Theor Appl Genet (2003) 106:1156–1163
DOI 10.1007/s00122-002-1167-y

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Brassica napus lines with rearranged *Arabidopsis* mitochondria display CMS and a range of developmental aberrations

Received: 18 April 2002 / Accepted: 16 September 2002 / Published online: 13 December 2002

Abstract Numerous *Brassica napus* (+) *Arabidopsis thaliana* somatic hybrids were screened for male sterility and aberrant flower phenotypes. Nine hybrids were selected and backcrossed recurrently to *B. napus*. The resulting lines displayed stable maternal inheritance of flower phenotypes. Nuclear and organellar genomes were characterized molecularly using RFLP analysis. No DNA from *A. thaliana* was found in the nuclear genome after six back-crosses, whilst the mitochondrial genomes contained rearranged DNA from both *A. thaliana* and *B. napus*. Each line tested had a unique RFLP pattern of the mitochondrial DNA (mtDNA) that remained unchanged between the BC_3 and BC_6 generation. The plastid genomes consisted of *B. napus* DNA. Five lines of the BC_5 generation were subjected to more comprehensive investigations of growth, morphology and fertility. On the basis of these investigations, the five CMS lines could be assigned to two groups, one represented by three lines displaying reduced vegetative development, complete male sterility, and homeotic conversions of stamens into feminized structures. The second group, represented by the other two lines, were not completely male-sterile but still displayed severely affected flower morphologies. These two lines did not display any reduction in vegetative development. For both groups only stamens and petals suffered from the morphological and functional aberrations, while the sepals and pistils displayed normal morphology. All plants were fully female-fertile. Different rearrangements of the mitochondrial genome disturbed nuclear-mitochondrial interactions and led to various types of aberrant growth and flower development. The existence of numerous CMS lines with different mitochondrial patterns involving a species with a sequenced genome offers new opportunities to investigate the genetic regulation of CMS and its associated developmental perturbations.

Keywords Cytoplasmic male sterility · RFLP · Morphology · Alloplasmy

Communicated by H.C. Becker
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Introduction

When protoplasts of two different species are fused, not only nuclear, but also organellar genomes are combined into one cell. As the fusion products divide, form calli, and are induced to regenerate plants the chloroplasts usually are inherited from only one of the parents, whereas the mtDNA is rearranged and may include DNA from both parents (Landgren and Glimelius 1994; Earle 1995). The novel cytoplasmic composition established in the hybrid plant remains essentially unchanged during subsequent sexual crossings and is maternally inherited. Recurrent back-crossing to either one of the parents rapidly results in elimination of the nuclear DNA of the other parent (Bohman et al. 1999). Thus, alloplasmic hybrids can be obtained with the nuclear genome from one species, with a rearranged mitochondrial genome including DNA from both species and with either of the parental chloroplast genomes. A frequent consequence of alloplasmy is perturbation of anther development resulting in reduced pollen production, a phenomenon known as cytoplasmic male sterility (CMS). In *Brassica* species the CMS trait is often utilized for hybrid seed production and a number of different alloplasmic combinations have been established for this purpose (reviewed by Makaroff 1995; Delourme and Budar 1999).

Additionally, *Brassica* species are excellent for studying the molecular interactions between the nucleus and cytoplasm due to their small and well-characterized genomes. CMS has been associated with alterations of the mitochondrial genome that disturb its interaction with the nuclear genome (Delourme and Budar 1999).

Since nuclear restorer genes can revert CMS plants to fertility, alloplasmic CMS is clearly due to a nuclear-mitochondrial incompatibility (Hanson 1991). In most cases, investigations have revealed that the CMS-associated regions of the mitochondrial genome contain an open reading frame (ORF) (Schnable and Wise 1998). These ORFs are usually cotranscribed with one of the known mitochondrial genes, but no homology between the different polypeptide sequences encoded by ORFs has been found and their function remains unknown. The abundance of the polypeptides is reduced upon restoration of fertility (Schnable and Wise 1998; Bellaoui et al. 1999). However, the basic mechanisms leading to CMS are still largely unknown.

Many reports of a dramatic mitochondrial influence on flower morphology and reproductive development have been presented. Different male-sterile *Brassica* cytoplasms affect flower morphology in a highly variable manner (reviewed by Delourme and Budar 1999). Anthers can be rudimentary, feathery or sepaloid, as well as fused with other organs; petals are often modified. *Brassica napus* with the Ogura cytoplasm has been reported to vary in morphology with temperature from almost normal to completely sterile anthers (Polowick and Sawhney 1987). Although reports about vegetative development are less frequent, mtDNA rearrangements associated with reduced growth and abnormal vegetative development have been reported in maize (Newton and Coe 1986), in tobacco (Bonnett et al. 1993) and in wheat (Ikeda and Tsuunewaki 1996). Furthermore, Kirti et al. (1995) and Malik et al. (1999) found variation in flowering time in *Brassica juncea* combined with cytoplasms from other *Brassica* species.

In this study we have characterized a novel CMS system derived from somatic hybrids between oilseed rape, *B. napus* and the genetic model organism *Arabidopsis thaliana*. The complete sequence of the *A. thaliana* nuclear (The Arabidopsis Genome Initiative 2000) and mitochondrial (Unseld et al. 1997) genomes make this material excellent for studies of nuclear-mitochondrial interactions leading to floral aberrations and CMS.

Materials and methods

Plant material

Somatic hybrids between *B. napus* cv Hanna and *A. thaliana* var. Landsberg erecta were produced by Forsberg et al. (1998) via protoplast fusion. The hybrids were backcrossed to *B. napus* cv Hanna, and the BC_1 generation, consisting of 170 lines derived from individual fusion products, was screened for male sterility and aberrant flower phenotypes. Out of the 22 lines displaying complete or partial male sterility nine were selected and subjected to recurrent backcrosses to *B. napus* cv Hanna as a pollinator to obtain a BC_6 generation.

Organelle and nuclear DNA analysis

For organelle analysis total DNA was isolated (Sharpe et al. 1995) from young leaves of the parental plants and three individual plants of each hybrid line from the BC_3 generation and two individual plants from the BC_4 (lines 8:2, 9:13, 14:4, 48:60) and BC_6 (lines 4:19, 14:103, 41:17, 4:55, 41:38) generation. DNA was cut with *Bam*HI and 3 µg loaded on gels. The DNA was electrophoresed in 0.8% agarose gels (1 V/cm, 16 h) and transferred to nylon filters (Hybond N+, Amersham Pharmacia Biotech) using 0.4 M NaOH (Sharpe et al. 1995). Labelling and hybridisations were performed according to Forsberg et al. (1998), but using 0.2 × SSC + 0.1% SDS for the final washing step. A 3.8-kb *Sac*I lettuce cpDNA fragment (Jansen and Palmer 1987) was used as a probe for chloroplast analysis. Probes for the mitochondrial genes *cob*, *cox1*, *cox2*, *cox3*, *atp1*, *atp6*, *atp9*, *nad5a*, *nad6* and *rrn26* were PCR amplified from *A. thaliana* DNA using primers designed by Giegé et al. (2000), except that *atp6* was amplified between primers UATP6 (5′TACTAAAAAGGGAGGAGGAAAC3′) and LATP6 (5′CTATAAATAAAGGACCAAGAGC3′). Hybridized filters were exposed to Kodak X-OMAT AR film using intensifying screens at –70 °C.

For nuclear analysis DNA from two individual plants of each line in the BC_6 generation was analysed. DNA isolation and Southern procedures were performed as above except that 10 µg of DNA was restricted with *Eco*RI and that hybridisation of filters was done according to the manufacturers instructions using Church and Gilbert buffer. Sixteen mapped *A. thaliana* RFLP markers (Liu et al. 1996) covering all five chromosomes (Forsberg et al. 1998) were used as probes. The *bar* gene, conferring resistance to the herbicide Basta and present in the *A. thaliana* parental line was also tested. Seeds were germinated on MS-medium with 200 mg/l of Basta and screened for resistance.

Morphology and fertility

For detailed morphological studies five lines of the BC_5 generation were grown in pots under controlled conditions in a climate chamber with 22 °C day/18 °C night temperatures and a photoperiod of 16 h. Light intensity was 400 µmol/m^2 s and air humidity kept at 85%. Five plants of each line were grown in a complete randomized design. Measurements of plant height and leaf size were performed every week until the inflorescence appeared (5 weeks). Flowers from the third branch of the inflorescence were collected and all floral organs were analysed. Dry weight of shoots was determined 2 and 5 weeks after sowing by drying five shoots of each line for 24 h at 105 °C.

Of each plant ten flowers were hand-emasculated and pollinated with *B. napus* cv Hanna. When feasible ten flowers of each plant were also self-pollinated. The number of ovaries per pistil was counted. Ripe pods were threshed and seeds counted and weighed.

Statistical significance of flower morphology and fertility characteristics was calculated with one-way ANOVA using the general linear model procedure in MINITAB software. Pairwise comparisons were performed using Bonferroni multiple *t*-tests.

Histological studies

The five lines that were investigated morphologically were also subjected to histological studies. Floral pieces (sepals, petals, stamens and pistils) were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at room temperature for 2 h, rinsed in the same buffer, then post-fixed in 2% osmium tetroxide (same buffer) for 2 h at room temperature. After three rinses with the same buffer, the material was dehydrated in a graded ethanol series (20, 40, 60, 80, 95 and 100%) and then gradually embedded in Spurr low viscosity resin. Polymerization of the resin was carried out at 70 °C for 12 h.

Thin sections were cut at 1 µm with a glass knife on a microtome (Heidelberg HM 350), floated on drops of distilled water and dried onto microscope slides. Sections were stained with Toluidine blue O, pH 4.5 in acetate buffer. All micrographs were taken on a Nikon Fuji Digital camera HC 300Zi.

Results

Nuclear-cytoplasmic composition

Out of a population of 170 BC_1 lines derived from individual protoplast fusions between *B. napus* (+) *A. thaliana* we found 22 lines displaying male-sterile or aberrant flower phenotypes. Nine lines, with distinct floral phenotypes and good seed-set after back-crossing with pollen from *B. napus* were selected and back-crossed further. These lines were investigated for nuclear and organellar composition using RFLP markers. The analysis revealed that the lines contained a mixed mitochondrial composition with both *B. napus* and *A. thaliana* mtDNA and frequent rearrangements (Table 1). Each line displayed a unique individual RFLP pattern of the mitochondrial genome that was stably inherited between generations. For most probes tested both *B. napus* and *A. thaliana*-specific fragments were present, except for line 41:17 in which the number of *A. thaliana*-specific fragments was lower. None of the lines, though, contained all the *B. napus* or *A. thaliana*-specific fragments, indicating that no pure parental mitochondria were present. Five of the nine tested lines contained recombined fragments; these were found in the regions of *cob*, *cox2*, *atp1*, *atp9* and *nad5a*. All lines contained chloroplasts from *B. napus*.

The primary hybrid lines contained most of the nuclear DNA of *A. thaliana* as investigated by Forsberg et al. (1998) using 16 nuclear *A. thaliana* RFLP markers covering all chromosome arms. The CMS lines, analysed in the BC_6 generation, contained none of these markers. Neither did any line possess resistance to Basta, indicating that the *bar* gene, present in the *A. thaliana* parental line, had been lost.

Plant development and flower morphology

In all lines, flower phenotypes remained stable during recurrent backcrosses, indicating consistent maternal inheritance. After five backcrosses we performed a more detailed morphological characterization in a controlled environment. Five CMS lines were investigated and compared with fertile *B. napus* cv Hanna during vegetative and reproductive development. All five lines differed in morphology, but two distinct groups of CMS lines could be established based on differences in morphology and size of the floral organs (Fig. 1 and Table 2) and in vegetative growth (Fig. 2).

Group 1 is comprised of Lines 4:19, 14:103 and 41:17. These lines were completely male-sterile and the stamens showed homeotic conversions to carpel-like structures, sometimes exhibiting ovule-like structures in the margins (Fig. 1e). These feminized stamens usually contained stigmatoid structures at their tips. The petals were significantly shorter in length and narrower in width but had the same colour as *B. napus* cv Hanna. Group 1 lines developed slowly, had shorter inflores-

Table 1 RFLP patterning of mitochondria of the nine investigated male-sterile *B. napus* (+) *A. thaliana* hybrid lines. A = *A. thaliana* fragment, B = *B. napus* fragment, R = recombined fragment. When the parental RFLP patterns consist of more than one fragment, each fragment is indicated in the line

Probe	Line										
	B. napus	*A. thaliana*	4:19	14:103	41:17	4:55	41:38	8:2	9:13	14:4	48:60
cob	B	A	A, B	A, B	A, B	A, B, R	A, B	A, B	A, B	A, B	A, B, R
cox1	B	A	A	A	A, B	A	A	A	A	A	A
cox2	B1, B2	A1, A2	A1, A2, B1, R	A1, B1, B2	B1, B2	A2, B2	A1, B1, B2	A1, A2, B2	A1, B1	A1, B2	A1, B2
cox3	B1, B2, B3	A	A, B1, B2, B3	A, B1, B2, B3	B1, B2, B3	A, B1, B2, B3	B1, B2, B3	A, B1, B2, B3	A, B1, B2, B3	A, B1, B2, B3	A, B1, B2, B3
atp1	B1, B2	A	A, B1, B2	A, B1, B2	B1, R	B1, B2	A, B1, B2	A, B2	A, B1, B2	A, B2	A, B1, B2
atp6	B	A	A, B	A, B	A	A	A	A	A, B	A	A
atp9	B	A	A	A	B, A	A	B, R	A	A	A	A
nad5a	B	A	A	B	B	A, R	B	B	B	B	B
nad6	B	A1, A2	B	A1, A2, B	A1, A2, B	A1, A2, B	A1, A2, B	A1, A2, B	A1, A2, B	A1, A2, B	A1, A2, B
rrn26	B	A	A, B	A, B	A, B	B	A, B	A	B	A, B	A, B

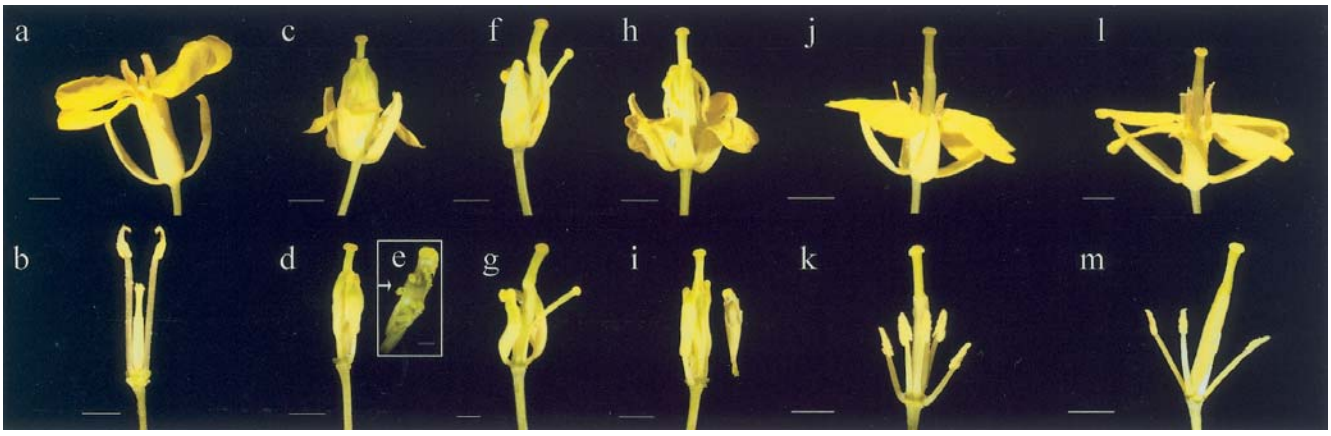

Fig. 1a–m Phenotypes of mature flowers. Intact flowers (*upper row*) and flowers with sepals and petals removed (*lower row*). **a–b** *B. napus* cv Hanna. **c–d** 4:19, **f–g** 14:103, **h–i** 41:17, **j–k** 4:55, **l–m** 41:38. **e** Detail of one stamen with homeotic conversions into carpel-like structures. Notice the ovule-like structures (*arrowhead*) and at the tip of the "anther" the stigmatic cells. *Bars*: b = 4 mm, e = 1.5 mm, others = 3 mm

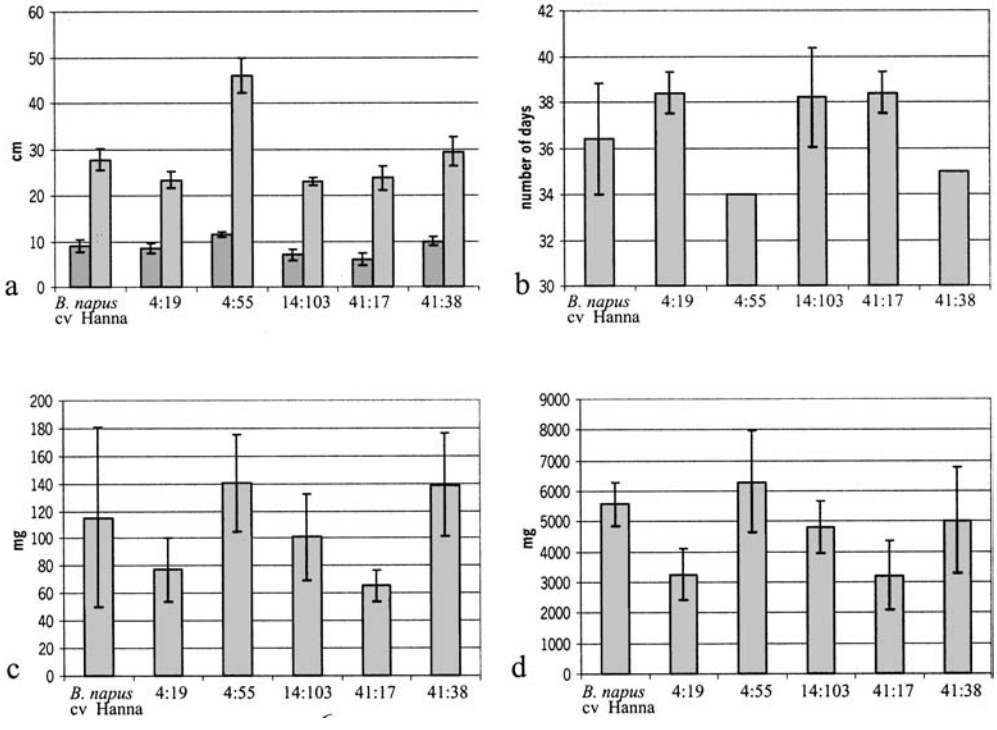

Fig. 2a–d Graphics of plant growth. **a** Shoot height after 2 and 5 weeks. **b** Number of days to flowering. Lines 4:55 and 41:38 have a standard deviation of 0. **c** Shoot dry weight after 2 weeks. **d** Shoot dry weight after 5 weeks

Table 2 Means of flower morphology and fertility characteristics of *B. napus* cv Hanna and CMS lines. Means with different letters within rows are significantly different at the $P < 0.05$ probability level

Character	Line					
	cv Hanna	4:19	14:103	41:17	4:55	41:38
Carpels (cm)	0.95 a	0.91 a	0.8 b	0.73 b	0.92 a	0.92 a
Stamens (cm)	0.97 a	0.59 b	0.56 b	0.5 b	0.5 b	0.5 b
Petals length (cm)	1.45 a	0.69 d	0.37 f	0.5 e	0.88 b	0.79 c
Petals width (cm)	0.58 a	0.29 c	0.13 e	0.2 d	0.41 b	0.38 b
Sepals length (cm)	0.8 a	0.73 a, b	0.69 b	0.7 b	0.75 a, b	0.68 b
Sepals width (cm)	0.2 a, b	0.23 a	0.19 b	0.2 a, b	0.2 a, b	0.2 a, b
Ovules/ovary	25.8 a	24.3 a	25.6 a	25.5 a	25.1 a	22.4 a
Seeds/pollinated flower	12.8 a	14.2 a	12.3 a	11.7 a	16.8 a	10.7 a
Weight of 100 seeds (g)	0.56 a	0.65 a	0.66 a	0.65 a	0.60 a	0.62 a

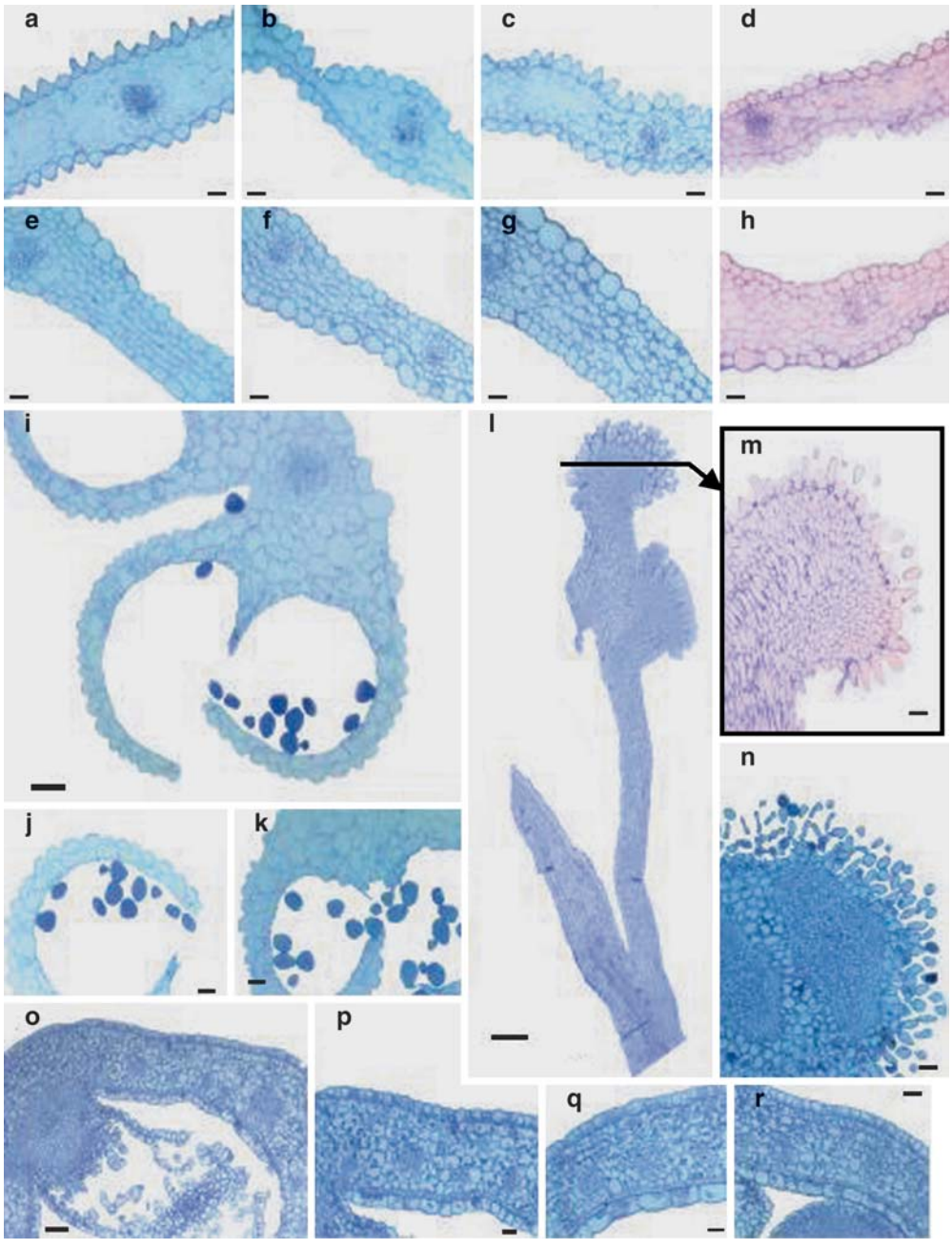

Fig. 3a–r Histological analysis of floral organs. **a–d** Transverse sections of the middle part of the petals from **a** *B. napus* cv Hanna, **b** 41:17, **c** 4:19, **d** 41:38. *Bars* = 2 µm. **e–h** Transverse sections of the middle part of the sepals from **e** *B. napus* cv Hanna, **f** 41:17, **g** 4:19, **h** 41:38. *Bars* = 2 µm. **i–k** Transverse sections of anthers from **i–j** *B. napus* cv Hanna and **k** 4:55. *Bars*: **i** = 5 µm, **j and k** = 2 µm. **l** Longitudinal section of an anther with homeotic conversions, line 4:19. *Bar* =10 µm. **m** Detail of the stigmatic-like cells formed at the tip of the anther. *Bar* = 2 µm. **n** Transverse section of the stigma from *B. napus* cv Hanna line. *Bar* = 2 µm. **o–r** Transverse sections from the middle part of the carpels of **o–p** *B. napus* cv Hanna, **q** 4:55 and **r** 4:19. *Bars* o = 5 µm, **p, q and r** = 2 µm

cences, flowered later and had a reduced shoot dry weight in comparison to *B. napus* cv Hanna (Fig. 2a–d). Leaves were small in the first 2 weeks, but by 5 weeks the plants contained large and broad leaves.

Group 2 is comprised of Lines 4:55 and 41:38. The stamens resembled to a large extent those from *B. napus* cv Hanna. However, these lines had significantly shorter stamens (half the size of *B. napus* cv Hanna) and shrunken anthers (Table 2 and Fig. 1j–m). Despite the fact that the anthers contain some viable pollen they don't extend to the stigma allowing self-pollination. Some flowers contained stamens that grew to a very reduced size (2–3 mm) and degenerated rapidly. The petals were significantly smaller than in *B. napus* cv Hanna, even though they were longer and broader than in the Group 1 plants. Group 2 lines grew as fast or faster than *B. napus* cv Hanna and the plants flowered more rapidly and very synchronized (Fig. 2b). Inflorescences were taller than in *B. napus* cv Hanna. In comparison to Group 1, the number of leaves was higher during the first 2 weeks of development; the leaves were also larger and wider. By 5 weeks, Group 2 plants contained smaller, but a higher number of leaves than Group 1 plants.

In all lines, sepals and carpels were largely unaffected in comparison to *B. napus* cv Hanna, except for lines 41:17 and 14:103 where these organs were somewhat smaller (Table 2). Neither did the CMS lines display any reduction in nectar production. Female fertility, evaluat-

ed by ovule number and seed set after cross-pollination, also remained unaffected (Table 2).

Histological studies

Histological studies were performed on sepals, petals, stamens and carpels. No critical differences were found for the sepals. Cell size, shape and arrangement of the epidermis and parenchyma were similar among all lines examined (Fig. 3e–h). Both abaxial and adaxial epidermal cells show the same distribution. In the petals the parenchyma was severely reduced in line 41:17 (Group 1). In regions of the petals between veins, the two epidermal layers were contiguous (Fig. 3b). However, in all other lines cell organisation between the epidermal cell layers was similar to the fertile *B. napus* cv Hanna. The epidermal cells are rounder when compared with *B. napus* cv Hanna. In sections of the CMS lines that displayed homeotic conversion of stamens into carpel-like structures, we found a stigma-like structure at the tip of the stamen. When we compared this structure to a carpel stigma, the cells of both organs displayed the same shape and pattern (Fig. 3e–h). Cross-sections from line 4:55 (Group 2) were made of the anthers at full maturity when the tapetum layer already had degenerated. No differences were found in the connective, vascular or epidermal tissues in comparison to *B. napus* cv Hanna (Fig. 3i–k). As expected, no differences were observed in cell shape or arrangement in carpels of all lines (Fig. 3o–r).

Discussion

The novel *B. napus* (+) *A. thaliana* alloplasmic lines, containing the nucleus of *B. napus* combined with rearranged mtDNA representing both species, display several interesting vegetative and reproductive aberrations. The reshuffling of the mitochondrial genomes obtained in the CMS lines is probably associated with impaired nuclear-mitochondrial interactions and normal mitochondrial function that affect plant development and morphology.

The extensive presence of *A. thaliana* mtDNA in these novel alloplasmic lines might be explained by the selection of male-sterile phenotypes, which probably biased the selection of lines having *A. thaliana* mtDNA, as these lines are more likely to display CMS in a rapeseed nuclear background. The distribution of rearrangements is, however, clearly not random. Certain breakpoint regions or hotspots in the *Brassica* mitochondrial genomes are more prone to rearrangements. For example, the *atp1* and *atp9* regions have been associated with intergenomic rearrangements after protoplast fusion in *Brassica* (Temple et al. 1992; Landgren and Glimelius 1994; Liu et al. 1999). Moreover, in this material, novel fragments were found in these regions together with novel fragments in the *cob*, *cox2* and *nad5a* regions. CMS in *B. napus* has previously been found to correlate with expression of chimeric genes in regions of *nad5c* (nap) (L'Homme et al. 1997), *atp6* (tour) (Landgren et al. 1996) and (pol) (Singh and Brown 1991), and *orfB* (Ogu) (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993). In the present RFLP study, we cannot associate any particular region in the mitochondrial genome to the CMS phenotype. Since all regions investigated contained recombined or *A. thaliana* DNA in most lines, any of these regions might express novel transcripts leading to CMS.

In this study it was shown that none of the nuclear *A. thaliana* DNA markers were present in the alloplasmic lines and that all intact *A. thaliana* chromosomes had been lost during backcrosses. Although the primary hybrids contained most chromosomes from both species (Forsberg et al. 1998), recurrent backcrossing to *B. napus* appears to sort out the *A. thaliana* DNA rapidly. This finding is in accordance with Bohman et al. (1999) who also showed that translocations in this material are rare events. The phenotypic stability within lines and between generations in the present study supports the assumption of minimal presence of *A. thaliana* nuclear DNA. However, the existence of *A. thaliana* nuclear DNA fragments in the *B. napus* chromosomes cannot be excluded completely.

The flower morphology analysis clearly shows that the floral organs affected are the petals and the stamens, and only to a limited extent sepals or carpels. In agreement with the results reported by Kofer et al. (1991) and Conley and Hanson (1995), all CMS lines in the present study demonstrate developmental aberrations in the organs of the two middle floral whorls. In Group 1 plants we found homeotic conversions of the anthers to carpels containing ovules and stigmatic cells in the tip. The carpel-like structures did not fuse, as occurs in normal carpel development. These aberrations correspond well with the feminisation of anthers, including stigmatoid structures and ovules in place of anther sacs, also found in other alloplasmic lines of rapeseed as reported by Pellan-Delourme and Renard (1987), Polowick and Sawhney (1987), Liu Clarke et al. (1999) as well as by Farbos et al. (2001) in tobacco. According to our investigations vegetative growth in the alloplasmic CMS plants was also affected. Other investigations have also reported reductions in vegetative growth even though most studies, focusing on the CMS trait, have ignored this aspect. Cybrids from e.g. *Nicotiana* (Aviv and Galun 1986; De Paepe et al. 1990; Gutierres et al. 1997) and *Brassica juncea* (Malik et al. 1999), resembled the fertile type in most features, but the male-sterile lines were usually shorter and retarded in growth. These alterations were stably transmitted through sexual generations.

Mitochondrial dysfunctions, associated with certain *orfs* and chimeric genes, have been linked to inhibited pollen production and, thus, male sterility (Schnable and Wise 1998). However, nuclear-mitochondrial communications are also involved in vegetative development as well as development of floral organs not directly correlated to pollen production. Recently, Farbos et

al. (2001) showed that the floral aberrations in the alloplasmic CMS line of *Nicotiana tabaccum*, containing the mitochondria of *Nicotiana repanda*, might be the result of floral meristem disorganization at a stage prior even to organ primordia formation. A linkage between mitochondrial function and expression of meristem and/or organ-identity genes has not yet been revealed; nor has a linkage been clarified to *orfs* and chimeric genes described in the CMS lines. In order to understand and differentiate whether there is one or several mechanisms involved in the nuclear-mitochondrial interactions affecting development, as proposed by Pelletier et al. (1987), it is necessary to establish and characterize a number of different alloplasmic lines showing a range of phenotypes. The present alloplasmic lines with the genetic model species *A. thaliana* as the cytoplasmic donor indeed display a range of different morphological aberrations and correspondingly heterogeneous mitochondrial genomes. By correlating an aberrant plant phenotype with a unique nuclear-mitochondrial composition, the genes regulating male sterility as well as flower and vegetative development may be discovered through analyses of RNA/DNA expression profiling.

Acknowledgements The authors thank L. Carlsson and I. Eriksson for excellent technical assistance. We also thank P. Giegé for providing primer sequences for PCR amplification of mitochondrial probes. This work was supported by the Swedish Research Council (VR), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Carl Tryggers Foundation and Svalöv Weibull AB. Rita Teixeira was supported by a fellowship from Fundação para a Ciência e Tecnologia Ministério da Ciência e Tecnologia, Portugal.

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