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Towards positional cloning in *Brassica napus*: generation and analysis of doubled haploid *B. rapa* possessing the *B. napus pol* CMS and *Rfp* nuclear restorer gene

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Abstract

The Polima (*pol*) system of cytoplasmic male sterility (CMS) and its fertility restorer gene *Rfp* are used in hybrid rapeseed production in *Brassica napus*. To facilitate map-based cloning of the *Rfp* gene, we have successfully transferred the *pol* cytoplasm and *Rfp* from the amphidiploid *B. napus* to the diploid species *B. rapa* and generated a doubled haploid *pol* cytoplasm *B. rapa* population that segregates for the *Rfp* gene. This was achieved through interspecific crosses, *in vitro* rescue of hybrid embryos, backcrosses, and microspore culture. Male fertility conditioned by *Rfp* was shown to co-segregate in this population with *Rfp*-specific mitochondrial transcript modifications and with DNA markers previously shown to be linked to *Rfp* in *B. napus*. The selfed-progeny of one doubled haploid plant were confirmed to be characteristic *B. rapa* diploids by cytogenetic analysis. Clones recovered from a genomic library derived from this plant line using the RFLP probe cRF1 fell into several distinct physical contigs, one of which contained *Rfp*-linked polymorphic restriction fragments detected by this probe. This indicates that chromosomal DNA segments anchored in the *Rfp* region can be recovered from this library and that the library may therefore prove to be a useful resource for the eventual isolation of the *Rfp* gene.

Abbreviations: CMS, cytoplasmic male sterility; DH, doubled haploid; h, hour; min, minute; kb, kilobase; PPR, pentatricopeptide; Rf, restorer of fertility

Introduction

For many organisms, map-based or positional cloning is the primary strategy for identifying and characterizing genes with unknown biochemical products. The complex genomes found in many crop plant species, however, render map-based cloning approaches difficult. For example, most segments of the *Arabidopsis* genome are represented multiple times in the genomes of the diploid

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Brassica species, a consequence of genome duplications and rearrangements that have occurred during the evolution of the *Brassicaceae* (Cavell *et al*., 1998; Lagercrantz, 1998; Lan *et al*., 2000). Despite this, several groups have recently used map-based cloning approaches to identify genes in *Raphanus*, which is expected to have a genome structure similar to that of the diploid *Brassica* (Brown *et al*., 2003; Desloire *et al*., 2003; Koizuka *et al*., 2003). Map-based cloning in the amphidiploid *Brassica* species such as *B. napus* is expected to be considerably more challenging, however, since these species arose through natural interspecific hybridizations of diploid *Brassicas*. Thus, if an average of three regions corresponding to each segment of the *Arabidopsis* genome are present in the diploid *Brassica*, approximately six distinct co-linear segments are expected to occur in the genomes of the amphidiploid species. The capacity to transfer genes among *Brassica* species through interspecific crosses (Williams and Hill, 1986) offers a potential means of circumventing the need for cloning directly from amphidiploid *Brassica*.

The trait of cytoplasmic male sterility (CMS) results from the expression of novel, often chimeric genes located in the mitochondrial genome. In many cases, the CMS phenotype can be suppressed by nuclear genes termed restorers of fertility (Rf), which usually act to down-regulate the expression of novel, CMS associated genes. Although CMS is widespread in flowering plants, the novel mitochondrial genes associated with the trait, are, in most cases, structurally unrelated. Several Rf genes have been cloned and most have been found to specify a protein with pentatricopeptide (PPR) repeats (Hanson and Bentolila, 2004). The gene family encoding PPR proteins is exceptionally large in plants, and most of these proteins are predicted to be targeted to the mitochondrion or plastid. PPR containing proteins in plants and fungi have been shown to post-transcriptionally regulate the expression of organelle genes (Small and Peeters, 2000; Hanson and Bentolila, 2004).

There are several novel features of the CMS trait in the oilseed rape species *Brassica napus*. The two CMS systems, *nap* and *pol*, that are native to this species are associated with novel mitochondrial genes that are highly similar in structure (L'Homme *et al*., 1997; Brown, 1999). In addition, unlike other species with more than one form of CMS, in *B. napus* the nuclear restorer genes for the *nap* and *pol* systems, *Rfn* and *Rfp*, respectively, map to the same nuclear locus belonging to the linkage group 18 of Landry et al. (1991), and are possibly alleles of one another (Li *et al*., 1998; Brown, 1999). Both genes act by mediating specific mitochondrial RNA processing events that decrease levels of their cognate, CMS-associated mitochondrial transcripts, but differ in their specificity: *Rfp* does not act on *orf222* transcripts and *Rfn* does not act on *orf224* transcripts (Li *et al*., 1998). Such considerations suggest that isolation and characterization of the *Rfp* locus and its counterpart in the *Rfn* haplotype could further our understanding of the specificity of restorer gene action and provide insight into the molecular features of the evolutionary processes through which new restorer genes appear in populations (Li *et al*., 1998; Brown, 1999; Budar *et al*., 2003).

Towards this goal, we report here the transfer of the *pol* CMS cytoplasm and the nuclear *Rfp* restorer gene from amphidiploid *B. napus* into a doubled haploid (DH) line of *Brassica rapa*, a diploid species. We show that the introduced *Rfp* locus maps to the same genomic region in both species and that fertility restoration in *B. rapa* is associated with the same *orf224* transcript processing pattern as in *B. napus*. Finally, we describe the construction of a cosmid library of the *B. rapa* line that is suitable for recovery and identification of the *Rfp* gene through map-based cloning.

Materials and methods

Plant materials

The *B. napus* line Westar-Rf (Singh and Brown, 1991) was used as the donor of the *pol* cytoplasm and the *Rfp* nuclear restorer gene. This line was originally obtained from Dr. Gerhard Rakow of Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan, and contains the *pol* cytoplasm and *Rfp* gene of the *B. napus* variety 'Italy' (Fang and McVetty, 1989). One *B. rapa* diploid line used in the interspecific crosses and in the backcrosses was a rapid cycling *B. rapa* developed by Wisconsin Fast Plants Inc. (Williams and Hill, 1986) and supplied by the Carolina Biological Supply Company, Burlington, North Carolina. Another line used in the introgression was CV2, a doubled haploid line of *B. rapa* canola (Ferrie and Keller, 1995).

Genetic crosses

Flowers were manually emasculated one to two days prior to the opening of the petals. The pistils of these flowers were manually pollinated, labeled and covered with waxed paper bags until the resultant seeds were harvested.

In vitro *rescue of interspecific hybrid embryos*

Thirty days after fertilizing the Westar-Rf line with pollen from rapid cycling *B. rapa*, the siliques were surface sterilized with 70% ethanol for 10 s and then transferred to a 1% hypochlorite solution for 10 min. The siliques were rinsed three times with autoclaved water under sterile conditions. Embryos (nearly aborted seeds) were removed from the siliques and placed on agar-solidified, half-strength MS medium (Murashige and Skoog, 1962) with or without 0.2 mg/l indoleacetic acid (IAA). The germinated seedlings from the rescued embryos were then placed in a mixture of soil and vermiculite and maintained in growth chambers with 15 °C/20 °C night/day temperature regime and a photoperiod of 16 h.

Microspore embryogenesis

Brassica rapa microspore embryogenesis was carried out as previously described (Ferrie and Keller, 1995) using sterile and fertile floral buds randomly collected from plants of the second backcross generation (BC2). The plantlets were treated with solution of 0.34% (w/v) cholchicine for 1.5 h before being planted to vermiculite potting mix in growth chambers. The plantlets in potting mix were partially covered with transparent plastic bags for the first three days to maintain high humidity.

Plant growth conditions

Parental plants and progeny were raised in growth chambers at 20 °C day, 15 °C night, with a 16-h photoperiod. The plants were watered once a day and fertilized once a week with a nitrogen–phosphate–potassium (NPK) solution. For the microspore-derived plantlets, the temperature regime was 15 °C during the day, 10 °C at night, with a 12-h photoperiod.

Cytogenetic analysis

Floral buds were fixed in Farmer's solution (3:1 of ethanol:acetic acid, v:v). Meiotic squashes were stained with acetocarmine for counting chromosome number and for evaluating meiotic behavior.

RFLP analysis

Total DNA of *Brassica rapa* plants was isolated by a modification (Li *et al*., 1996) of the method of Dellaporta *et al*. (1983). Restriction enzyme analysis, electrophoresis, blotting, DNA probe labeling, and Southern blot hybridization were as described (Jean *et al*., 1997). Co-segregation analyses were performed with Mapmaker v.2.01 (Lander *et al*., 1987). Linkage association and map distance were determined using a minimum LOD of 2.0.

Preparation and analysis of mitochondrial RNA

Mitochondrial RNA was prepared according to Singh and Brown (1991) from a mixture of flowers and young leaves. RNA electrophoresis using urea-agarose gels, blotting, probe labeling, and northern blot hybridization analysis were as previously described (Li *et al*., 1998).

Isolation of nuclei and nuclear DNA preparation

Nuclei were isolated as described by Olszewski *et al*. (1988), except that ethidium bromide was omitted from the isolation buffer. Purified nuclei were incubated at 55 °C for 2 h with Proteinase K (200 μg/ml), then extracted successively with equal volumes of phenol, phenol/chloroform, and chloroform. Each extraction was performed for 10 min at room temperature by gentle inversion of the tube. DNA was then precipitated by an addition of 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). The resulting DNA clot was spooled on a glass capillary, washed in 70% ethanol, dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and stored at 4 °C.

Cosmid library construction

Genomic DNA was partially cleaved with the restriction endonuclease *Taq*I and fractionated in a low-melting agarose gel (Cambrex BioScience,

Rockland, ME) using a pulsed-field gel electrophoresis apparatus (Amersham Biosciences, Piscataway, NJ). An agarose plug containing DNA of the desired size was excised from the gel and extracted using GELase (Epicentre, Madison WI) according to the manufacturer's recommendations. 200 ng of this insert DNA was then ligated with 1 μg of *Cla*I-digested dephosphorylated pOCA18 vector at 16 °C overnight using 100 units of T4 DNA ligase (New England Biolabs, Beverly MA). The ligated mixture was immediately packaged *in vitro* into phage lambda heads (Gigapack Gold, Stratagene, La Jolla, CA) according to the manufacturer's recommendations and the encapsidated products were used to transfect *E.coli* strain XL1-Blue MR (Stratagene, La Jolla, CA). Resulting colonies were counted and the titer of the primary library estimated.

Cosmid library screening

Approximately 200,000 cfu (colony forming units) of the primary library were plated on ten 128 mm nylon membrane filters (Hybond-N+, Amersham Biosciences, Piscataway NJ) which had been placed on agar plates; bacterial colonies were then grown on the filters at 37 °C for up to 24 h. Colony lifts and hybridizations were performed as described by Weiss (1999). Due to a large density of colonies it was not always possible to align positive signals on autoradiographs with corresponding single colonies on filters. When necessary, a small area of the nylon filter was excised, and placed in 1 ml of LB media on ice for 6 h. The resulting bacterial suspension was then subjected to secondary screening as described above except that 82 mm nylon filters were used. Occasionally, tertiary screening was necessary to obtain a pure clone.

Results

Transfer of pol cytoplasm and Rfp *to* B. rapa

To address the problem of using a positional cloning approach for the isolation and identification of genes from the complex, amphidiploid genome of *B. napus*, we first introduced the gene in question, *Rfp*, and the associated *pol* cytoplasm, into the diploid species *B. rapa* through three successive sexual crosses. We then generated a *pol* cytoplasm doubled haploid *B. rapa* population in which the *Rfp* gene was segregating, and selected a single, fertile plant homozygous for *Rfp* from this population to serve as a source of genomic DNA for construction of a library from which the *Rfp* gene could eventually be recovered.

To achieve the transfer of the *pol* cytoplasm and the *Rfp* restorer gene to *B. rapa*, Westar-Rf *B. napus* (genome: AACC; cytoplasm: *pol*; genotype: *Rfp*/*Rfp*) was crossed as the female parent with a rapid cycling *B. rapa* line (genome AA; cytoplasm: *cam*; genotype: *rfp*/*rfp*). The siliques resulting from this cross appeared severely shrunken and partially dehydrated through the fourth week post-fertilization. To rescue the hybrid embryos/seeds, the siliques were surface sterilized and the seeds were placed on agar-solidified, half-strength MS medium. Despite their abnormal appearance, the seeds of Westar-Rf x *B. rapa* germinated well on the medium (Figure 1: top panel). Plants from the rescued F1 embryos were planted and maintained in a growth chamber.

As a control for evaluating the male fertility of the hybrid progeny, crosses were also made between *pol* CMS *B. napus* Westar, which contains *pol* cytoplasm but lacks *Rfp*, and the *B. rapa* rapid cycling line. Ten interspecific F1 hybrid plants were recovered from this cross and all were male sterile, indicating there was no major restorer gene in the rapid cycling *B. rapa* line. In contrast, flowers of the interspecific F1 hybrid plants formed from the cross between Westar-Rf and *B. rapa* appeared normal and shed abundant pollen. These F1 interspecific hybrids were, however, partially female sterile – only 41 BC1 seeds were obtained from 397 floral buds fertilized with pollen of the same rapid cycling *B. rapa* line. By comparison, normal diploid or tetraploid *Brassica* plants produce about 20 seeds per flower. It was hypothesized that the female sterility of the hybrid plants was likely due to chromosomal aneuploidy.

The efficiency with which doubled-haploid (DH) *B. rapa* plants can be recovered through microspore culture is highly genotype dependent (Ferrie and Keller, 1995). To facilitate the generation of a DH *B. rapa* population that would segregate for *Rfp*, the three individual BC1 hybrid plants (Plants A, B, and C) that appeared most morphologically similar to the diploid *B. rapa* line (Figure 1, bottom) were therefore crossed as

Figure 1. Hybrid embryo rescue and plant morphology. Top panel: Germination of *in vitro* rescued interspecific hybrid embryos. Bottom panel: BC1 plants derived from [(Westar-Rf × *B. rapa*) × *B. rapa*] that were used to produce the BC2 population. The middle two plants were male fertile; and the plants on the two sides were male sterile. The female flowers on these plants were fertile, and the overall morphology and growth of the plants was normal.

females with the microspore culture-amenable doubled haploid *B. rapa* line CV2 (Ferrie and Keller, 1995). Like the rapid cycling line, CV2 has a *cam* cytoplasm and *rfp*/*rfp* restorer genotype. We experienced no difficulty in recovering seeds from these crosses, presumably because the three BC1 plants behaved essentially as diploids. For convenience we refer to the families (Lines A, B, and C, respectively for the three parental BC1 plants) derived from these crosses as BC2 generations, although this may depart from conventional usage, since the *B. rapa* parent was different from that used to obtain the BC1 generation. In a preliminary grow-out of Line B seeds, three fertile plants and three sterile plants were observed, confirming that there is no *Rfp* gene in the *B. rapa* line CV2 and that the BC1 plant indeed had the *Rfp*/*rfp* genotype. We then planted three to five seeds of each BC2 family (Lines A, B, and C,) all of which germinated and gave rise to adult, flowering plants. Line B was found to provide the best response to microspore culture and was subsequently used for microspore embryogenesis to develop a doubled haploid *pol* CMS *B. rapa* population that segregated for the *Rfp* restorer gene.

Because these plants were relatively small, it was necessary to sterilize nearly all their inflorescences before the floral buds opened and we were thus unable to determine their fertility characteristics.

Characteristics of the microspore-derived plant population

The plants derived from microspore embryogenesis of the BC2 *B. rapa* family varied in size. Most were smaller than 20 cm at the time of flowering, although a few grew to as tall as 60 cm. Of the 78 *in vitro* plantlets recovered, 59 survived transfer to planting and further cultivation in a growth chamber. Among these 59 plants, 40 were sufficiently robust as to allow flowering and the recovery of sufficient plant material to permit the isolation of mitochondria and nucleic acids for molecular analysis. Only the data concerning these plants is considered further.

The *pol* CMS phenotype in *B. napus* is characterized by flowers with small petals and stamens that do not grow to the height of the stigma. These CMS flowers possess small, pale anthers which can, in certain nuclear genetic backgrounds, shed limited amounts of pollen (Fan and Stefansson, 1986). Flowers of fertility restored plants, by contrast, have normal sized petals that are usually as large as those of the fertile maintainer line, stamens that are as high as or higher than the stigma, and deep yellow anthers that shed abundant pollen. Among the 40 microspore plants raised to maturity in the present study, 14 had normal sized petals, stamens that reached at least to the level of the stigma, and anthers that were normal in size and deep yellow. All these features imply the presence of the nuclear restorer gene *Rfp*. Among these 14 plants, 11 released large amounts of pollen but three failed to release any pollen. The male sterility observed among these morphologically ''fertility-restored'' plants is assumed to result from homozygosity for a recessive nuclear male sterile mutation that we have

observed in the rapid cycling *B. rapa* line and that we know acts independently of the *pol* cytoplasm (unpublished results). All 14 of the plants with morphologically normal flowers were therefore considered to possess the *Rfp* gene.

Among the same 40 plants, 26 had smaller petals and stamens, and of these, 14 shed no pollen while 12 had anthers that produced a small amount of pollen. This partially sterile phenotype is consistent with *pol* CMS in *B. napus* cv Westar and many other cultivars (Fan and Stefansson, 1986; Chuong *et al*., 1988; Fang and McVetty, 1989). Therefore, these 26 plants were considered to form a ''sterile group'' because they very likely lacked the major restorer gene *Rfp*. In summary, of the 40 plants analyzed at the morphological level, 14 were classified as likely having the nuclear restorer gene *Rfp*, and 26 were classified as sterile and unlikely to have the nuclear restorer.

If all the cultured microspores were derived from fertile BC2 plants (heterozygous genotype *Rfp*/*rfp*), equal numbers of fertile and sterile plants would be expected in the resulting doubled haploid (DH) population. In our experiments, however, the BC1 plant used to generate the BC2 population was heterozygous for *Rfp* gene, and hence the BC2 population was segregating for fertility. Since floral buds used in microspore culture were taken from the BC2 plants before the flowers opened, some of the parental plants for the DH population were expected to be sterile or partially sterile and lacked *Rfp*. High efficiency regeneration of microspore plants has previously been reported for haploid culture of *pol* CMS lines (Chuong *et al*., 1988), and therefore microspores from the partially sterile BC2 plants (without the major restorer gene *Rfp*) probably also regenerated to form doubled haploid plants. Consequently, it is not unexpected that the number of sterile plants in this population exceeds the number of fertile plants.

DNA marker analysis

Since the ultimate goal of this work was to generate a DH *B. rapa* line suitable for map-based cloning of *Rfp*, it was critical to determine if the gene had integrated into the chromosomal site that corresponds to its location in *B. napus*. To assess this, we analyzed the DH plants with the marker probe cRF1, which has previously been shown to be tightly linked to *Rfp* in *B. napus* (Jean *et al*., 1997; Li *et al*., 1998). Among the 40 microspore-derived plants, all 14 plants of the fertile group but only one (plant B40) of the 26 sterile plants carried the cRF1 RFLP tightly associated with the *Rfp* gene. The fertile-specific restriction fragment detected by this probe in the *B. napus* parental line and its segregation in a portion of the analyzed population is shown in Figure 2. It is noteworthy that the single sterile plant (B40) with the *Rfp*-linked polymorphism was only about 15 cm tall and produced few flowers, all of which were both male and female sterile; it was therefore judged to be a non-doubled haploid. These results show that the cRF1 RFLP co-segregated with fertility restoration in the DH population.

As shown in Figure 2, several other polymorphisms detected by the cRF1 probe were observed to segregate in the DH population. This is not unexpected, since coding sequence probes detect, on average, three different genetic loci in the diploid *Brassica* species. Similar observations were made for two other markers, 5NE12 and 4ND7 (data not shown), which have also been shown to be linked to *Rfp* in *B. napus* (Jean *et al*., 1997).

As shown in the genetic maps (Figure 3), the segregating polymorphisms detected by these three markers defined three independently segregating linkage sets. This suggests that sequences falling between the sites detected by probes 5NE12 and 4ND7 are represented at least three distinct sites in the *B. rapa* genome, only one of which corresponds to the region surrounding the *Rfp* gene. This finding indicates that there are multiple chromosomal regions spanning these three markers, and is consistent with the view that diploid *Brassica* genomes contain many large scale duplications, apparently as a result of ancient polyploidization events that occurred during the evolution of the *Brassicaceae*.

Rfp-*specific mitochondrial transcript modifications in* pol B. rapa

To determine if the *Rfp* gene that had been introgressed into *B. rapa* acts as it does in *B. napus* to modify transcripts of the *pol* CMS-associated mitochondrial gene *orf224*, we performed northern blot analysis of mitochondrial RNA from the parental lines and from 16 of the microspore-

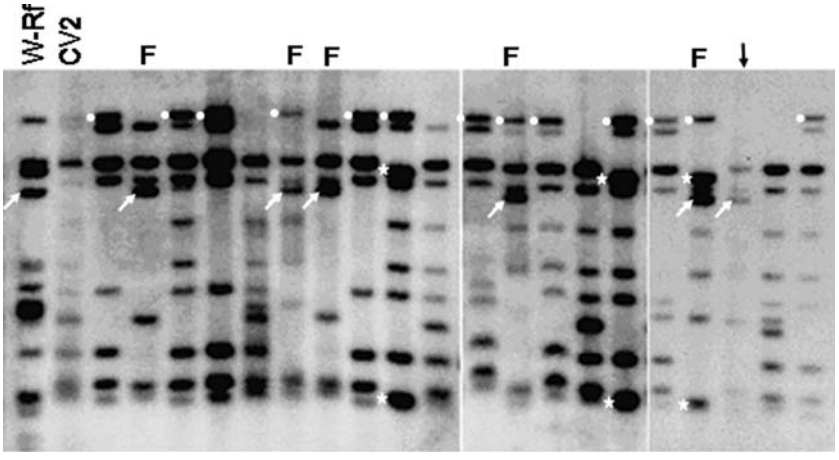

Figure 2. Southern blot analysis of the DH *B. rapa* population with *Rfp*-linked RFLP marker cRF1. W-Rf: Westar-Rf (*pol* cytoplasm, *B. napus*); CV2: a microspore-culture amenable *B. rapa*. Westar-Rf and CV2 are two parents for the DH *B. rapa* population. The white arrow indicates the polymorphic restriction fragment that co-segregates with male fertility restoration in *B. napus* as well as in the DH population. The downward pointing arrow indicates the *B. rapa* line B40, which contains the *Rfp*-linked cRF1 polymorphism but is both male and female sterile. Fragments corresponding to the segregating mapped polymorphic loci cRF1(2) and cRF1(3) are indicated by asterisks (*) and bullets (•), respectively. Genomic DNA was digested with *Hin*dIII.

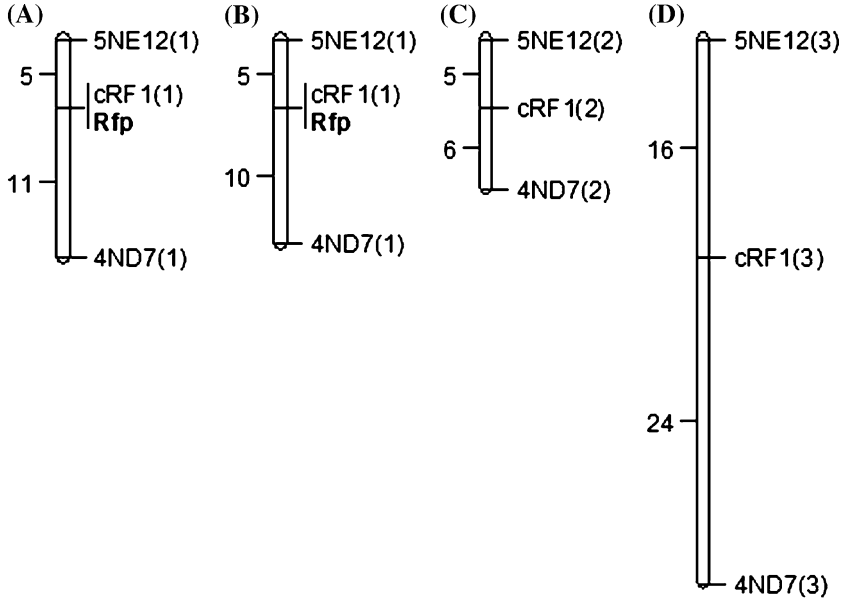

Figure 3. RFLP linkage maps of the *Rfp* gene region and homologous regions in *B. rapa*. (A) linkage map surrounding *Rfp* in *B. napus* (adapted from Jean *et al*., 1997). (B) linkage map of the *Rfp* region in the *B. rapa* DH population. Linkage maps C and D are also based on the *B. rapa* population and represent chromosomal regions that are paralogous to the *Rfp* region but that do not contain the *Rfp* gene. cRF1, 5NE12, and 4ND7 are marker probes used for mapping analysis.

derived plants. The *atp6* gene is located immediately downstream of *orf224* in *pol* CMS but not in *nap* or *cam* mitochondrial DNA (Singh and Brown 1991; L'Homme and Brown, 1993). In *pol* CMS plants, mitochondrial *atp6* probes detect transcripts of 2.2, 1.9 and 1.1 kb; in fertility restored plants, levels of the 2.2 and 1.9 transcripts are reduced and additional transcripts of 1.4 and 1.3 kb appear. In plants with *nap* or *cam* cytoplasm, only a single prominent *atp6* transcript of 1.1 kb is observed.

As shown in Figure 4 only a single prominent transcript of 1.1 kb was detected by the *atp6* probe in mtRNA of the rapid cycling and CV2 lines of *B. rapa*, as expected. The same *atp6* probe detected transcripts of 2.2, 1.9 and 1.1 kb characteristic of *pol* cytoplasm in all analyzed plants of the *B. rapa* DH population, indicating that, as expected, these plants all contain the *pol* CMS cytoplasm. Importantly, the same probe also detected the *Rfp*-specific transcripts of 1.3 kb and 1.4 kb in the *B. napus* Westar *pol*-Rf line and all seven of the analyzed male fertile microspore-derived plants. These two transcripts were also present in plant B40, the likely non-doubled haploid that also possessed the *Rf*- specific cRF1 polymorphism, but not in the *pol* CMS *B. napus* Westar parent (Menassa *et al*., 1999; data not shown) or any other male sterile plant of the DH population. These data indicate that the introgressed *Rfp* gene acts to modify *orf224*/*atp6* transcripts in *B. rapa* in the same manner as it does in *B. napus*.

Selection of doubled haploid lines for genomic library construction

Most *Brassica* chromosomal segments occur in multiple paralogous copies distributed at different sites in the genome; coding sequences are thus present in multiple genomic copies. It is therefore necessary to develop a strategy that allows clones to be identified as containing a genomic region of interest versus one or more closely related paralogous regions. One means of achieving this is to identify a clone contig representing the region of interest on the basis of the presence of a discriminating DNA marker tightly linked to the gene of interest (Brown *et al*., 2003). This strategy requires that the source plant for the genomic library be homozygous at virtually all genetic loci to avoid problems in contig assembly that arise from the presence of DNA polymorphisms between homologous regions.

The fertile DH lines of *pol* cytoplasm *B. rapa* are, by definition, homozygous at all loci and therefore ideal for the construction of such a library. In particular, the microspore-derived plant B8 appeared to be highly suitable for this purpose. This plant was nearly as large as the parent cultivar CV2, and was about three times taller than the rapid cycling lines. The plant carried the DNA marker cRF1 (Figures 2 and 3) and *Rfp*-specific mitochondrial transcript modifications (Figure 4). Cytological analysis of meiotic cells confirmed that the plant was diploid, having 10 bivalents

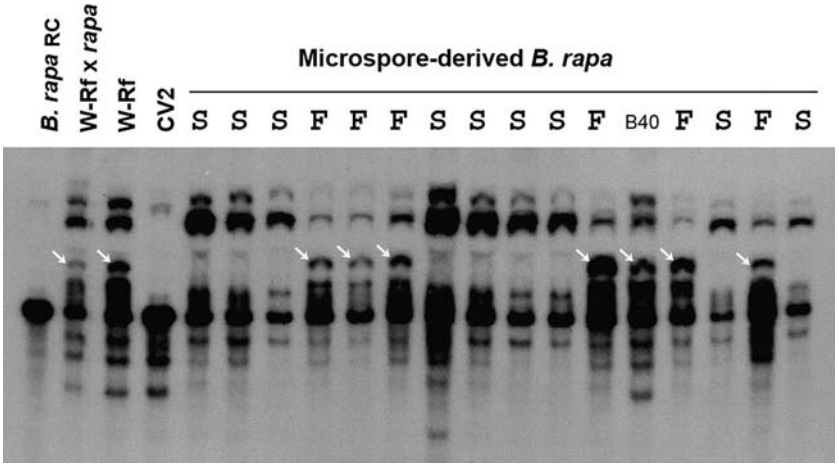

Figure 4. Northern hybridization analysis of mitochondrial transcripts of the DH population of *pol B. rapa* and its parental lines. *B. rapa* RC: the rapid cycling *B. rapa* line used as a male parent during the introgression of *pol* CMS and *Rfp* from Westar-Rf to *B. rapa*; W-Rf x *rapa*: a hybrid between Westar-Rf as the female parent and the *B. rapa* RC line as the male parent; W-Rf: Westar-Rf (*B. napus* line used as the source of the *pol* cytoplasm and *Rfp* gene); CV2: a microspore-culture amenable *B. rapa* line used as the male parent to generate parental plants of the DH population; S: male sterile DH plant; F: male fertile DH plant; B40: the *B. rapa* plant B40, which contains the *Rfp*-linked cRF1 polymorphism but is both male and female sterile. The blots were probed with the mitochondrial *atp6* gene. Both *B. rapa* parental lines possess *cam* cytoplasm and express only a single prominent *atp6* transcript of 1.1 kb (asterisk). The remaining plants carry *pol* cytoplasm. Arrows indicate the 1.4 kb transcript specific to fertility restored plants.

characteristic of *B. rapa* (Figure 5A) with 10 corresponding chromosomes in haploid cells of microspore tetrads (Figure 5B). Six selfed progeny of B8 were raised to maturity, and all were identical to one another and to B8 for both morphological traits and fertility. This phenotypic uniformity is in agreement with the expectation that the B8 plant and its selfed progeny, as a doubled haploid line, were genetically homozygous. B8 and its selfed progeny were therefore chosen for genomic library construction.

Construction and analysis of a transformation-competent cosmid library from DH Rfp/Rfp B. rapa

We chose the transformation-competent cosmid pOCA18 (Olszewski *et al*., 1988) as the vector to generate a genomic library suitable for cloning the *Rfp* gene from the DH *B. rapa* line B8. This vector can be used for *Agrobacterium*-mediated transformation of *B. napus* (Li *et al*., 1996, Brown *et al*., 2003); cosmid clones from the library can therefore be directly tested for the presence of *Rfp* based on its capacity to rescue the *pol* CMS phenotype. Genomic DNA for library construction was isolated from purified nuclei in order to minimize contamination with chloroplast and mitochondrial DNA. This nuclear DNA was partially digested with the restriction endonuclease *Taq*I and subjected to pulsed-field gel electrophoresis. Genomic DNA fragments in the range of 20–30 kb were excised from the gel and ligated into *Cla*I digested pOCA18. The resulting products were encapsidated *in vitro* into phage lambda heads to generate a primary genomic library of the B8 line (Materials and Methods) that contained 3.5×10^6 cosmids. The average size of inserted DNA in individual cosmids was found to be 21 kb. On the basis of an estimated size for the *B. rapa* genome of 500 Mb (Rana *et al*., 2004), each segment of the *B.rapa* genome should be represented >100 times in this library.

To further investigate the characteristics of the library, we screened *ca*. 200,000 colonies with the *Rfp*-linked probe cRF1. Seventeen colonies that reproducibly hybridized to this probe were recovered after secondary and tertiary cloning steps (see Materials and Methods). Restriction digests of the cloned cosmids were used to group these 14 clones into 4 physical contigs, each of which contained one or more *Hin*dIII restriction fragments of a size consistent with those observed in Southern blots of B8 DNA probed with cRF1 (Figure 6). Interestingly, limited sequence analysis of one clone from the *Rfp*-linked contig "f" (data not shown) indicates that it is co-linear with a region of the Arabidopsis genome that is distinct from that corresponding to the cloned radish restorer gene *Rfo*. Contig "f" corresponds to an *Arabidopsis* region located near coordinate 4.6 Mb on the short arm of chromosome 1. By mapping markers derived from the flanking *Arabidopsis* sequences in *B. napus* populations segregating for *Rfp* (Jean *et al*., 1997), we have been able to show that synteny between *Arabidopsis* and *Brassica* extends from this locus to as far as 1.8 Mb towards the closest *Arabidopsis* chromosome 1 telomere (data not shown).

Discussion

Despite difficulties such as seed abortion and the low degree of seed setting that were encountered

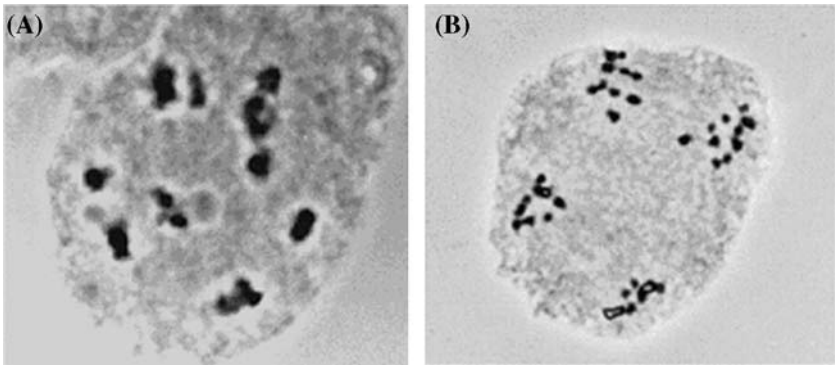

Figure 5. Chromosomes ($n=10$) of the *B. rapa pol Rfp* microspore plant B8. (A) A cell undergoing meiosis I; (B) A cell undergoing meiosis II.

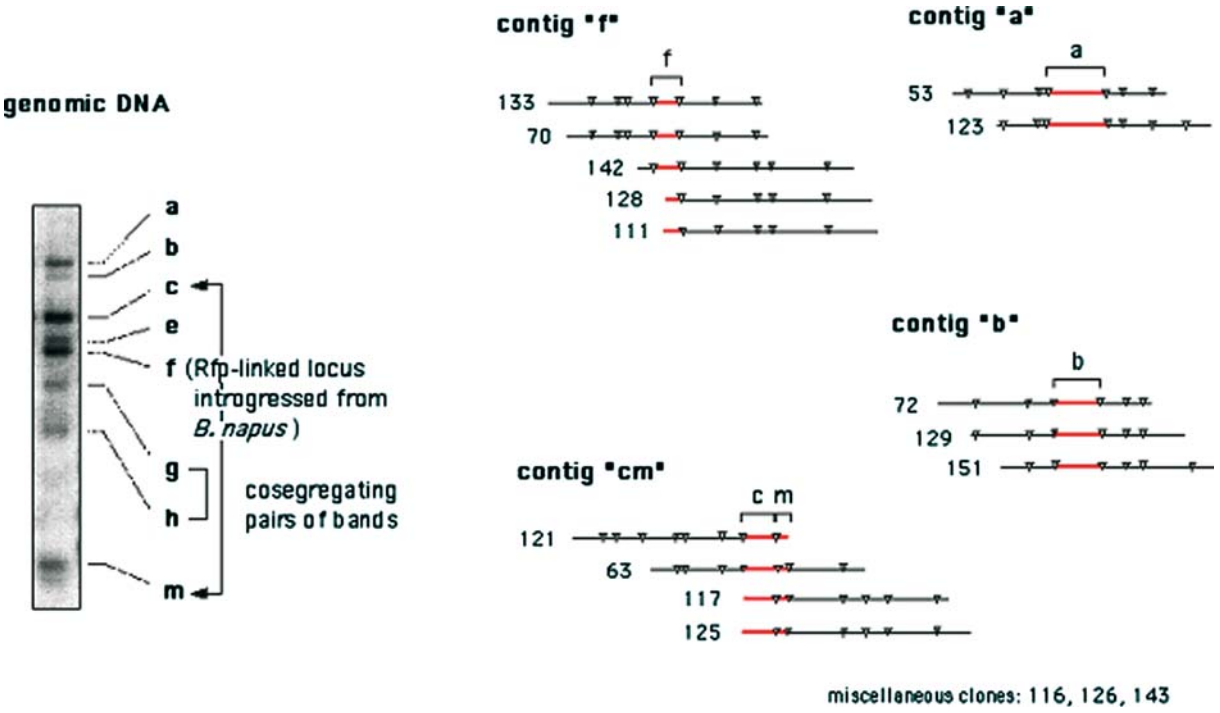

Figure 6. Cosmid clones recovered from the fertile DH line B8 using the *Rfp*-linked cRF1 RFLP probe. Left: Southern blot of *Hin*dIII-digested B8 DNA; different restriction fragments are designated by lower case letters, and co-segregating fragments are indicated. Right: *Hin*dIII restriction maps of recovered clones, with fragments hybridizing to the cRF1 probe indicated in red; clones are grouped into contigs on the basis of overlapping restriction maps which are designated according to the hybridizing fragments. Contigs f, a and cm correspond to the cRF1(1), cRF1(2) and cRF1(3) loci, respectively.

during the backcrossing process, we were able to successfully transfer the *pol* cytoplasm and the *Rfp* gene from *B. napus* into *B. rapa*. For the B8 line in particular, the chromosomes observed during both meiosis I and meiosis II were characteristic of those of a typical *B. rapa* plant with 10 haploid and 20 diploid chromosomes, respectively. In addition, the plant B of the BC1 family used to produce BC2 seeds was likely to be a normal diploid because the plant was fully fertile. Because the C genome represents only 50% of the *B. napus* parent (AACC) and because each backcrossing step eliminates more than 50% of the remaining non-recurrent parental genome following an interspecific cross (Grant, 1975), close to 100% of the new DH lines' genomes were expected to be of the AA type (*B. rapa*). It is possible, however, that some homeologous pairing may have taken place between the A and C genomes during meiosis of the F1 interspecific hybrid plants, which could have increased the C genome content of the DH lines beyond the expected values.

In *B. napus*, the *Rfp* gene functions to restore fertility by conditioning specific endonucleolytic cleavage events within transcripts of the *pol* CMS-associated gene *orf224* (Li *et al*., 1998; Menassa *et al*., 1999). These *Rfp*-mediated transcript modifications result in reduced *orf224* expression and can be most easily detected by probing with the *atp6* gene, with which *orf224* is co-transcribed (Singh and Brown, 1991; Menassa *et al*., 1999). The results presented here indicate that *Rfp* acts similarly in the genetic background of *B. rapa* and further support the notion that the mitochondrial *orf224*/*atp6* region is associated with the *pol* CMS (Singh and Brown, 1991; Witt *et al*., 1991; Handa and Nakajima, 1992; L'Homme and Brown, 1993; Wang *et al*., 1995). Verma and coworkers (Verma *et al*., 2000) have identified several *B. rapa* varieties that contain a single gene capable of fully restoring fertility to *pol* CMS *B. rapa*. It would be of interest to determine whether this gene maps to the same location as our introgressed *Rfp* locus and whether it has similar effects on transcripts of the *orf224*/*atp6* mitochondrial gene region.

Both genetic and physical mapping experiments indicated that the cRF1 marker sequence detects

multiple loci in *B. rapa*. This was not unexpected, since there are several paralogous copies of most chromosomal regions in diploid *Brassica* and since the probe hybridized to a relatively large number of restriction fragments in Southern blots with genomic DNA. The degree of sequence divergence among these paralogous DNA segments is adequate to generate sufficient dissimilarity in the restriction digestion patterns of the selected clones to allow the different copies to be clearly distinguished from each other and to allow their grouping into distinct contigs.

The clones selected by the cRF1 probe could be grouped on the basis of overlapping restriction digestion patterns into four physical contigs. It is likely that additional cRF1 paralogs exist in the B8 genome since not all the restriction fragments detected in Southern blots were present in these four contigs. It is possible that one or more such copies may be represented in the "singleton" clones selected by the probe that did not group into a contig. The contig comprising the largest number of clones contained a polymorphic *Hin*dIII restriction fragment (fragment f, Figure 6) that closely co-segregates with *Rfp* in the DH *B. rapa* population and in two *B. napus* BC1 populations in which the *Rfp* gene segregates (Jean *et al.*, 1997). This contig is therefore anchored in the *Rfp* genomic region and may represent a starting point for the isolation of this gene through chromosome walking strategies (e.g. Brown *et al.*, 2003).

Physical mapping and blot hybridization experiments indicate that the cRF1 sequence resides in at least 4 distinct loci in the B8 plant that served as the source of the genomic library. Only three loci that span the linked markers 5NE12, cRF1 and 4ND7, however, were revealed through segregation analysis of the DH population. Several factors could contribute to this discrepancy. First, it should, in principle, be possible to recover all chromosomal loci containing the cRF1 by screening a sufficiently large number of genomic clones. To show genetic linkage between a given cRF1 locus and flanking loci such as 4ND7 and 5NE12, however, it is necessary that the particular cRF1 locus as well as the flanking loci all possess detectable polymorphisms that segregate in the DH population. Although the level of polymorphism in the DH population was high, at least one monomorphic band was seen with each probe, and these could represent additional unmapped loci. In addition, while most portions of the *Arabidopsis* genome appear to reside at multiple, usually three, co-linear sites in diploid *Brassica* genomes, the *Brassica* counterparts often lack one or more of the genes found in the co-linear *Arabidopsis* segment (O'Neil and Bancroft, 2000; Rana *et al.*, 2004). Thus it is possible that the 5NE12 or 4ND7 sequences might be missing in one or more of the co-linear homeologous segments surrounding cRF1 sequences in *B. rapa*.

In conclusion, we have successfully transferred the *pol* CMS system and the RFLP marker linkage group for *Rfp* gene from the amphidiploid *B. napus* into the diploid *B. rapa*. Further, we have shown that the transferred *Rfp* gene functions to restore male fertility and modify mitochondrial transcripts in a *B. rapa* genetic background in the same manner as in *B. napus*. In particular we have generated a homozygous doubled haploid *B. rapa* line containing the *pol* cytoplasm and its restorer, and we have generated a chromosomal library from this line from which it should be possible to clone the *Rfp* gene through genetic and physical mapping approaches similar to those used to clone the *Rfo* gene from radish (Brown *et al.*, 2003). It should be possible to extend this approach to facilitate map-based cloning of other genes from amphidiploid *Brassica* species. It should be noted that many major crop species, such as wheat, have polyploid genomes. The process of introgressing genes into diploid relatives or progenitors may therefore be widely applicable for the cloning of agriculturally important genes from polyploid crop species.

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