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ORIGINAL PAPER

Mitochondrial genotypes with variable parts of *Arabidopsis thaliana* DNA affect development in *Brassica napus* lines

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Abstract Phenotypic, genetic and molecular studies were made of *Brassica napus* lines with mitochondrial genomes consisting of DNA from both *B. napus* and *Arabidopsis thaliana*. The lines were isogenic regarding the nuclear and plastid genomes. Out of 21 lines, 10 were male-sterile, 3 semi-sterile and 8 male-fertile. Screening of the mitochondrial genomes with a dense set of *A. thaliana* specific markers showed that most lines contained large but variable portions of *A. thaliana* mitochondrial DNA. Several of the *A. thaliana* sequences in the mitochondrial genomes lead to the accumulation of novel transcripts. In addition, the restorer line showed different ability to restore male-fertility in the male-sterile lines. These results indicate that CMS is caused by several mitochondrial loci or combinations of loci. Beside petal and stamen morphology, growth rate and adenylate content varied among the lines. Furthermore, we found that the mitochondrial background had a distinct influence on nuclear gene expression. A clear example is the reduced expression of the two B-genes *APETALA3* and *PISTILATA* in the male-sterile lines. From the studies made comparing the mitochondrial loci and the observed phenotypic alterations, our interpretation is that different loci in the mitochondrial genome influence nuclear gene expression via several retrograde signalling pathways.

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Jenny Carlsson and Matti Leino have contributed equally to this study.

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Introduction

Cytoplasmic male-sterility (CMS) is a maternally inherited trait featured as a failure of plants to produce functional anthers and/or pollen. When novel nuclear–cytoplasmic combinations are produced, e.g., after interspecific crosses, cytoplasmic male-sterile plants are frequently obtained (reviewed by Kaul 1988). This type of CMS, termed alloplasmic male-sterility, suggests that CMS-inducing genes can be present in mitochondrial (mt) genomes, which normally are suppressed by nuclear genes in the original species (reviewed by Kaul 1988; Pelletier and Budar 2007). Genetic variation of mt-genomes and alloplasmy can also result in genetic and phenotypic effects not correlated with CMS. Examples of such traits are the non-chromosomal stripe mutants in maize (Newton and Coe 1986), variegated leaves in tobacco (Bonnett et al. 1993) and variation in starch production in potato (Lössl et al. 1994).

In several CMS-systems the trait has been associated with transcription and translation of certain *orf*s (open reading frames) in the mt-genome. Although no sequence similarity between the CMS-associated *orf*s has been found, the *orf*s are characterized by chimeric structures involving fragments of mt-genes and/or co-transcription with mt-genes (reviewed by Hanson and Bentolila 2004; Chase 2007; Pelletier and Budar 2007). In analyses of alloplasmic or cybrid plants expression of such *orf*s is often found

(Håkansson and Glimelius 1991; de la Canal et al. 2001; Leino et al. 2005).

In addition to male-sterility, homeotic changes of stamens can be observed in alloplasmic CMS-lines. For example, the *Brassica napus* CMS-lines analysed in this study have stamens replaced by carpelloid organs with ovule-like structures found at the internal margins of the unfused carpelloid structures (Leino et al. 2003; Teixeira et al. 2005a). Similar homeotic modifications have also been found in other CMS-systems such as *Nicotiana tabacum* (Farbos et al. 2001; Kofer et al. 1991; Zubko et al. 1996), *Daucus carota* (Linke et al. 1999, 2003), and *Triticum aestivum* (Murai et al. 2002; Ogihara et al. 1997). These CMS-phenotypes resemble the homeotic conversion of stamens to carpels found in *A. thaliana apetala3* (*ap3*) and *pistillata* (*pi*) mutants (Bowman et al. 1989, 1991). Several recent reports suggest that the CMS-inducing gene or genes indeed cause alterations in the expression of transcription factors regulating floral development (Zubko et al. 2001; Linke et al. 2003; Murai et al. 2002; Hama et al. 2004; Geddy et al. 2005; Teixeira et al. 2005a; Carlsson et al. 2007).

The phenomenon of mitochondrial regulation of nuclear gene expression is referred to as retrograde signalling (Liao and Butow 1993), and CMS is one of the responses. Mutations and alterations in the mitochondrial genome, inhibition of different complexes in the electron transport chain or the TCA cycle and abiotic or biotic stress induce altered expression of nuclear genes (reviewed by Rhoads and Subbaiah 2007). Besides the flower specific modifications, other aberrations have been associated with CMS; e.g., reduced vegetative development (Malik et al. 1999; Leino et al. 2003) and impaired ATP production (Bergman et al. 2000; Sabar et al. 2003; Teixeira et al. 2005b). However, conclusive results have not been found to distinguish if the observed reduction in vegetative development and impaired ATP-production caused by a novel mitochondrial background are linked to expression of the male-sterile phenotype or not.

In the present study a unique plant material consisting of 21 lines generated via protoplast fusions between *B. napus* cv. Hanna and *A. thaliana* Landsberg *erecta* have been examined. The lines are isogenic regarding the nuclear and plastid genomes (both genomes originating from *B. napus*), while the mitochondrial genome is distinct to each line. Most lines contain mitochondria with variable amounts of DNA from *B. napus* and *A. thaliana*. This study provides detailed analysis of the mitochondrial genome and we establish which parts of the *A. thaliana* mtDNA are present in the lines. In addition, we show that the lines differ in respect to male-sterility, growth rate, adenylate content and flower and vegetative development.

Materials and methods

Plant material and phenotypic characterisation

The plant material consists of a progeny derived from somatic hybrids between *B. napus* cv. Hanna and *A. thaliana* var. Landsberg *erecta* (Forsberg et al. 1998). About 21 lines obtained from individual calli were backcrossed to *B. napus* cv. Hanna for at least 4 generations; however, several lines have been backcrossed for up to 12 generations (Table 1; Fig. 1). A number of these lines are described in Leino et al. (2003) and Teixeira et al. (2005a, b). A fertility restored line was produced that is isogenic with the CMS line 4:19 with respect to the mitochondrial genome (Leino et al. 2004). The parental and fertile *B. napus* cv. Hanna was included as a reference. All plants were grown under controlled conditions in a culture chamber with 22°C/18°C day/night temperatures and a photoperiod of 16 h. Flower organs were measured on the five most recently opened flowers, which represent approximately flower developmental stage 14/B4 (Müller 1961; Smyth et al. 1990). The flowers were picked from the primary shoot of six plants within each line. Flowering time was scored as the first open flower (stages 13–14) in days after sowing. Fresh and dry weight of shoots was determined 52 days after sowing by drying 6 shoots of each line for 24 h at 105°C.

Isolation of buds and qRT-PCR

Buds were isolated from *B. napus* and the somatic hybrid lines and sorted according to their developmental stage. The isolated stages were 0–5 and 8 in accordance to previous defined stages in *A. thaliana* (Smyth et al. 1990). Buds were picked from four to six inflorescences on each plant and from three different plants within each line. They were picked and sorted starting at approximately the same time of the day at each occasion.

Total RNA was isolated and cDNA was synthesized as described earlier (Carlsson et al. 2007). Quantitative real time reverse transcriptase polymerase chain reactions (qRT-PCR) of *LEAFY* (*LFY*), *UNUSUAL FLORAL ORGAN* (*UFO*), *APETALA1* (*AP1*), *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) were carried out as described earlier (Carlsson et al. 2007), and the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes Oy, Espoo, Finland) was used. The primers used are described in Table S1 in Supplemental Data. Relative quantification values and standard deviations were calculated using the $\Delta\Delta$-C_T method according to the manufacturer's instructions (User Bulletin #2, ABI Prism 7700 Sequence Detection system, updated 10/2001). Values were normalized to the expression of the reference gene actin as well as the calibrator

Table 1 Measurements of floral organs

Line[a]	Generation	Phenotype	Sepals			Petals			Stamens, long		Stamens, short		Pistil	
			Number	Length (mm)	Width (mm)	Number	Length (mm)	Width (mm)	Number	Length (mm)	Number	Length (mm)	Number	Length (mm)
cv. Hanna		f	4.0 ab[b]	8.3 abc[b]	2.7 abcdefg[b]	4.0 a[b]	14.2 ab[b]	5.9 cd[b]	4.0 ab[b]	10.9 a[b]	2.0 a[b]	9.4 a[b]	1 a[b]	9.2 ab[b]
4:19	BC12	s	4.0 ab	6.9 g	2.4 cdefg	4.0 a	6.5 f	2.9 ghi	4.0 ab	5.9 bc	2.0 a	5.3 de	1 a	8.7 ab
4:55	BC12	ses	4.0 ab	7.0 gf	2.6 abcdefg	3.9 a	9.3 c	5.2 de	4.0 ab	5.4 bc	2.0 a	4.4 e	1 a	8.5 ab
4:64	BC4	f	4.0 ab	8.6 ab	3.2 a	4.0 a	13.8 ab	7.2 abc	4.0 ab	10.4 a	2.0 a	8.1 ab	1 a	9.8 ab
8:2	BC10	ses	4.0 ab	6.7 g	2.2 fg	4.0 a	4.1 h	1.7 ij	3.8 bc	5.7 bc	1.8 a	5.6 de	1 a	8.5 ab
8:31	BC4	f	4.0 ab	7.5 bcdefg	2.8 abcdefg	4.0 a	13.8 ab	6.8 abc	4.0 ab	10.3 a	2.0 a	8.5 a	1 a	9.5 ab
9:20	BC4	f	4.0 ab	8.2 abc	3.1 abc	4.0 a	15.1 a	6.8 abc	4.0 ab	10.8 a	2.0 a	9.0 a	1 a	9.6 ab
14:4	BC10	s	4.0 ab	7.2 cdefg	2.4 cdefg	3.8 ab	4.8 fgh	2.3 ghij	3.5 bc	4.7 cd	2.0 a	4.5 e	1 a	8.2 b
14:13	BC4	f	4.0 ab	8.1 abcde	2.9 abcdef	4.0 a	13.9 ab	7.1 abc	4.0 ab	10.0 a	2.0 a	7.8 abc	1 a	9.5 ab
14:15	BC4	ses	4.0 ab	6.9 fg	2.6 abcdefg	4.0 a	8.7 cde	4.3 ef	4.0 ab	5.2 cd	2.0 a	4.6 de	1 a	9.5 ab
14:92	BC4	f	4.0 ab	8.2 abcd	2.9 abcdefg	4.0 a	12.9 b	6.4 bcd	4.0 ab	10.1 a	2.0 a	7.7 abc	1 a	9.4 ab
14:103	BC12	s	4.0 ab	7.3 cdefg	2.2 g	4.0 a	3.0 h	1.1 j	3.0 c	5.9 bc	nd	nd	1 a	8.4 ab
14:109	BC4	s	4.0 ab	7.2 cdefg	2.3 efg	3.9 a	4.0 h	1.7 ij	3.8 bc	5.8 bc	1.8 a	4.8 de	1 a	9.2 ab
15:62	BC4	f	4.0 ab	8.9 a	3.2 ab	4.0 a	15.1 a	8.0 a	4.0 ab	11.0 a	2.0 a	8.9 a	1 a	10.4 a
41:16	BC4	s	4.0 ab	7.1 defg	2.3 defg	4.0 a	4.3 gh	2.1 hij	3.6 bc	5.1 cd	2.0 a	5.7 de	1 a	8.3 ab
41:17	BC12	s	4.0 ab	7.0 efg	2.4 bcdefg	4.0 a	6.6 def	2.9 ghi	4.0 ab	6.3 bc	2.0 a	6.4 bcd	1 a	8.6 ab
41:38	BC12	ses	4.0 ab	6.5 g	2.2 fg	3.4 b	4.7 fgh	2.4 ghij	4.0 ab	3.5 d	2.0 a	2.3 f	1 a	8.2 b
41:94	BC4	f	4.0 b	8.3 abc	3.0 abcde	4.0 a	14.9 ab	7.5 ab	4.0 ab	10.6 a	2.0 a	8.7 a	1 a	10.3 ab
43:60	BC4	s	4.0 b	6.9 fg	2.4 defg	4.0 a	6.2 gf	2.8 ghi	4.7 a	5.3 bc	1.7 a	4.4 e	1 a	9.0 ab
48:37	BC4	s	4.0 ab	7.3 cdefg	2.5 abcdefg	4.0 a	6.7 def	2.9 ghi	4.2 ab	6.2 bc	nd	nd	1 a	9.6 ab
48:48	BC4	f	4.0 ab	8.1 abcdef	3.1 abcd	4.0 a	14.3 ab	7.5 ab	4.0 ab	10.1 a	2.0 a	8.4 a	1 a	9.5 ab
48:60	BC10	s	4.0 ab	7.4 cdefg	2.5 abcdefg	4.0 a	6.5 ef	3.4 fgh	4.0 ab	6.0 bc	2.0 a	5.2 de	1 a	8.9 ab
Restored		ses	4.1 a	6.9 fg	2.3 efg	3.7 ab	8.7 cd	3.5 fg	3.8 b	6.8 b	1.9 a	6.0 cde	1 a	9.8 ab
All f (n = 8)			4.0 x	8.2 x	3.0 x	4.0 x	14.2 x	7.1 x	4.0 x	10.4 x	2.0 x	8.4 x	1 x	9.8 x
All ses (n = 3)			4.0 x	6.8 y	2.4 y	3.8 y	7.9 y	4.1 y	4.0 x	4.8 y	2.0 x	4.1 y	1 x	8.9 y
All s (n = 10)			4.0 x	7.1 y	2.4 y	4.0 x	5.3 z	2.4 z	3.9 x	5.7 z	1.9 y	5.3 z	1 x	8.7 y

f fertile, *s* male-sterile, *ses* semi-sterile

[a] The values of the fertile *Brassica napus* cv. Hanna, the 21 alloplasmic lines and the restored line are presented, as well as the mean values of the 8 fertile alloplasmic lines, the 3 semi-sterile lines and the 10 male-sterile lines

[b] Lines with different letters are significantly different for the specific characteristics measured. Lines with the same combination of letters group together

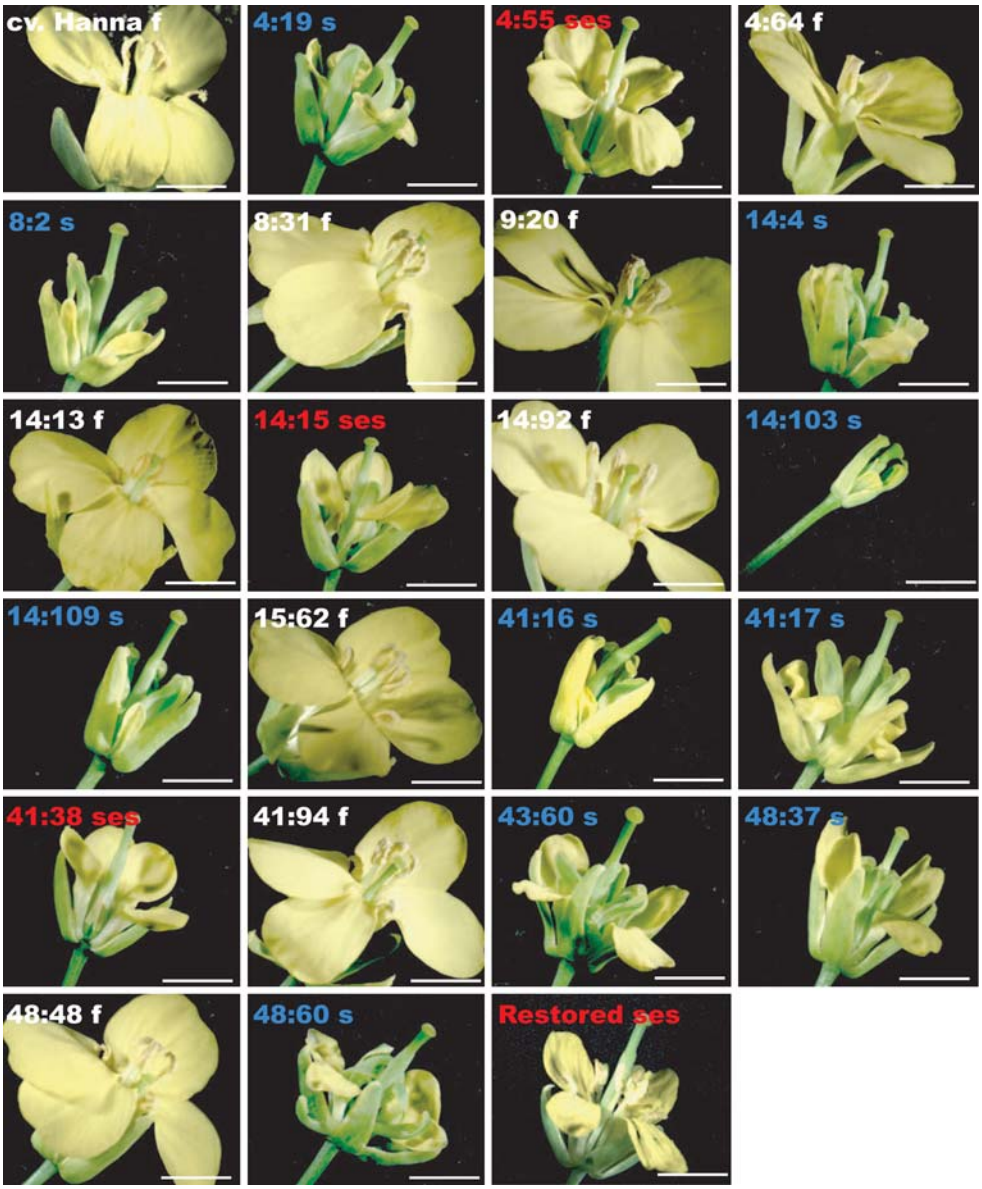

Fig. 1 Phenotypes of mature flowers of the lines described in Table 1. *s* male-sterile line; *ses* semi-sterile line; *f* fertile line

line *B. napus*. Differences in expression were identified using the Student's *t*-test.

DNA analysis

Total DNA was isolated (Sharpe et al. 1995) from leaf tissue and subjected to PCR analysis with *A. thaliana* mt-genome specific primers. The primers were chosen to exclusively amplify *A. thaliana* mtDNA, however, occasionally result in a simple sequence length polymorphism (SSLP) if amplified from *B. napus* mtDNA. Primer sequences and positions are indicated in Table S1 in Supplemental Data. In each PCR reaction, as a positive internal control, the nuclear *B. napus* specific primers *co16* (5′ TAG CCT TTC TCT CCA TTG 3′) and *co39* (5′ CTG CCG AGC TGA TTC TGC 3′) resulting in a 750 bp fragment was included. The reaction mixture (20 µl) contained 0.2 µM of each primer (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 U Taq-polymerase (Fermentas, Burlington, ON, Canada), 1× of the supplemented buffer containing $(NH_4)_2SO_4$ and 20 ng DNA. Amplifications were run at 2 min, initial denaturation at 94°C, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR products were electrophorised on 1.2% agarose gels and visualised with EtBr.

Northern analysis

Mitochondrial RNA was isolated from fresh flower buds as described by Leino et al. (2005). Probes were PCR amplified

from *A. thaliana* DNA using the same primers applied in the DNA analysis (Table S1) or in Leino et al. (2005). Northern analysis was performed as described by Leino et al. (2005).

ATP measurements

Fresh flower buds at stages 0–12, were analysed for ATP and ADP content. One fraction (0.2–0.3 g) was used for dry matter determination and with the remaining buds snap frozen in liquid N_2. The buds were ground to a powder and 0.05–0.1 g was extracted in 600 ml 10% perchloric acid and 0.04–0.06 g insoluble polyvinylpolypyrrolidone. After thawing, extracts were neutralized with buffer (5 M KOH, 1 M triethanolamine) and the ATP and ADP content was determined luminometrically employing the firefly luciferase method essentially according to Gardeström and Wigge (1988), using the ATP Kit SL (BioThema, Haninge, Sweden) and the supplied buffer. From each line three extractions were made from individual plants. Measurements were made twice for each extraction.

Statistical methods

The GLM (general linear model) procedure in SAS® 9.1 (SAS Institute Inc, NC, USA) were used for statistical tests ($\alpha = 0.05$). For pair-wise comparisons Bonferroni corrections were used ($\alpha = 0.05$). In pair-wise comparisons lines, or classes, with no significant differences are given the same letter. Lines, or classes, with the same set of letters belong to the same sub-group. The statistical analyses were calculated on fertile (eight lines), male-sterile (ten lines) and semi-sterile (three lines) lines.

The statistical comparisons for the floral organs were made between lines as well as between classes. Within each line six plants were evaluated (with a mean value from five flowers per plant), except lines 4:19, 8:31 and 14:4 (five plants) and 14:103, 41:38 (four plants).

For the dry-weight of the shoots, comparisons were made between lines as well as between classes. Within each line six plants were evaluated, except for lines 4:55, 8:2, 9:20 and 14:92 (five plants). Line 14:103 was excluded.

The statistical comparisons for the adenylates were made between lines as well as between classes. In this case we used three biological replicates and two technical replicates per line. Within each line three plants were included, except lines 41:16 and 48:37 (two plants), and two technical repetitions giving six (or four) measurements.

The statistical comparisons for the qRT-PCR were made between classes and calculated for fertile (eight lines), male-sterile (ten lines) and semi-sterile (three lines) lines.

Results

Twenty-one lines derived from somatic hybrids between *B. napus* and *A. thaliana* were used in the analyses (Fig. 1). Eight lines were categorized as male-fertile with normal fertile stamens, ten lines as male-sterile with sterile homeotically converted stamens and three lines as semi-sterile with relatively short but male-fertile stamens. All lines were fully female-fertile. Additionally, a restored line containing *A. thaliana* chromosome III (At3) was included. This line is isogenic to the male-sterile line 4:19 with respect of the mitochondrial genome. In the restored line the anthers developed into normal anther bags with pollen albeit with shorter filaments compared to the fertile *B. napus* cultivar. The parental and fertile *B. napus* cultivar was included as a reference. Pair-wise comparisons were made between all lines and between the classes of male-sterile, semi-sterile and male-fertile plants. In pair-wise comparisons differences were considered significant if *P* values were less than, or much less than 0.0500, as described in "Materials and methods". Lines, or classes, with no significant differences were given the same letter and belongs to the same group. Lines, or classes, with the same set of letters belong to the same sub-group.

The lines could be clustered into different groups according to distinguishable flower morphologies

Since several lines displayed homeotically affected flowers it was of interest to study the flower morphology more closely. The number of organs was calculated and the organ-size was measured. The aim was to correlate the morphology of the different floral organs with male-sterility. All lines show differences in organ sizes, giving a wide range of phenotypes clustering together (Table 1; Fig. 1).

Based on the pair-wise comparisons between lines regarding the number of sepals, three groups were found (Table 1). Two of the three groups contained fertile, male- and semi-sterile lines. The restored line was the only representative in the third group. In this line an increased number of sepals were commonly found. The restored line differed significantly from all but two lines. No significant differences in the number of sepals between the three classes of fertile, male- and semi-sterile plants were found, which is in agreement with the pair-wise comparisons made between lines. The sepal was significantly longer and wider in the class of fertile lines in comparison to the male- and semi-sterile lines. Also, in pair-wise comparisons across lines the sepal length of the fertile lines had a tendency, according to the sub-groups, to differ from male- and semi-sterile lines. This pattern was not as pronounced, if present at all, for the sepal width. No morphological or colour differences regarding the sepals were observed.

The class of semi-sterile lines had significantly fewer petals than the fertile and male-sterile lines. The lines 4:55, 14:4, 14:109 and the restored line had reduced petal number although not significantly different from the other lines, while the semi-sterile line 41:38 had significantly fewer petals. The petal size was significantly longer and wider in the class of fertile lines in comparison to the semi- and male-sterile lines. The semi-sterile lines as well as the restored line had petals of intermediate size. Among the fertile lines, lines 9:20 and 15:62 had longer petals than the fertile line 14:92. Among the male-sterile lines large differences in petal size were found, where the lines 8:2, 14:103 and 14:109 had exceptionally small petals. The petals of the male-sterile lines had the same colour as the fertile and semi-sterile lines.

Interestingly, both the long and short stamens were significantly longer in the class of male-sterile lines in comparison to the semi-sterile lines. Furthermore, the stamens of the male-sterile lines were homeotically converted into female structures, in general with stigmatoid tissues and ovules. The stamens of the semi-sterile and fertile lines hade a similar morphology, i.e. normal stamen morphology, however, the semi-sterile stamens were relatively short in comparison to the pistil when compared to the fertile lines. The number of long stamens was similar in the three classes. The long stamens were also significantly longer in the fertile lines in comparison to male- and semi-sterile lines. The class of male-sterile lines had significantly fewer short stamens than the fertile lines, and the short stamens were significantly longer in the fertile lines in comparison to male- and semi-sterile lines.

All lines had one pistil. However, the pistil was significantly longer in the class of fertile lines in comparison to the male- and semi-sterile lines. This difference between classes could probably be explained by the fertile line 15:62 which had a very long pistil and the two lines 14:4 (male-sterile) and 41:38 (semi-sterile) that had very short pistils. These three lines form two distinct groups, while all other lines are found in a third group, according to pair-wise comparisons between lines. No obvious morphological differences were detected.

The results of the floral organ measurements show that the reduced size of stamens and petals have a high correlation with male-sterility. The number and size of these organs also varies among the male- and semi-sterile lines, resulting in a wide range of distinguishable phenotypes within these two classes. The size of the sepals and the pistil had a much lower correlation to male-sterility.

Growth rate and flowering time varied largely within and between lines with a low correlation to male-sterility

To analyse if growth rate was affected in the alloplasmic lines and correlated to fertility, the dry-weight of 52-days-old plants was determined for each line (Table 2). The semi-sterile lines as a class had a significantly higher dry-weight than the fertile and male-sterile lines. Pair-wise comparisons of all lines showed that the semi-sterile line 4:55 had a significantly higher dry-weight than the fertile lines 14:92, 15:62 and the male-sterile line 4:19. In summary, although differences between lines were observed, giving three sub-groups (*a*, *ab* and *b*), growth rate showed a low correlation with male-sterility.

Flowering time was analysed in order to study whether the mitochondrial background had an influence on the onset of flowering. No significant differences in flowering time were observed between the three classes (Table 2). The three early flowering fertile lines 9:20, 14:13 and 15:62 flowered significantly earlier than the late flowering lines 48:48 (fertile), 41:16 (male-sterile) and the restored line. Seven sub-groups with no direct correlation to male-sterility were found. In addition, the flowering time varies highly within each class indicating that flowering time has low or no correlation with male-sterility.

No clear correlation to the levels of adenylates and ATP/ADP-ratios with male-sterility was found

Impairment of the ATP synthase complex in plant mitochondria has been one of the major hypotheses regarding a mechanism behind the CMS trait. Thus, it was of interest to analyse the levels of ATP and ADP in the alloplasmic lines.

The fertile line 48:48 had significantly more adenylates than most lines except for the fertile lines 4:64, 14:92, 15:62, 41:94, the male-sterile line 14:4, the restored line and the *B. napus* cultivar (Table 2). The male-sterile line 14:103 had the lowest amount of adenylates and the levels were significantly lower than in the fertile lines 15:62, 48:48 and the male-sterile line 14:4, i.e. the lines with the highest levels (Table 2). Five sub-groups were found between lines considering adenylates. According to pair-wise comparisons between lines no significant differences were found regarding the ATP/ADP-ratios. However, the fertile lines had a significantly higher amount of adenylates and a higher ATP/ADP-ratio than the male-sterile lines.

The fertile line 48:48 had the highest adenylate content (166.96 nmol/g) and the male-sterile line 14:103 the lowest (34.89 nmol/g). Since these lines had extreme values in comparison with the other lines we removed these lines and made a new comparison (data not shown). We found no significant differences between lines regarding total amount of adenylates or the ATP/ADP-ratio. No significant differences were found between classes regarding total amount of adenylates. However, the fertile lines had a significantly higher ATP/ADP-ratio than the male-sterile lines.

Table 2 Measurements of plant characteristics

Line[a]	Phenotype	Flowering time (days after sowing)	Shoot dry-weight (g)	Adenylates in buds (nmol/g dry-weight)	ATP/ADP-ratio
cv. Hanna	f	43.3 abcd[b]	5.0 ab[b]	106.2 abc[b]	3.5 a[b]
4:19	s	45.2 abcd	3.1 b	76.4 bc	2.6 a
4:55	ses	42.8 abcd	6.7 a	78.3 bc	3.3 a
4:64	f	43.7 abcd	4.9 ab	98.0 abc	3.6 a
8:2	s	44.5 abcd	4.2 ab	68.7 bc	2.6 a
8:31	f	41.6 bcd	5.0 ab	86.3 bc	4.0 a
9:20	f	38.5 cd	3.6 ab	79.1 bc	3.1 a
14:4	s	41.8 bcd	5.8 ab	124.5 ab	2.4 a
14:13	f	38.2 d	5.8 ab	74.5 bc	3.9 a
14:15	ses	40.8 bcd	5.6 ab	78.2 bc	3.6 a
14:92	f	46.0 abc	3.1 b	110.0 abc	3.3 a
14:103	s	42.3 bcd	nd	34.9 c	2.6 a
14:109	s	42.2 bcd	4.4 ab	69.5 bc	3.2 a
15:62	f	38.5 cd	3.2 b	132.7 ab	3.9 a
41:16	s	50.0 a	3.8 ab	76.3 bc	2.4 a
41:17	s	45.5 abcd	3.8 ab	70.8 bc	3.1 a
41:38	ses	42.3 bcd	6.4 ab	78.1 bc	3.3 a
41:94	f	41.0 bcd	4.0 ab	98.8 abc	3.3 a
43:60	s	44.7 abcd	5.6 ab	88.9 bc	3.2 a
48:37	s	43.3 abcd	5.3 ab	66.7 bc	2.8 a
48:48	f	46.5 ab	3.5 ab	167.0 a	3.9 a
48:60	s	41.5 bcd	5.8 ab	88.2 bc	3.3 a
Restored	ses	48.2 ab	3.7 ab	95.7 abc	3.6 a
All f (n = 8)		41.7 x	4.1 x	105.8 x	3.7 x
All ses (n = 3)		41.9 x	6.4 y	78.2 y	3.4 xy
All s (n = 10)		44.2 x	4.6 x	76.5 y	2.8 y

f fertile, *s* male-sterile, *ses* semi-sterile

[a] The values of the fertile *Brassica napus* cv. Hanna, the 21 alloplasmic lines and the restored line are presented, as well as the mean values of the 8 fertile alloplasmic lines, the 3 semi-sterile lines and the 10 male-sterile lines

[b] Lines with the same letter are not significantly different regarding the mean-value of the specific characteristics measured. Lines with the same combination of letters group together

The male-sterile lines contained large but variable portions of *A. thaliana* mt-DNA

We have previously demonstrated by RFLP that some of the male-sterile lines contain vast amounts of *A. thaliana* DNA (Leino et al. 2003). In order to determine which of the *A. thaliana* mtDNA fragments were present in all lines, the population was screened with a dense set of *A. thaliana* specific mtDNA PCR-markers. These markers are located approximately every tenth kb throughout the 367 kb *A. thaliana* mt-genome (annotated I-XXXVI). Markers located in *orf*s were preferentially chosen, with three markers (V, XI and XXIX) positioned in repeated regions in the *A. thaliana* mt-genome. Examples of PCR analyses are shown in Fig. 2.

The presence or absence of each *A. thaliana* mtDNA marker is illustrated in Table 3. All male- and semi-sterile lines contained mtDNA from most of the *A. thaliana* genome. The semi-sterile lines did not differ from the male-sterile lines considering the amount of *A. thaliana* mtDNA. In addition two fertile lines (9:20 and 14:92) contained scattered fragments of *A. thaliana* mtDNA. The other six fertile lines did not contain any of *A. thaliana* specific mtDNA markers tested. The marker XIII could not be found in any of the analysed lines, and the surrounding markers XII (*orf122a*) and XIV (*orf109a*) were absent in most lines. The markers VI, XXI and XXXV were found in all male- and semi-sterile lines, but not in any fertile line. Thus, these three positions in the *A. thaliana* mtDNA genome are putative sites for CMS-associated sequences.

A. thaliana sequences in the mt-genome lead to the accumulation of several novel transcripts

Northern analysis was performed to examine if the *A. thaliana* mt-DNA, corresponding to the putatively male sterility-associated markers, was expressed. Mitochondrial RNA was isolated from flower buds of all lines, and analysed in gel blots and hybridised to mt-probes (Fig. 3). Hybridization with the probes directly correlating to VI, XXI and XXXV did not result in any detectable signal. In the proximity of marker XXXV, no open reading frames were found in the sequence (NC_001284), and therefore we did not study this region further. However, markers VI

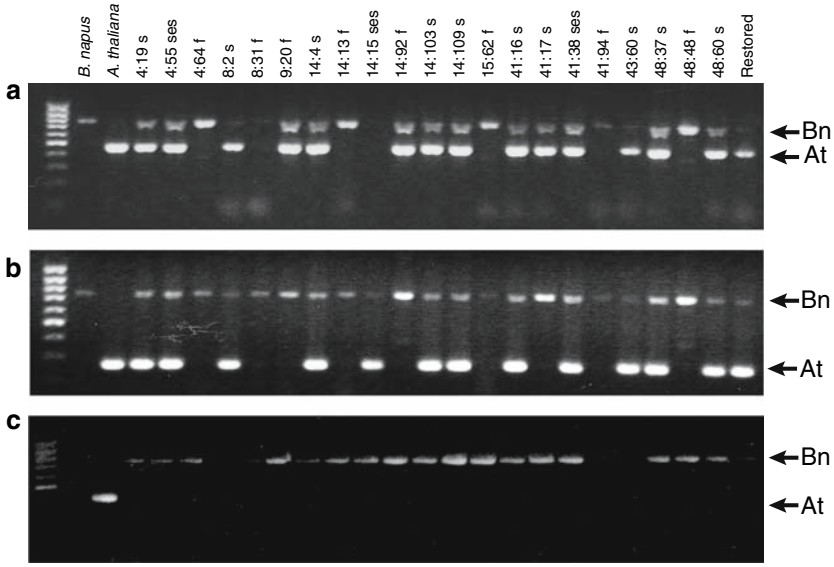

Fig. 2 Ethidium bromide stained gels of PCR products amplified with *A. thaliana* mtDNA specific primers (*At*). As a control, *B. napus* mtDNA specific primers resulting in a 750 bp fragment were included in each PCR (*Bn*). **a** marker XIX, **b** marker XXIX, **c** marker XIII

and XXI are closely positioned to *rpl5* and *cox3*, respectively. Thus, we analysed if these genes exhibited novel transcription patterns. Hybridization with an *rpl5* probe resulted in a transcript pattern where only transcripts similar in size to either or both of *A. thaliana* and *B. napus* were found and no novel transcripts were observed (data not shown). The expression pattern of *rpl5* was not specific for the male-sterile lines. Hybridization with a *cox3* probe (Fig. 3a) displayed a novel transcript of approximately 5 kb in size in all lines where marker XXI was found (all male- and semi-sterile lines) except in the semi-sterile line 41:38.

In addition, hybridizations were performed with gene probes where we previously have shown that presence of *A. thaliana* mtDNA in a *B. napus* nuclear background results in novel transcripts (Leino et al. 2005). All male- and semi-sterile lines as well as the fertile lines 9:20 and 14:92 contain marker V (*orf139*). Transcripts of *orf139* (marker V) were found in all male- and semi-sterile lines, and in the fertile line 14:92 (Fig. 3b). All male- and semi-sterile lines as well as the fertile line 14:92 contain marker XX (*orf240a*). The *orf240a* (marker XX) transcripts were detected in all male- and semi-sterile lines and in the fertile line 14:92 (Fig. 3c). The *orf294* was present in all male- and semi-sterile lines except lines 4:55 and 41:17. Transcripts of *orf294* (marker XXX), were found in all male- and semi-sterile lines except for the male-sterile line 41:17 and the semi-sterile line 4:55 (Fig. 3d). The mitochondria in each line contain a specific set of *A. thaliana* mt-markers. In addition, each of these markers can be transcribed or not. In conclusion, presence of *A. thaliana* mtDNA from several regions, in the *B. napus* nuclear background, resulted all together in enhanced transcript accumulation.

The restorer line displays different ability to restore male-fertility

We have previously shown that a 4:19 addition line with *A. thaliana* chromosome III (At3) has the ability to partially restore fertility and flower morphology of the male-sterile line 4:19 (Leino et al. 2004). To test whether the restorer gene/genes present on At3 also could restore fertility in other CMS-lines, the restored line was used to pollinate all male- and semi-sterile lines described here. The offspring were screened concerning changes in flower fertility and morphology.

Offspring of line 4:19 displayed a partial restoration of flower fertility and morphology as expected, although less pollen was formed compared to the restored line. This indicates that a monosomic addition of At3 to the male-sterile line 4:19 is not satisfactory to generate a complete restoration. In addition, the offspring from the male-sterile lines 9:13, 14:4 and 48:60 were restored to some extent. Although some flowers produced homeotically converted anthers, other flowers on the same plant produced short anthers with fertile pollen. No differences in fertility and flower morphology could be noted in the offspring from the other lines crossed with the restored line. Consequently, as the restorer gene/genes present on At3 has an effect on flower phenotype in some CMS-lines but not in others, it seems likely that the male-sterile lines differ between each other and might have different sets of CMS-associated genes.

AP3 and *PI* had a low expression level in male-sterile lines

Since the male-sterile lines have a phenotype that resembles the *ap3* and *pi* mutant phenotypes in *A. thaliana* we

Table 3 Presence (+) or absence (−) of *A. thaliana* specific mitochondrial markers in the alloplasmic lines

Line																							
	4:19	4:55	4:64	8:2	8:31	9:20	14:4	14:13	14:15	14:92	14:103	14:109	15:62	41:16	41:17	41:38	41:94	43:60	48:37	48:48	48:60	R
Marker	s	ses	f	s	f	f	s	f	ses	f	s	s	f	s	s	ses	f	s	s	f	s	ses
I	+	+	−	+	−	−	+	−	+	+	+	−	−	+	+	+	−	+	+	−	+	+
II	+	−	−	+	−	+	+	−	+	+	+	−	−	+	+	+	−	+	+	−	+	+
III	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+
IV	+	+	−	+	−	−	+	−	−	+	+	+	−	+	+	+	−	+	+	−	+	+
V	+	+	−	+	−	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
VI	+	+	−	+	−	−	+	−	+	−	+	+	−	+	+	+	−	+	+	−	+	+
VII	+	+	−	+	−	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
VIII	+	−	−	+	−	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
IX	+	−	−	+	−	+	+	−	+	+	+	−	−	+	+	+	−	+	+	−	+	+
X	+	+	−	+	−	−	+	−	+	−	+	−	−	+	+	+	−	+	+	−	+	+
XI	+	+	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	+	+	−	+	+
XII	+	−	−	+	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+
XIII	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
XIV	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−
XV	−	−	−	+	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
XVI	+	+	−	+	−	−	−	−	+	−	−	+	−	+	+	+	−	+	−	−	+	+
XVII	+	+	−	+	−	+	+	−	+	−	−	+	−	+	+	+	−	+	−	−	+	+
XVIII	+	+	−	+	−	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
XIX	+	+	−	+	−	+	+	−	−	+	+	+	−	+	+	+	−	+	+	−	+	+
XX	+	+	−	+	−	−	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
XXI	+	+	−	+	−	−	+	−	+	−	+	+	−	+	+	+	−	+	+	−	+	+
XXII	+	+	−	+	−	−	+	−	+	−	+	+	−	+	+	−	−	+	+	−	+	+
XXIII	+	+	−	+	−	−	+	−	+	−	+	+	−	+	−	−	−	+	+	−	+	+
XXIV	+	+	−	+	−	−	+	−	+	−	+	+	−	+	−	−	−	+	+	−	+	+
XXV	+	+	−	+	−	−	+	−	−	−	+	−	−	+	−	−	−	−	+	−	−	+
XXVI	+	+	−	+	−	−	+	−	+	−	+	−	−	+	−	−	−	+	+	−	+	+
XXVII	+	+	−	+	−	−	−	−	+	−	+	+	−	+	−	−	−	−	+	−	−	+
XXVIII	+	−	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	+	+	−	+	+
XXIX	+	+	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	+	+	−	+	+
XXX	+	−	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	+	+	−	+	+
XXXI	+	−	−	+	−	−	+	−	+	−	+	+	−	+	+	+	−	+	+	−	+	+
XXXII	+	−	−	+	−	−	+	−	+	−	+	+	−	+	+	+	−	+	+	−	+	+
XXXIII	+	+	−	+	−	−	+	−	+	+	+	−	−	+	+	+	−	+	+	−	+	+
XXXIV	+	+	−	+	−	−	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+
XXXV	+	+	−	+	−	−	+	−	+	−	+	+	−	+	+	+	−	+	+	−	+	+
XXXVI	+	+	−	+	−	−	+	−	+	+	+	−	−	+	+	+	−	+	+	−	+	+

Markers (I-XXXVI) are ordered as positioned in the *A. thaliana* mitochondrial genome. Markers V, XI and XXIX are located in repeated regions. For details about the markers see Table S1.

R restored line; *s* male-sterile line; *ses* semi-sterile line; *f* fertile line

studied the expression profiles of *AP3* and *PI* in all lines. In addition *AP1*, *LFY* and *UFO* were chosen since they regulate the expression of *AP3* and *PI*. *AP2* was added to the study since it is important for several steps during flower development. In previous studies (Teixeira et al. 2005a; Carlsson et al. 2007) it was shown that *AP3*, *PI*, *AG*, *LFY* and *UFO* had an altered expression profile in the male-sterile lines 4:19 and 41:17. Our aim was to explore if the expression profile for these five genes as well as *AP1* and *AP2* were in common for all male-sterile lines, and how similar or different these profiles would be in the fertile and semi-sterile lines.

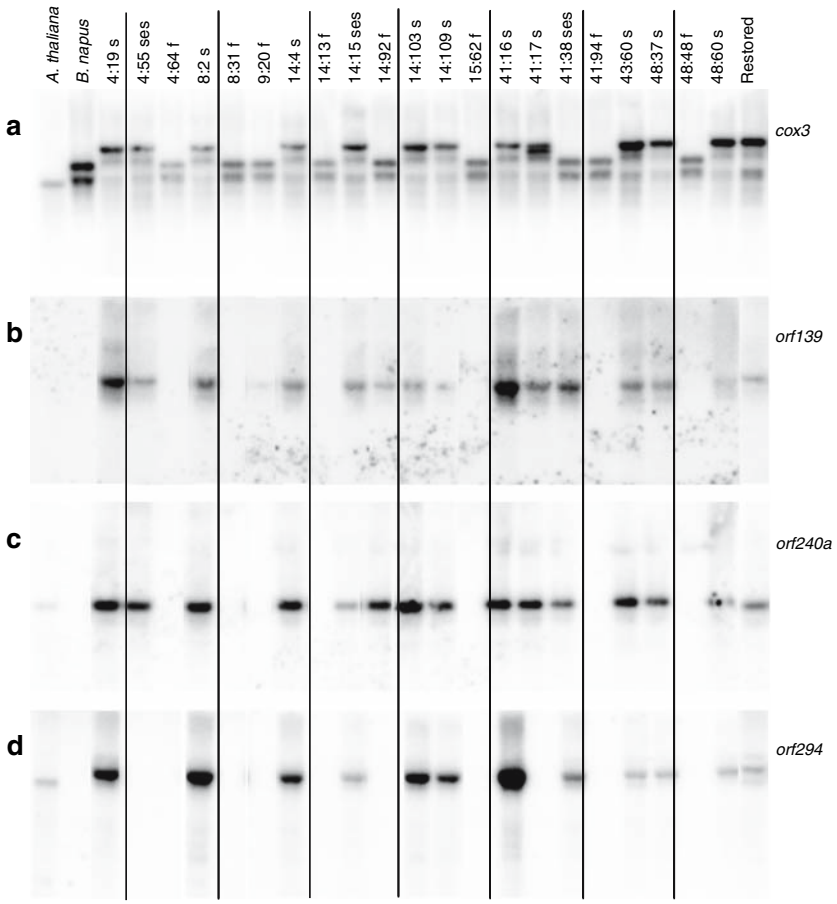

Fig. 3 Northern analysis of mitochondrial transcripts. Flower bud mtRNA was separated on a denaturing agarose gel, transferred to a nylon membrane and hybridised with [^{32}P]-labelled probes for **a** *cox3*, **b** *orf139*, **c** *orf240a*, **d** *orf294*

By qRT-PCR we quantified the expression of these seven flower development genes in all lines at two different stages of developing flower buds. The results were compared to the expression of the calibrator line *B. napus* cv Hanna and are shown in Fig. 4 and5. No significant differences were found between the three classes fertile, semi- and male-sterile for any examined gene during the early stages 0–5 (Fig. 4a). *AP1* expression was significantly lower in the class of fertile lines in relation to the male-sterile lines during stage 8, while no significant differences were observed for the semi-sterile lines (Fig. 4b). The *AP1* gene had a tendency to have a lower expression in all lines in comparison to *B. napus* at stages 0–5 and 8. The male- and semi-sterile lines were in general closer to the expression levels detected in *B. napus*, while the fertile lines had a significantly lower expression (Fig. 5a). The *AP2* gene had a significantly lower expression in the fertile lines in comparison to the semi- and male-sterile lines at stage 8 (Fig. 4b). The *AP2* gene had a slightly lower expression in male- and semi-sterile lines and some of the fertile lines at stages 0–5 compared to *B. napus*. During stage 8 it appeared as if the male- and semi-sterile lines in general had a significantly higher expression, while the fertile lines had a significantly lower expression (Fig. 5b). The expression profiles of the two B-genes *AP3* and *PI* were similar. The two genes had a significantly higher expression in the group of fertile lines compared to the male- and semi-sterile lines at stage 8 (Fig. 4b). For *AP3*, eight out of ten, and for *PI*, six out of ten male-sterile lines had a significantly lower expression in correlation to cv. Hanna during stages 0–5 (Fig. 5c, d). All male-sterile lines had a significantly lower expression of *AP3* and *PI* relatively cv. Hanna during stage 8. Thus, a low expression of *AP3* and *PI* is correlated with male-sterility. The *AG* gene did not display any significant differences between the three classes either at stages 0–5 or stage 8 (Fig. 4). The *AG* gene expression in general appeared to be lower in all lines at both stages in comparison to *B. napus*. Although significant differences in comparison to cv Hanna were found no obvious patterns was observed (Fig. 5e). *LFY* expression was significantly lower in the classes of fertile and semi-sterile lines in relation to the male-sterile lines during stage 8 (Fig. 4b). In general the *LFY* expression, at both stages and in all lines, was higher or equal to the expression of cv. Hanna. The *LFY* expression during stage 8 in the different male-sterile lines was in general significantly higher (Fig. 5f). No significant differences were observed in the *UFO* expression at stages 0–5 and stage 8 (Fig. 4a, b). With a few exceptions, the expression of *UFO* in all lines was close to cv. Hanna (Fig. 5g).

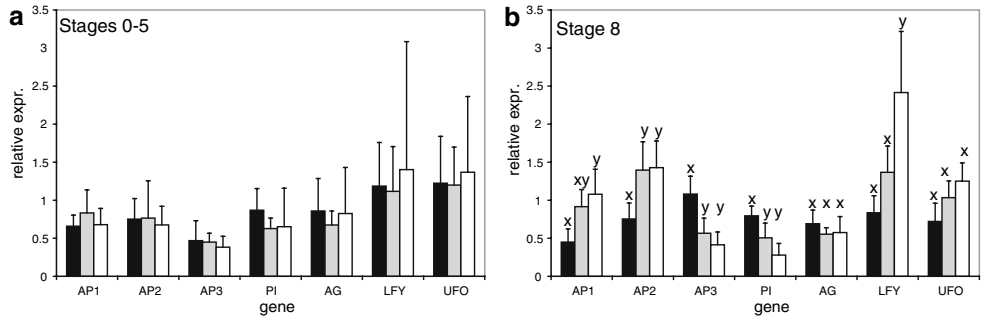

Fig. 4 Relative gene expression in relation to cv Hanna (relative expr.; mean values for each class) in stages 0–5 (**a**) and stage 8 (**b**). No significant differences were observed between classes at stages 0–5 (**a**). The significant differences found between classes at stage 8 (**b**) are indicated with *letters* (the *same letter* indicates no difference). *Black bars* fertile lines; *grey bars* semi-sterile lines; *white bars* male-sterile lines

Since the restored line and the sterile line 4:19 are isogenic regarding the mitochondrial background and the restored line has an addition of *A. thaliana* chromosome III (At3) in the nuclear genome it was of interest to compare the nuclear gene expression of the two lines. The expression of *AP1*, *AP2* and *AG* was more or less similar in the two lines, while the expression of *AP3*, *PI*, *LFY* and *UFO* was significantly different (data not shown). The expression of *AP3*, *PI*, *LFY* and *UFO* in the restored line, compared to 4:19, was closer to the expression in cv Hanna.

AP3, *PI* and *AG* had more or less a lower expression in lines 4:19 and 41:17, while *LFY* and *UFO* had a higher expression, all in agreement with previous studies (Teixeira et al. 2005a; Carlsson et al. 2007). It appears as if only the lower expression of *AP3* and *PI* is in common for all male-sterile lines, supporting that the two B-genes, *AP3* and *PI*, are affected by retrograde signalling from the mitochondria. The relative expression of *AP3*, *PI*, *LFY* and *UFO* in the restored line is closer to one, i.e. closer to the relative expression in cv Hanna, than the relative expression of these genes in line 4:19.

Discussion

Although many novel mitochondrial genes and transcripts, in addition to physiological and morphological alterations have been associated with CMS in various systems clear evidence for the link between the male-sterility phenotype and the mitochondrial locus is often lacking (reviewed by Hanson and Bentolila 2004; Pelletier and Budar 2007). In our study we have utilized 21 protoplast fusion lines most of which contain various parts of the mtDNA from *A. thaliana* displaying different flower morphologies and fertility aberrations. It is clear from our results that certain observations made, e.g. floral organ morphology, has a high correlation with CMS, whereas others, e.g. adenylate content, has a low or even no correlation with male-sterility.

The mitochondrial genomes of the lines studied differ in amount of retained *A. thaliana* DNA. Lines with large portions of *A. thaliana* mtDNA were male- or semi-sterile, while lines with few or no markers were fertile. We assume that the likelihood to get a male-sterile line is increasing with an increasing number of *A. thaliana* mtDNA markers. In the same way we assume that the likelihood to receive alteration in adenylate content, growth rate, etc increases with a rising number of markers. But, it is not necessarily the same piece or pieces of mtDNA that cause the aberrations.

The most obvious morphological difference among the lines is the stamen phenotype. Normal pollen producing stamens, shrunken but pollen producing stamens or homeotically converted stamens without pollen production divides the lines into three classes of fertile, semi- and male-sterile. Correlation of male-sterility with presence of *A. thaliana* mtDNA showed that markers VI, XXI and XXXV were present in all male- and semi-sterile but not in any fertile plants. Northern analysis with these markers as probes could not detect any expression signal. However, we cannot exclude that sequences in close proximity to the markers might be expressed. For example hybridization with a *cox3* probe, close to marker XXI, showed a novel transcript present in all male- and semi-sterile lines except the semi-sterile line 41:38. We have previously shown that the *A. thaliana* mitochondrial *orfs* such as *orf139*, *orf240a* and *orf294* (corresponding to markers V, XX and XXX, respectively) had an increased expression level in the male-sterile line 4:19 compared to the *B. napus* cultivar (Leino et al. 2005). Here, we found an increased expression of these three *orf*s in all male- and semi-sterile lines with two exceptions; *orf294* had no expression in lines 4:55 and 41:17. There were no *orf294* marker in lines 4:55 and 41:17, so the lack of expression was not surprising. Of the fertile lines two lines (9:20 and 14:92) contained marker V (*orf139*) and one line (14:92) contained marker XX (*orf240a*). Only line 14:92 expressed *orf139* and *orf240a*.

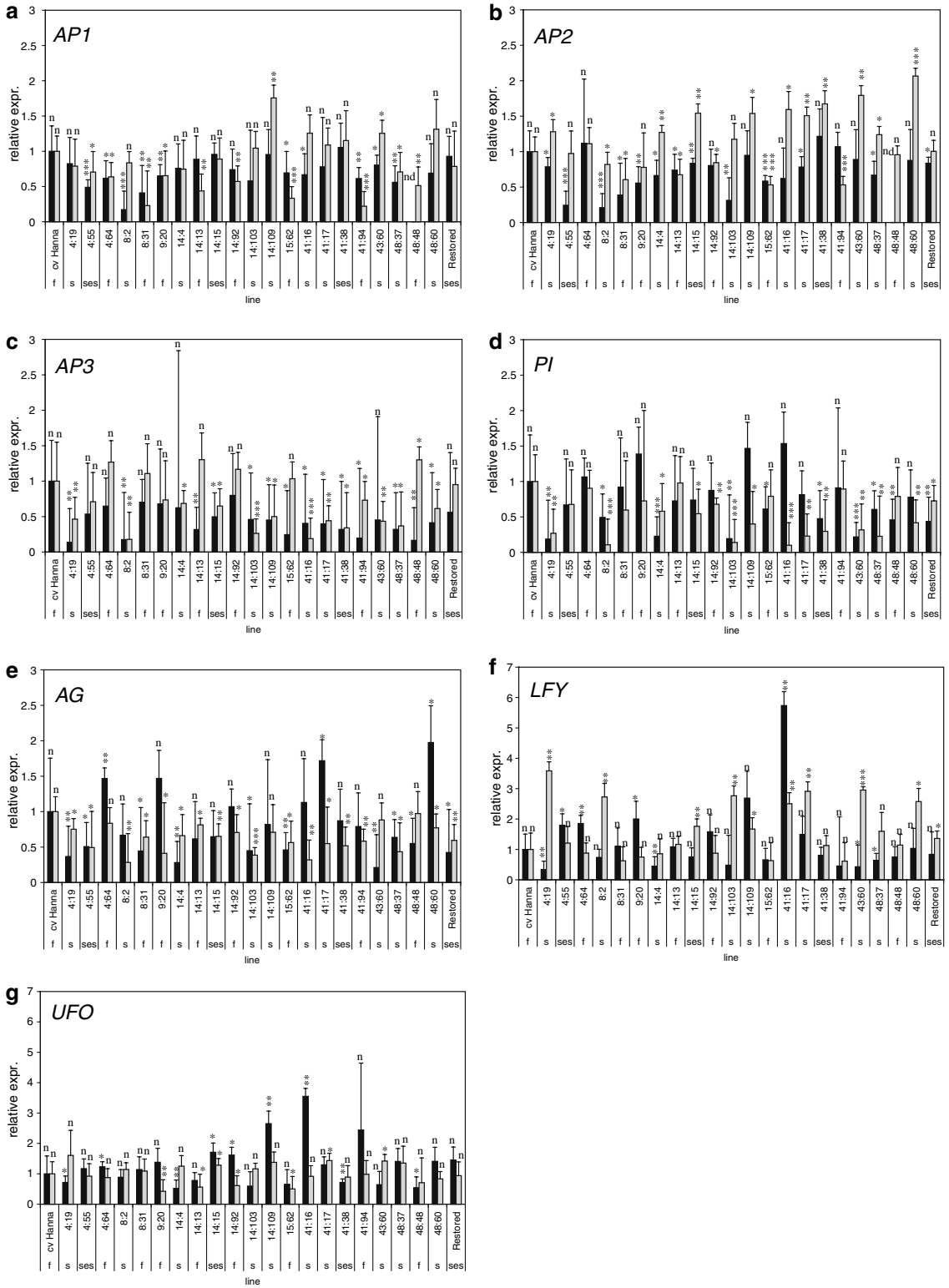

Fig. 5 Relative gene expression in relation to cv Hanna (relative expr.) for the genes *AP1* (**a**), *AP2* (**b**), *AP3* (**c**), *PI* (**d**), *AG* (**e**), *LFY* (**f**) and *UFO* (**g**). For each gene, the gene expression in each line were statistical tested (using a Student's *t*-test) in comparison to in *B. napus* cv. Hanna (with a relative gene expression of 1). *Black bars* stages 0–5; *grey bars* stage 8. *ND* not determined; *N* not significant, $P > 0.05$; *, $0.01 < P \leq 0.5$; **, $0.001 < P \leq 0.01$; *** $P \leq 0.001$

Although no single transcript could be ultimately linked to CMS, several putative candidate genes have been identified in this study. In addition, restoration to fertility by introducing *A. thaliana* chromosome III (At3) by sexual crossings was only possible in a few lines. These results indicate that regulation of male-sterility in this system is complex and most probably affected by several loci.

Line 14:103 had the most extreme flower phenotype. The flowers were very small and the buds hardly opened at all. It also had a very low adenylate content and low ATP/ADP-ratios. This line contained 29 out of 36 mt-markers from *A. thaliana*. This resembled the phenotypes of lines 8:2 (that had 35 out of 36 markers), 14:109 (that had 23 out of 36 markers) and 41:16 (that had 34 out of 36 markers). Line 8:2 had the least abnormal phenotype of these four lines. Line 4:19 had 33 out of 36 markers and it had slightly larger flowers (e.g. significant larger petals) than the four lines mentioned above. Lines 8:2, 4:19 and 41:16 had almost all markers, but they differ slightly in phenotype. Hence, the amount of retained *A. thaliana* mtDNA does not explain the severity of the male-sterility phenotype.

Notably, the homeotic conversions of the third whorl organs observed in our system and other CMS-systems such as *N. tabacum* (Kofer et al. 1991; Zubko et al. 1996), *D. carota* (Linke et al. 1999, 2003), and *T. aestivum* (Murai et al. 2002; Ogihara et al. 1997) show striking similarities with the third whorl organ phenotypes of *A. thaliana* mutants affected in the B-class genes *AP3* and *PI* (reviewed by Jack 2004; Kramer and Hall 2005; Krizek and Fletcher 2005). This suggests that the regulation of the B-class genes, or other components in the same pathway, is affected in many CMS-systems. Studies of CMS-lines from *N. tabacum*, *D. carota*, *T. aestivum*, and *B. napus* could indeed show a correlation between down-regulation of the B-class genes and the CMS-phenotype (Linke et al. 2003; Murai et al. 2002; Teixeira et al. 2005a; Zubko et al. 2001). All male-sterile lines had a significantly lower expression of *AP3* and *PI*, especially at stage 8, in comparison to *B. napus* cv. Hanna. In contrast, all fertile lines had a significantly higher expression at least at stage 8 (except line 48:48). Homeotic conversions of stamens, and thus male-sterility, are caused by down-regulation of *AP3* and *PI*, e.g. in mutants of *A. thaliana*. Our male-sterile lines are sterile due to alterations in the mitochondrial genome. Hence, the down-regulation of *AP3* and *PI* is due to alterations in the mitochondrial genome, indicating that the homeotic changes of the stamens in the male-sterile lines are regulated via retrograde signalling.

The gene expression profiles are in general similar for the male- and semi-sterile lines and the profiles often differ from the fertile lines. The gene expression differs more or less for all genes and all lines studied in comparison to *B. napus*. The gene expression of *AP3* and *PI* clearly correlates with male-sterility. The expression of *LFY* showed a slight correlation with male-sterility, while the expression of the remaining five genes has a low or no correlation to male-sterility. All lines in this study have flowers with four distinct whorls, normal sepals in whorl one and a normal pistil in whorl four. Petals are present in all flowers in the second whorl as expected, although the size of the petals varies between lines, with large petals in fertile lines and small in male-sterile lines. The stamens are found in whorl three in all lines. Fertile and semi-sterile lines have stamens with pollen while the male-sterile lines have stamens homeotically converted into carpelloid structures. *AP1* is expressed in the meristem and later on in whorls one and two. *AP2* is expressed in all four whorls. *AP3* and *PI* are expressed in whorl two and three. *AG* is expressed in whorl three and four. The combined expression of these five genes is the basis for the so called ABC-model that describes the floral-organ patterning in e.g. *A. thaliana* (reviewed by Krizek and Fletcher 2005). The distinct pattern of whorls and normal development of whorl one and four organs may account for the low correlation of male-sterility and the expression of *AP1*, *AP2* and *AG*. On the other hand, the *AP3* and *PI* expression is highly reduced in male-sterile lines, i.e. lines with small petals in whorl two and with no true stamens in whorl four. *LFY*, together with other genes, is the link between the flowering inducing pathways and the transition from shoot apical meristem to floral meristem identity (reviewed by Krizek and Fletcher 2005), and together with *AP1*, it has been shown to activate *AP3* (Lamb et al. 2002). The correlation between *LFY* and male-sterility may reflect an attempt to activate *AP3* in order to rescue stamen development in the male-sterile lines. Perhaps the change in flowering time could explain the change of expression of *LFY*, but that would only account for differences in expression at early stages. *UFO* is involved in the regulation of *AP3* and *PI* (Zhao et al. 2001), and we expected it to have a similar expression profile as *LFY* but this was not the case.

A high adenylate content and ATP/ADP-ratio is generally found in the fertile lines with little or no *A. thaliana* mtDNA. However, the semi-sterile lines did not contain significantly more adenylates than the male-sterile lines. Thus, our results do not support a direct link between energy production of mitochondria and a certain phenotype. Regarding growth rate and flowering time, a large variation for these features was observed among the lines. This variation seems not to be associated with fertility. Furthermore, significantly higher growth rates of semi-sterile lines in comparison to fertile and male-sterile line indicate a different mitochondrial influence on growth and plant development in these lines. Consequently, many observed phenotypic alterations can be correlated to alterations of mitochondrial genomes, independent of fertility.

A general conclusion is that the mitochondrial genome affects many features in the plant, such as flower morphology, nuclear gene expression, energy production, growth, flowering time and male-sterility. With induction of species-specific incompatibilities between nuclear and mitochondrial genomes more aberrations were found. From our studies we show that specific elements of the mt-genome could be correlated to the modifications and that the alterations not only were due to a dose effect. Thus, more than one locus in the *A. thaliana* mitochondrial genome can result in CMS when transferred to the nuclear background of *B. napus.* It is also likely from this study that transcriptional aberrations often observed in alloplasmic lines (Håkansson and Glimelius 1991; de la Canal et al. 2001; Leino et al. 2005) can induce a variety of phenotypic alterations.

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