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ORIGINAL PAPER

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Arabidopsis thaliana chromosome III restores fertility in a cytoplasmic male-sterile *Brassica napus* line with *A. thaliana* mitochondrial DNA

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Abstract Somatic *Brassica napus* (+) *Arabidopsis thaliana* hybrids with a cytoplasmic male sterility (CMS)-inducing cytoplasm were screened for fertility-restored plants. One line was selected and recurrently backcrossed with the maintainer line, *B. napus*, resulting in fertile/sterile segregating populations. Restriction fragment length polymorphism mapping showed the co-segregation of *A. thaliana* chromosome (chr) III markers with the fertility trait. As it was not possible to stabilise the fertility trait via selfings, a dihaploidisation strategy was assessed. Ninety haploid plants were regenerated and analysed with numerous simple sequence length polymorphism (SSLP) markers. Markers covering both arms of *A. thaliana* chr III were present in two plants, whereas no *A. thaliana* DNA could be detected in the other plants. Following colchicine-induced chromosome doubling only these two plants with *A. thaliana* DNA produced fertile offspring. In one of the two lines, however, the *A. thaliana*-specific DNA markers and fertility were lost in subsequent generations. The other line remained fertile after repeated selfings. Using genomic in situ hybridisation (GISH) we were able to demonstrate that this latter line possessed a disomic addition of the *A. thaliana* chromosome. The restored line was comparable to the maintainer line with respect to flower morphology, but the petals and stamens were slightly reduced in size. The homeotic conversion of stamens to pistil-like structures, which is typical for the CMS line, was reversed, and stamens with a normal appearance with viable pollen appeared. Flowering time was as in the CMS line—in both lines it was delayed in comparison to the maintainer line. The introgressed chromosome also contributes to several pleiotropic effects, such as reduced leaf crinkling and shorter stems. The ability to restore fertility through the introgression of nuclear genes from the main cytoplasmic donor species indicates that the CMS trait in this system mainly is due to *B. napus*/*A. thaliana* alloplasmic incompatibility and not mitochondrial DNA rearrangements. Further exploitation of the material is discussed.

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Introduction

Alloplasmy, the combination of the cytoplasm from one species with the nucleus from another, often results in cytoplasmic male sterility (CMS). The trait is maternally inherited, and the inability to produce functional anthers or pollen is considered to be due to nuclear-mitochondrial incompatibility, as plants with specific nuclear genes have the ability to suppress the male-sterile phenotype in plants carrying CMS-inducing cytoplasm. These nuclear genes, termed restorer of fertility (*Rf*) genes, have been found to alter the expression of specific regions in the mitochondrial genome that are associated with CMS (Schnable and Wise 1998). The action of restorer genes have been found to involve several different mechanisms, such as copy number abundance of the CMS gene (Janska et al. 1998), transcript initiation (Edqvist and Bergman 2002), maturation of transcripts (Dill et al. 1997; Singh et al. 1996), post-transcriptional expression of the CMS gene product (Bellaoui et al. 1999) or compensatory metabolic effects (Cui et al. 1996; Liu et al. 2001). However, the exact mode of action of the restorer genes remains to be elucidated.

One obvious strategy to restore the nuclear-mitochondrial incompatibility achieved in alloplasmic CMS systems is the introgression of nuclear DNA from the cytoplasm donor species. This has been accomplished in several species with alien cytoplasms, for example in tobacco (Burns et al. 1978; Gerstel et al. 1978) and wheat (Livers 1964). In *Brassica* species the CMS trait is often observed in intergeneric or intertribal species derived from sexual crossings or somatic hybridisation. Alloplasmic CMS lines have been produced in *Brassica juncea* with cytoplasm from *Erucastrum canariense*

(Prakash et al. 2001), *Diplotaxis catholica* (Pathania et al. 2003) and *Enarthrocarpus lyratus* (Banga et al. 2003). Nuclear DNA fragments from the cytoplasm donor species act as restorers in these systems. In *Brassica napus* with the CMS-inducing Ogura cytoplasm from *Raphanus sativus* restoration of fertility was achieved by introgressing nuclear radish DNA (Heyn 1976). For most wild relatives, however, molecular markers or detailed information on the genomes are lacking, which consequently impairs the possibilities to perform marker-assisted selection or isolation of the restorer gene(s). In contrast, the situation is different in hybrids where *Arabidopsis thaliana* is one of the parental species. The availability of the full genome sequence (The Arabidopsis Genome Initiative 2000) offers unique possibilities to perform genetic and functional genomic studies.

The production of a novel CMS system consisting of *Brassica napus* with rearranged *Arabidopsis thaliana* mitochondria has been reported recently (Leino et al. 2003). In this system, *A. thaliana*, the cytoplasm donor, is the source for nuclear *Rf*-genes. Therefore, *B. napus* (+) *A. thaliana* somatic hybrid lines with CMS-inducing cytoplasm were screened for fertility-restored plants. We describe here a strategy for stabilising a fertility-restored line and present evidence that the introgression of *A. thaliana* chromosome III into the *B. napus* nucleus restores male-fertility in this system.

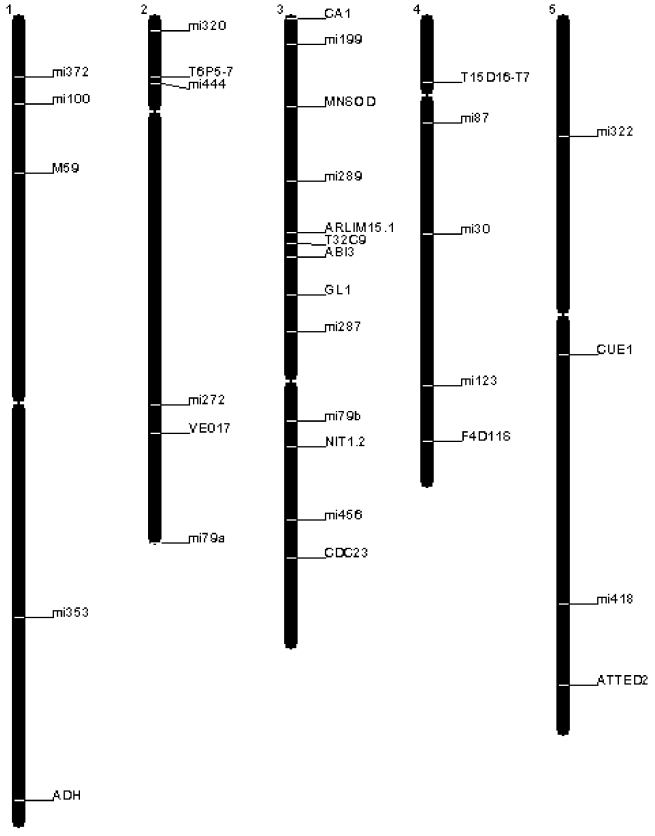

Fig. 1 A schematic map of the *Arabidopsis thaliana* chromosomes indicating the positions of the mi RFLP markers (Liu et al. 1996) used in the screening of the segregating BC_6 generation and the PCR markers (available at http://www.arabidopsis.org) used in the screening of the haploid plants

Materials and methods

Plant material and phenotypic characterisation

CMS lines were obtained from somatic hybrids produced between *Brassica napus* cv. Hanna and *Arabidopsis thaliana* var. *Landsberg erecta* (Forsberg et al. 1998) that were backcrossed to *B. napus* cv. Hanna as a maintainer line (Leino et al. 2003). Fertile plants in a fertile/sterile segregating BC_1 population of line 4:19 were recurrently backcrossed with the maintainer line to obtain a BC_6 generation. Fertile BC_3 plants were selfed, and a BC_3F_3 generation was raised. Fertile plants were selected for all backcrossings and selfings. Microspores were isolated from a fertile BC_5 plant (Nehlin et al. 1995), and 90 haploid plants were generated that were subsequently chromosome doubled with colchicine and selfed when feasible.

All plants were grown under controlled conditions in a greenhouse or culture chamber at 22°C/18°C (day/night) and under a photoperiod of 16/8 h (day/night). Fertility was assessed as the ability to set seed after selfing. Flower organs were measured on flowers taken from the primary shoot for ten individual plants of each line.

Nuclear DNA analysis

Southern analysis was performed on 30 plants of the BC_6 generation as described by Leino et al. (2003) using mapped *A. thaliana* restriction fragment length polymorphic (RFLP) markers (Liu et al. 1996) (Fig. 1). PCR analysis of the 90 haploid plants was performed on crude DNA extracts (Edwards et al. 1991) from leaf tissue. Cleaved amplified polymorphic sequence (CAPS) marker primer pairs from The Arabidopsis Information Resource (TAIR) database that could detect simple sequence length polymorphism (SSLP) between *A. thaliana* and *B. napus* were used as markers (Fig. 1). The primer sequences are available at http://www.arabidopsis.org. Except for markers MNSOD, CA1 and CUE1 the primers only amplify fragments from *A. thaliana*. Therefore, as a positive internal control, the *B. napus*-specific primers co16 (5′-TAG CCT TTC TCT CCA TTG-3′) and co39 (5′-CTG CCG AGC TGA TTC GC-3′) were used in the other PCR reactions, which results in a 750-bp fragment. The reaction mixture (25 μl) contained 0.2 m*M* of each primer (Invitrogen, Carlsbad, Calif.), 2.5 m*M* $MgCl_2$, 1.25 U *Taq*-polymerase (Fermentas), 1× the supplemented buffer containing $(NH_4)_2SO_4$ and 1 μl crude DNA extract. The amplification protocol consisted of a 2-min initial denaturation at 94°C, 35 cycles of 94°C for 30 s, 50–61°C for 30 s and 72°C for 2 min and a 5-min final elongation step at 72°C. The PCR products were electrophoresed on 1.2% agarose gels and visualised with ethidium bromide (EtBr).

Cytogenetic characterisation

To obtain suitable material for cytogenetic studies and genomic in situ hybridisation (GISH) we produced *Agrobacterium rhizogenes*-transformed root cultures as described by Fahleson et al. (1997). To synchronise cell division, we transferred actively growing roots to 4°C overnight followed by 24 h at 22°C. Cell divisions were blocked at metaphase in 2 m*M* 8-hydroxyquinoline at room temperature for 2 h followed by 2 h at 4°C according to Schwarzacher et al. (1994). Roots were fixed in ethanol:acetic acid (3:1) at room temperature for 2 h and stored in fixative at −20°C until used. Root tips were excised, and single cells were released from the root tissues by enzyme treatment according to Fahleson et al. (1997). The enzyme-digested cells were washed and fixed according to

Schwarzacher et al. (1994). Slides were prepared by placing 5-μl drops of cell suspension onto the slide and slowly adding 10 μl fixative (methanol:acetic acid, 3:1), after which they were exposed to drying and stored at 4°C until used. For karyotype determination, slides were stained with 2% Giemsa, and chromosomes in metaphase were subsequently counted and photographed in a Nikon Microphot-FXA microscope. At least 15 metaphases of each line were examined.

The *A. thaliana* probe for GISH was labelled with Cy3-dCTP by nick-translation using the N5500 Nick Translation kit from Amersham Biosciences (Piscataway, N.J.) according to their instructions with an additional 0.0023 U DNAse I. One microgram of total *A. thaliana* DNA was used as template. Prior to hybridisation the probe was pre-annealed (Anamthawat-Jónson and Reader 1995) at 37°C for 30 min (Snowdon et al. 2000) with a 50× excess of unlabelled *Eco*RV/*Hin*dIII-digested total *B. napus* DNA to remove common sequences. The pre-treatment and denaturation of slides were performed according to Anamthawat-Jónson and Reader (1995), and hybridisation was carried out overnight at 37°C in a humid chamber. The slides were washed at 42°C in 2× SCC for 5 min and in 0.2× SSC for 10 min, rinsed with 4× SSC containing 0.2% Tween 20 and counterstained with 1 μg/ml DAPI for 1 min. Coverslip mounting was done with antifade VECTASHIELD Hard set medium (Vector Laboratories). The chromosome spreads were examined and photographed using an epifluorescence microscope (Nikon Microphot-FXA) with an excitation filter of 330–380 nm for DAPI fluorescence and filterblock G1-B (excitation filter 546/10) for Cy-3. At least ten metaphase plates of each genotype were evaluated.

Mitochondrial DNA analysis

For analysis of mitochondrial (mt)DNA a crude fraction of mitochondria was isolated by differential centrifugation. Between 3 g and 10 g of fresh flower buds was ground in a mortar with 50 ml of grinding buffer (0.3 *M* sucrose, 50 m*M* MOPS-KOH pH 7.8, 2 m*M* EDTA, 1% bovine serum albumin, 1% PVP and 20 m*M* cysteine). The suspension was filtered through miracloth, centrifuged twice for 3 min at 7,000 rpm in a SS-34 rotor (approx. 3,800 *g*) and the supernatant saved. Crude mitochondria were collected by centrifugation for 15 min at 12,500 rpm in the same rotor (approx. 12,800 *g*) and DNA was isolated by lysing the crude mitochondrial fraction with three volumes of buffer containing 50 m*M* Tris-HCl pH 8.0, 20 m*M* EDTA, 4% (w/v) sodium *N*-laurylsarcosine and 0.5 mg/ml proteinase K at +60°C for 1 h. Proteins were removed by phenol/chisam extractions. Following RNAse treatment, the mtDNA was ethanol-precipitated. Southern analysis was performed on *Bam*HI-, *Eco*RI- or *Hin*dIII-restricted DNA as in Leino et al. (2003) using random primed CsCl-purified *B. napus* mtDNA (Landgren and Glimelius 1990) as a probe.

Results

A BC_1 population of the *B. napus* (+) *A. thaliana* somatic hybrid line 4:19 (Forsberg et al. 1998) was found to segregate into fertile and male-sterile plants. The male-sterile plants displayed a stable completely male-sterile flower with severe anther modifications upon recurrent backcrosses with the maintainer line *B. napus* cv. Hanna (Leino et al. 2003), whereas the fertile plants continued to segregate into fertile and sterile plants upon repeated backcrosses with the same line. The production of a stable non-segregating fertile offspring by repeated selfings was not successful.

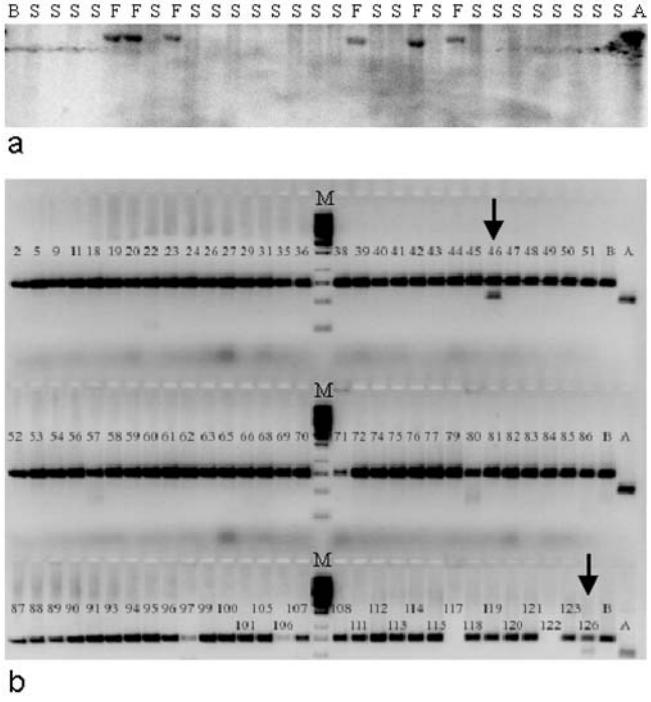

Fig. 2 a Example of RFLP analysis of the 30 segregating BC_6 plants using the mi289 marker as a probe showing co-segregation of the *A. thaliana* 4-kb *Eco*RI fragment with fertility. *A A. thaliana*, *B Brassica napus*, *F* fertile plant, *S* sterile plant. **b** Example of SSLP analysis of the 90 haploid plants. Inverted EtBr-stained gel of PCR products amplified with *A. thaliana*-specific primers GL1 and internal control *B napus*-specific primers co16–co39. *A A. thaliana*, *B B. napus*, *M* size marker; the haploid plant ID numbers are indicated. *Arrows* indicate plants with the *A. thaliana* marker present

Male-fertility co-segregates with *A. thaliana* chr III RFLP markers

A segregating BC_6 generation was analysed with RFLP markers covering each arm of the five *A. thaliana* chromosomes (Fig. 1). In the population of BC_6 plants investigated here, 24 were male-sterile and six male-fertile, which is significantly different from a 1:1 ratio (χ^2=10.8, P<0.01). All of the tested *A. thaliana* markers were absent in the sterile plants, while markers mi199, mi289, mi287, mi79b and mi456 were present in the fertile plants (Fig. 2a). These markers are positioned along most of *A. thaliana* chr III. None of the markers located on *A. thaliana* chromosomes I, II, IV and V was found to be present in any of the plants (data not shown).

PCR analysis of microspore-derived plants

The RFLP analysis indicated that an addition of chr III of *A. thaliana* had occurred in the fertile plants. The inability to produce a stable, non-segregating fertile line via selfings indicated that a monosomic addition line was obtained. Thus, disomic addition lines were synthesised through chromosome doubling of haploid plants. Microspores were isolated from a fertile BC_5 plant, and haploid

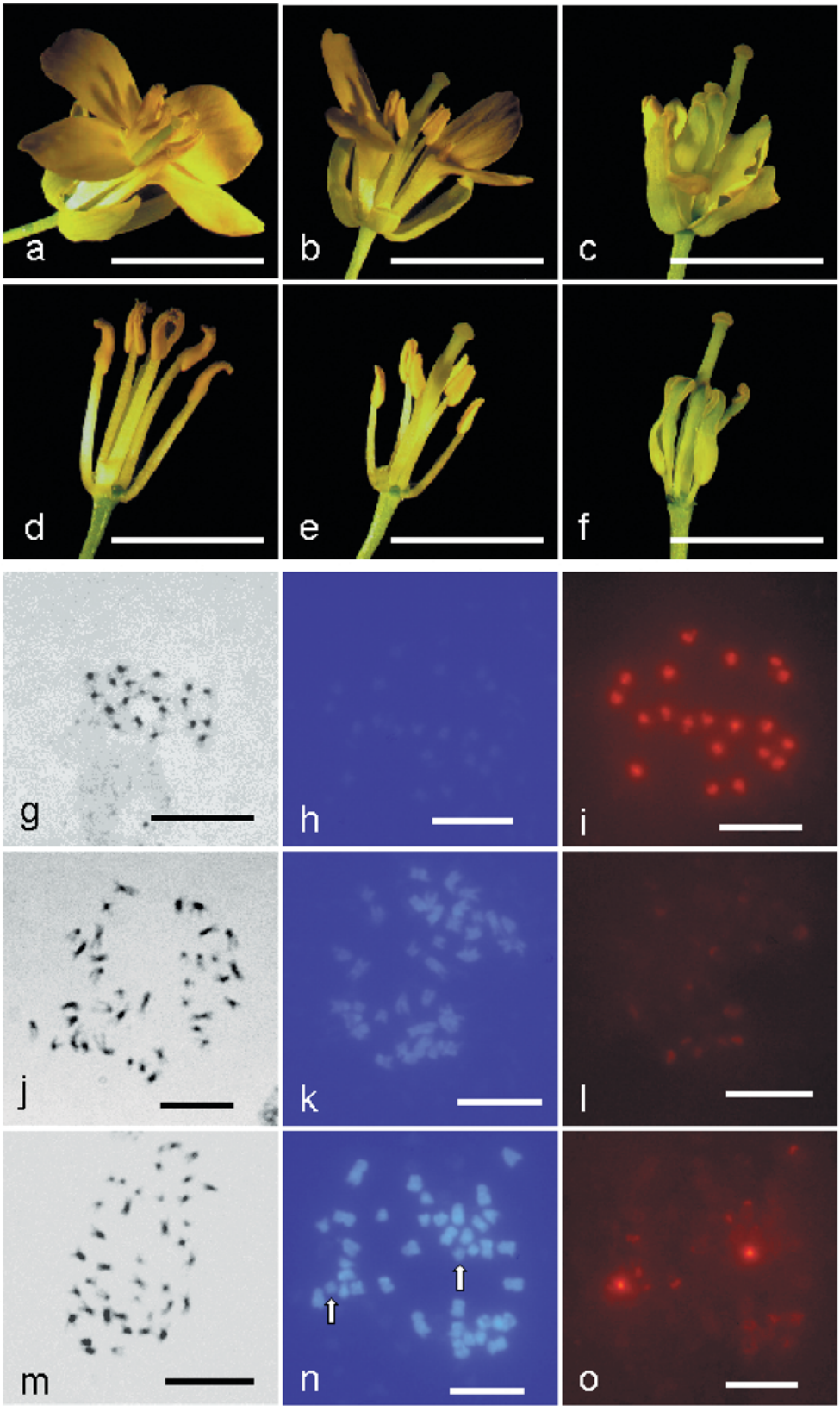

Fig. 3a–o Phenotypic and cytogenetic characterisation of the plant material. **a** Mature flower from *B. napus* cv. Hanna. **b** Mature flower from restored line 46. **c** Mature flower from CMS line 4:19. **d–f** The same flowers as in **a–c** but with sepals and petals removed. **g** Metaphase chromosome plate of *A. thaliana*. **h** *A. thaliana* chromosomes stained with DAPI. **i** The same chromosomes as in **h** hybridised to Cy3-dCTP-labelled *A. thaliana* total DNA. **j** Metaphase chromosome plate of *B. napus*. **k** *B. napus* chromosomes stained with DAPI. **l** The same chromosomes as in **k** hybridised to Cy3-dCTP-labelled *A. thaliana* total DNA. **m** Metaphase chromosome plate of restored line 46. **n** Chromosomes from restored line 46 stained with DAPI. *Arrows* indicate the *A. thaliana* chromosomes. **o** The same chromosomes as in **n** hybridised to Cy3-dCTP-labelled *A. thaliana* total DNA. *Bar*: (**a–f**) 1 cm, (**g–o**) 0.01 mm

plants were subsequently generated. To screen this population of 90 plants for the presence of *A. thaliana* DNA, we used 16 SSLP markers (Fig. 1)—eight from chr III and eight from other chromosomes. These markers either exclusively amplified *A. thaliana* DNA, or they gave fragment length polymorphism between *B. napus* and *A. thaliana* (Fig. 2b). The presence of *A. thaliana* DNA was demonstrated in only two of the 90 haploid (C_0) plants, denoted "46" and "126". These plants were found to be positive for all eight markers (CA1, MNSOD, ARLIM15.1, T32C9, ABI3, GL1, NIT1.2 and CDC23) tested for from chr III. Markers from the other *A. thaliana* chromosomes were not found in any of the plants analysed (data not shown). Following colchicine-induced chromosome doubling of the haploid plants (C_0), the fertile plants carrying the markers from chr III were selfed. All of the plants (n=10) in the offspring populations (C_1) of line 46 and line 126 were male-fertile. When we evaluated flower morphology, we found that the offspring from line 46 plants displayed a better restoration with larger flowers that resembled those of the maintainer line more closely than the offspring from line 126. One C_1 plant from each line was selfed to obtain a C_2 generation. In C_2, all of the progeny from line 46 remained fertile, while all progeny from line 126 were sterile. PCR tests with the SSLP-markers ARLIM15.1 and NIT1.2 demonstrated that the *A. thaliana* chr III markers had been lost in the progeny obtained from line 126, while they were still present in the progeny from line 46.

Chromosome and GISH analysis

To determine the chromosome composition of stably restored line 46 we determined chromosome numbers. Metaphase plates with well-spread chromosomes were obtained from synchronised roots. The chromosome number in the restored line was 2n=40, while the maintainer line contained 2n=38 (Fig. 3j, m). In the *A. thaliana* parental line the detected chromosome number varied between cells, although most cells contained 20 chromosomes (Fig. 3g). The finding of *A. thaliana* cells with 20 chromosomes or more indicates frequent endopolyploidisation as has been reported earlier in this species (Galbraith et al. 1991; Maluszynska and Helsop-Harrison 1991).

A. thaliana chromosomes were specifically labelled by hybridising Cy3-labelled total *A. thaliana* DNA to the chromosome spreads. Prior to the hybridisation the labelled DNA was pre-annealed to an excess of *B. napus* total DNA to block homologous sequences that eventually would result in false positive signals. In *A. thaliana*, the labelled probe hybridised to all of the chromosomes (Fig. 3h, i), whereas no hybridisation signal was obtained in the *B. napus* chromosome plates (Fig. 3k, l). In the restored line a distinct *A. thaliana* hybridisation signal was obtained from two *A. thaliana* chromosomes of equal size (Fig. 3n, o).

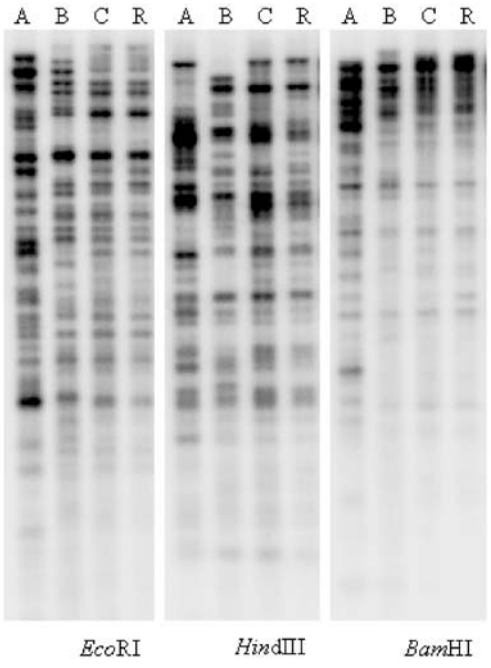

Fig. 4 Southern blot hybridisation of mtDNA, digested with *Eco*RI, *Bam*HI and *Hin*dIII. *A A. thaliana*, *B B. napus*, *C* CMS, *R* restored

MtDNA restriction patterns

To exclude the possibility that mitochondrial rearrangements or segregations occurred during the microspore regeneration procedures or sexual crossings, we analysed mtDNA restriction patterns. MtDNA from restored line 46, the CMS line and the parental lines were restricted with *Bam*HI, *Eco*RI, and *Hin*dIII, respectively, electrophoresed, blotted and hybridised with pure *B. napus* mtDNA as a probe. The patterns clearly demonstrated the presence of a recombined *B. napus* (+) *A. thaliana* mtDNA composition in the CMS line as suggested earlier (Leino et al. 2003). We were unable to detect any clear differences in the mitochondrial RFLP pattern between the restored lineand the CMS line (Fig. 4).

Plant characteristics

Line 46 (C_1) was characterised morphologically. In comparison to the maintainer and CMS lines, it showed good plant vigour but had reduced leaf crinkling and shorter stems. Flowering time was as delayed as in the CMS line (Table 1). The severely aberrant petals and stamens observed in the CMS line were restored, although they were smaller than in those of the maintainer line (Table 1, Fig. 3a–f). Most noteworthy is our observation that the carpel-like homeotic conversions of the stamens found in the CMS line were fully converted into stamens of normal appearance. The anther bags contained mature pollen but the filament length was still reduced (Fig. 3a–f). The fact

Table 1 Comparative morphology of the maintainer line *Brassica napus* cv. Hanna, the CMS line 4:19 and the fertility-restored line 46 (C_1)

Character	Line		
	B. napus	CMS line 4:19	Fertility-restored line 46
Shoot height after 5 weeks (cm)	73±2.8[a]	41±8.3	29±6.6
Days to flowering	31±1.3	39±1.8	39±1.9
Carpels (cm)	1.0±0.13	1.0±0.06	1.1±0.12
Short stamens (cm)	0.8±0.05	0.6±0.09	0.6±0.05
Long stamens (cm)	1.0±0.03	0.7±0.07	0.8±0.05
Petals width (cm)	0.7±0.08	0.3±0.06	0.4±0.05
Petals length (cm)	1.5±0.6	0.7±0.11	1.0±0.11
Sepals width (cm)	0.2±0.04	0.2±0.05	0.2±0.03
Sepals length (cm)	0.8±0.04	0.7±0.05	0.7±0.07

[a] Values are means of ten plants (n=10) ± the standard deviation

that the anthers do not fully reach the stigma reduces seed-set upon natural selfing. When artificially pollinated, however, seed-set is normal and comparable to seed-set in *B. napus*.

Discussion

CMS lines are frequently obtained after interspecific and intergeneric protoplast fusions (Earle 1995) and are due to disturbed nuclear-mitochondrial interactions. These could be the result of DNA rearrangements in the recombined mitochondrial genomes and/or alloplasmic incompatibility between the nuclear and mitochondrial genomes. The results presented in this article, which show that introgression of nuclear DNA from the main cytoplasm donor species restores fertility, are in agreement with the latter hypothesis.

The large genetic distance between *A. thaliana* and *B. napus* does not facilitate introgression of foreign DNA through chromosome association. Indeed Bohman et al. (1999) showed that translocations or substitutions are very rare events in this material. Therefore, difficulties in stabilising a male-fertile line were expected, especially as the restoration seemed to be the result of a monosomic addition of one chromosome from *A. thaliana.* Restricted competitiveness of gametes carrying an alien chromosome is a well-known phenomenon in, for example, numerous wheat hybrids (reviewed by Gale and Miller 1987) and greatly impairs the possibility to create disomics via selfings. Thus, only by screening large populations of selfed individuals might it be possible to obtain disomic addition lines. As an alternative strategy, dihaploidisation was performed. It turned out, however, that microspores carrying an alien chromosome were also eliminated or regenerated to haploid plants only with difficulty, since the addition was found in only two out of 90 plants. Furthermore, in one of the two restored lines, the chromosome markers—and the corresponding fertility—were lost during subsequent selfings. Why the extra *A. thaliana* chromosome(s) were eliminated in this line is unclear. Nevertheless, the other line displayed stable inheritance of the chromosomal addition through sexual generations. The GISH assay clearly demonstrated that the chromosome addition is present in disomic state in this line, which enables a stable inheritance. Additionally, a tally of 40 chromosomes indicates that the *B. napus* genome is intact and has not undergone any substitution with the foreign chromosomes.

Though GISH is an excellent technique to detect additional chromosomes in intergeneric hybrids of *Brassica*, as has been shown earlier (Fahleson et al. 1997; Skarzhinskaya et al. 1998; Snowdon et al. 2000), the possibility of detecting translocations is perhaps limited. Snowdon et al. (1999) found a translocation in a *B. napus*-*R. sativus* hybrid by GISH, but smaller translocations or translocations in non-heterochromic regions might be impossible to detect. Thus, even though neither *A. thaliana* DNA markers from other chromosomes other than chr III nor any GISH signals indicating translocation were found, it cannot be totally excluded that minor *A. thaliana* DNA fragments could be included in the *B. napus* genome of the restored line. The obvious and clear correlation between fertility restoration and presence of *A. thaliana* chr III does, however, demonstrate that the *Rf* gene(s) are located on the additional chromosome.

During the initial protoplast fusion the mtDNA was mixed and reshuffled from the two parental genomes (Leino et al. 2003), which is clearly seen in the restriction patterns of the CMS and restored line (Fig. 4) These lines have been separated for many sexual generations, but no distinct differences in their mitochondrial genome structures have been detected. Landgren and Glimelius (1990) and Leino et al. (2003) showed that protoplast fusion-induced mtDNA rearrangements are fixed rapidly following plant regeneration. They also showed that mtDNA rearrangements are stably inherited through sexual generations. A few hybridising fragments seem to be present in different stoichiometries in the restored line in comparison to the CMS line. One possibility is that the additional chromosome itself induces stoichiometric shifts in the mitochondrial genome. Nuclear genes affecting mtDNA have been reported at least twice—the *Fr* gene in common bean (Janska et al. 1998) and the *CHM* gene in *A. thaliana* (Abdelnoor et al. 2003). Noteworthy, the *CHM* gene is indeed located on chr III.

In spite of the fact that some floral parts in the restored line do not fully resemble the maintainer line in size, a major reversion of stamen phenotype was found. In the CMS line all stamens display homeotic conversions into carpel-like structures with stigmatic tissues and ovules as described in detail by Leino et al. (2003). In the restored line stamens revert to normal morphology and function. The phenomenon of anther homeotic conversions in an alloplasmic line and the reversion of the stamens to a normal appearance following the introgression of nuclear restorer genes—most commonly from the cytoplasmic donor species—are interesting results that have been found in other CMS systems—for example, tobacco (Gerstel et al. 1978; Kofer et al. 1991) and wheat (Murai

et al. 2002). The additional chromosome causes, not unexpectedly, several pleiotrophic effects in addition to fertility restoration such as the reduced leaf crinkling. In other aspects, such as flowering time, the fertility-restored line resembles the CMS line. This similarity to the CMS line could be due to incomplete restoration of mitochondrial function or pleiotrophic effects not involved in the nuclear-mitochondrial interactions.

To our knowledge, this is the first report that introgressed *A. thaliana* DNA can act as the restorer of fertility in a CMS system. Through the production of a restored line, we have obtained a complete *B. napus* (*A. thaliana*) CMS system in which the CMS line, the restored line and the two parental species can serve as research material for further studies of nuclear-mitochondrial interactions and development of the CMS trait. *A. thaliana* chr III contains 5,220 genes spread over 23 Mbp (The Arabidopsis Genome Initiative 2000). Although this constitutes an enormous addition of foreign DNA, it is very tempting to speculate about putative genes on chr III that could act as fertility restorer genes. With the recent identification of the *Petunia Rf* gene as a pentatricopeptide repeat protein (PPR) (Bentolila et al. 2002), a breakthrough in the isolation of the long elusive *Rf* genes was achieved. To date, the *Brassica napus* (*Ogu*) *Rfo* gene (Brown et al. 2003; Desloire et al. 2003), Kosena radish *Rfk1* gene (Koizuka et al. 2003) and rice *(ms-bo) Rf-1* gene (Kazama and Toriyama 2003) have also been identified as PPR genes. The PPR proteins are normally targeted to organelles and probably have RNA-binding structures (Small and Peeters 2000). Consequently, these proteins are putative candidates for *Rf* genes. Among the genes of the PPR protein family, more than 450 genes have been found in *A. thaliana.* Aligning these genes to the identified radish *Rfo* and petunia *Rf* genes restricts the number to 20, out of which most are located on chr I and only one on chr III of *A. thaliana* (Desloire et al. 2003). Additionally, Elo et al. (2003) recently reported that a large cluster of genes located on *A. thaliana* chr III encodes proteins targeted to the mitochondria and associated with DNA and RNA maintenance functions. Transformation experiments with these genes and screening for changes in mitochondrial gene expression and floral phenotype provide means to identify the genes involved in the nuclear-mitochondrial regulation of CMS.

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