parents
Aos meus Pais

To my sister
À minha irmã

Abstract

Teixeira, R. T. 2005. Development studies of cytoplasmic male-sterile *Brassica napus* lines.
Doctor's dissertation
ISSN: 1652-6880, ISBN: 91-576-7007-2

The presence of male sterile plants in the wild gained the attention of breeders to utilize the trait for hybrid seed production in economically important crops. For scientists, cytoplasmic male sterile (CMS) plants are regarded as an excellent tool to study the genetic interactions between mitochondria and nucleus in flower development. CMS plants can be obtained after sexual crosses between different species of the same family or by somatic hybridisations between unrelated species. The *Brassica napus* CMS lines investigated in this thesis were obtained after protoplast fusion between *B. napus* cv. Hanna and *Arabidopsis thaliana* var. Landsberg erecta. After several backcrosses using *B. napus* as the male parent, the cells of the CMS lines contain the nucleus and the plastids from *B. napus* while the mitochondria have a rearranged mitochondrial (mt) DNA between the two species.
The vegetative and flower development of the two CMS lines was compared with *B. napus*. The CMS plants showed a reduced seedling growth. This slower growth rate was present throughout the vegetative development of the CMS plants. They also bolted later than *B. napus*. However, when fully matured they were of the same size as *B. napus*. The reduced number of stem cells and its smaller size during the first six weeks of growth seems to be the reason for the shorter stature of the CMS plants. Metabolic studies revealed that the CMS plants had an abnormal starch accumulation with a concomitant reduction of sucrose levels when compared to *B. napus*.
The CMS plants are mainly characterized by the presence of carpelloid structures in the third whorl of the flower, replacing the stamens of *B. napus*, and by petals reduced in size. Histological and ultrastructural studies made of young flower buds showed that the cell division pattern in the putative whorls two and three was altered. Cells in the CMS lines had divided in several directions instead of the typical anticlinal cell division present in the two first layers of the *B. napus* young flower bud. The same alterations in cell division patterning were also observed in the vegetative meristem of the CMS lines. In both these two meristematic tissues (vegetative and floral), two mitochondrial populations were found in the CMS lines. One population of mitochondria resembled the *B. napus* ones at the ultrastructural level but were always smaller in size. The other population showed disrupted inner-membrane systems and the density of the matrix was strongly reduced. In accordance with the disrupted mitochondria, flower tissues from the CMS plants displayed reduced levels of ATP in comparison to *B. napus*.
All the homeotic genes and their upstream genes showed the same expression pattern in young flower buds between the three lines until third whorl organs had differentiated. Even though the pattern was similar, the expression levels of the same genes showed differences between the two CMS lines and *B. napus*. By the time third whorl organs started to differentiate, *BnAP3* and *BnPI* expression levels were strongly reduced while the upstream genes like *BnUFO* and *BnLFY* were up-regulated. This up-regulation suggest that the action of *BnUFO* and *BnLFY* is interrupted when activating *BnAP3* and *BnPI* that in turn, seems to reflect a feedback up-regulation mechanism by the nucleus. This feedback regulation can be explained by the fact that the nuclear genes in the CMS cells are not mutated. The transcription of nuclear genes coding for non-functional proteins will then be up-regulated. The hypothesis developed in this study relates the reduced levels of ATP in the flower tissues of the CMS lines with the protein degradation of key proteins necessary for correct flower development and cell division.

Key words: *Brassica napus*, cytoplasmic male sterility, flower development, homeotic genes

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Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to as their roman numerals.

I. Leino, M.*, Teixeira, R.*, Landgren, M. and Glimelius, K. (2003) *Brassica napus* lines with rearranged *Arabidopsis* mitochondria display CMS and a range of developmental aberrations. *Theor. Appl. Genet.* 106: 1156-1163.

II. Teixeira, R.T., Knorpp, C. and Glimelius, K. (2004) Modified sucrose, starch and ATP levels in two alloplasmic male-sterile lines of *B. napus*. *J. Exp. Bot*. (submitted).

III. Teixeira, R.T., Farbos, I. and Glimelius, K. (2004) Expression levels of meristem identity and homeotic genes are modified by nuclear-mitochondrial interactions in alloplasmic male-sterile lines of *Brassica napus*. *Plant J*. (accepted pending revision)

IV. Carlsson, J., Teixeira, R.T., Sundström, J., Lagercrantz, U. and Glimelius, K. (2004) Cytoplasmic male sterility *Brassica napus* displays altered expression in a diverse range of nuclear genes according to microarray analysis. (Manuscript).

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Contents

Introduction

Brassica napus L.

Brassica napus L., is an allotetraploid or amphidiploid species with n=19 chromosomes that is derived from crosses between *B. oleracea* (n=9) and *B. rapa* (n=10) (U, 1935; Song and Osborn, 1992). These crosses are most probably ancient and may have occurred at different places in the world (Rakow, 2004). Molecular data showed that both *B. rapa* and *B. oleracea* have evolved from one progenitor (Gómez-Campo and Prakash, 1999). *B. napus* is one of 37 species of the genus *Brassica* belonging to the Cruciferae or Brassicaceae family (Rakow, 2004). The genus *Brassica* includes many crop species of economic importance (Rakow, 2004) such as turnip rape (*B. rapa*), Indian mustard (*B. juncea*) and black mustard (*B. nigra*). *B. oleracea* includes very important crops such as kales, savoy cabbage, Brussels sprouts, cauliflower, broccoli and calabrese (Gómez-Campo and Prakash, 1999).

Due to their ability to germinate and grow at low temperatures, the oilseed *Brassicae* is a few of the edible oil crops that can be cultivated in cooler agricultural regions and at higher elevations (Downey and Röbbelen, 1989). Both winter and summer annual forms of *B. napus* are grown as oilseeds in many countries of the world, it is the most productive *Brassica* oilseed species under cultivation (Rakow, 2004). Wild *B. napus* has been reported to grow on the beaches of Gotland, Sweden, the Netherlands and Britain (Rakow, 2004). The rapeseed oil produced from varieties of *B. napus* in Canada (known as canola), European Union (known as rapeseed) and Australia have a high oleic acid content of about 60%. Its fatty acid composition is of superior nutritional value for human nutrition, and rapeseed oil is therefore considered one of the healthiest vegetable oils. The content of saturated fat is low (about 7%) which is also beneficial for human health (Rakow, 2004). Before rapeseed-quality varieties of *B. napus* were developed (zero erucic acid content, low glucosinolate), rapeseed oil was primarily used for industrial applications such as lubricant for steam engines and as a lamp oil (Downey and Röbbelen, 1989).

Cytoplasmic male sterility (CMS)

Cytoplasmic male sterile (CMS) plants have been found in over 150 wild species (Laser and Lersten, 1972, a phenomenon already noticed by Darwin (1877). The natural occurrence of male sterile plants in a given population is the result of the need hermaphrodite plants have to promote crossing of one plant with pollen from another plant to avoid inbreeding depression (reviwed by Budar and Pelletier, 2001, Budar et al., 2003). As was stated once by Darwin, the dimorphism imposed by several male sterile plants is a step towards the separation of the different sexual organs into different individuals (diocy), which represents the achievement of an obligate cross-fertilizing mating type (discussed in Budar and Pelletier, 2001). In nature, the presence and co-existence of hermaphrodite and male sterile plants of the same species in a population is a phenomenon called gynodioecy (Budar and Pelletier, 2001; Budar et al., 2003).

The importance of CMS systems was noticed by its agricultural value for the production of hybrid seeds. CMS plants are used as female parents in crosses ensuring that all seeds produced, are the result of cross-pollination (Hanson and Conde, 1985). Beside the CMS systems, the production of hybrid seeds can be performed using other mechanisms: nuclear genetic male sterility and hand-emasculation. However, these two methods are not economically viable since it would require an enormous amount of man labour in the field. Using nuclear genetic male sterile plants in the field, all the male fertile plants have to be removed. The elimination of these male fertile plants from a population segregating for nuclear recessive male sterility creates the risk of leaving some plants behind interfering with the desirable cross hybridisation. The hand emasculation of the crop plants is extremely time consuming (Hanson and Conde, 1985). Hybrid seed production in *Brassica* can also be obtained using self-incompatible plants, since a large number of species are self-incompatible by expressing the self-incompatible genes (SI) (Watanabe and Hinata, 1999). Male sterile plants obtained by genetic transformation is another method used in order to obtain a system where pollination can be controlled and produce hybrid seeds (Mariani et al., 1990; reviewed by Budar et al., 2004). Application of chemical hybrididizing agents (CHAs) also known as male gametocides induce transitory male sterility promoting the outcrossing (reviewed by Tu and Banga, 1998).

Spontaneous mutations can also give rise to naturally occurring plants that, through interspecific exchange of nuclear and cytoplasmic genomes, produce male sterile plants. In the case of the cytoplasmic male sterile plants, the trait has been synthesized as a result of crosses in which the nuclear genome of one species has been moved into the cytoplasmic background of another (Hanson, 1991). The resulting combination of the nucleus and cytoplasm is called “alloplasmy”. Several CMS lines have occurred in breeding lines without intentional intervention of man being used for agriculture purposes later on: for example, the maize T-cytoplasm (Levings, 1990), the *pol* cytoplasm of *B. napus* (Singh and Brown, 1991), the male sterile cytoplasm of common bean (Mackenzie, 1991) and the S-cytoplasm of onion (Tatlioglu, 1982)

A typical way to produce alloplasmic CMS systems is via interspecific crosses. By this, it is common to obtain both male and female sterile plants in the F1 generations as a result of chromosomal and meiotic aberrations unrelated to CMS. The resulting interspecific hybrid is then backcrossed with the crop species of interest in successive generations allowing the elimination of nuclear genes from the wild type species until the alloplasmic plant reaches a nearly pure nuclear genome from the cultivated species into the wild cytoplasm background (Hanson and Conde, 1985). Interspecific hybridisations can be achieved between otherwise incompatible species via sexual crosses followed by embryo rescue (Glimelius, 1999) or via protoplast fusion with the production of cybrids (Pelletier et al., 1983, Earle, 1995, Glimelius, 1999). Production of hybrids via sexual crosses or sexual hybridisations is limited by the distance between the two species used in the crosses (Glimelius, 1999). One of the problems is the reduction in fertility that can result from the difference of the non-homologous chromosomes of the two parental plants (Chopra et al., 1996). Leaf chlorosis and poorly developed nectaries (Bannerot et al., 1997) are other defects that can appear together with CMS (Edwardson, 1970). Such vegetative alterations appear because, usually, the

organelles are inherited maternally, and the combination with a different nuclear background leads to some incompatibilities. The *nap* system in *B. napus* (Banga and Banga, 1998) and the *B. campestri* and *B. juncea* alloplasmic lines created after introduction of *B. oxyrrhima* cytoplasm (Prakash and Chopra, 1990) are examples of chlorosis associated with CMS. One method to overcome these problems obtained after sexual hybridisation is the use of protoplast fusion (Glimelius, 1999). Protoplast fusion is an effective method to obtain a high frequency of CMS phenotypes and to create novel nuclear-cytoplasmic combinations from sexually incompatible and distantly related species (Glimelius and Bonnett, 1981; Pelletier et al., 1983; Zubko et al., 1996; 2003). After protoplast fusion, several cells contain the nucleus of one species imbedded in a hybrid cytoplasm of the two species used for protoplast isolation (Glimelius, 1999). The mitochondrial genome presents extensive recombination from the two species combined (Pelletier et al., 1983; Kofer et al., 1991, Earle et al., 1992). Even though the original cytoplasm contains the plastids and mitochondria from the two species fused, elimination of plastids from one of the parents will occur during the following cell divisions (Pelletier et al., 1983, Landgren et al., 1996).

Several CMS-inducing cytoplasms have been reported. In sunflower, for example, the appearance of the cytoplasm inducing CMS was derived from an interspecific cross between *Helianthus petiolaris* and *H. annus* (Siculella and Palmer, 1988). Similarly, the *ogu* cytoplasm derived from radish confers male sterility in *B. napus* (Bannerot et al., 1974) as also in *B. juncea* (Kirti et al., 1995). Other CMS inducing cytoplasms in *B. napus* are derived from *A. thaliana* as reported from Forsberg et al. (1998) and used in this study as well as the cytoplasm from *B. tournefortii* (Stiewe and Röbbelen, 1994; Liu et al. 1996). Several different *Nicotiana* cytoplasms can cause CMS in *N. tabacum* plants (Bonnett et al., 1991).
All the CMS systems analysed so far have shown that the mitochondrion is the organelle in the cell that is associated with CMS (Vedel et al., 1982; Earle et al., 1992; Hanson and Bentolila, 2004). Plant mitochondrial genomes are very stable in sequence but highly variable in organization (Palmer and Herbon, 1988) and have an unusual recombination activity (Small et al., 1987). Mitochondria have a tendency to accumulate dominant mutations as a consequence of intergenic recombination events (Bonen and Brown, 1992). Such mutations have been associated with pollen sterility (reviwed by Hanson, 1991; Schnable and Wise, 1998). Compared to animals, plant mitochondrial genomes are large in size although, they encode only a fraction of the genetic information required for mitochondrial biogenesis and function (Marienfeld et al., 1997). Plant mitochondria possess a large number of ORFs of unknown function (Marienfeld et al., 1997). Some of these may regulate or possibly induce CMS.
The CMS trait can be described as a mitochondrial mutation, which impairs the proper function of the mitochondria, leading to male sterility (reviwed by Budar and Pelletier, 2001). The mitochondrial “mutations” are related to rearrangements of mtDNA, often containing novel open reading frames (ORFs) that can be physically associated and co-transcribed with standard mitochondrial genes. The mitochondrial rearrangements can also result in production of chimeric genes where a given *orf* contains fragments of standard genes (reviewed by Schnable and Wise, 1998).
A common feature shared by several of the CMS-associated genes is the fact that they encode proteins containing large hydrophobic domains (Schnable and Wise,

1998). Fig. 1 illustrates some of the rearranged regions of the mtDNA associated with CMS. Examples of such novel genes can be found in common bean where the *atpA* gene is co-transcribed with *orf239* (Mackenzie and Chase, 1990) and in sunflower where the same gene is co-transcribed with *orf522* (Horn et al., 1996). In CMS tobacco lines *atpA* is co-transcribed with *orf274* (Bergman et al., 2000). In *B. napus* or *B. juncea* containing the *B. tournefortii* cytoplasm (Landgren et al., 1996), *atp6* and *orf263* are co-transcribed.

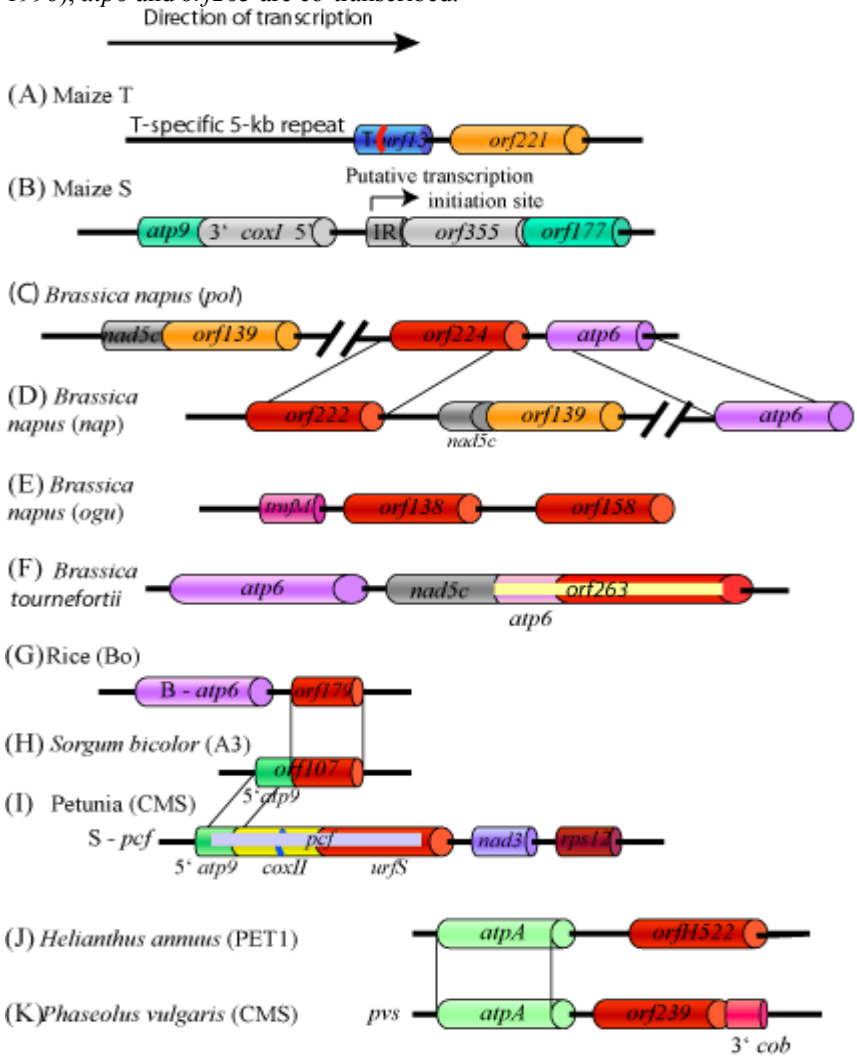

Fig. 1. Chimeric cytoplasm male sterile (CMS)-associated regions in the mitochondrial genomes of various species (marked next to the corresponding CMS-associated *orf*). IR – Inverted region. Red regions – ORFs. The direction of transcription (from 5' – to the 3'-end) is as indicated at the top of the figure. Adapted from Schnable and Wise (1998).

With the exception of the similarity between *orf222* and *orf224* from two CMS-inducing cytoplasms in *B. napus* (L'Homme et al., 1997; Brown, 1999), all other CMS-associated ORFs share no similarity among them (Schnable and Wise, 1998). Most of the mtDNA regions associated with CMS have involved gene promoter regions of the ATP synthase subunit and portions of coding regions

(Hanson and Bentolila, 2004). In most cases, the abnormal mtDNA region in the CMS plants is expressed in all plant tissues. In common bean, the CMS-associated gene product is apparently degraded by a protease in the mitochondria of vegetative tissue and hence the product only accumulates in reproductive tissues (Abad et al., 1995; Sarria et al., 1998).
The polypeptides encoded by CMS associated genes have in some cases been associated with the mitochondrial membranes. This is the fact with the URF13 protein from CMS-T maize (Levings, 1993), with ORF138 from the *ogura* CMS cytoplasm induced in *B. napus* (Grelon et al., 1994) and the 16 kDa protein encoded by *orfH522* in the male sterile PET1-cytoplasm of sunflower (Horn et al., 1996).

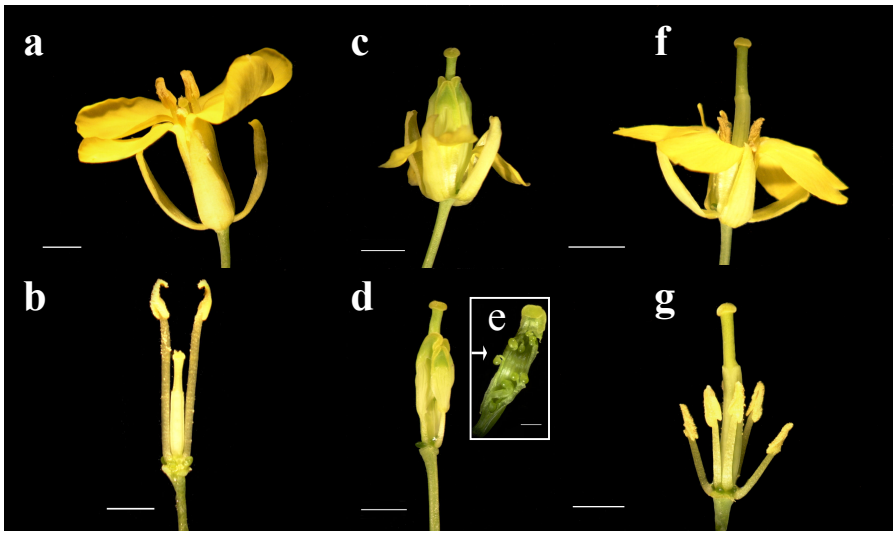

Fig. 2. **a** and **b** *Brassica napus* cv. Hanna flowers. **c** and **d** CMS line 4:19 displaying homeotic conversions of the third whorl organs into carpelloid structures. **e** magnification of the carpelloid organs. Ovule-like formations can be seen at the unfused margins of the organ. **f** and **g** CMS line 4:55 where the anthers are reduced in size and the levels of pollen production are less than in *B. napus* cv. Hanna. Petals were removed in **b**, **d** and **g**. Bar: **a** and **b**: 4mm, others: 3mm

The mechanism of CMS is still unclear. At this point, two observations seem to be relevant: 1 - most CMS-associated genes are expressed in all tissues of the plant throughout development. 2 – the observed phenotype is not uniform among all types of CMS, although in a majority of cases the first cytological anomalies appear in the tapetal cell layer, followed by the abortion of microspores (Budar et al., 2003). However, in some species, male sterility is manifested by homeotic alterations of the anthers (reviewed by Hanson and Bentolila, 2004). In the original *ogura B. napus* CMS plants growing at low temperatures (18ºC light/15ºC dark), the anthers in the male fertile lines were replaced by carpelloid structures bearing ovule-like structures (Polowick and Sawhney, 1986). This flower morphology closely resembles the flowers produced by the CMS plants used in this study (Leino et al., 2003, Fig. 2). In carrot lines containing CMS cytoplasm, flowers may exhibit either a petalloid or a carpelloid phenotype depending on the nuclear background (Linke et al., 2003). CMS tobacco plants obtained both by sexual and somatic hybridisation produce flowers with several degrees of

deformations of the male organs: from the complete absence of anthers to petalloid filaments tipped with stigmatoid structures (Kofer et al., 1992). Corolla length and form has also been altered in CMS lines of tobacco (Bonnett et al., 1991; Farbos et al., 2001). Homeotic conversions of anthers into carpelloid structures were also found in CMS lines of wheat (Murai et al., 2002). In *B. juncea* CMS lines induced by *D. berthautii* cytoplasm, several floral morphologies were obtained from smaller and indehiscent anthers containing empty, sterile pollen to petalloid stamens or antherless stamens (Malik et al., 1999).

The restoration of CMS

Nuclear genes called restorers of fertility (*Rf*) have the ability to suppress the male sterile phenotype and hence restore the production of pollen to plants carrying the deleterious mitochondrial genome. Restorer genes are present in response to selective pressure created by the spread of a male sterile cytoplasm in a plant population (Li et al., 1998). Nuclear restorer genes are also used to differentiate CMS systems within the same species indicating that restoration of fertility is specific for each CMS system (Bellaoui et al., 1999). This is due to the fact that several restorer genes, mapped to different chromosomal positions may restore one type of CMS-induced cytoplasm. The three different CMS-inducing cytoplasms in maize are good examples. In this case, each type of the cytoplasm inducing CMS is restored by a distinct nuclear gene (Braun et al., 1992; Levings, 1993). The opposite situation, in which more than one type of CMS can be restored by a gene or genes present at a single locus is rare but has been shown to occur in some *Brassica* CMS systems (Li et al., 1998).

Little is known about the molecular features of the restorer genes and the proteins they encode. In some CMS systems, fertility restoration is associated with decrease of expression of the CMS-associated transcript or protein. Altered RNA maturation of the CMS-associated transcripts in restored plants has also been demonstrated (Singh and Brown, 1991; Wise et al., 1996). Bellaoui et al. (1999) suggested that *Rfo* acts post-translationally in *B. napus* by selectively targeting the protein of ORF138 for proteolysis. Other restores mediate processing or translation of transcripts of CMS-associated genes (Schnable and Wise, 1998, Budar et al., 2003). The restorer *Fr* of bean CMS is the only known restorer gene acting at the level of mitochondrial DNA (He et al., 1996). It eliminates a 25 kb molecule bearing the CMS determinant, the *atpA-orf239* region. The *atpA* function is maintained because there is a second copy of this gene not associated with *orf239* (Janska and Mackenzie, 1993). Thus, Fr apparently reduces the expression of CMS-associated *orf239* by reducing the amount of the encoding DNA rather than the RNA or protein (Janska et al., 1998).

In maize, nuclear restorer genes reduce the amount of the unique polypeptides associated with the CMS type. *Rf1* affects the transcriptional profile of the T-urf13 and decreases the abundance of URF13 protein by about 80% but needs the action of Rf2 for complete restoration (Dewey et al., 1987; Levings, 1993). Compensatory mechanisms for the action of restorer gene products were advanced when the *Rf2* gene necessary for restoration in T-cytoplasm maize was cloned. The gene encodes for an aldehyde dehydrogenase suggesting that the role of the RF2 protein would be to detoxify the additional aldehydes produced in the cell (Cui et al., 1996). In summary, with the exception of maize *Rf2*, all restorers are known to affect either the transcript profiles or the protein accumulation of the

CMS-associated locus, and some have been observed to affect both RNA and protein products (Hanson and Bentolila, 2004).

Besides the maize *Rf2* that encodes for an aldehyde dehydrogenase (Cui et al., 1996; Liu et al., 2001), all other restorer genes cloned so far, encode for pentatricopeptide repeat (PPR) containing proteins. This is the case for *Petunia Rf* (Bentolila et al., 2002), for the kosena radish and *ogura Rfo* gene (Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003) and for Bo-CMS *Rf-1* in rice (Kazama and Toryama, 2003).
Petunia Rf regulates the expression of the abnormal CMS-associated *Petunia* mitochondrial gene *pcf* by affecting both the profiles of the transcripts and reducing the protein levels (Bentolila et al., 2002). As it was recently characterized by Lurin et al. (2004), the PPR motif proteins comprises a large family that are likely to be targeted to mitochondria and/or chloroplasts. They are characterized by the presence of tandem arrays of a degenerated 35 amino acid repeat. These proteins are thought to act at the level of RNA processing in plant organelles like RNA editing, which appears to require a potentially large number of undiscovered nuclear-encoded sequence-specific factors (Small and Peeters, 2000). Most of the PPR genes are assumed to modify the translation and/or processing of specific organelle-encoded RNA. The radish *Rfo* locus contains multiple, related and tightly linked genes that arose through evolutionarily recent gene duplication events and encodes proteins with repeat domains. (Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003). In the case of *Petunia*, the *Rf* locus is composed of duplicated genes containing a pentatricopeptide repeat (PPR) motif. This restorer gene is a mitochondrially-targeted protein that is almost entirely composed of 14 repeats of the 35 amino acid pentatriopeptide repeat motif (Bentolila et al., 2002).

CMS in *Brassica* species

Wild *Brassica* species are of great importance for breeding purposes because they are the source of a vast cytoplasmic variability for production of alloplasmic male sterile lines (Prakash et al., 1995; Banga and Banga, 1998). This genetic variability of *Brassica* cytoplasm is necessary to preserve and enlarge the cytoplasmic diversity in order to provide breeders with new and improved CMS systems and eliminate the possible problem of epidemic diseases (Cardi and Earle, 1997). CMS in *Brassica napus* can be conferred by alien cytoplasm, such as the *ogura* cytoplasm of radish (Ogura, 1968), *B. tournefortii* or the *B. oxyrrhima* cytoplasm (Shiga and Baba, 1980; Stiewe and Röbbelen, 1994; Liu et al., 1996; Banga and Banga, 1998) or by endogenous cytoplasm like the *polima* or *nap* cytoplasm from *B. napus* (Li et al., 1998; Brown, 1999).
The *ogura* cytoplasm also confers male sterility to *B. juncea* (Labana and Banga, 1989) and "CMS *juncea*" promotes CMS in *B. napus* (Pradham et al., 1991; Liu et al., 1996). In the case of *B. oleracea*, CMS plants were obtained by transfer of the CMS *B. juncea* cytoplasm from *B. rapa* through protoplast fusion (Cardi and Earle, 1997). In the *B. tournefortii* system, the expression of *B. tournefortii* mitochondrial genes is variably affected in different nuclear backgrounds so that sterility is either expressed (*B. juncea, B. napus*) or not (*B. tournefortii*, *B. napus* restored) (Liu et al., 1996).
The *ogu* system is thought to have a significant potential for large-scale hybrid seed production in *B. napus* because it expresses a highly stable male sterility

especially under European weather conditions (Pelletier et al., 1983, Delourme et al., 1991). The implementation of this system, however, has encountered certain difficulties in the development of restorer lines free of the negative agronomic characters that were introduced from radish into *B. napus* along with the restorer gene *Rfo* (Brown et al., 2003). Such negative characteristics include high levels of glucosinolates limiting the cultivation of these lines as canola plants (Delourme et al., 1998).
The *pol* cytoplasm is correlated with the chimeric gene *orf224/atp6* and confers male sterility in most *B. napus* cultivars (L'Homme et al., 1997). The *nap* cytoplasm is associated with the *orf222/nad5c/orf139* region conferring male sterility in a limited number of cultivars that lack the corresponding restorer gene (*Rf*) (L'Homme et al., 1997). Unlike the *orf*s associated with different forms of CMS in other plant species, *orf222* and *orf224* are highly similar in sequence over the entire length (Li et al., 1998). The *ogura* CMS cytoplasm induced in *B. napus* is characterized by the presence of the *orf138* gene. This gene is also present in male-sterile radish lines carring the *ogura* cytoplasm and its protein accumulates in all plant tissues (Bellaoui et al., 1999). The accumulation of ORF138 protein is dramatically reduced in the presence of the nuclear restorer gene although the *orf138* RNA levels are not affected. The action of the restorer gene is hence posttranscriptional and acts either on the translation efficiency of the RNA or on the stability of the protein (Krishnasamy and Makarott, 1994; Bellaoui et al., 1999).

In *B. napus*, the restorer locus has been shown to affect the transcripts of several mitochondrial genes, two of them being associated with the *nap* and *pol* CMS (Brown, 1999). Nuclear restoration of *pol* cytoplasmic male sterility is conditioned by the gene *Rfp* that is also involved in modifying transcripts of the *pol* CMS-associated *orf224/atp6* mtDNA. Analysis of the transcripts of the *Brassica napus nap* and *pol* CMS-associated regions has revealed that *Rfp* results in enhanced processing of dicistronic *orf224/atp6* transcripts so that the monocistronic *atp6* transcripts increase in abundance (Singh and Brown, 1991; Li et al., 1998). The restorer genes *Rfn* and *Rfp*, for the two cytoplasms represent different alleles or haplotypes of a single nuclear locus. Therefore, different forms of *Brassica* CMS are restored by alleles of a single nuclear locus and the restoration properties of the alleles reflect their involvement in the modification of transcripts of corresponding CMS-associated mtDNA regions (Li et al., 1998; Brown, 1999). Recently, Brown et al. (2003) cloned the *Rfo* gene, a single radish nuclear gene that restores *ogura* CMS in *B. napus* (Brown, 1999). Sequence analysis of the cloned *Rfo* gene revealed that it codes for a PPR-motif containing protein (Brown et al., 2003, see section *The restoration of CMS*- p. 13). In the *B. napus* CMS line obtained after somatic hybridization between *B. napus* and *A. thaliana* (Forsberg et al., 1998) where the nucleus and plastids are from *B. napus* and the mtDNA is recombined (Leino et al., 2003), the CMS line 4:19 showed a segregation into fertile and sterile plants in the first backcross with *B. napus*. Both lines were maintained after several backcrosses with *B. napus*. Fertile lines were analysed using RFLPs and showed that fertility co-segregated with chromosome III (chr III) from *A. thaliana* (Leino et al., 2004). This restorer line is an interesting line for further studies because *Arabidopsis* chr III contains a large cluster of genes encoding proteins that are targeted to the mitochondria (Elo et al., 2003). Alignment of the restorer PPR genes of radish (*Rfo*), petunia (*Rf*) and the PPR

gene family present in *Arabidopsis*, demonstrated that, in *Arabidopsis*, one of the PPR gene is localized in chr III (Desloire et al., 2003).
Some of the nuclear restorer genes seem to act at post-transcriptional levels (Menassa et al., 1999). For example, protein levels of the radish CMS-associated mitochondrial protein, ORF125, were considerably reduced in plants in which fertility was restored, although, mRNA expression was normal indicating that the *Rf* gene regulates expression of *orf125* at the post-transcriptional level (Koizuka et al., 2003).

Seedling development

Shoot apical meristem (SAM)

Shoot apical meristems (SAMs) are small groups of pluripotent cells responsible for making leaves, stems and flowers while axillary SAMs gives rise to branches (McConnell and Barton, 1998). The primary SAM is formed during embryogenesis and gives rise to the main axis of the plant (McConnell and Barton, 1998). Upon germination, SAMs initiate their growth and maintenance using a highly coordinated cell division program that continues throughout vegetative development (Hamada et al., 2000; Nakajima and Benfey, 2002). These coordinated cell divisions form distinct clonal cell layers such as L1-L3 that maintain an intimate intercellular communication allowing programmed development of organs with fixed shape and size (Scheres, 2001; Nakajima and Benfey, 2002; Sharma and Fletcher, 2002). The fates of SAM cells are determined by their positions in the meristem rather than by the information inherited from their ancestors (Marcotrigiano and Bernatzky, 1995; Sharma and Fletcher, 2002).
The SAM serves two main functions in the central zone. A population of undifferentiated, pluripotent stem cells is maintained, and in the peripheral zone, lateral organ primordia are initiated (Lenhard et al., 2002). These two main functions are balanced in the SAM: 1- maintenance of the pool of undifferentiated cells allocated to organ primordia, which takes place in the central zone. 2 – Initiation of organ primordia on the flanks of the meristem, with predictable size, position and timing (determination of phyllotaxy) (Clark, 1997; Gallois et al., 2002).

Root apical meristem (RAM)

Just like the shoot, the RAM contains a pool of pluripotent stem cells, which display self-maintenance and produce the cells required for organ initiation (reviewed by Nakajima and Benfey, 2002). The RAM is formed in embryos and its subsequent growth gives rise to the primary roots of the plant body (Cary et al., 2002). The RAM contains the only cells in the plant embryo that are derived from the suspensor rather than the embryo proper (van der Berg et al., 1997). In contrast to what happens in the SAM, RAM provide cells in two directions. In one direction, cells produce a tissue called the root cap, which covers the distal tip of the root. In the opposite direction, cells are produced that contribute to the root proper (van der Berg., 1995). Root meristems contain a distinct central region of mitotically inactive cells, the quiescent center, which has a low mitotic activity and

inhibits the differentiation of the initial cells by short-range control (van der Berg et al., 1997). The pattern of cell division in roots, at least in *Brassicaceae*, is almost completely stereotyped. Each initial cell divides with a strictly programmed plane of division and the daughter cell adjacent to the quiescent center is maintained in an undifferentiated state, while the other daughter cell differentiates according to a positional signal from adjacent more mature cells (Meyerowitz, 1997; Nakajima and Benfey, 2002).

Flower development

Flower development and mitochondria

Plant cells are characterized by the presence of three different genomes: nuclear, mitochondrial and plastid. The co-evolution of these three sets of genomes results in mitochondria with particular genetic and biochemical features, as compared to mitochondria from other eukaryotic organisms (Mackenzie and McIntosh, 1999). Since eukaryotic cells integrated mitochondria as an organelle, their biogenesis has been under control of nuclear genes. However, by retrograde regulation, mitochondria are able to control the expression of a subset of nuclear genes (Surpin and Chory, 1997; Yu et al., 2001). Different nuclear backgrounds strongly affect the mitochondrial transcription and editing (Leon et al., 1998). CMS plants are good examples of such nuclear-mitochondrial incompatibilities. The bidirectional communication between organelles and nuclei is accomplished essentially by polypeptides (Mackenzie and McIntosh, 1999). These polypeptides, involved in the communication between mitochondria and the nucleus, result from the fact that most of the organellar proteins are nuclear encoded and imported into the mitochondria where they frequently are assembled with proteins synthesized inside the organelle. In turn, mitochondria send signals to the nucleus about the proteins required to carry out respiration (Bonen and Brown, 1992). Genome recombination is a phenomenon that occurs frequently in this organelle resulting in a high level of heterogeneity which permits the plant cell an opportunity for specific regulation in different tissues or organs (Smart et al., 1994; Mackenzie and McIntosh, 1999). In particular for male organs, a consensus box called a "pollen box" was found in the promoter region of several genes that encode specific pollen proteins (Twell et al., 1991). This pollen box region is also present in genes encoding one of the complex I subunits and confers specifically high transcription levels in the anthers (reviewed by Brennicke et al., 1999).

The transition from vegetative to reproductive growth is the most dramatic phase change in plant development. At this point a new set of genetic actions has to take place in a precisely time-controlled manner. Production of elevated levels of ATP is one of the prerequisites (Scortecci et al., 2001; Araki, 2001). Plant tissues like meristems and anthers demand high respiratory rates, as evidenced by a tissue-specific difference in mitochondrial-related transcript levels for particular loci (Huang et al., 1994; Smart et al., 1994). In fact, increased levels of transcripts from nuclear genes coding for mitochondrial proteins and from mitochondrial genes, are related to the elevated number of mitochondria per cell that can be found in the male organs (Smart et al., 1994), suggesting a coordination between mitochondrial activity and microsporogenesis. This mitochondrial activity, in turn,

contributes to a tissue-specific increase in ATP synthesis (reviewed by Brennicke et al., 1999). For example, the transcripts of the nuclear encoded complex I subunit are more abundant in flowers than in other tissues (Rasmusson et al., 1998). In young flower buds, especially in anther development, the number of mitochondria increases with a concomitant increase of mitochondrial transcripts and ATP production (Lee and Warmke, 1979; Huang et al., 1994, Smart et al., 1994). In fact, of the four floral organs, stamens are the ones that have the highest respiratory demands and sporogenous tissue has the highest demand in the stamen (Smart et al., 1994). *In situ* hybridisation studies of maize seedling tissue, showed that particular mitochondrial transcripts are detected at different levels depending on tissue type (Li et al., 1996). Likewise, studies of developing anthers of sunflower demonstrate a marked accumulation of *atpA*, *atp9*, *cob* and *rrn26* transcripts in young meiotic cells with a concomitant increase in their respective protein products (Smart et al., 1994). Several nuclear genes encoding mitochondrial proteins demonstrate higher levels of mRNA accumulation in flower tissues, indicating increased mitochondrial activity in these tissues (Huang et al., 1994). Even though different plant tissues, such as meristems (Li et al., 1996) and microspores (Smart et al. 1994) have higher levels of mitochondrial transcripts, the regulatory mechanism that controls transcription acts in a global manner since all mitochondrial transcripts appear to be up-regulated.

Establishment of flower development

Initial flower development depends on meristems, the group of dividing cells that are the source of new plant structures. In the apical meristems, additional collections of cells are set aside in defined sequential patterns. These collections of cells become either new meristems or primordia for organs such as petals or stamens (Steeves and Sussex, 1989). Each floral organ starts its development as a little bulge on the floral meristem, a tiny clump of undifferentiated cells (Meyerowitz, 1997). During floral patterning, several processes need to occur coordinately including proper positioning of floral organs and specification of their identity in a position dependent manner (Reviewed by Meyerowitz, 1996).
Cell division in the flower meristem is well coordinated. In the first two layers of the meristem, cells divide anticlinally and remain clonally distinct forming the layers L1 and L2 (Kaya et al., 2001). Beneath these two layers, the orientation of cell division is more variable (Meyerowitz, 1997). Organogenesis in flowering plants results almost entirely from patterned control of the numbers, loci and planes of cell divisions coupled with regulated and coordinated cellular expansion in the early floral meristem rather than by lineage (Lucas et al., 1995; Hase et al., 2000). Correct cell division patterns in flower organogenesis are important since intercellular interactions are indispensable for organ development (Hase et al., 2000; Jenik and Irish, 2001). Whorl boundaries appear to be defined by coordinated patterns of cell division before the identity genes are expressed (Irish, 1999; Jenik and Irish, 2000). Later on, during floral development, the number of cells contributing to organ formation is controlled by the genes responsible for floral organ identity, (Jenik and Irish, 2000; Lohmann and Weigel, 2002) which, to some extent, also appear to regulate cell division and proliferation.

The ABC model

The basic plan of flower formation is quite constant among all flowering species, despite enormous diversity in flower morphology (Lohmann and Weigel, 2002). The floral meristem is divided into four concentric regions wherein each one is characterized by a unique pattern of organ identity gene expression, corresponding to the regions in which sepals, petals, stamens and carpels will arise (Meyerowitz, 1996; 1997).

Before the action of the floral identity genes, other classes of genes have to be activated. Flowering time genes function to control the activity of a much smaller group of floral meristem identity genes. These latter genes specify lateral meristems in *Arabidopsis* to develop into flowers rather than leaves or shoots. *LEAFY* (*LFY*) and *APETALA1* (*AP1*) are the genes that specify lateral primordia to develop as flowers in *Arabidopsis*. Both *lfy* and *ap1* single mutants exhibit a partial conversion of flowers to shoots (Irish and Sussex, 1990; Weigel et al., 1992; Bowman et al., 1993). *LFY* and *AP1* encode sequence-specific DNA binding transcription factors (Mandel et al., 1992), whereas *LFY* encodes a plant-specific protein without similarity to other genes in *Arabidopsis* (Weigel et al., 1992). Genetic and molecular investigations have demonstrated that the plant-specific transcription factor and meristem identity gene *LFY*, controls the transition from vegetative to reproductive development by directly inducing *AP1* and regulating the expression of additional genes like the *AP1*-related factor, *CAULIFLOWER* (*CAL*) (Bowman et al., 1993; Wagner et al., 1999; William et al., 2004). After initiation, the meristem identity gene *LFY* has a second role in activation of the floral homeotic genes that specify identity of organs in the flower (Weigel and Meyerowitz, 1994). These two roles of *LFY* are separated genetically and molecularly (Parcy et al., 1998).

According to the ABC model, the identity of the different floral organs is determined by three classes of homeotic genes: class A *APETALA1* (*AP1*) and *APETALA2* (*AP2*); class B *PISTILATA* (*PI*) and *APETALA3* (*AP3*) and class C *AGAMOUS* (*AG*). According to the model, the class A genes specify sepals, the A and B class genes together specify petals, the action of B and C class genes establishes the formation of stamens. Carpels are formed by the action of the C gene alone (Schwarz-Sommer et al., 1990; Bowman et al., 1989b; 1991; Coen and Meyerowitz, 1991; Jack et al., 1992). A and C activity are mutually exclusive and repress each other (Fig. 3) (Bowman et al., 1991b). The mutation of these genes results in homeotic phenotypes in which one or more whorls of the flower develop into organs characteristic of another whorl of the flower (reviewed by Jack et al., 2004). In class A mutants, C class activity expands into all whorls, with sepals being replaced by carpels and petals by stamens. Besides the role of *AG* in specifying carpels and stamens, it establishes flower determinacy. Therefore, C-mutants flowers consists of (sepals/petals/petals)$_n$ (Bowman et al., 1989b; 1991b; Meyerowitz, 1994). In B-mutant plants, anthers are replaced by carpelloid structures and petals are converted into sepals, resulting in homeotic conversions of both organs (Bowman et al, 1989, 1991a).
With the exception of *AP2*, all other ABC class genes are members of the MADS family of transcription factors (Riechmann et al., 1996; Theissen et al., 2000). Three other MADS-box genes, *SEPALLATA1*, *SEPALLATA 2* and *SEPALLATA 3* (*SEP1*, *SEP2*, *SEP3*) have been added to the ABC model and, together, they are necessary for proper development of petals, stamens and carpels (Fig. 4) (Pelaz et

al., 2000; Theissen, 2001; Jack, 2001). B and C class genes cannot function without this trio of MADS-box genes (*SEPALLATA* genes), whose combined knockout phenotype resembles that of plants without B and C function (Pelaz et al., 2000).

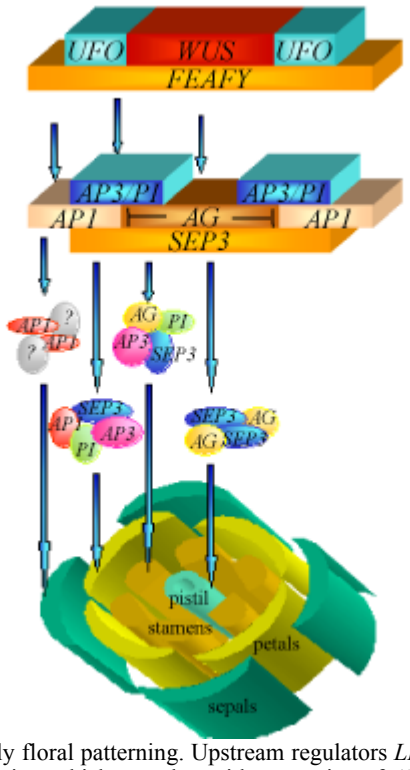

Fig. 3. Flow chart of early floral patterning. Upstream regulators *LFY*, *WUS*, and *UFO* are expressed in specific domains, which, together with repression of *AP1* by *AG*, results in the ABC pattern. How the *SEP* pattern is regulated is not known. ABC gene products and SEP proteins, all of which are MADS domain proteins, assemble into higher order, most likely quaternary complexes, which specify different organ identities. It is not known whether AP1 assembles into higher order complexes. Adapted from Lohmann and Weigel. (2002).

With the finding that all flower organs resembled sepals in mutants in which the three *SEPALLATA* genes had lost their function, the quartet model was advanced based on the hypothesis that the formation of each floral organ requires the action of four assembled proteins (Fig. 3) (Pelaz et al., 2000; Honma and Goto, 2001; Theissen and Saedler, 2001). This new class of genes forms the class E genes. Together, the class B and C specifies petals, (B+C+E) stamens and (C+E) carpels (Theissen and Saedler, 2001; Theissen, 2001; Jack, 2001).

The flower phenotype of the two CMS lines investigated in this study (Leino et al., 2003) resembles flowers formed in plants where the B- class genes *AP3* and *PI* were mutated (Bowman et al., 1991a; Coen and Meyerowitz, 1991). Plants mutated in several of the genes upstream of the B-class genes like *ASK1* (Zhao et al., 1999), *UFO* (Levin and Meyerowitz, 1995) or *LFY* (Weigel and Meyerowitz, 1993) also show phenotypic similarities to the *B. napus* CMS plants analysed here.

Whorl three organs

Stamens are the male reproductive organs of flowering plants and consist of a filament topped by a complex anther structure. The development and function of the anther require the coordinated activity of many different cells and tissues (Chasan and Walbot, 1993). The filament is a tube of vascular tissue that anchors the stamen to the flower and serves as a conduit for water and nutrients. The anther contains the reproductive and nonreproductive tissues that are responsible for producing and releasing pollen grains (Goldberg et al., 1993). Pollen development involves an array of extraordinary events, including cell division and differentiation independent of a conventional meristem. The transition from sporophytic to gametophytic generation and the modifications that must take place concerning cell division in order to produce new cell types such as pollen grains, are mechanism unusual for plant development when compared to the type of cell divisions involved in all the other vegetative organs formation (Scott et al., 2004). Just after stamen primordia start to elongate, microsporogenesis begins to occur with the meiotic division of a diploid pollen mother cell. The haploid microspores then, divide mitotically to produce the three-celled pollen grain (Chasan and Walbot, 1993).

The role of *LEAFY* (*LFY*), *UNUSUAL FLOWER ORGANS* (*UFO*) and *Arabidopsis Skp1*-Like gene (*ASK1*)

The activation of the organ identity genes in specific domains is still not completely clear but we know now that the activity of several floral meristem identity genes like *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*) are necessary for the initiation of *AP1*, *AP3*, *PI* and *AG* expression. *LFY*, in combination with *UFO* are, in part, responsible for the establishment of the B domain (Weigel et al., 1992; Wilkinson and Haughn, 1995; Parcy et al., 1998). LFY activates the expression of *AG* and *AP3* in a nonautonomous fashion that is independent of the mechanisms used to specifying flower meristem (Parcy et al., 1998; Wagner et al., 1999, Sessions et al., 2000). ASK1 (*Arabidopsis SKP1-LIKE1* gene) is also involved in the activation of *AP3* and *PI* by interacting genetically with LFY (Samach et al., 1999; Zhao et al., 2001; Wang et al., 2003).

ASK1 is expressed in all actively dividing cells of the plant and especially in plant meristems. In the flower, this gene is initially expressed in the floral organ primordia and later, it becomes restricted to the tapetum layer (Bai et al., 1996). *ASK1* is a homologue of the yeast and human Skp1 protein (Ingram et al., 1995; Yang et al., 1999) that interacts with *UFO*, an F-box protein, to control floral organ identity in whorls 2 and 3 (Samach et al., 1999; Zhao et al., 1999; Yang at al., 1999). The specificity of ASK genes comes from the F-box proteins they interact with. Only when the two specific proteins are expressed in the same plant at the same time, can they interact *in vivo* to confer a biological function (Zhao et al., 2003). Plants with different ASK RNAi constructs have distinct phenotypes suggesting that members of the *ASK* gene family may have diverse functions in plant development and physiology (Zhao et al., 2003). ASK1 is also essential for normal male meiosis for separation of homologous chromosomes (Yang and Ma, 2001). The ask1-1 mutant of *Arabidopsis* exhibits a male-sterile phenotype, shorter internodes and smaller leaves, suggesting that the *ASK1* gene is involved not only in vegetative development but also in the establishment of the identity of the floral organs in the second and third whorls (Zhao et al., 1999).

UFO acts upstream of *AP3*, *PI*, *AP2* and *AG*. Together with LFY, UFO confers a pattern in gene activation in the flower meristem by establishing domains of differential cell division necessary for proper patterning in the young floral meristem (Samach et al., 1999; Laufs et al., 2003). Mutations in these two genes lead to a reduction in B-gene expression (Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Ectopic expression of *AP3* and *PI* in *ufo* mutants restores B-function both in *Arabidopsis* and *Antirrhinum* when *FIM*, an orthologue of *UFO*, is mutated (Weigel and Meyerowitz, 1993; Ingram et al., 1997).

Proper timing is required for UFO functions during flower development (Laufs et al., 2003). The late function of *UFO* is to promote growth of the petals and regulate the size of the third whorl through the positive affect on B-activity. At later floral stages, *UFO* may be necessary to degrade a repressor modulating the level or maintenance of *AP3* and *PI* transcription (Samach et al., 1999), by physically interacting with *ASK1* and *AtCUL1* (*Arabidopsis CULLIN* gene) genes (Zhao et al., 2001; Wang et al., 2003). The interaction of the proteins encoded by these genes forms a complex called SCF^{UFO} that interacts genetically with LFY to regulate B-function gene expression by promoting the targeting of the *LFY* negative regulator for destruction (Samach et al., 1999; Zhao et al., 2001; Wang et al., 2003). Both SKP1 and F-box containing proteins are subunits of the SCF ubiquitin ligase complex termed SCF (Skp1/cdc53/F-box protein), suggesting that ASK1 and UFO might be components of the SCF complex. *AtRbx1* mutated plants (part of the SCF complex) produced flowers with reduced petal size and filamentous/carpelloid organs in the third whorl (Ni et al., 2004). The *ufo-6axr6-2* double mutant showed an increased number of carpelloid organs and filaments in the third whorl (Ni et al., 2004). Flower development in *ask1-1* results in smaller petals and stamens with shorter filaments. No differences were found in shape or size of the inflorescence meristem until stage 6, as compared to wild type (Zhao et al., 2001).

Weak double mutants of *ufo6* with *ask1-1* develop flowers with reduced petals and carpelloid organs in the third whorl (Zhao et al., 2001). All these mutants closely resemble the *B. napus* CMS lines investigated in this study, concerning not only the flower phenotype but also some vegetative growth features.

The role of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*)

The phenotype of the *ap3* and *pi* single mutants leads to the hypothesis that simultaneous presence of both *AP3* and *PI* is required for normal organ formation (Bowman et al., 1989, 1991a, Jack et al., 1992). When *AP3* is expressed constitutively, the RNA is detected throughout the plant but the phenotypic changes occur only where both the AP3 and the PI proteins are expressed (Jack et al., 1994). Both *PI* and *AP3* regulation have two distinct stages. The first stage is dependent on at least two *cis*-acting elements that requires the action of the meristem identity genes, *AP1*, *UFO* and *LFY* (Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995; Hill et al., 1998; Ng and Yanofsky, 2001). During the second phase, *PI* and *AP3* expression is maintained through the interaction of *PI* and *AP3* autoregulation (Goto and Meyerowitz, 1994, Jack et al., 1994, Samach et al., 1997, Hill et al., 1998, Tilly et al., 1998, Honma and Goto, 2000). The *AP3*

promoter region contains three close matches to the consensus CArG box sequence that binds with high affinity to MADS domain proteins (Hill et al., 1998). MADS is an acronym for the four founder proteins MCM1 (from *Saccharomyces cerevisiae*), AGAMOUS (from *A. thaliana*), DEFICIENS (from *Antirrhinum*) and SRF (a human protein). The MADS domain is determinant for DNA binding sites by its consensus sequence $(CCA/T)_6GG$, called CArG box present in the promoter regions of many genes regulated by MADS-box genes (Reviewed by Theissen et al., 2000; Theissen, 2001).
The different sites in the *AP3* promoter for the binding of MADS-domain containing proteins, indicate that different regulatory gene products may mediate *AP3* transcription differently (Hill et al., 1998). Mutation in two of the *AP3* promoter CArG boxes results in decreased gene expression, while a mutation in the third CArG region results in enhanced gene expression (Tilly et al., 1998). For example, LFY seems to directly regulate *AP3* expression during early floral stages by binding to a LFY binding site in the promoter region of *AP3* (Hill et al., 1998).

AP3 and *PI* expression are necessary for the autoregulatory maintenance of *AP3* transcription after stage 6 (when sepals completely enclose the bud and stamen primordia have arisen) (Smyth et al., 1990) of flower development (Jack et al., 1994). Class B proteins bind to their own promoters as heterodimers with specific cofactors for each of the B-class genes in order to maintain correct expression levels of the B-class genes (Samach et al., 1997). The levels of AP3 expression are critical for organ formation (Jenik and Irish, 2001). As stated by Samach et al. (1999) the relative concentration of both AP3 and PI proteins is critical for development. Low level of one of the two proteins makes the plant sensitive to fluctuations in the level of the other (Jack et al., 1994).

The *PI* promoter consists of discrete *cis*-elements. One in the distal region is responsive to induction signals mediated by the meristem identity genes *LFY* and *UFO*, and a second element in the proximal region is responsible for autoregulatory signals produced by the PI/AP3 complex necessary to up-regulate *PI* transcription. This autoregulatory mechanism occurs via a proximal promoter by an indirect pathway, since there is no CArG box-like sequences in the promoter region (Honma and Goto, 2000). This indirect regulation that takes place after floral stage 6 requires *de novo* protein synthesis (Tilly et al., 1998; Honma and Goto, 2000). In pi-1 mutant flowers, *PI* RNA accumulates normally in stages 3 to 6, before the primordia of second and third whorl organs begin to differentiate (Goto and Meyerowitz, 1994).

From all the organs formed throughout a plant's life, stamens are the ones that integrate the highest number of genes, not only genes involved in B-class genes activation (Bowman et al., 1993; Ng and Yanofsky, 2001; Lamb et al., 2002), but also genes down-regulated by AP3/PI (Wellmer et al., 2004). Many AP3 and PI downstream genes are associated with basic metabolic processes and stress-related events suggesting that both petal and stamen development require rapid cellular responses (Zik and Irish, 2003). The findings of these authors suggest that AP3 and PI act in a very direct way in regulating the basic cellular functions during petal and stamen morphogenesis (Zik and Irish, 2003).

Cell division during flower development

The well established coordination of plant cell division uses the same general machinery as do other eukaryotic cells. It involves the activation of cyclin-dependent protein kinases (CDKs) by different families of cyclins. Each cyclin type is activated, depending on the phase of the cell cycle, by specific phosphorylation/desphosphorylation events (Fig. 4A) (Hemerly et al., 1999; Stals and Inzé, 2001). Cyclins are controlled by a variety of internal and external signals (Hemerly et al., 2000). Internal signals include sucrose concentration (Ohto et al., 2001; Riou-Khamlichi et al., 2000; Healy et al., 2001) and plant hormones such as cytokinins and brassinosteroids (Stals and Inzé, 2001).

Cyclin proteins contain a box that targets them to destruction through ubiquitin-dependent degradation (Kaiser et al., 2000; Stals and Inzé, 2001). It is this pathway that regulates the levels of cyclins, allowing the cell to progress through the different phases of the cell cycle. Regulation of proteolysis and the different stages of the cell cycle are then connected by promoting the degradation of key proteins involved in the cell division cycle (Bai et al., 1996; Koepp et al., 1999). This cell cycle progression depends on protein degradation as illustrated by the *CycB1* gene. *CycB1* expression is linked to active cell division. Its transcripts are exclusively detected in newly forming organs and tissues at a precise time of the cell cycle (Ferreira et al., 1994). The protein encoded by this gene is expressed at the G2/M transition (Fig. 4A) and its destruction is required to allow cells to exit mitosis (Colón-Carmona et al., 1999, Capron et al., 2003).

Ubiquitinylation

Ubiquitinylation or proteolysis is a complex process with a tightly temporal control that regulates a broad array of cellular processes, such as auxin responses (Gray et al., 1999), jasmonate siganaling (Xu et al., 2002), flower development (Zhao et al., 1999), photocontrol of circadian clocks (Somers et al., 2000) and cell cycle control by promoting unidirectional progression in the cell cycle (Bai et al., 1996). Protein degradation is carried out by a complex cascade of enzymes and is highly specific for its numerous substrates (Shen et al., 2002, Gagne at al., 2002). Ubiquitination pathways are highly dependent on ATP, for initial activation of the ubiquitin-activating enzyme (E1), for progression through the pathway, and for release of ubiquitin monomers from various adducts, a key step for proteolysis (Ciechanover, 1998). The ubiquitin-conjugating pathway involves the activity of three enzymes or protein complexes called ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3) (del Pozo and Estelle, 2000). The E1 enzyme is activated in an ATP-dependent manner with the formation of a high-energy thiolester intermediate and an AMP molecule. The cascade works in a hierarchical way amplifying the energy requirement, since a single E1 enzyme catalyses the activation of ubiquitin and transfers it to several E2 enzymes followed by E2 transfer of ubiquitin to several E3 complexes, until the protein targeted for degradation is polyubiquitinated (del Pozo and Estelle, 2000; Xiao and Jang, 2000). The polyubiquitin chain is recognized by the 26S proteosome (Fig 4B), the target-protein is degraded and ubiquitin monomers are released (Gagne et al., 2002). The last component of the proteolysis cascade, the

E3 complex, provides the key to regulate ubiquitinylation because it is this complex that selectively targets proteins for degradation (Koepp et al., 1999). Two E3 complexes have been discovered so far: the cyclosome/anaphase-promoting complex (APC), necessary to degrade CycB1 proteins, and the Skp1-Cullin-F-box protein ligase complexes (SCF), the complex involved in flower development (Fig. 4B) (Hershko and Ciechanover, 1998; Koepp et al., 1999). The APC/C is a multiple-subunit E3 complex that controls important transitions during mitotic progression and exit. APC/C activity is precisely regulated to control cell-cycle progression, being active during mitosis and G1. APC/C activation requires phosphorylation for its role in the cell cycle (Harper et al., 2002; Capron et al., 2003). The SCF ubiquitin ligase acts by targeting a number of cell cycle regulators, transcription factors and other proteins for degradation (Shen et al., 2002). Targeted phosphorylation is a prerequisite for recognition by the SCF complex (Gagne et al., 2002). In fact, all the processes of protein degradation by the SCF complex are phosphorylation driven (Fig. 4B) (Ciechanover, 1998; Koepp et al., 1999).

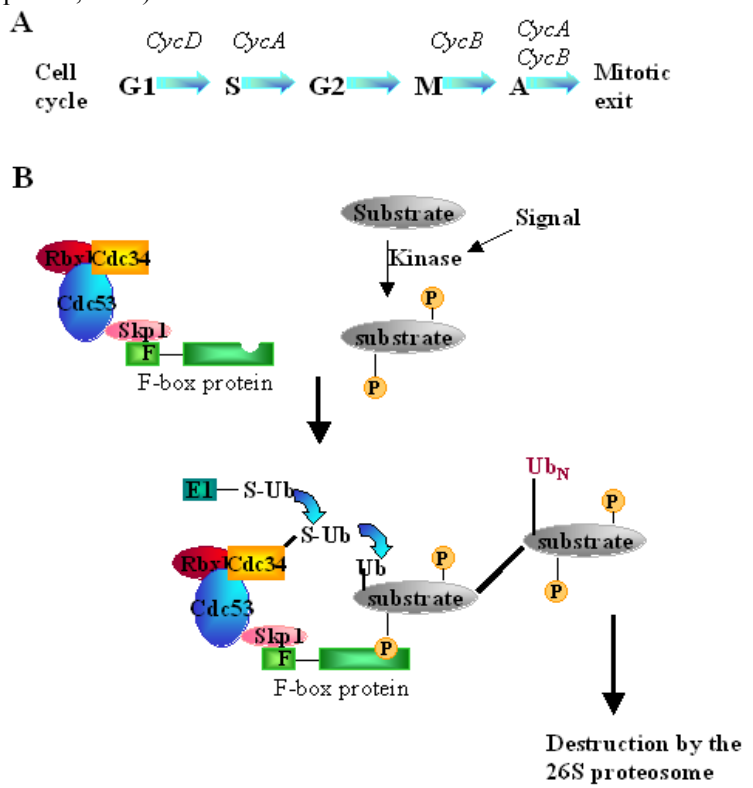

Fig. 4. Ubiquitinylation pathways in the cell cycle. **A**- ubiquitinylation substrates and the time of the cell cycle in which they are degraded. **Cyc** Cyclins. **B**-model for phosphorylation-driven ubiquitinylation through the SCF pathway. **P** phosphate, Ccc53, Skp1 and F-box proteins form the E3 complex. Rbx1 and cdc34 forms the E2 complex. **Cdk** cyclin dependent kinase; **Sic** CDK inhibitor; **Ub** ubiquitin. Adapted from Koepp et al. (1999).

Aims of this study

The main scope of this thesis was to study the nuclear-mitochondrial interactions involved in vegetative and floral development of two CMS lines of *B. napus* with mitochondrial DNA derived mainly from *A. thaliana*. In all studies performed, comparative analyses were made with the fertile *B. napus* cultivar constituting the nuclear donor of the CMS lines. Some metabolic features were also investigated. Specific aims were to:

- monitor the vegetative growth from seedling to full maturity
- assess the energetic status of the plant by measuring ATP and sucrose contents
- characterize the homeotic conversions of stamens morphologically, histologically and ultrastructurally
- analyze the expression of genes related with flower development using microarray cDNA slides
- establish the expression levels and patterns of the genes responsible for stamen and petal development in the CMS lines
- establish the expression levels and patterns of the genes involved in cell division

The overall aim was to correlate phenotypic features of CMS with alloplasmy and mitochondrial DNA and try to explain the influence of the rearranged mtDNA in the overall plant development.

Plant material

Several CMS lines were obtained after protoplast fusion of *Brassica napus* cv. Hanna and *Arabidopsis thaliana*, var. Landsberg erecta (Forsberg et al., 1998). Several lines displaying male sterility were identified and selected for further backcrosses to *B. napus* cv. Hanna until BC_9 was achieved. The resulting plants have the nucleus of *B. napus*. The stable inheritance of the phenotypic features of CMS through several generations indicates that the nucleus does not contain portions of *Arabidopsis* DNA influencing the vegetative and floral development. RFLP analysis of the two CMS lines demonstrated that the chloroplast genomes were of *B. napus* origin, while the mitochondrial genomes had rearranged mtDNA inherited from *B. napus* and *A. thaliana* (**I** and data not shown). From the several CMS lines phenotypically described in paper **I**, lines 4:19 and 41:17, displaying the most abnormal floral phenotype, were selected for the studies in this thesis.

Results and discussion

Vegetative alterations observed in the CMS lines

Seedling and vegetative growth

Alterations in vegetative development are not commonly reported in cytoplasmic male sterile plants. The agronomic importance of male sterile plants for hybrid seed production demands that CMS plants behave as well as the fertile lines except for pollen production. Thus, selection has been made of the most suitable lines for agronomic use. However, in order to more clearly analyse and further understand the mitochondrial impact on vegetative and floral development, we have chosen to investigate CMS lines of *B. napus* displaying both modified vegetative and reproductive behaviour.

Germination was slightly retarded in the CMS lines, but the seedlings were phenotypically similar to *B. napus* (data not shown). The delay in seedling growth in the CMS plants was observed throughout plant growth where the CMS plants of the same age as *B. napus* plants always showed a shorter height (**I** and **II**). Despite this delay in growth, the CMS plants were able to reach *B. napus* height by the time plants reached full maturity (**II**). The pigmentation in the cotyledons and in the leaves of CMS plants was similar to *B. napus* with no signs of chlorosis in any part of the plant (data not shown). The plastid genome in the CMS lines studied was of *B. napus* origin, which thus would be fully compatible with the nuclear genome. In CMS lines reported to have growth defects due to chlorosis, the plastid genome was derived from the cytoplasmic donor. Thus, the reduced growth could not be attributed to chlorosis, which has been reported for several of the CMS lines in *Brassica* (Bannerot et al., 1977; Pelletier et al., 1983; Kirti et al., 1995). Reduced plant height has been reported for alloplasmic CMS lines of *B. juncea* (Malik et al., 1999), cybrids of *N. tabacum* nucleus with *Hyoscyamus niger* cytoplasm (Zubko et al., 2003), alloplasmic *N. tabacum* lines with *N. repanda* cytoplasm (Farbos, unpublished) and for *T. aestivum* (Ikeda and Tsunewaki, 1996). Even though these lines have both the plastid and mitochondrial genomes of the cytoplasmic donor, no chlorosis defects were reported. Thus, the vegetative alterations reported for several CMS plants constituting distinct alloplasmic systems support the idea that the presence of recombined mtDNA can affect not only flower male organ formation, but also other parts of plant development.

In order to determine if the reduced plant size of the CMS lines was due to lower cell number or to reduced cell elongation, measurements of cell size and number

were performed in leaves and stems. These studies revealed that CMS leaves contain fewer cells both in the epidermis and mesophyll layers (**II**). The mesophyll cells were also smaller. The stem cells of CMS plants, measured between leaves three and four, were almost half the size of *B. napus* (**II**).
Cell division was monitored indirectly by cultivation of protoplasts in order to estimate the amount of *CycD2* and *CycD3* transcripts in the three lines. Protoplasts isolated from the CMS lines had a slower rate of division and the expression levels of *CycD2* and *CycD3* were lower. However, the alterations in cell division rate and elongation do not result in phenotypic modifications. This is in agreement with previous studies of plants mutated in cell regulators, in which the final phenotype of the plant does not change from the wild type except for their shorter stature (Hemerly et al., 1995, 1999). In contrast to plants mutated in one of the cyclin genes, plants expressing *CycD2* constitutively have an accelerated division rate but still retain a normal morphology (Cockroft et al., 2000).

Throughout the vegetative characterization of the two CMS lines and *B. napus*, not only structural but also metabolic differences were found. By analysing cross sections of CMS leaves, an abnormal accumulation of starch was noticed. Further starch and sucrose measurements were performed demonstrating that in fact, CMS plants had a higher concentration of starch with a concomitant reduction in the sucrose content. In order to establish a link between starch degradation and sucrose synthesis, and because the CMS traits are mitochondrially associated, measurements of total ATP were conducted in several tissues.
Green leaves did not show a statistical difference in ATP content between the three lines. However, etiolated CMS leaves showed a reduction in ATP levels (**II**). The differential ATP levels observed in green and etiolated leaves suggest that the ATP produced by the chloroplast may compensate for the reduction of adenylates produced by the mitochondria when total ATP was measured. Although, only the ATP produced by the mitochondria is exported to the cytosol (Leon et al., 1998) and then, used for several metabolic mechanisms like sucrose synthesis (reviewed by Raghavendra and Padmasree, 2003).

Starch is degraded in the chloroplast and transported to the cytosol in the form of triose-P during both the light and dark period. However, in the dark, no photosynthesis takes place resulting in neither ATP nor starch production in the chloroplasts. After starch breakdown in the chloroplast, sucrose precursors are phosphorylated and enter the sucrose biosynthesis pathway making use of large amounts of ATP produced by the mitochondria (reviewed by Raghavendra and Padmasree, 2003; Chia et al., 2004). The low ATP levels present in the leaves of plants growing for three days in etiolated conditions suggest that in green leaves the mitochondria may synthesise less ATP just like in etiolated leaves. Therefore, the amount of ATP exported to the cytoplasm from the mitochondria may not be sufficient for efficient synthesis of sucrose.
When CMS seeds were allowed to germinate in the dark they obtained longer hypocotyl extension compared to *B. napus* (data not shown). This probably reflects the reduced capacity to synthesize sucrose as was found for leaves (**II**). Plants mutated in the first enzyme in the hexose assimilation pathway are not able to sense sucrose acting as if there was no sucrose in the cells. These mutant plants mimicked our etiolated seedlings in that they showed longer hypocotyl extension compared to the control (Jang et al., 1997).

One-week etiolated plants were transferred back to normal photoperiod conditions to investigate how CMS plants would recover from energy depletion after a period of etiolation. The alloplasmic lines demonstrated a slower recovery rate than *B. napus* plants. *B. napus* plants returned to a photoperiodic regime of 16 h day length after the etiolation period formed new green leaves after 7 days, while the CMS plants did not show any signs of recovering. The etiolated CMS plants started to develop small green leaves two weeks after being transferred to normal photoperiod conditions, but they never reached *B. napus* plant size (**II**). The recovery of the etiolated plants after being transferred back to a normal photoperiod, mainly involved an increased cell division rate stimulated by external and internal signals such as sucrose (Riou-Khamlichi et al., 2000).
Besides the role of sucrose as a second messenger, sucrose availability in the cytoplasm also affects cell division. Both *CycD2* and *CycD3* genes are regulated by sucrose (Riou-Khamlichi et al., 2000; Healy et al., 2001). The reduced levels of *CycD2* and *CycD3* RNA expressed in the protoplasts of the CMS lines could reflect the low levels of sucrose in the cytoplasm and thus cause a reduction of cell division rates in leaves and stems.

Modified cell division patterning in the CMS lines

The two CMS lines showed a misregulation in cell patterning both in the SAM and in the young floral meristems. In *B. napus*, the controlled orientation of cell divisions occurring in the apical meristems forms the clonally distinct cell layers L1 and L2. Contrary to what is found in L1 and L2 in *B. napus*, where cells divided anticlinally, in the CMS lines L2 cells divided in all planes, partly abolishing the cell layer organization. The alterations occurred in the peripheral zone of the shoot apical meristem (Fig. 5) and in the presumptive whorls 2 and 3 of the flower meristem (**III**). In both these meristematic zones, cell division is faster than in the center of the meristem (Meyerowitz, 1997; Bäurle and Laux, 2003), resulting in that in the meristem of the two CMS lines, cell division patterning was affected in the zones where a more rapid cell division takes place.

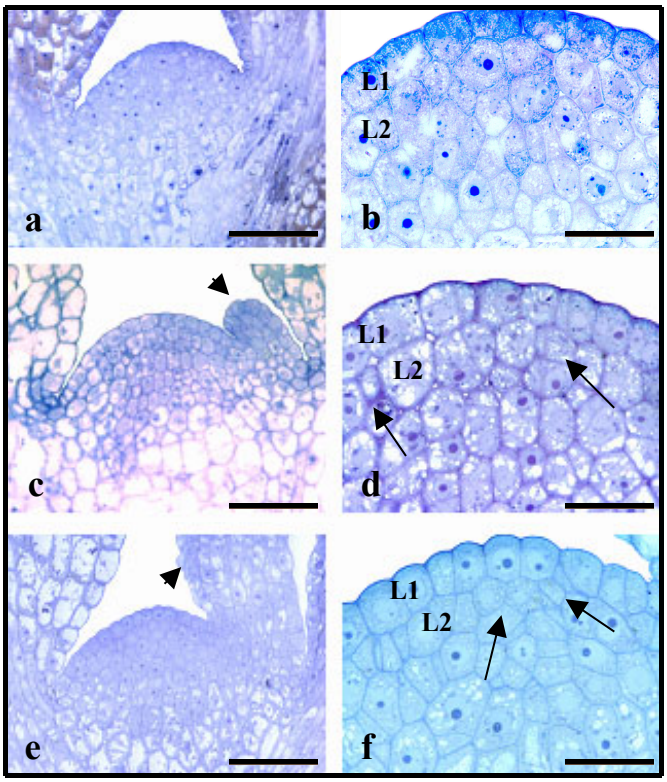

Fig. 5. Histological studies of the SAM **a)** Longitudinal thin section of a *B. napus* seedling after three days of germination. **b)** SAM under higher magnification showing the well arranged cell layers L1 and L2. **c)** Longitudinal thin section of CMS line 4:19, with one leaf primordium (arrowhead). **d)** Magnification of c) showing the alterated cell divisions in the L2 layer with some cells dividing anticlinally (arrow). **f)** Longitudinal thin section of CMS line 41:17 seedling, with an already formed leaf (arrowhead). **g)** Magnification of f) showing the pronounced cell division alterations in the L2 layer. **L1** and **L2** meristematic cell layer 1 and 2; Scale bars **a)**, **d)** and **g)** 100µm. **b)**, **e)** and **h)** 30 µm.

Cell pattern modifications were also observed in the roots. Columella cells and cortex cells were misshapen (Fig. 6). Similar cell pattern modifications were also noticed by Weingartner et al. (2004) in tobacco plants transformed with a nondegradable form of CyclinB1. Besides the alterations of cell shape and shorter *Nicotiana* plants, the expression level of the nondegradable *CycB1* mRNA was higher when compared to control plants transformed with the gene for a degradable form of the same protein. In our material, young CMS seedlings showed an accumulation of *CycB1* mRNA (data not shown). Young CMS flower buds also displayed an increased accumulation of *CycB1* transcripts (**III**). Later in flower development, the levels were reduced, related to the fact that cell division is replaced by cell elongation at later floral stages (Bossinger and Smyth, 1996). *CycB1* expression is linked to active cell division with its mRNA detected in newly forming organs and tissues such as microsporogenesis tissue (Ferreira et al., 1994, **III**). The reduced expression levels of *CycB1* in the CMS flowers at later stages in comparison to *B. napus* flower tissues at the same developmental stages possibly reflect the lack of male reproductive tissues in the CMS flowers.

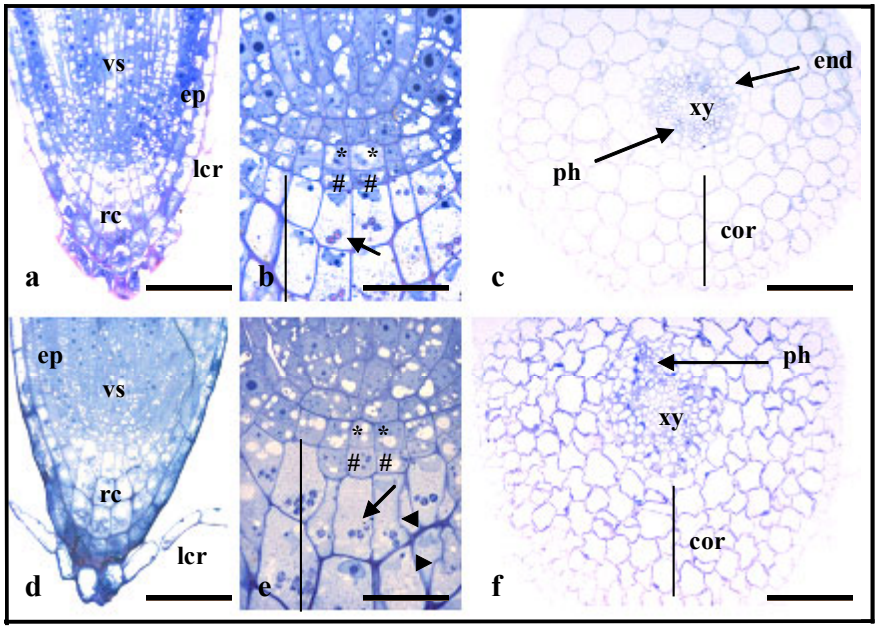

Fig. 6. Histological studies of the RAM. Longitudinal sections of roots of **a)** *B. napus* and **d)** CMS line 4:19 showing the vascular system (**vs**), root meristem and root columella (**rc**). **b)** Magnification of the root meristem in *B. napus*. The quiescent center cells (*****) and the columella initials (**#**) are indicated. The columella cells are marked by a vertical line. Starch granules are indicated with arrows. **e)** Same as in b) but corresponding to e) CMS line 4:19. Alterations in the plane of cell division in the columella are obvious (arrowheads). Cross section of roots from **c)** *B. napus* and **f)** CMS line 4:19. All sections were done 2 mm from the root tip. In **c)** the uniform roundly shaped cortex cells (**cor**) are clearly distinguished from the endodermis layer (**end**). Cell shape of the cortex in **f)** CMS line 4:19 is irregular and differ from the *B. napus* cortex cell shape. In all lines, central xylem cells are surrounded by phloem tissue. **cor** cortex; **end** endodermis; **lar** lateral root cap; **ph** phloem; **pl** plastid; **rc** root cap; **s** starch. **vs** vasculature system; **xy** xylem.. Scale bars **a)** and **d)** 100µm. **b)** and **e)** 30 µm. **c)** and **f)** 200µm.

The levels of cyclins are regulated by ubiquitination (Koepp et al., 1999; Peters, 2002). Degradation of mitotic proteins is a hallmark of the exit from mitosis in all eukaryotes (Weingartner et al., 2004). Cyclin dependent-kinases (CDK) activity needs to be switched off during mitotic exit in order to promote spindle disassembly, cytokinesis and licensing of replication origins during G1. All these mechanisms need to occur in order to promote new rounds of DNA synthesis (Zachariae and Nasmyth, 1999). CDK inactivation is believed to occur essentially through proteolysis of the B-type cyclins by the multisubunit ubiquitin protein ligase, termed anaphase-promoting complex or cyclosome (APC/C) (Peters, 2002).

The process necessary to allow cells to progress from one cell stage to another is highly ATP dependent, a mitochondrial product that is present at reduced levels in the CMS lines (**II**). It was the most active tissues in the CMS plants, i.e., the inflorescences and young flower buds, that had the lowest ATP levels according to our measurements (**II**). According to what is proposed by us, the amount of ATP available in these CMS cells may not meet the cells demand for an "energy check-

point", thereby failing to drive the process of protein degradation necessary for proper cell division progression. As a result, the rate of cell division might be slower and that, in turn, will lead to an accumulation of RNA of the *CycB1* gene by feedback regulation, in a similar way to what happens with the nondegradable form of CycB1 protein in the experiments performed by Weingartner et al. (2004). The action of TON1 is to control microtubule dynamics during mitosis in order to promote correct cell patterning after nuclear lamina disassembling and nuclear envelope breakdown induced by the CycB1/CDK complex (Vasques et al., 1999; Nigg, 2001). Considering that the dividing cell cycling does not occur in a proper way due to incomplete or slower degradation of CycB1 protein, the action of TON1 will therefore be incomplete, signaling the nucleus to up-regulate the *CycB1* and *TON1* genes. Accumulation of transcripts after treatment with cyclohexamide, a protein synthesis inhibiter, was also observed by Sablowski and Meyerowitz (1998).
CycB1 and *TON1* genes were not the only ones related to cell division that were missregulated in the CMS lines. Wide genome comparison between *B. napus* and the CMS line 4:19 using microarrays revealed that some genes involved in cell wall remodelling belonging to the arabinogalactan protein family were down-regulated in the CMS line during the earliest floral stages (**IV**). Arabinogalactan proteins (AGPs) are present in the plasmalemma, bound to the cell wall or in a soluble form in the intercellular space. It is believe that they function by signaling neighbouring cells during cell division (Majewska-Sawka and Nothnagel, 2000). The fact that the CMS lines 4:19 and 41:17 displayed an irregular plane of cell division in young flower buds may also reflect the down-regulation of the AGPs genes.

Ultrastructural analysis of the mitochondria

All CMS meristematic tissues analysed showed two distinct mitochondrial families. One type resembled the organelles present in *B. napus*, although smaller in size, and a second type was characterized by loss of inner-membrane integrity and a strong reduction in matrix density (**III** and Fig. 7). Some of these mitochondria in the meristematic cells of the CMS lines analysed, were even ruptured in the outer-membrane (**III** and Fig. 7). Structurally modified mitochondria associated with CMS plants were also noted by Farbos et al. (2001) in alloplasmic lines of tobacco. The same kind of structural mitochondrial disruption was observed by Hernould et al. (1998) in tobacco plants transformed with an unedited copy of the mitochondrial *atp9* gene.

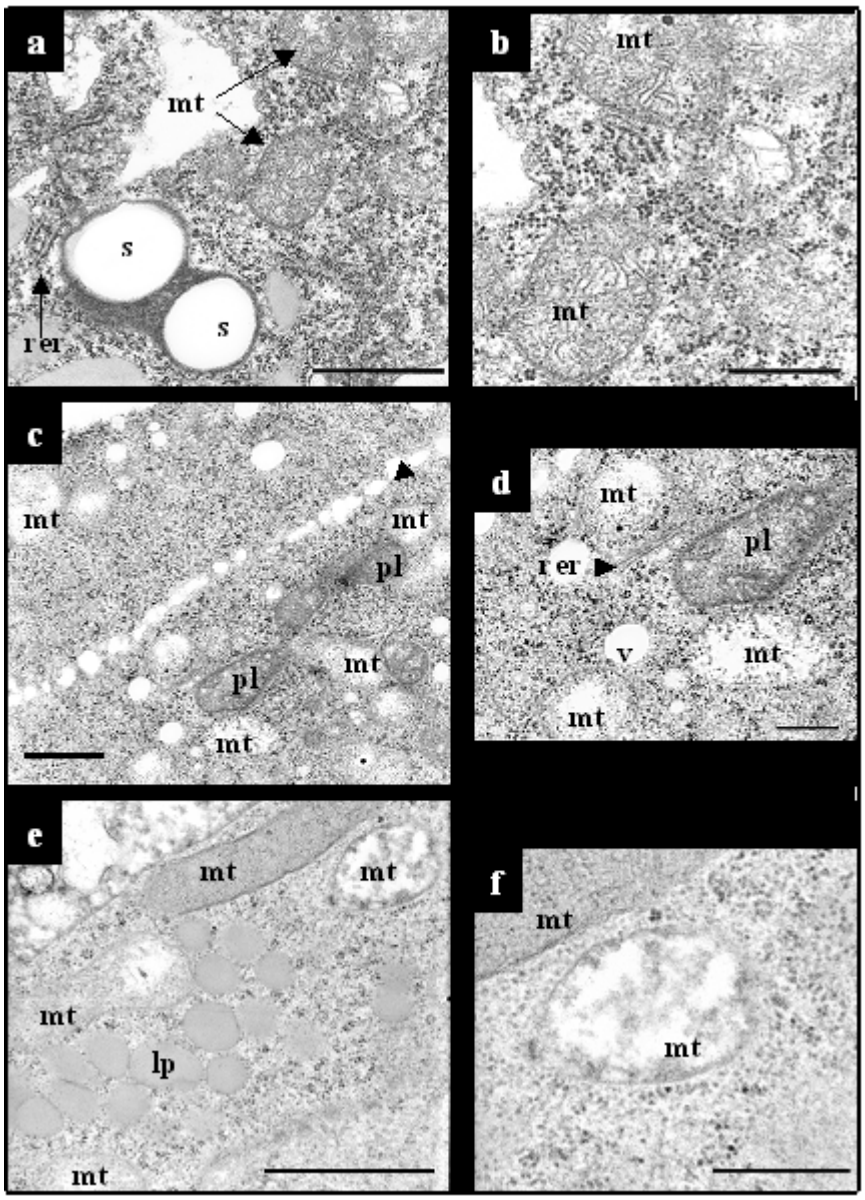

Fig. 7. Ultrastructural studies of SAM cells. **a)** Ultra thin section of shoot apical meristematic cells of *B. napus*, revealing the cytoplasm-enriched cells containing numerous well development mitochondria (arrows). **b)** Magnification of a). **c)** SAM cells of the CMS line 4:19 containing numerous small vacuoles and mitochondria lacking the inner membranes system and matrix content. A new cell wall is starting to be formed in the line formed by the small vacuole system in the middle of the picture. **d)** Magnification of c). **e)** SAM cell of the CMS line 41:17 containing mitochondria displaying the same features as in c). **f)** Magnification of e). **lp** lipid bodies; **mt** mitochondria; **pl** plastid; **rer** rough endoplasmic reticule **s** starch. **v** vacuole. Scale a), c) and e) 1 μm; b), d) and f) 0.5 μm.

The mitochondria in the green CMS leaves showed no ultrastructural differences compared to *B. napus*. This may result from the fact that leaves are fully mature tissues and the few mitochondria present in the cytoplasm showed no disruption in integrity. The structure of plastids and chloroplasts concerning the thylakoid formation and the stroma density in the CMS lines were similar to the ones of *B. napus*.
It was in the most energy-demanding tissues that mitochondria displayed the most dramatic changes, especially in stamen primordia and flower tissues at early stages of flower development. These observations are in agreement with the fact that anther formation, together with microsporogenesis, are extremely energy-demanding processes (reviewed by Brennicke et al., 1999). An increase in mitochondrial number has been reported to occur during early stages of anther development (Warmke and Lee, 1978; Lee and Warmke, 1979). The fact that mitochondrial gene transcripts accumulate more in meiocytes and tapetal cells than in other plant tissues with a concomitant increase in their respective protein content (Smart et al., 1994), and that several nuclear genes encoding mitochondrial proteins also demonstrated higher levels of mRNA accumulation in flower tissues, are indicative of increased mitochondrial activity in these particular tissues (Huang et al., 1994).

CMS systems have been related to novel mitochondrially encoded proteins. Each system has been associated with a specific mtDNA region often co-transcribed with standard mitochondrial genes. Most of the co-transcribed or chimeric genes involve promoter regions and portions of coding regions of one of the ATP synthase subunit genes (Schnable and Wise, 1998; Budar and Pelletier, 2001; Budar et al., 2003). Several of these new proteins are localized in the mitochondrial membrane fractions (Reviewed by Hanson and Bentolila, 2004, see introduction for examples). When cDNA microarray slides were hybridized with mRNA from *B. napus* and CMS line 4:19 flower buds, a group of three genes present in the mitochondrial and nuclear genome that code for the same mitochondrial product, showed a higher expression level in the CMS line (**IV**). Part of the genes present in the slides correspond to the *atp9* gene and to *orf139a/b*. This *orf* is specific of *A. thaliana* not found in the nuclear or mitochondrial genome of *B. napus*. The elevated expression of this mitochondria-associated *orf139a/b* in the CMS line is similar to what occurs in other CMS systems and seems to be directly related with the CMS trait in our system.
It is not very surprising that in tissues where mitochondrial activity has to increase in a short and controlled period of time, strange polypeptides incorporated in the mitochondrial membranes will affect the organelle inner-membrane system possibly creating a decoupling effect of the membrane potential. The effect of oxidative phosphorylation decoupling was observed in maize when a chimeric T-URF13 protein was incorporated in the inner mitochondrial membrane (Klein and Koeppe, 1985). In addition to our CMS system, reduced levels of ATP in flower tissues were also reported for CMS tobacco plants by Bergman et al. (2000). Hypotheses claiming that the energy levels in the CMS cells are related to CMS can also be found in Budar et al. (2003) and in Farbos et al. (2001). Sabar et al. (2003) have also shown that ATPase activity of the F_1F_0 complex in mitochondria was significantly reduced in the CMS plants of sunflower expressing the CMS-associated ORF522 with decreasing ATP hydrolysis by the ATP synthase.

Flower development

The most striking phenotype of the two *B. napus* alloplasmic lines in these studies relates to the flower. Sepals and carpels of CMS flowers were phenotypically indistinguishable from those of *B. napus*. However, petals were reduced in size due to fewer and smaller cells but they retained true petal identity. The strongest alterations relate to stamens that were replaced by carpelloid organs in the CMS plants (**I** and **III**). Ovule-like formations were found at the internal margins of the unfused carpelloid structures (Fig. 1) (**I** and **III**). In spite of the homeotic conversions all organs in the CMS flowers were distributed typically within the flower: four sepals surrounding four petals, six stamens i.e. carpelloid organs occupying the third whorl and two fused carpels in the fourth whorl. The phenotypic differences in flower development between the CMS lines and *B. napus* start to be visible by the time third whorl organs begin to differentiate (**IV**). The development of the CMS flower buds until stage 6 resembles the ones of *B. napus* with sepal primordia appearing before the rest of floral organs (**III** and **IV**). Stamens are the next organs to be formed followed by the two carpels (**IV**). The flower development described here for the CMS line 4:19 and 41:17 was similar in another abnormal CMS line (line 14:103) (**IV**, data not shown). This line has been grouped together with the lines 4:19 and 41:17 (**I**). Two of the less abnormal CMS lines described in paper I, namely 4:55 and 41:38, had initially a similar phenotypic floral development as in the other CMS lines (**IV**, Fig. 8 l and m). However, compared to the other CMS lines, differences appeared when anthers started to differentiate resembling more closely *B. napus* (**IV**, Fig. 8 l and m). In contrast to what happens in *Arabidopsis* flower development, where petal and stamen primordia arise at the same time (Smyth et al., 1990), in *B. napus* petals are the last floral organs to be formed (**IV**).

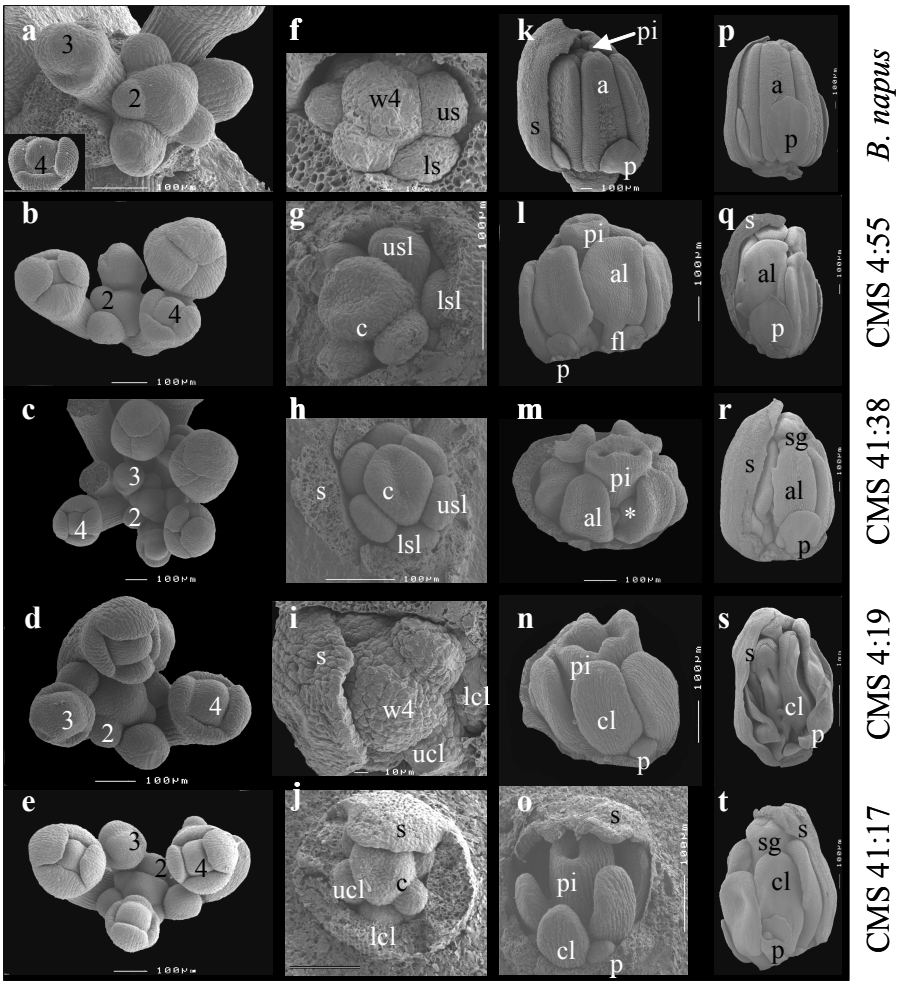

Fig. 8. Flower development study using SEM of *B. napus* and four different CMS lines.
(a-e) Inflorescence containing flower buds from stage 1 to stage 4.
(f-j) Young flower bud at stage 6. In all the lines, the four upper and the two lower stamens/stamens-like and carpelloid-like structures primordial are formed. The two carpels start to be distinguished.
(k-o) Flower buds at late stage 9. Petal primordial are already formed between the insertion point of the third whorl organs.
(p-t) Flower buds at early stage 10. Petals reach half the size of third whorl organs. Flower stages are numbered. Bar **(j)**: 100 μm. (*) - ovule-like structure. **a** – anther; **al** – anther-like organ; **cl** – carpelloid-like organ; **fl** – filament-like structure; **lcl** – lower carpelloid-like primordial; **ls**- lower stamen primordial; **lsl** – lower stamen-like primordial; **p** – petal; **pi** – pistil; **sg** – stigma; **ucl** – upper carpelloid-like primordial; **us** – upper stamen primordial; **usl** – upper stamen-like primordial; **w 4** – whorl four.

The CMS flowers resemble, in many aspects, the *Arabidopsis* flowers of plants mutated in *AP3* or *PI* genes (Bowman et al., 1991a; Krizek and Meyerowitz, 1996). The phenotypic similarity between the CMS lines and *ap3*/*pi* mutants suggested that the B-class genes might not be properly regulated. *In situ* hybridisation studies using Dig-labeled probes for *BnAG*, *BnAP3* and *BnPI*, three homeotic genes responsible for stamen and petal formation, showed no differences in expression patterns between the CMS lines and *B. napus* during the first six floral stages (**III**). However, later in flower development, the expression patterns observed in the CMS third whorl organs mimicked the expression patterns observed in the carpels. For example, *BnAP3* mRNA accumulated in the tip of the homeotic organs and in the ovule-like structures (**III**) just as in true carpels and ovules. *AP3* signals in ovules were also observed in *A. thaliana* (Jack et al., 1994). The indistinguishable expression pattern of the homeotic genes detected early in flower development between all the lines, was also noted by Farbos et al. (2001) in alloplasmic tobacco lines and by Linke et al. (2003) in CMS carrot lines.
Expression levels of *BnAP3* and *BnPI* were investigated by real time RT-PCR showing no statistical alterations during the first stages (1-6) of flower development, although, CMS line 41:17 showed a reduction in *BnPI* expression levels at early flower stages. After stage 6, however, the levels were drastically reduced (**III**). Wide expression profiles using microarrays also showed the same results (**IV**). The results from real time RT-PCR using the mRNA utilized in the hydridization with the microarray slides confirmed the differences of the B-genes expression levels in *B. napus* and the CMS line 4:19 (**IV**). Thus, the homeotic conversion of anthers into carpelloid structures in the CMS plants seems to be correlated with the strong reduction of *BnAP3* and *BnPI* expression levels after stage 6 of floral development. Microarray experiments of flower buds between stages 1 and 5 showed that several genes belonging to the gene families pectinesterases, polygalacturonase and multi cupper oxidase were down-regulated in the CMS line (**IV**). Distinct functions have been attributed to these genes and most of them are involved in pollen production (**IV**). Even though these genes act in flower buds after stage 6, their expression is detected earlier. For example,this is the case for the *EXTRA SPOROGENOUS CELLS* (*EXS*) gene involved in the formation of meiocytes which transcript is detected in the inflorescence (Canales et al., 2002).

Genes responsible for *AP3* and *PI* activation were also studied by real time RT-PCR. The *BnLFY*, *BnUFO* and *BnASK1* genes were chosen. *LFY* is necessary for floral meristem identity and is later required for *AP3* and *PI* activation (Weigel et al., 1992; Weigel and Meyerowitz, 1994). The direct binding of LFY to an *AP3* promoter element activates the expression of this gene during early floral stages (Hill et al., 1998). However, LFY also acts indirectly at later floral stages for *AP3* activation. This indirect activation requires *de novo* protein synthesis (Lamb et al., 2002).
The *UFO* and *ASK1* genes were also studied because weak double mutants of *ufo-6 ask1* mimicked the phenotypes of the CMS-lines. In these *Arabidopsis* mutants, carpelloid structures replaced the stamens and the petals were smaller in size. In the mutated plants the shorter stature observed was due to shorter internode distances. Leaves were smaller owing to reduced cell number and no differences

in shape or size of the inflorescence meristem was noticed until stage 6 (Zhao et al., 1999, 2001). In weak double mutants of *UFO* and any other gene coding for proteins belonging to the SCF^{UFO} complex (Zhao et al., 2001; Ni et al., 2004), the petals are reduced in size. In conclusion, these mutants more closely resemble the CMS plants analysed in this study (Leino et al., 2003), rather than the *ap3/pi* mutants in which the petals are modified to sepal-like organs (Bowman et al., 1989, 1991a).

Analysis of *BnLFY*, *BnUFO* and *BnASK1* expression levels in our material suggests that the activation of the B-class gene by the SCF^{UFO} complex and LFY is interrupted. We observed a higher accumulation of *BnLFY*, *BnUFO* and *BnASK* RNA during the first floral stages, especially in the CMS lines 4:19. *AP3* transcripts did not show a significant reduction at these stages most probably due to the direct activation of *LFY* and *AP1* (Ng and Yanofsky, 2001). The *BnPI* transcripts, however, were reduced already during the first floral stages, most probably because initial activation of this gene by *UFO* and *LFY* does not occur as it does for *AP3* (Honma and Goto, 2000). Once this activation is on during the first floral stages, the autoregulatory pathway is active but in this phase, protein synthesis is necessary for *PI* activation. In the case of *AP3*, its activation is carried out by direct binding of an AP3/PI heterodimer to the promoter (Tilly et al., 1998). Later in flower development (stage 7-9), *BnLFY* and *BnUFO* levels are even higher with a stronger reduction of AP3/PI levels. Especially in the CMS line 41:17 when compared with the values during the first six floral stages. This could reflect a delay in gene activation occurring in the CMS line 41:17. When confirming the microarray results by real time RT-PCR for *BnLFY*, *BnPI* and *BnAP3*, the same tendency was found with the more abnormal CMS lines such as 14:103 and 41:17 showing the strongest reduction in *BnAP3* and *BnPI* mRNA levels (**IV**).

By considering the model proposed by Zhao et al. (2001), the SCF^{UFO} complex will target the negative regulator of LFY for destruction through ubiquitinylation, promoting the correct transcription of *AP3*. Our results of the transcription levels of *BnASK1*, *BnUFO* and *BnLFY* genes involved in the activation of *BnAP3* and *BnPI*, suggest that the involvement of the SCF^{UFO} complex and LFY in activating the B-genes is affected in the CMS plants (Fig. 9). The results of Zhao et al. (1999, 2001) and Ni et al. (2004) supported the hypothesis of the role of SCF^{UFO} complex in the regulation of the B-function. This is accomplished by genetic interaction of *UFO*, *ASK1* and *AtCul1* that regulate the expression of *AP3* and *PI* (Zhao et al., 2001; Wang et al., 2003).

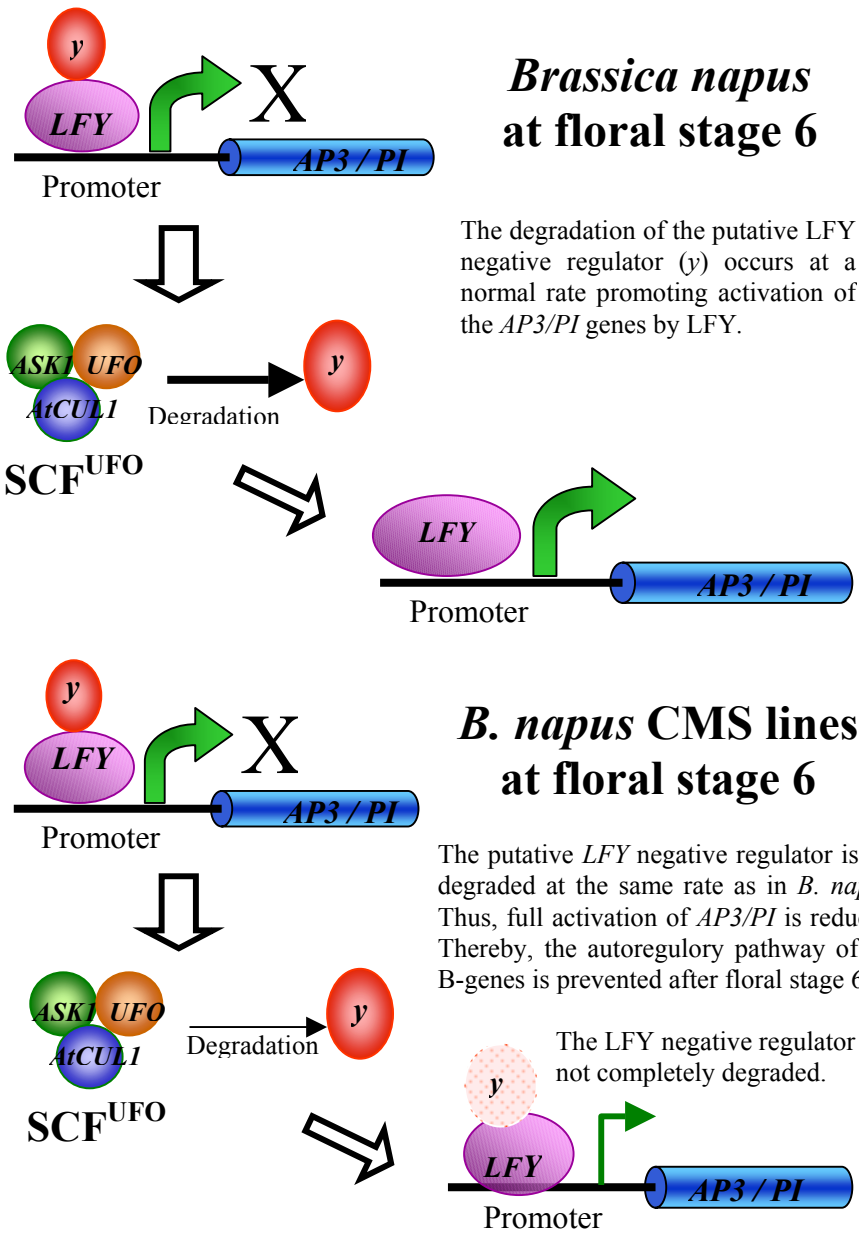

Fig. 9. Schematic representation of the mechanism involved in *AP3* and *PI* activation by the SCF^{UFO} complex in *B. napus* and in the two CMS *B. napus* lines according to our hypothesis and based on the model proposed by Zhao et al. (2001). ***y*** – putative LFY negative regulator.

The resulting low levels of AP3 and PI proteins produced will influence the auto-regulatory pathway of B-genes since the levels of AP3 and PI proteins have to reach a critical point in order to promote the activation of both the *AP3* and *PI*

genes (Goto and Meyerowitz, 1994; Jack et al., 1994; Samach et al., 1997). The amount of *BnASK1* transcripts at these later stages may reflect the redundancy of this class of genes. The *ASK* genes belong to a large family and are expressed mostly in actively dividing cells such as meristems (Bai et al., 1996). They are also required for homologous chromosome separation at anaphase I during male meiosis. *ASK1* gene is highly expressed in the microsporogenic tissue (Yang et al., 1999). This tissue is missing in the CMS lines, which influences the expression levels at these later floral stages. The same explanation can be applied to the values of AG after stage six. Later in flower development, AG is only highly expressed in the connective tissue of the anther and at lower levels in the anther walls and the filament. In carpels it is highly expressed in stigmatic papillae and developing ovules (Bowman et al., 1991b). In our CMS plants, anthers are converted into carpelloid structures even though these organs do not have true ovules. Thus, the number of cells expressing AG in the CMS flowers is reduced. Other floral genes are down-regulated in the CMS line 4:19 at later floral stages such as *SEPALLATA3* (*SEP3*) or *AGAMOUS LIKE GENE 18* (*AGL18*) (**IV**).

Activation and regulation of B-genes is the most complex mechanism within all the homeotic genes and have to occur in a very precise time frame once flower development is initiated (Bowman et al., 1993; Honma and Goto, 2000; Ng and Yanofsky, 2001; Lamb et al., 2002). In the CMS system, the elevated number of disrupted mitochondria apparently cannot provide enough ATP to meet the high energy-demand of anther formation. As a result, regulatory pathways such as protein degradation promoted by the SCF^{UFO} complex could occur at a slower rate interrupting the activation of *BnAP3* and *BnPI*.

In the work of Weingartner et al. (2004), where a nondegradable version of CycB1 was expressed in tobacco plants, greater mRNA accumulation of *CycB1* was observed. In analogy to the observations of Weingartner et al. (2004), the high accumulation of RNA in the CMS lines from genes involved in the SCF^{UFO} complex could be related to the action of the corresponding proteins. If the degradation of the *LFY* negative modulator through proteolysis would occur at a slower rate due to lower levels of energy in the flower tissues (**II**) *AP3* and *PI* levels may not be able to reach the threshold value necessary to induce the AP3/PI autoregulatory pathway (Fig. 7). This slower process of the B-genes activation by the SCF^{UFO} complex will promote the accumulation of RNA of all the genes encoding for the proteins belonging to the SCF^{UFO} complex and of *LFY* through a feedback up-regulation when the action of the product of these genes is not carried out. Such feedback regulation is possible to occur since nuclear genes are not mutated in the CMS plants.

In order to elucidate and to confirm the hypothesis brought forward in this work, additional experiments at the protein level are required (see future perspectives).
Besides the energetic hypothesis formulated throughout this thesis, other possible effects from the presence of disrupted mitochondria should be considered. Mitochondrial proteins and RNA might be exported to the cytosol. These exported mitochondrial products may then interfere with other proteins or even with nuclearly transcribed RNA present in the cytoplasm. These possibilities require further experiments.

Conclusions and future perspectives

Conclusions

After analysis of the results reported in this thesis, the main conclusions are the following:

- Several cytoplasmic male sterile lines were obtained in *B. napus* after somatic hybridisations between *B. napus* cv. Hanna and *A. thaliana* ecotype Landsberg erecta followed by recurrent backcrosses with *B. napus* as the pollinator. Some of the CMS lines displayed homeotic conversions of the anthers into carpelloid structures.
- The CMS lines exhibiting strongly modified floral organs and chosen for further studies, were also characterized by reduced growth rate and a limited capacity to recover after being submitted to etiolation i.e. a stress condition.
- The metabolism in the CMS plants was changed in comparison to *B. napus* with higher starch accumulation in the chloroplasts and reduced sucrose levels in the leaves.
- Actively dividing tissues in the CMS plants, such as young flower buds, showed a significant reduction in the ATP levels. In these tissues, the structure of most of the mitochondria appeared to be disrupted, missing the inner-membrane system of cristae and the matrix.
- The homeotic genes *BnAP3* and *BnPI,* responsible for petal and stamen formation, were down-regulated in the CMS flowers after stage 6 according to real time RT-PCR.
- Upstream genes of *BnAP3* and *BnPI* such as *BnASK1*, *BnUFO* and *BnLFY* showed an abnormal RNA accumulation throughout flower development, suggesting that the activation of the B-class genes may not be correctly regulated by the SCF^{UFO} complex together with LFY.
- The two mitosis-related genes, *BnCycB1* and *BnTON1*, showed an expression profile similar to *BnASK1* in the floral tissues. They were up-regulated during the first six floral stages when cell division is the main mechanism involved in the growth of the flower buds. After stage 6, cell division is replaced mainly by cell elongation and the RNA levels of *BnCycB1* and *BnTON1* were reduced in the CMS lines as compared to *B. napus*. *BnUFO* and *BnLFY* were also up-regulated throughout flower development. The fact that genes regulating the second and third whorl organs as well as genes involved in cell division rate and patterning display similar expression profiles, suggests that a common mechanism may be influencing these two distinct cellular events of stamen formation and cell division in flower development.
- Microarray cDNA slides hybridized with mRNA from flower buds at different floral stages revealed that 90 genes displayed a significant expression level in the CMS line 4:19 compared to *B. napus*.
- A group of carpel-related genes were up-regulated in the CMS line at later floral stages probably reflecting the CMS phenotype. Another group of genes showed a down-regulation in the CMS. Half of these genes are predominantly expressed in the stamens.
- Microarray experiments also demonstrated that a group of stamen-specific genes were down-regulated during earlier floral stages in the CMS line.

These genes belong to the gene family of pectinesterases, polygalacturonases and multi cupper oxidases type I and known to be involved in pollen formation.

Future perspectives

Protoplast fusion between *B. napus* and *A. thaliana* and recurrent backcrossing to *B. napus* resulted in the formation of cybrids lines. These alloplasmic lines contain mitochondria with rearranged DNA between the two species. The largest portions are derived from *Arabidopsis*. As a result, several new *orf*s not present in *B. napus* mitochondria are expressed (Leino et al., personal communication). After analysing the transcriptional profile of the putative CMS-associated *orf*s, the best candidate to be associated with CMS should be chosen to investigate further. By producing antibodies against the putative proteins encoded by these *orf*s Western blots and EM immunolocalization studies can be performed. Discovering where in the cell, the possible CMS-associated proteins are accumulating, may shed some light on the role of these proteins. If the cellular localization of the CMS-associated proteins is mainly within the mitochondrial membrane, it might explain the hypothesis formulated throughout this thesis. It is proposed in this work that the disrupted mitochondria present in the CMS plants cannot produce enough ATP to meet the energy demands required by specific tissue types during development of the young flowers. If the CMS-associated proteins accumulate mainly in the cytoplasm or in the nucleus, it would be interesting to perform studies to show whether these proteins interact with other proteins or with DNA.

Performing Western blots with antibodies against LFY, UFO and ASK might bridge the gap between our knowledge concerning gene expression and the CMS phenotype since the expression levels of these genes are up-regulated in the CMS lines even though their action in activating the *AP3* and *PI* genes seems to be interrupted. It may also help to elucidate the hypothesis brought forward regarding a slower ubiquitination rate and to verify whether this caused the modified levels of AP3 and PI proteins during floral stages 3-6 and during stage 7 and onwards.
By extracting mRNA from specific cell types at a precise floral stage with Laser Capture microdissection (LCM), would help to narrow down the number of genes differentially expressed between the CMS lines and *B. napus* that were obtained from the microarray experiments. These putative genes should then be analysed further by *in situ* hybridisations and by real time RT-PCR.

In this study we tested the effects of one stress condition (etiolation) on the plant material and as a result, the vegetative growth of the CMS plants was reduced when compared to *B. napus*. Other stress conditions such as drought, cold or different photoperiods would be of interest to study in respect of the CMS plants response to the proposed environmental conditions. By studying the alloplasmic lines, the importance of the mitochondrial genome for the metabolic compounds produced during the stress conditions would be possible to analyze.

Proteomic analysis, using protein extracts from specific tissue types such as third whorl organs, can be used to identify if there are differences in the CMS lines and *B. napus* regarding the class or amount of proteins being expressed in a given

tissue type. This method may also permit the identification of putative CMS-associated proteins specifically expressed in floral organs.

Another line of study would be to continue with the RNA studies being carried out in the lab. Such studies consist in trying to find out if the disrupted mitochondria present in the meristematic cells of the CMS lines allow the release of portions of strange RNA to the cytoplasm. These small portions of RNA may influence translation by binding to the RNA that encodes key proteins involved in developmental processes such as flower development.

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Acknowledgements

Because without some very special persons this work would never be accomplish, my sincere gratitude goes to:

My supervisor Professor **Kristina Glimelius** by accepting me without knowing me at all, for always pushing me to do a better job, for the respect demonstrated concerning my ideas and on top of everything, my very strange working hours. Thank you for always receiving me with a sincere smile.

My co-supervisor **Isabelle Farbos** who shared my frustrations, my anxieties and my ideas. Thank you for all the new techniques and the acute vision over science.

Ingrid Eriksson for being my friend, my technical master and my partner in the experiments performed in this work. Thank you for never complaining about all the mistakes and all the times I have interrupted for very basic questions.

To **Birgitta Eriksson** for always taking care of my papers where I got completely lost. Thank you for all the nice words, support and time spent with my problems always with a beautiful smile.

To **Gun** for all the help with the protoplasts, keeping the *in vitro* plants always in shape and for sharing the lab with me.

My working colleges **Matti Leino** and **Jenny Carlsson** for the support, the friendship, scientific and non-scientific discussions, for all the cakes and for being who you are. For both of you my deepest thank you and the best of luck. I believe we will meet again in some lab.

To **Per Bergman** and **Per Olof Lundquist** for all the help and comments in ATP measurements.

To **Carina Knorpp,** for all the help with the corrections, support and friendship.

To **Lars-Olof Hansson** for solving all my library problems with such sympathy.

To **Maria**, **Jan**, **Johan**, **Monika, Jens** for never declining to help wherever I needed.
To all the ladies that kept the department a place so enjoyable to work in.

To my friends that I cherish so strongly: **Leticia Pizzul** for being my first neighbour and friend in Sweden and for every time listening to my complains always with a positive view of the world. **Oksana,** my office mate who always respected me and made me company during the long nights in the department. **Vasilios** for all the support and friendship. **Gosia** for all the company during the late hours and the conversations between experiements. **Lada** for being such a good scientist and such a good person to speak with. **Nandi**, for allowing me to speak another language than English and all the scientific support. **Peter** and **David** for being in the department when nobody else is and for always being nice.

Above all, I would like to express my gratitude to The Swedish University of Agricultural Sciences for accepting me as PhD student and to Fundação para a Ciência e a Tecnologia – Ministério da Ciência e do Ensino Superior – Portugal, as well as to FORMAS and VR for financial support.

Finally, my Portuguese friends living in Stockholm. Without you, I would never have made it.
A todos agradeço do fundo do coração por me terem aturado nas frustações nas crises e terem partilhado a minha experiência na Suécia. Obrigada por todos os jantares, almoços, churrascos, viagens, etc. **Andreia**, um profundo obriagada pelas noites de longas conversas, por partilhares o gosto por café e bolo de chocolate e sobretudo pelo modo extremamente sincero com que sempre encaraste a nossa amizade. **J**, por te teres sempre preocupado e por sempre me teres apoiado e compreendido. **Gonçalo** por podo o apoio, carinho e amizade demostradas. Obrigada por todas as viagens, saídas e concertos que organizaste e fez com que eu saísse de Uppsala. **Mónica**, pelos fantásticos chocolates quentes partilhados em Gamla Stan, pelas longas conversas e por sempre perguntares como correm as coisas. **Zé**, companheiro de viagens a quem devo um grande carinho por sempre me ter ouvido e respeitado as minhas ideias mesmo as mais estranhas. **Quintó**, pela extrema simpatia e amizade. **Levi**, por seres tão positivo e receptivo a novas ideias.
A todos, devo-vos as boas horas que recordarei para sempre da minha passagem por terras escandinavas.

Thank you, Obrigada, Tack