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Genetics

Restorer genes for different forms of *Brassica* cytoplasmic male sterility map to a single nuclear locus that modifies transcripts of several mitochondrial genes

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ABSTRACT **The oilseed rape plant, *Brassica napus*, possesses two endogenous male sterile cytoplasms, *nap* and *pol*. Previous studies have shown that nuclear restoration of *pol* cytoplasmic male sterility (CMS) is conditioned by a gene, *Rfp*, that is also involved in modifying transcripts of the *pol* CMS-associated *orf224/atp6* mtDNA region. We now find that the *nap* nuclear restorer gene *Rfn* apparently is identical to *Mmt*, a gene that conditions the modification of transcripts from several different mtDNA regions, including one that is associated with *nap* CMS and contains *orf222*, a chimeric gene related to *orf224*. *Mmt*, in turn, is found to be allelic to *Rfp*, suggesting that restorer genes for the two cytoplasms represent different alleles or haplotypes of a single nuclear locus. This view is supported by restriction fragment length polymorphism mapping studies that indicate that *Rfn* and *Rfp* map to the same chromosomal position. Thus, in contrast to CMS in other species, different forms of Brassica CMS are restored by alleles of a single nuclear locus, and the restoration properties of these alleles reflect their involvement in the modification of transcripts of corresponding CMS-associated mtDNA regions. A survey of 51 varieties from 8 *Brassica* and *Sinapis* species failed to find evidence of *Rfn(Mmt)* in other than fertility-restored, *nap* cytoplasm *B. napus*. This suggests that *Rfn(Mmt)* arose in Brassica with *nap* cytoplasm and that the necessity for fertility restoration may have provided the selective pressure for its origin and maintenance.**

The control of the expression of individual mitochondrial genes by specific nuclear genes represents a key mechanism for ensuring the cooperative function of the nuclear and mitochondrial genomes (1, 2). In flowering plants, the suppression of cytoplasmic male sterility (CMS) by nuclear restorer of fertility (Rf) genes represents a striking example of this type of nuclear–mitochondrial gene interaction. CMS is a maternally transmitted failure in pollen production. In several plant species, mitochondrial gene regions have been identified whose expression is associated with CMS. These regions contain unusual ORFs that often are cotranscribed with conventional mitochondrial genes (3). In general, the nuclear restorer genes that suppress CMS specifically modify expression of the CMS-associated regions, but not other mitochondrial genes.

In certain plant species, multiple forms of CMS are found. These forms can be distinguished by their associated novel mitochondrial ORFs and by the nuclear Rf genes that restore their fertility (3, 4). In such cases, the restorers for the different forms represent distinct genes that map to different chromosomal loci. In maize, for example, there are three forms of CMS: T, S, and C. Restoration of *cms*-T requires two genes, *Rf1* and *Rf2*, that map to chromosomes 3 and 9, respectively (5, 6), whereas *cms*-S is restored by *Rf3* on chromosome 3 (7, 8) and *cms*-C is restored by *Rf4* on chromosome 8 (9). In rice, as well, restorer genes for three different forms of CMS have been found to map to different chromosomes (10). Even when linkage is observed between restorers for different forms of CMS, on close examination these have been found to segregate as distinct loci, as in the case of the wheat *Rfv1* and *Rf3* genes (11). Although a single type of CMS may be restored by genes mapping to different chromosomal positions (7, 12), the converse situation, in which more than one type of CMS can be restored by a gene or genes present at a single locus, has not been shown to occur.

Two forms of CMS, designated *nap* and *pol*, are endogenous to the oilseed rape plant, *Brassica napus* (13). Most *B. napus* varieties contain the *nap* cytoplasm but are male-fertile because they possess a restorer gene for *nap* CMS, designated here as *Rfn* (14). The *nap* cytoplasm confers male sterility on a few exceptional varieties that lack *Rfn*, such as the cultivar "Bronowski" (13, 15). The male fertile "maintainer" strains of these varieties contain the fertile *cam* cytoplasm derived from the related species *Brassica campestris* (13, 16). Most *B. napus* varieties lack a restorer gene for *pol* CMS and, hence, are sterilized by *pol* cytoplasm (14). Restorer genes for *pol* CMS have been identified in various strains (14, 17) and map to a single nuclear locus designated *Rfp* (18). The relationship between the various *B. napus* cytoplasms, restorer genes, and male sterility or fertility are outlined in Table 1.

Analysis of mtDNA organization and expression in fertile, sterile, and nuclear restored lines has indicated that the *pol* CMS is likely to be specified by the *atp6* gene region (16, 19–21). In *pol* mtDNA, *atp6* is cotranscribed with a chimeric ORF, *orf224*. The *Rfp* restorer gene acts in a dominant manner to modify transcripts of the region (19, 20, 22). In *pol* CMS plants, dicistronic *orf224/atp6* transcripts predominate; in the presence of *Rfp*, monocistronic *atp6* transcripts predominate. Similar analyses have shown that *nap* CMS is correlated with expression of an mtDNA region containing a different chimeric ORF, *orf222*, that is cotranscribed with an exon of a trans-spliced gene, *nad5*c, and another ORF, *orf139* (23). Nuclear restoration of *nap* CMS has both quantitative and qualitative effects on *orf222/nad5*c*/orf139* transcripts. Unlike the ORFs associated with different forms of CMS in other plant species, *orf222* and *orf224* are highly similar in sequence over their entire length.

Abbreviations: CMS, cytoplasmic male sterility; RFLP, restriction fragment length polymorphism; cM, centimorgans.

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Table 1. Nuclear–cytoplasmic interactions in *Brassica napus*

Cytoplasm	Restorer genotype and fertility status		
	rfn, rfp	*Rfn, rfp*	*rfn, Rfp*
cam	Fertile	Fertile	Fertile
nap	Sterile	Fertile	Sterile
pol	Sterile	Sterile	Fertile

A survey of the transcripts detected by cloned DNAs comprising approximately 90% of the *Brassica* mitochondrial genome indicated that, as with all other nuclear restorer genes identified thus far, the effects of *Rfp* on mitochondrial gene expression appear to be restricted to a single mtDNA region (22). However, the recessive allele of this gene (*rfp*) is linked to a gene, *Mmt* (modifier of mitochondrial transcripts), that affects the transcripts of a pseudogene (*ccl1*-l; refs. 22 and 24) and the *nad4* gene (22). We now find *Mmt* (*rfp*) to be indistinguishable from a gene that is responsible for both the modification of *orf222*/*nad5*c/*orf139* transcripts and for nuclear restoration of *nap* CMS. This indicates that *Rfn* and *Rfp* are different alleles or haplotypes of a single, possibly complex, nuclear genetic locus. Mapping analysis using nuclear DNA markers linked to *Rfp* (18) is consistent with this possibility. In a survey of 50 different varieties of *B. napus* and related species, *Mmt (Rfn)* was found only in fertile lines with *nap* cytoplasm, suggesting that the sterile *nap* cytoplasm has provided the selection pressure for the origin and/or maintenance of the *Mmt(Rfn)* gene in *B. napus*.

MATERIALS AND METHODS

Plant Material. Fifty strains of nine different crucifer species (the three italic letters in parentheses indicate the cytoplasm) were used in the present study (sources are available on request). Of the *B. napus* strains, the *nap* CMS and maintainer strains Bronowski (*nap*) and Bronowski (*cam*), the *pol* CMS strains Karat (*pol*) and Westar (*pol*), the *pol* fertility-restored strains Italy (*pol*), UM2353 (*pol*), and Westar-Rf (*pol*), and the male fertile strains Karat (*nap*), Regent (*nap*), and Westar (*nap*) have all been described previously (16, 18, 19, 22). The additional male fertile *B. napus* strains analyzed for *nad4* transcripts, all of which possess the *nap* cytoplasm, were: AC Elect, Amazon, Arctik, Cyclone, Defender, Ebony, Falcon, GrGc 5–1, GrGc 5–2, Korell, LG3310, Pearl, and Topas. The male fertile *B. campestris* (synonymous with *B. rapa*) lines analyzed, all of which possess the *cam* cytoplasm, were: AC Parkland, AC Sunshine, Aiyou, Cash, CV2, Eldorodo, F6–15-8810, Goldrush, GrGc 1–9, Hysyn 110, Klondike, Reward, and SRS 753. The *B. oleracea* (*ole*) strains analyzed were: GrGc 3–8Y (fertile), GrGc 3–8W (nuclear male sterile), green cabbage, red cabbage, and broccoli. We also analyzed the *B. carinata* (*car*, fertile) strains Dodolla and GrGc 6–1, the *B. juncea* (*jun,* fertile) strains AC Vulcan, Commercial Brown, GrGc4–1, and IM41, the *B. nigra* (*nig,* fertile) strains AC Type 1 and GrGc 2–1, the *Sinapis alba* (*sin*, fertile) strain AC Pennant, and the *S. arvensis* (*arv,* fertile) strain SRS35.

Fertility Assessment. Plants were grown to maturity in the McGill University Phytotron under conditions of a 16-hr photoperiod and day/night temperatures of 20/15°C. Fertility was assessed by observing five flowers per plant, at least two times during the flowering period. The overall morphology of the flowers was noted as well as the production of pollen on anthers. Both *pol* and *nap* CMS anthers have shorter filaments and poorly developed or absent pollen sacs and produce little or no pollen. In addition, flowers of *pol* CMS often have dramatically shrunken petals. The morphological contrast between CMS and fertile flowers was great enough that, for all the populations examined, CMS and fertility-restored plants could be discriminated without ambiguity.

RNA Purification and Analysis. Mitochondrial RNA was isolated from flowers/inflorescences as described (23) except the LiCl precipitation step was omitted. RNAs were size-fragmented on agarose-urea gels, transferred to Gene Screen-Plus (DuPont) hybridization membranes in 10× SSC (1.5 M NaCl/0.15 M sodium citrate), and fixed by UV cross-linking. DNA probe labeling, RNA blot hybridization, and washes were performed as described (25). The clones used as probes were the from the coding regions of the *B. napus atp6* (19), *nad4* exon 2 (22), and *orf139* (23) genes of the mitochondrial genome of *B. campestris*.

DNA Purification and Restriction Fragment Length Polymorphism (RFLP) Analysis. DNA extraction, RFLP analysis, and cosegregation analysis by the MAPMAKER program were as described (18).

RESULTS

A Brassica Nuclear Genotype That Fails to Restore *nap* CMS Lacks *Mmt* and *Rfp*. A survey of *B. napus* varieties indicated that a *nap* CMS line and its (nuclear) isogenic maintainer strain, the cultivar Bronowski, both of which lack the *Rfn* gene, did not possess the *nad4* and *ccl1*-l transcript modifications conditioned by the *Mmt* gene. Because these modifications were found previously to be absent only in lines homozygous for *pol* restorer genes, we checked the ability of the *nap* CMS and maintainer lines to restore male fertility in the F1 in crosses with *pol* CMS lines. No fertility restoration was observed, and, hence, the genotype of these varieties is *mmt, rfp*/*mmt, rfp*, a configuration of genes in the Rfp-Mmt chromosomal region that had not been observed previously. Because these lines also lack the *nap* restorer gene *Rfn*, they possess recessive, maintainer alleles for both the *nap* and *pol* CMS systems. Therefore, we chose to designate their restorer genotype simply as *rf*/*rf*.

Allelism of *rfp* and *Mmt*. *nap* maintainer genotypes, by definition, lack *Rfn*. Because *Mmt* and *rfp* are linked and because the *nap* maintainer lacked both *Mmt* and *Rfp*, it seemed possible that the three genes, *Mmt, Rfp* and *Rfn*, might all reside at the same genetic locus. To test this possibility we examined allelism of the three genes through the analysis of two different types of genetic populations. We first examined the allelism of *rfp* and *Mmt* through the analysis of a test-cross involving the double-recessive genotype *mmt, rfp*/*mmt, rfp* (cross I of Table 2). This provides a much more powerful allelism test than the F2 populations we analyzed previously (22). To generate this population, a Westar *pol* CMS plant (*rfp, Mmt*/*rfp, Mmt*) first was fertilized with pollen from the near isogenic *pol* fertility restorer line Westar-Rf (*Rfp, mmt*/*Rfp, mmt)*. We recovered fertile individuals that contained *pol* cytoplasm and were highly homozygous except at the Rfp-Mmt locus.

One such plant then was test-crossed as a female with the male fertile *nap* maintainer line Bronowski (*rf, mmt*/*rf, mmt*). The resulting test-cross population was expected to contain approximately equal numbers of sterile (*rfp*/*rf*) and fertile (*Rfp*/*rf*) *pol* cytoplasm individuals. If *Mmt* and *rfp* act as the same allele, all fertile progeny would be expected to lack the 1.6-kb *nad4* transcript that defines the presence of the *Mmt* allele, and to possess the 1.4- and 1.3-kb *orf224*/*atp6 Rfp*-specific transcripts. In contrast, all sterile progeny should possess the 1.6-kb *nad4* transcript and lack the 1.4- and 1.3-kb *orf224*/*atp6* transcripts. The results obtained were entirely consistent with these predictions. Thirty-nine of the 75 test-cross progeny obtained were fertile, and all of these lacked the transcript, whereas 36 were sterile and possessed this transcript (cross I, Table 2). An example of the transcript analysis data is shown in the top two panels of Fig. 1. From these results we

Table 2. Genetic crosses used to assess allelism relationships among *rfp, Rfn*, and *Mmt* and the map position of *Rfn*

Cross	No. of plants		Ratio	*Mmt*-specific 1.6-kb *nad4* transcript detected in	
	Fertile	Sterile		Fertile, %	Sterile, %
pol cytoplasm					
I. Westar (*pol*) *Rfp, mmt/rfp, Mmt* × Bronowski (*cam*) *rf, mmt/rf, mmt*	39	36	1:1	0	100
Nap cytoplasm					
II. *nap* CMS* × Westar (*pol*) *Rfp, mmt/rfp, Mmt*	4	3	1:1	100	0
III. BC1: *nap* CMS × a fertile progeny from cross II	16	14	1:1	100	0
IV. BC2: *nap* CMS × a fertile progeny from cross III	17	17	1:1	100	0
V. BC2: *nap* CMS × [*nap* CMS × (*nap* CMS × Karat†)]	4	5	1:1	100	0
VI. BC1: *nap* CMS × (*nap* CMS × Karat)	18	10	1:1‡	ND	ND

ND, not determined.
**nap* CMS: male sterile Brownowski (*nap*) *rf, mmt/rf, mmt.*
†Genotype of the variety Karat: *Rfn, rfp, Mmt/Rfn, rfp, Mmt* (*nap* cytoplasm).
‡Deviance from the expected 1:1 ratio is not statistically significant ($\chi^2 = 1.725$).

estimate that, at a 95% confidence level, *rfp* and *Mmt* map no more than 3.6 cM from one another. Thus, *rfp* and *Mmt* appear to represent the same allele or haplotype of a single restorer locus.

Allelism of *Rfn*, *Mmt*, and *rfp*. To test for allelism between all three genes, it was necessary to generate *nap* cytoplasm populations in which the segregation of both *Rfn* and *Mmt* (*rfp*) could be observed. To achieve this, we first crossed a Westar (*Rfp/rfp*) individual as male to a *nap* CMS plant possessing the Bronowski nuclear genotype (*rf, mmt/rf, mmt*) and *nap* cytoplasm (cross II of Table 2). BC_1 and BC_2 *nap* cytoplasm populations then were generated by backcrossing fertile progeny as males with the *nap* CMS line (crosses III and IV of Table 2). If *Mmt (rfp)* were to act as *Rfn* and map to the *Rfn* locus, we would expect to obtain only two progeny classes in these populations and to obtain these in approximately equal numbers: fertile individuals heterozygous for both *Rfn* and *Mmt* and sterile individuals homozygous for *rf* and lacking *Mmt*. The results were completely consistent with these predictions: all fertile progeny possessed the 1.6-kb *nad4* transcript indicative of *Mmt*, whereas all the sterile progeny lacked this transcript. Partial transcript analysis data are shown in the third panel of Fig. 1. Similar results were obtained upon analysis of a second BC2 population in which a different *B. napus* variety, "Karat," was used as the source of *Rfn* (*Mmt*) (cross V, Table 2). Perfect cosegregation of *Rfn* and *Mmt* was observed in a total of 80 individual progeny of these crosses. Thus, within the limits of this analysis, *Rfn* is indistinguishable from *Mmt*.‖ Because the analysis of cross I indicated that *rfp* is indistinguishable from *Mmt*, the restorer genes for the *nap* and *pol* CMS systems map to a single nuclear genetic locus. The *nap* maintainer genotype

‖At a 95% confidence level, Z-test, *Rfn*, and *Mmt* map no more than 3.5 cM apart.

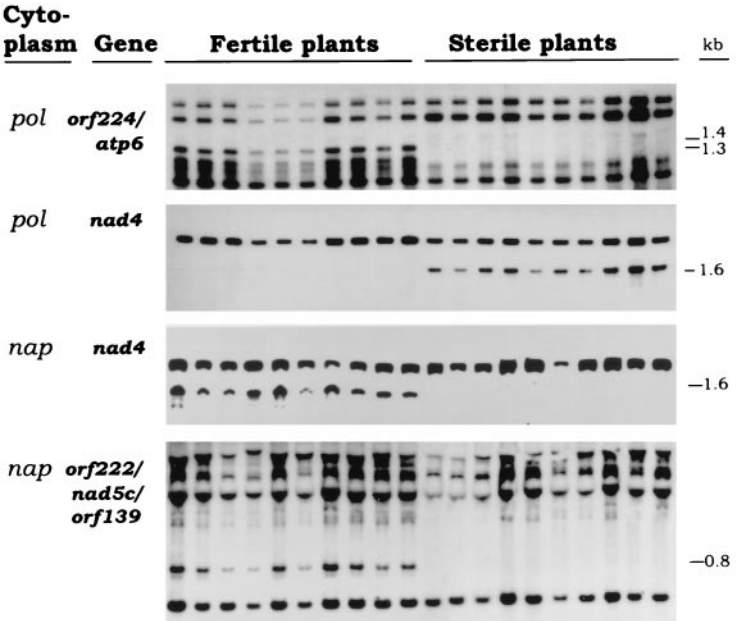

FIG. 1. RNA gel blot analysis of mitochondrial transcripts in test-cross or backcross populations segregating for male sterility and sterility restoration. For the top two panels, mtRNA samples from cross I progeny (Table 2) were probed with the *Brassica atp6* (top panel) or *nad4* (second panel) coding sequences to follow the segregation of *Rfp* and *Mmt*, respectively. For the bottom two panels, mtRNA samples from cross III progeny (Table 2) were probed with the *nad4* (top panel) or *orf139* (bottom panel) coding sequences. The sizes of transcripts specific to *Rfp (mmt)* plants (top panel) or *Rfn (Mmt)* plants (bottom three panels) are indicated to the right of the autoradiograms.

Bronowski lacks *Rfp*, *Rfn*, and *Mmt* and, hence, possesses a third allele of this locus, *rf*. The locus thus has three alleles or haplotypes: *Rfp (mmt)*, *Rfn (Mmt)*, and *rf (mmt)*.

***Rfp* and *Rfn* Map to the Same Site on the *B. napus* Genome.** Genetic mapping studies have provided further evidence that *Rfn* and *Rfp* map to the same locus. RFLP linkage analysis has shown that *Rfp* maps to *B. napus* linkage group 18 (18) [see Landry *et al.* (26)], between the markers 4ND7b and 4NB6. These mapping studies employed a *pol* cytoplasm BC1 population (termed the KW population) derived by crossing a *pol* CMS female with a restorer line to generate a fertile F_1 individual that was then used to pollinate the corresponding CMS parent. A similar strategy was used to map *Rfn*. The variety Karat (*rfp, Rfn*/*rfp, Rfn*), which served as the genotype of the CMS parent in the *Rfp* mapping cross, was used as the source of *Rfn*. Karat (*nap*) was crossed as male to the Bronowski *nap* CMS line, and the resulting F_1 was used to pollinate the *nap* CMS parent to generate a *nap* cytoplasm BC1 population segregating for *Rfn* (cross VI, Table 2). Eighteen male fertile and 10 male sterile plants were recovered; these progeny numbers fall within the statistically acceptable limits of the expected 1:1 pattern ($\chi^2 = 1.725$).

If *Rfp* and *Rfn* are indeed allelic, they will map to the same nuclear chromosomal locus. Because these genes are expected to be in repulsion, the polymorphic fragment detected by a linked RFLP probe that segregates with male fertility in *pol* cytoplasm crosses should be different from the fragment detected by the same probe that segregates with fertility in *nap* cytoplasm crosses. Thirteen linkage group 18 markers were tested on the cross VI population. Four of these proved to be monomorphic and could not be mapped. The map of *Rfn* and the nine remaining markers, all of which segregated in a 1:1 ratio, showed that *Rfn* occupies the same position on linkage group 18 as *Rfp*. The location of *Rfn* with respect to the six most closely flanking markers is shown in Fig. 2*A Right*. The position of *Rfp* with respect to the same set of markers, as derived from the analysis of the KW BC_1 *pol* cytoplasm population of Jean *et al.* (18), is shown in Fig. 2 *A Left*.

Fig. 2*B* shows the segregation of RFLPs detected by the marker 3NF2, which was mapped at 12.7 cM from *Rfp* in the KW BC1 population (18), in selected progeny from the *pol* and *nap* cytoplasm crosses used to map *Rfp* and *Rfn*, respectively. The 3NF2 probe detects polymorphic *Eco*RV fragments of 6.7, 10.0, and 10.2 kb specific to the nuclear genotypes of Karat, Westar-Rf, and Bronowski, respectively. Fig. 2 *B Upper* shows that in the *pol* cross used to map *Rfp*, where the genotype Karat was used as the CMS (*rfp*/*rfp*) parent, the 10.0-kb Westar-Rf-specific 3NF2 *Eco*RV fragment segregates with male fertility. In contrast, Fig. 2 *B Lower* shows that in the *nap* cross, where Karat was used to provide the *Rfn* restorer allele, the Karat-specific, 6.7-kb 3NF2 *Eco*RV fragment segregates with male fertility. This is consistent with the prediction that a single, linked probe will detect different polymorphic fragments segregating with male fertility in the two mapping populations. The RFLP mapping data therefore provide strong independent support for the view that *Rfn* and *Rfp* represent alternative alleles or haplotypes of a single nuclear locus.

***Rfn (Mmt)* Conditions Modification of Transcripts of the *nap* CMS-Associated Mitochondrial Gene Region.** Although previous studies have shown that transcripts of the *nap* CMS-associated *orf222*/*nad5*c/*orf139* mtDNA region differ between a restored and a *nap* CMS variety (23), it is not known whether these transcript differences result from the presence of the restorer gene *per se*. The availability of the cross II, III, IV, and V populations allowed us to assess the coinheritance of *orf222*/*nad5*c/*orf139* transcript modifications with *Rfn*-induced fertility restoration. *orf139*-specific probes detect a 0.8-kb transcript in fertility-restored *nap* cytoplasm plants that is not detected in *nap* CMS plants (23). This transcript was found in all the fertile progeny and in none of the sterile

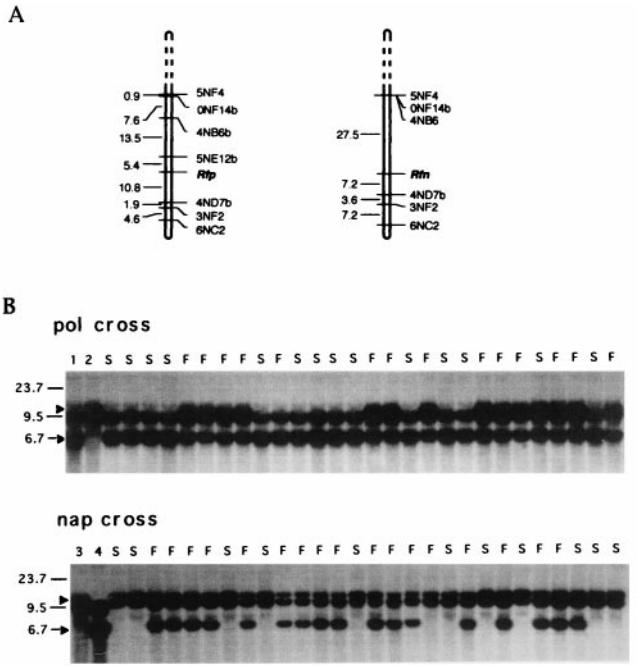

FIG. 2. RFLP mapping of *Rfp* and *Rfn*. (*A*) Maps of linkage group 18 in the vicinity of *Rfp* and *Rfn* constructed from backcross populations (described in *B*, below), which allowed segregation of *Rfp* (the *pol* cross, *Left*) and *Rfn* (the *nap* cross, *Right*) to be followed; note the similar map locations of the two restorer genes. The marker 5NE12b was not polymorphic in the *nap* cross and could not be mapped in this population. (*B*) A single RFLP marker detects alleles associated with both *Rfp* and *Rfn*. The RFLP marker 3NF2 (18) was used to probe *Eco*RV digests of genomic DNA from sterile (S) and fertile (F) individuals of two *B. napus* backcross populations. The *pol* cross allowed segregation of the *pol* restorer *Rfp* to be followed. It was generated first by crossing a Karat (*pol*) CMS plant (lane 1) with Westar-Rf (lane 2) and then crossing an F_1 individual with the Karat (*pol*) parent; this corresponds to the KW population of Jean *et al.* (18). The *nap* cross (cross VI of Table 2) allowed segregation of the *nap* restorer *Rfn* to be followed. The genotype Karat (lane 4), which served as the CMS parent in the *pol* cross, was used as the source of the *Rfn* gene in a cross to male sterile Bronowski (*nap*) (lane 3). Note that the 10.0-kb fragment specific to Westar-Rf segregates with male fertility restoration (F) in the *pol* cross, where Karat was used as the CMS parent, whereas the 6.7-kb fragment specific to Karat segregates with male fertility restoration in the *nap* cross, where Karat was used as the fertility restored parent. Because 3NF2 maps approximately 10 cM from *Rfp* and *Rfn*, segregation with male sterility in both cases is incomplete.

progeny of these crosses, indicating that *Rfn (Mmt)* does indeed condition the modification of transcripts of the *nap* CMS-associated mtDNA region. A representative portion of this analysis is shown in the bottom panel of Fig. 1. These results indicate that not only do different alleles of a single nuclear locus restore fertility to different male sterile cytoplasms but also that the same alleles of this locus are responsible for the modification of transcripts of the respective CMS-associated mitochondrial gene regions: *Rfp, rfn, mmt* for the *pol* mtDNA-specific *orf224*/*atp6* region, and *rfp, Rfn, Mmt* for the *nap* mtDNA-specific *orf222*/*nad5*c/*orf139* region.

***Rfn (Mmt)* Is Not a Common Gene in Most *Brassica* Species.** It is possible that new restorer genes are generated in response to selective pressure created by the spread of a male sterile cytoplasm in a plant population (27). If this were the case, a particular restorer gene might not be widely distributed in varieties and species in which the male sterile cytoplasm does not occur. Genetic testing for the presence of a particular restorer gene is time consuming and limited to the species in which the male sterile cytoplasm is available. With the excep-

tion of *Rfn*, the effects of restorer genes on mitochondrial gene expression thus far have been observed to be limited to male sterile cytoplasm-specific, CMS-associated mitochondrial gene regions. However, the apparent identity of *Rfn* and *Mmt* allowed us to easily test for the presence of *Rfn* in a relatively large number of strains by examining the transcripts of the *nad4* gene, which is not associated with CMS, for *Mmt*-specific modifications.

We used this strategy to survey a total of 50 different crucifer varieties for the presence of the *Mmt(Rfn)* (see *Materials and Methods*). These included 16 fertile and 1 sterile *B. napus nap* cytoplasm varieties, 3 fertile and 2 sterile *B. napus pol* cytoplasm varieties, 1 fertile *B. napus cam* cytoplasm variety, 1 male sterile and 4 male fertile *B. oleracea* varieties, and male fertile *B. carinata* (2 varieties), *B. juncea* (4 varieties), *B. nigra* (2 varieties), *B. rapa* (13 varieties), *Sinapis alba* (1 variety), and *Sinapis arvensis* (1 variety). Only the 16 male fertile lines of *B. napus* with *nap* cytoplasm possessed the additional *Mmt(Rfn)*-specific transcript (not shown). The data indicate that, apart from fertility-restored *nap* cytoplasm *B. napus* lines (the majority of *B. napus* varieties), *Mmt(Rfn)* is not widely distributed in *Brassica* and related genera.

DISCUSSION

Our results identify three different forms of a single *Brassica napus* nuclear genetic locus that we now designate as Rf-Mmt. One form, *Rfp(mmt)*, restores male fertility to plants with *pol* cytoplasm and modifies transcripts of the *pol* CMS-associated *orf224/atp6* gene region. A second form, *Rfn(Mmt)*, restores fertility to *nap* cytoplasm plants and modifies transcripts of the *nad4* gene, the *ccl1*-l pseudogene, and the *nap* CMS-associated *orf222/nad5*c/*orf139* gene region. A third form, *rf(mmt)*, is unable to restore fertility to either *pol* or *nap* cytoplasm plants and apparently is unable to condition any mitochondrial transcript alterations. *Rfp(mmt)* is dominant to *Rfn* with respect to *pol* restoration and modification of *orf224/atp6* transcripts, and *Rfn(Mmt)* is dominant to *Rfp(mmt)* with respect to *nap* restoration and modification of *orf222/nad5*c/*orf139*, *ccl1*-l and *nad4* transcripts.

In other plant species where more than one form of CMS is found, restorer genes for the different systems map to different nuclear genetic loci, and, in those cases where the locus is known to have effects on mitochondrial gene expression, these effects have been observed only on corresponding CMS-associated mitochondrial gene region. The Rf-Mmt locus therefore is novel in two respects: different forms of this single locus represent restorer genes for two distinct CMS systems, and one form of the locus, *Rfn(Mmt),* affects the transcripts of three different mitochondrial gene regions.

We have suggested previously that the transcript modifications conditioned by *Rfp* and *Mmt* may result from the selective destabilization of the 5′ termini of specific mitochondrial transcripts, and we proposed a model in which the specificity of these transcript modifications is conferred by the capacity of the corresponding gene products to recognize different but related hexanucleotide motifs (22). However, a sequence resembling the *Rfn(Mmt)* motif is not found in the *orf222/nad5*c/*orf139* region, and the effects *Rfn* has on this region are qualitatively quite different from the effects *Rfp* has on transcripts of the *orf224/atp6* region. Although it seems likely that *Rfn(Mmt)* acts to modify *orf222/nad5*c/*orf139* by mediating specific RNA processing events, the mechanism and/or recognition processes through which the these transcript modifications take place therefore may be more complex than was suggested initially.

The finding that *Rfn(Mmt)* is, within the limits of the varieties analyzed, found only in association with the *nap* cytoplasm suggests that the evolutionary appearance of the *nap* cytoplasm and the attending male sterility may have provided the selective pressure for the origin, and possibly the continued presence, of *Rfn (Mmt)* in *B. napus*. Similarly, *Rfp(mmt)* could have arisen as a variant of the same locus, with a slightly different mtRNA-processing specificity, in response to the appearance of *pol* cytoplasm. One or the other of these genes could have evolved as a neomorph or as a variant of yet another gene capable of influencing mitochondrial RNA-processing events.

It is possible that *Rfp(mmt)* and *Rfn(Mmt)* simply represent different alleles of a single gene. It also is possible, because of its multiple associated RNA processing and nuclear restoration properties, that the Rf-Mmt locus may be more complex and contain multiple, related, tightly linked genes. In this respect, it could resemble certain complex plant disease resistance loci for which the capacity to respond to a particular pathogen or chemical stimulus is conferred through a particular configuration of individual genes encoding related proteins (28, 29). If so, the evolution of new restorer genes in response to the appearance of new male sterile cytoplasms might occur via recombination within the locus, in the same manner that new resistance specificities have been proposed to arise in response to the appearance of new races of a pathogen (29, 30). The isolation and characterization of the various forms of Rf-Mmt therefore may provide insight not only into the mechanistic basis of fertility restoration, but also into the mechanisms by which these genes have evolved.

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