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Chapter 9
Brassica Ogu-INRA Cytoplasmic Male Sterility: An Example of Successful Plant Somatic Fusion for Hybrid Seed Production

Georges Pelletier and Francoise Budar

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9.1 Introduction

The concept of hybrid vigor has gradually emerged since the eighteenth century when J.G. Koelreuter observed individual plants resulting from interspecific crosses in various genera, such as *Nicotiana, Dianthus, Verbascum, Mirabilis,* and *Datura* (Zirckle 1952). These observations were confirmed a century later by Darwin (1876) in vegetables, and by Beal (1880) in maize. In 1914, Schull coined the term "heterosis" to define this physiological state of vigor of plants produced from crosses between parental lines that originate from sufficiently different genetic pools (Schull 1952). Relatively high heterosis has been reported as a general feature of F_1 hybrids in *Brassica* species: up to 80 % higher seed production compared to the mid-parent value in winter oilseed rape (*Brassica napus*; Lefort-Buson and Dattée 1982), 90 % in Indian mustard (*Brassica juncea*; Pradhan et al. 1993), and 60 % in summer turnip rape (*Brassica rapa*; Falk et al. 1994).

However, it is only relatively recently that breeders have been able to take advantage of heterosis for plant breeding. In the case of plants that reproduce by seed, obtaining large amounts of F_1 hybrid seed requires controlling cross-fertilization on a very large scale. The best way to do so is to grow large female stocks that will be pollinated by a male parent and harvest F_1 seed from these stocks. Female stocks can be obtained mechanically in monoecious species and sometimes by chemical means in hermaphrodite species. However, the most efficient system involves maternally determined male sterility. Jones and Clarke described the first male-sterility system used for the production of hybrid varieties in onion (*Allium cepa*; Jones and Clarke 1943). In the following decades, similar systems were developed in a wide range of species such as sorghum (*Sorghum bicolor*), sugar beet (*Beta vulgaris*), corn (*Zea mays*), sunflower (*Helianthus annuus*), rice (*Oryza sativa*), carrot (*Daucus carota*), etc.

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Studies on heterosis in *Brassica* have stimulated research on male-sterility systems, and they have greatly benefited from a wide range of biotechnological approaches such as interspecific/intergeneric crosses, in vitro culture, somatic hybridization, and genetic engineering. Only a few cases of spontaneous cytoplasmic male sterility (CMS) have been described in *Brassica* species: in *B. rapa* (Chinese cabbage; Okhawa and Shiga 1981) and in *B. napus* (rapeseed) with the "Polima" (*pol*) system (Fu 1981) and the "Shaan 2A" (*nap*) system (Li 1986 communication in Chinese cited in Fu and Yang 1995).

The first CMS system described in an open-pollinated radish (*Raphanus sativus*) cultivar (Ogura 1968) has since been reported to be widespread in wild radish populations in Japan (Yamagishi and Terachi 1994a, 1996) and in *Raphanus raphanistrum* in Asia as well as in Europe (Yamagishi and Terachi 1997; Giancola et al. 2007). This CMS therefore predates the divergence between the two radish species.

9.2 Somatic Hybridization in Brassicaceae for the Manipulation of Sex

9.2.1 Transfer of the Ogura Radish Sterility-inducing Cytoplasm into Brassica

In 1974, Bannerot et al. introduced cytoplasmic male-sterile radish cytoplasm into *Brassica oleracea* (cabbage) using interspecific crosses and in vitro embryo culture (Bannerot et al. 1974; Yamagishi and Terachi 1994a, 1996), followed by several backcrosses with *B. oleracea* as the male parent until the *B. oleracea* genome (nine chromosomes) was stabilized in the Ogura radish cytoplasm (Fig. 9.1). Unfortunately, the resulting male-sterile plants with a complete *Brassica* genome in an Ogura radish cytoplasmic background showed chlorophyll deficiency, which was particularly expressed at low temperatures, and therefore rendered this combination unsuitable for commercial hybrid variety production (Fig. 9.2). Moreover, *Brassica* plants possessing this cytoplasm had underdeveloped nectaries, with reduced nectar production and malformed ovaries and pods (Fig. 9.3). Therefore, their flowers were not attractive to honeybees, the natural pollinators needed to ensure hybrid seed production. Female fertility, in terms of seed set, of these initial Ogura CMS *B. oleracea* plants was therefore reduced.

Furthermore, hybridization of these Ogura CMS *B. oleracea* plants with a *B. napus* male parent resulted, after cytoplasm transfer, in an Ogura CMS *B. napus* that showed the same range of defects (Pelletier et al. 1983).

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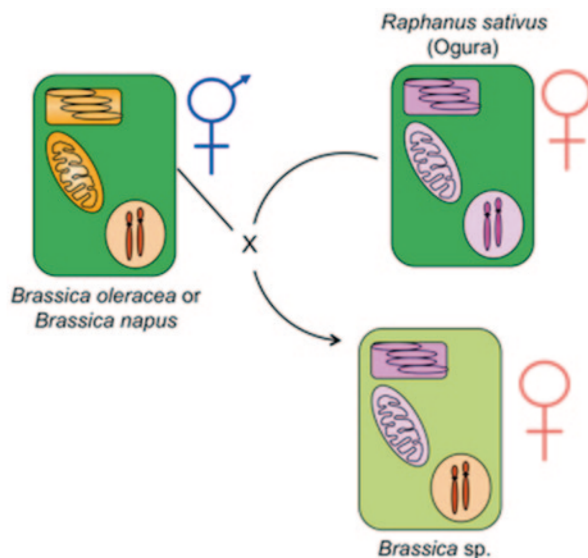

Fig. 9.1 An interspecific cross between an Ogura male-sterile radish with pollen of *Brassica* sp. followed by a series of backcrosses to *Brassica,* resulted in a male-sterile *Brassica* with Ogura radish plastids and mitochondria

Fig. 9.2 Chlorophyll deficiency (Y) observed in *Brassica* plants with (Ogura) radish cytoplasm, compared to normal plants (G)

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62 63 ### *9.2.2 Somatic Hybridization for Eliminating Ogura Cytoplasm Defects*

64 The defects in chloroplast biogenesis and floral morphology in *Brassica* plants with
65 the Ogura cytoplasm were assumed to result from negative interactions between the
66 *Raphanus* cytoplasm and the *Brassica* nucleus. For eliminating them, protoplasts

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Fig. 9.3 Malformed pods observed in *Brassica* plants with (Ogura) radish cytoplasm

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from a normal *B. napus* line (Table 9.1) were fused with protoplasts from a CMS (Ogura radish cytoplasm) *B. napus,* and protoplasts from a normal *B. oleracea* line were fused with protoplasts from a CMS (Ogura radish cytoplasm) *B. oleracea* in two independent experiments (Pelletier et al. 1983, 1988).

In parallel, an atrazine-resistant *B. rapa* (previously known as *B. campestris*) biotype was discovered in Canada (Maltais and Bouchard 1978). This resistance is encoded by plastid DNA. Alloplasmic *B. napus* lines with *B. rapa* atrazine-resistant cytoplasm have been produced and have shown to be fully resistant to the herbicide (Beversdorf et al. 1980). Material carrying this plastid marker trait was instrumental in protoplast fusion experiments for selecting cybrids that had successfully received this particular *Brassica* type of chloroplasts.

9.2.3 Experimental Procedure for Brassica Protoplast Isolation and Fusion

To isolate protoplasts for cybrid production, seeds of parental lines were surface-sterilized and then germinated in vitro. Shoot tip cultures were generated and cultured on the same medium (medium A, see Table 9.2) under a 16-h photoperiod, 150 $\mu mol.m^{-2}.s^{-1}$ light intensity, and 24°C, and repeatedly subcultured at 3-4-week intervals. Protoplasts were then isolated from scarified leaf fragments from these axenic in vitro plants by overnight maceration in 0.2% cellulase R10 and 0.1%

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Table 9.1 Recombination of cytoplasmic-associated traits in cybrids

Genotype	Cytoplasm	Nucleus	Parents of the fusion[a]	Associated traits
1	*B. napus*	*B. napus*	–	Male fertile, green leaves, normal flowers, atrazine susceptible
2	*B. oleracea*	*B. oleracea*	–	Male fertile, green leaves, normal flowers, atrazine susceptible
3	*B. rapa atr*^{R}	*B. napus*	–	Male fertile, green leaves, normal flowers, atrazine tolerant
4	*R. sativus*	*B. oleracea*	–	Male sterile, yellow leaves, abnormal flowers,[c] atrazine susceptible
5	Cybrid 58	*B. napus*	1+4[b]	Male sterile, green leaves, normal flowers, atrazine susceptible
6	Cybrid 17	*B. oleracea*	2+4	Male sterile, green leaves, normal flowers, atrazine susceptible
7	Cybrid 77	*B. napus*	3+4	Male sterile, green leaves, abnormal flowers, atrazine tolerant
8	Cybrid 13	*B. napus*	1+7	Unstable male sterile,[d] green leaves, normal flowers, atrazine susceptible
9	Cybrid 18	*B. napus*	1+7	Unstable male fertile,[d] green leaves, normal flowers, atrazine tolerant

R. sativus Raphanus sativus, B. napus Brassica napus, B. oleracea Brassica oleracea, B. rapa atr^R *Brassica rapa atr*^{R}
[a] When applicable
[b] Numbers refer to column 1
[c] Abnormal flowers means no nectar and or malformed ovaries (see Fig. 9.3)
[d] Cybrids 13 and 18 produced sexual progeny and somatic segregation of fully male-sterile and fully male-fertile plants, hence their phenotype was considered unstable

Table 9.2 The various media used in vitro for protoplast, tissue, and plant culture

Medium	Composition
A	MS medium, 10 g.l^{-1} glucose, 10 g.l^{-1} sucrose, 10 mg.l^{-1} gentamicin sulfate, 8 g.l^{-1} agar
B	Gamborg (B5) medium, 20 g.l^{-1} glucose, 70 g.l^{-1} mannitol, 1 mg.l^{-1} NAA, 1 mg.l^{-1} BA, O.25 mg.l^{-1} 2,4D, 10 mg.l^{-1} Tween 80
C	Half-strength B5 medium plus 200 mg.l^{-1} NH_4NO_3, 20 g.l^{-1} sucrose, 40 g.l^{-1} mannitol, 0.2 mg.l^{-1} NAA, 1 mg.l^{-1} BA
D	Half-strength B5 macro salts, MS microelements, 20 g.l^{-1} sucrose, 1 mg.l^{-1} 2,4D, 30 mg.l^{-1} adenine sulfate
E	MS medium, 10 g.l^{-1} sucrose, 20 g.l^{-1} mannitol, 1 mg.l^{-1} NAA, 1 mg.l^{-1} IPA, 0.02 mg.l^{-1} GA3, 8 g.l^{-1} agarose
F	MS medium, 10 g.l^{-1} sucrose, 0.1 mg.l^{-1} NAA, 0.5 mg.l^{-1} BA, 8 g.l^{-1} agarose
G	Half-strength MS medium, 10 g.l^{-1} glucose, 0.01 mg.l^{-1} NAA, 8 g.l^{-1} agar; pH adjusted to 5.8 before autoclaving

MS Murashige and Skoog 1962, B5, (Gamborg et al. 1968), *NAA* naphthalene acetic acid, *BA* benzyl aminopurine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *IPA*, N^6 -(Δ^2 isopentenyl)-adenine, *GA*$_3$ gibberellic acid

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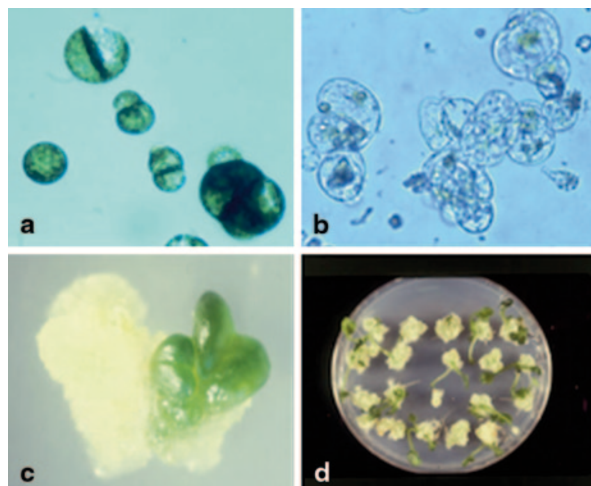

Fig. 9.4 **a** *Brassica* protoplast fusion, **b** cell colony formation, **c** derived callus with bud regeneration, and **d** bud growth into plantlets

pectolyase Y23 in culture medium B (Table 9.2). After washing in 2.5 % KCl, 0.2 % $CaCl_2.2H_2O$, protoplasts were fused (Fig. 9.4a) in drops of 25 % polyethylene glycol 6000 in 3 % $CaCl_2.2H_2O$ and subsequently further diluted directly in the petri dish with culture medium B to a cell density of $5 \times 10^4.ml^{-1}$.

A succession of different media was used (shown in Table 9.2) to stimulate the growth of protoplast-formed colonies and bud regeneration (Fig. 9.4b c, d). Protoplasts were grown in the dark for 3 days after fusion and then transferred to an illuminated growth chamber, then diluted on day 12 with an equal volume of medium C, and then with medium D on day 20. On day 30, colonies were plated onto medium E and emerging buds were placed onto medium F; after shoots developed, they were rooted on medium G.

No nucleus or cytoplasm inactivation is needed before protoplast fusion because the purpose was not to obtain a new combination of nuclei and cytoplasm but to allow genetic recombination between the two species' cytoplasms, as discovered previously in *Nicotiana tabacum* (Belliard et al. 1978, 1979).

AQ1

9.2.4 Selection, Characterization, and Plastid Identification of Cybrids

Selection was carried out on regenerated plants by screening for new combinations of cytoplasmic traits that resulted from fusion events, as described in Table 9.1. More specifically, from the combination by protoplast fusion of *B. napus* or *B. oleracea* cytoplasms with Ogura *R. sativus* cytoplasm, cybrids were selected to have not only normal greening of leaves at low temperature but also well-formed male-sterile flowers. These cybrids were promising for developing male-sterile lines for F_1 hybrid seed production.

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110 *Raphanus* and *Brassica* plastid DNAs are easily distinguished by their restric-
111 tion patterns (Vedel et al. 1982; Yamagishi and Terachi 1997; Giancola et al. 2007)
112 and the selected green, male-sterile plants contained only the *B. napus, B. rapa,* or
113 *B. oleracea* chloroplast genomes (e.g., see cybrids 58, 17, 77 in Table 9.1). These
114 results demonstrate that the chlorophyll deficiency in parental male-sterile *Brassica*
115 lines was due to a detrimental interaction between *Raphanus* plastids and *Brassica*
116 nuclei.

117 The plastid genomes of cybrids were identical to one parental genome in the
118 case of *B. napus/B. rapa* cytoplasmic hybridization (e.g., cybrid 18 has *B. rapa*
119 plastids and cybrid 13 has *B. napus* plastids; both were derived from the same fu-
120 sion described in Table 9.1). Harboring one or the other parental plastid at random
121 is a general feature of cybrid plants, indicating that the heteroplasmic state is very
122 transitory. Nevertheless, in the cases where Ogura *Raphanus* plastids and *Brassica*
123 plastids were combined by fusion in a *Brassica* nuclear background, the plastid
124 type in recovered cybrids was always that of *Brassica,* indicating strong selection
125 pressure against Ogura *Raphanus* plastids, which could result from the incompat-
126 ible nucleo-plastid interaction observed in plant development (i.e., the dramatically
127 reduced chlorophyll content).

128 The progeny of cybrids resulting from the combination of *B. napus* or *B. olera-*
129 *cea* cytoplasm with Ogura *R. sativus* cytoplasm shows complete stability of chlo-
130 roplastic traits, i.e., normally green leaves and male sterility in subsequent sexual
131 generations, indicating that the genetic determinants of the reverse traits (i.e., chlo-
132 rophyll deficiency and male fertility) are lost after the cytoplasms are combined in
133 protoplast fusion, and before the development of reproductive organs. Moreover,
134 regarding flower morphology, abundant nectar production was recovered to some
135 extent in cybrids 58 and 17 (and several others) but not in cybrid 77 (and several
136 others), indicating that nectar production is not correlated with the plastid genome
137 as is the case for leaf chlorophyll content.

138 Similar experiments on Ogura male sterility were performed by other laborato-
139 ries in receipt of plant material obtained by Bannerot et al. in *B. napus* (Menczel
140 et al. 1987; Jarl and Bornman 1988; Jarl et al. 1989;), *B. oleracea* (Kao et al. 1992;
141 Walters et al. 1992; Walters and Earle 1993), and *B. juncea* (Kirti et al. 1995). When
142 successful, these improved Ogura cytoplasms were introduced into other species
143 such as *B. rapa* and *B. juncea* (Delourme et al. 1994) and vegetable *B. rapa* (Heath
144 et al. 1994) via sexual crosses to produce male-sterile lines.

145 Protoplast fusions have also been used to transfer, or modify, other sterility-in-
146 ducing cytoplasms into *Brassica,* such as the Kosena radish cytoplasm, subsequent-
147 ly shown to be a variant of the Ogura cytoplasm, in *B. napus* (Sakai and Imamura
148 1990, 1992), the "Anand" *B. rapa* cytoplasm in *B. oleracea* (Cardi and Earle 1997),
149 the "polima" *B. napus* cytoplasm in *B. oleracea* (Yarrow et al. 1990), *Diplotaxis*
150 *muralis* cytoplasm in *B. juncea* (Chatterjee et al. 1988), *Moricandia arvensis* cyto-
151 plasm in *B. juncea* (Prakash et al. 1998), and *Brassica tournefortii* cytoplasm in *B.*
152 *napus* (Liu et al. 1995).

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9.3 Molecular Analyses of Cybrid Mitochondrial Genomes

9.3.1 Mitochondrial Genomes of Cybrids

Restriction profiles of cybrid and parental mitochondrial DNAs have been compared (Chetrit et al. 1985). As observed in tobacco, each cybrid profile is unique and different from those of the parents (except cybrid 77, which is apparently identical to the Ogura radish mitochondrial profile). In these restriction profiles, there are some parental restriction fragments and some new ones, a signature of molecular exchanges and recombination between the two parental genomes.

The fact that cybrid mitochondrial genomes are recombined is valuable in two ways. First, the male-sterile determinant originating from the Ogura radish can be segregated from undesirable traits that likely result from a detrimental interaction between radish mitochondria and *Brassica* nuclei, such as low nectar production and abnormal flower development. Second, it opened up the possibility of associating a specific DNA sequence with the male-sterile trait, this sequence being present in sterile cybrids and absent or modified in fertile ones. The molecular characterization of genomic fragments originating from the radish mitochondrial genome that were linked to undesirable traits, and the identification of the male-sterility mitochondrial gene (see below) allowed the definition and patenting of the Ogu-INRA system in *Brassica* crops.

9.3.2 Identification of the Ogura CMS Gene

CMS-associated genes are most often unconserved mitochondrial open reading frames (ORFs) created by intragenomic recombination (Hanson 1991; Braun et al. 1992; Kubo et al. 2011). They have a chimeric structure in which parts of normal mitochondrial genes can be recognized (Hanson and Bentolila 2004). The Brassicaceae family may be the one that has benefitted the most from efforts to identify mitochondrial male-sterility genes, because at least five different CMS systems have been studied at the molecular level: "Ogura" (Bonhomme et al. 1991), *pol* (L'Homme and Brown 1993), *nap* (L'Homme et al. 1997), *tour* (Landgren et al. 1996), and *Mori* (Ashutosh et al. 2008).

The mitochondrial determinant for Ogura-derived male sterility was one of the first CMS genes identified. It was discovered by studying *Brassica* cybrids obtained via the protoplast fusion experiments listed in Table 9.1. The first set of fusion experiments gave *B. napus* and *B. oleracea* cybrid cytotypes, which were used in a second series of fusion experiments involving cybrids and parental lines ("back-fusion"; Pelletier et al. 1986). Cybrid 13, derived from such an experiment, provided the material for the molecular characterization of the sterility determinant. This cybrid was selected as male-sterile, but it repeatedly reverted to male fertility

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191 with constant minor variation in mitochondrial DNA restriction profiles. It was
192 therefore possible to correlate a mitochondrial DNA region with the male-sterile
193 trait and a DNA sequence (*orf138*) whose expression at the RNA level is strictly
194 correlated with the sterile phenotype of *B. napus* and *B. oleracea* cybrids (Bon-
195 homme et al. 1991, 1992). Expression of this mitochondrial gene was subsequently
196 associated with the sterile phenotype of the original "Ogura" radish (Krishnasamy
197 and Makaroff 1993). Interestingly, *orf125,* a gene very similar to *orf138,* has been
198 identified in the mitochondrial genome of the Kosena radish and assumed to be
199 responsible for male sterility in this cytoplasm (Iwabuchi et al. 1999). Furthermore,
200 Kosena CMS is very likely a variant form of Ogura CMS, and *orf125* a sequence
201 variant of *orf138* (Koizuka et al. 2000; Yamagishi and Terachi, 2001).

202 *9.3.3 Contribution of New Recombined Genomic Arrangements* 203 *to Our Understanding of Mitochondrial Gene Expression*

204 *orf138* is a chimeric gene. The end of its coding sequence and the 3′ flanking region
205 are identical (70 nucleotides including the last 12 codons) to the 3′ untranslated re-
206 gion of *Arabidopsis thaliana orf557* (*ccb206*) that likely encodes a protein thought
207 to be involved in cytochrome c biogenesis (Bellaoui et al. 1998). The *B. napus*
208 *orf577* gene, homologous to the bacterial *ccl1* gene, is very similar to *A. thaliana*
209 *orf557* and has in its 3′ untranslated region the same short and perfect homology to
210 *orf138* (Menassa et al. 1997; Handa et al. 1996).

211 The *orf138* gene is co-transcribed with a gene called *orfB,* conserved among
212 plant mitochondrial genomes, and which has been demonstrated to correspond to
213 the subunit 8 of the ATP synthase complex (Gray et al. 1998; Sabar et al. 2003). The
214 bi-cistronic structure of the locus is responsible for the stability of the CMS trait in
215 the Ogura radish and in stable cybrids. Instability has been observed in cybrids har-
216 boring a second copy of the *orfB* gene, introduced through back-fusion to normal *B.*
217 *napus* and derived from the *B. napus* mitochondrial genome, or in cybrids in which
218 the *orf138* and *orfB* genes are no longer associated and the *orf138* gene is expressed
219 as a monocistron, such as male-sterile plants derived from the unstable cybrid 18
220 (Bonhomme et al. 1991, 1992; Grelon et al. 1994; Bellaoui et al. 1998).

221 The unstable cybrid 13 not only helped identify the sterility gene but also shed
222 light on the importance of posttranscriptional events such as degradation/stabiliza-
223 tion of RNA in plant mitochondrial gene expression (Binder and Brennicke 2003).
224 Indeed, in fertile revertants of cybrid 13 (13F plants), the *orf138* gene is present but
225 found upstream of the *atpA* gene instead of the *orfB* gene, due to recombination
226 that deleted the Ogura radish *orfB* gene. As mentioned above, these plants carry
227 a *B. napus orfB* gene, unlinked to *orf138* that compensated this loss. In this new
228 genomic organization, the *orf138* gene is no longer expressed (no detectable level
229 of mRNA or protein), although it is transcribed as efficiently as in the sterile cybrid
230 13 plants because the transcript is rapidly degraded due to the lack of the stabilizing
231 sequences found in the *orfB* 3′ UTR (Bonhomme et al. 1992; Bellaoui et al. 1997).

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232 In contrast, male-sterile plants derived from cybrid 18, which carry a monocistron-
233 ic *orf138* with very efficient stabilizing sequences in its 3′UTR, over-accumulate
234 *orf138* mRNA and the ORF138 protein (Grelon et al. 1994; Bellaoui et al. 1997).
235 The *orf125* gene from Kosena radish is present in the same genomic organization
236 as the *orf138* gene and is co-transcribed with *orfB* (Iwabuchi et al. 1999). Iwabuchi
237 et al. (1999) also described a monocistronic *orf125* genomic organization in a sterile
238 *B. napus* cybrid.

239 *9.3.4 Deciphering the Mechanism of CMS in Brassica Cybrids*

240 The ORF138 protein is bound to the inner mitochondrial membrane and forms
241 oligomers (Grelon et al. 1994; Krishnasamy and Makaroff 1994; Duroc et al. 2005).
242 It is present in all plant organs. In *B. napus* cybrids, its accumulation appears to
243 follow the abundance of normal mitochondrial proteins in the different organs of
244 the plant (Bellaoui et al. 1999). This result is not very surprising given that *orf138*
245 expression is mediated by the promoter and posttranscriptional expression signals
246 that drive the expression of the *orfB* gene, which encodes a subunit of the ATP
247 synthase complex. However, as in many other CMS systems, it raises an intriguing
248 question: Why is the phenotypic consequence of ORF138 accumulation restricted
249 to pollen development?
250 In the tapetal cells of sterile anthers, mitochondria swell and lose their internal
251 membrane structure at the tetrad stage (González-Melendi et al. 2008). This leads to
252 premature degeneration of the tapetum and subsequent abortion of developing pol-
253 len. ORF138 forms an apparently homo-oligomeric complex in the inner membrane
254 of mitochondria (Duroc et al. 2005, 2009). In contrast to what has been shown for
255 G-CMS in beet (Ducos et al., 2001), sunflower CMS (Sabar et al. 2003), and WA-
256 CMS in rice (Luo et al. 2013), ORF138 does not interfere with complexes of the
257 electron transport chain (Duroc et al. 2009). Nevertheless, measurements of oxygen
258 consumption on whole anthers and on mitochondria isolated from buds suggested
259 that mitochondria are partially uncoupled in sterile reproductive organs, whereas no
260 sign of decoupling is detected in vegetative tissues (Duroc et al. 2009).

261 9.4 The Restoration of Fertility in Ogura-related Systems

262 *9.4.1 The Genetics of Restoration in Ogura and Ogu-INRA*
263 *Plants*

264 Restorer genes are generally dominant, except for the unique homozygous lethal
265 restorer *rfl1* of maize, which was obtained by mutagenesis (Wen et al. 2003). De-
266 terminism is monogenic or oligogenic according to the male-sterility-inducing cy-
267 toplasm. In the case of the Ogura radish system, the genetics of restoration differs

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268 among crosses, suggesting that there are several redundant restoration loci in radish
269 nuclear genotypes (Yamagishi and Terachi 1994b). In some cases, a single domi-
270 nant restorer is sufficient to completely restore fertility of radish plants with the
271 Ogura cytoplasm.

272 A restored *B. napus* has been obtained after crossing an alloplasmic rapeseed
273 possessing the Ogura cytoplasm with a *Raphano-brassica* hybrid from an interge-
274 neric cross, and subsequent chromosome doubling. The genetics of restoration in
275 rapeseed carrying the unmodified Ogura cytoplasm is complex, involving several
276 loci of which one is linked to a gene involved in petal color: white-flowered plants,
277 a *Raphanus*-based character, were more fertile than yellow-flowered plants. Inter-
278 estingly, the restoration of rapeseed cybrids with a corrected phenotype involves a
279 unique locus, unlinked to petal color (Pelletier et al. 1986). The obtained restorer
280 rapeseed genotypes show a unique radish introgression that carries the restoration
281 locus, named *Rfo* (Delourme and Eber 1992; Delourme et al. 1998).

282 Two complementary genes were found to be necessary for restoring the Kosena
283 radish CMS, whereas only one of them, named *Rfk1* or *Rfk,* is sufficient for the
284 restoration of *B. napus* cybrids (Koizuka et al. 2000). This gene was subsequently
285 found to be identical to *Rfo* (see below).

286 9.4.2 Effect of Restorer Nuclear Background on the Expression 287 of the Ogura CMS Mitochondrial Gene

288 In the vast majority of CMS systems, fertility restoration acts via the inhibition of
289 male-sterility gene expression, most often at the RNA level, and sometimes at the
290 protein level (Budar and Pelletier 2001; Budar et al. 2003). The influence of a res-
291 toration gene on the expression of a mitochondrial chimeric gene is sometimes the
292 only evidence of the implication of this mitochondrial gene in the sterile phenotype
293 (Kubo et al. 2011).

294 Nuclear restorers have a dramatic effect on the accumulation of the ORF138
295 protein in buds and leaves in Ogura radish (Krishnasamy and Makaroff 1994). In
296 Ogu-INRA *B. napus,* restoration is characterized by the decrease of ORF138 ac-
297 cumulation in anthers and its complete depletion from the tapetum (Bellaoui et al.
298 1999; Uyttewaal et al. 2008). In both species, the accumulation of *orf138* mRNA
299 is not affected by the nuclear restorer gene indicating that the restorer acts either
300 on mRNA translation efficiency or on ORF138 protein stability (Krishnasamy and
301 Makaroff 1994; Bellaoui et al. 1999; Uyttewaal et al. 2008).

302 The *orf125* gene has an expression profile in Kosena CMS plants that is similar
303 to that of the *orf138* gene in Ogura radish or Ogu-INRA Brassicas (Iwabuchi et al.
304 1999). In addition, as in the Ogura radish CMS or the Ogu-INRA *Brassica* cybrids,
305 the restorer nuclear background induces a strong decrease in ORF125 protein ac-
306 cumulation, but does not affect the accumulation of *orf125* mRNA, either in Kosena
307 radish or *B. napus* cybrids (Iwabuchi et al. 1999).

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9.4.3 Identification of the Male-Fertility Restorer Gene

Rfo and *Rfk,* which are in fact the same gene, were independently identified by fine genetic mapping in radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). This restorer gene encodes a pentatricopeptide repeat (PPR) protein which has been further characterized in a restored *B. napus* cybrid (Uyttewaal et al. 2008). The restoring protein interacts with *orf138* mRNA, and probably impairs its translation, thus preventing the deleterious effects of the ORF138 protein on tapetal cells.

9.5 The Use of the Ogu-INRA Hybridization System for Seed Production in Brassica Crops

Cabbage and rapeseed cybrids with the Ogura sterility determinant and corrected phenotypes have been evaluated in regard to their seed production and attractiveness to pollinators. These studies helped select two to three recombined cytoplasms in each crop, which were then provided to breeders in the early 1990s.

In cabbage, only the vegetative parts of the plant are of agricultural interest; therefore, there was no need to introduce the restorer gene in hybrids. The first cabbage F_1 cultivars using the Ogu-INRA system were registered in the official French seed catalogue in 1993. They display ~40% of heterosis, giving yields of around 200 tons per hectare (sauerkraut cabbage). In 1999, the French catalogue listed 65 F_1 cultivars of different cabbage types that have been obtained using the Ogu-INRA CMS system.

The emergence of restored rapeseed hybrids was impaired by undesirable traits linked to the introgression of the radish *Rfo* gene, such as high glucosinolates, poor female fertility, and poor genetic stability of the restorer rapeseed genotypes. Nevertheless, cultivars containing a mixture of female hybrid plants and fertile plants from one of the parental lines in a ~85:15% ratio (line–hybrid composite, LHC) led to the registration of the first rapeseed hybrid cultivar (Synergy) in 1994. However, this type of cultivar may induce losses in yield when temperatures are unfavorable during the pollination period, which limits pollinator visits.

Considering the opposition of European authorities to transgenic rapeseed and the very high cost of complying with regulations, it is not possible to use restored genotypes in which only the radish *Rfo* gene has been introduced via transformation in Europe, even though this would eliminate all the developmental defects. This is an example of regulation inconsistencies and society's general attitude towards genetically modified organisms because the same gene (together with many other unknown genes) is already present in restored hybrid rapeseed cultivars with introgressed radish genes for male-fertility restoration.

An intense breeding effort to produce restorer lines has led to an improved R113 restorer line, followed by R2000 (Primard-Brisset et al. 2005). The first restored hybrid cultivar was authorized in 1999 in France. In 2002, 14% of rapeseed crops

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347 were planted with restored hybrids versus 8% with LHC. The improvement of re-
348 storers has completely supplanted LHC cultivars. The introduction of a dwarf mu-
349 tation in one of the hybrid parents allowed the development of half-dwarf hybrid
350 cultivars in the mid-2000s.
351 Plant material has been distributed without exclusivity to a large number of seed
352 companies in the world and license fees represent only a small percentage of the
353 commercial price of seed. This material is widely used in *Brassica* vegetables, but
354 much more in rapeseed. According to our calculations, in 2009, 5.4×10^6 ha were
355 planted with varieties containing the Ogu-INRA cytoplasm worldwide. Considering
356 that the total rapeseed crop surface area of the main continents that use this system
357 (Europe, North America, Australia) covers $\sim 15 \times 10^6$ ha, we estimate that the cyto-
358 plasms resulting from somatic hybridization are found in approximately one third
359 of the rapeseed crops grown in these countries.

360 9.6 Conclusion

361 The main way of creating new CMS systems is to associate the nucleus of one spe-
362 cies with the cytoplasm of a different origin, e.g., another species. This new associa-
363 tion can be achieved either by crosses and backcrosses with the nuclear donor or by
364 somatic cell genetics (cybrid production). The first method leads to several sources
365 of CMS, which remain generally underexploited due to the multiple interactions be-
366 tween the cytoplasmic genes and the (foreign) nucleus with negative consequences
367 on plant productivity. Cybrid production can result in improved CMS systems, due
368 to plastid exchange and mitochondrial recombination.
369 More generally, in the absence of a method for transforming mitochondria in
370 higher plants, the only possibility for manipulating the mitochondrial genome lies
371 in cybrid production by protoplast fusion, which can be considered to be "cyto-
372 plasm breeding." This requires expertise in plant protoplast culture, which is be-
373 coming regrettably increasingly rare in today's plant biology laboratories.
374 Outside the family of Brassicaceae, examples of cybrid production for agricul-
375 tural purposes can be found in tobacco (Horlow et al. 1993), rice (Akagi et al. 1995),
376 or *Citrus* species (Bassene et al. 2008), illustrating how cellular and molecular biol-
377 ogy can contribute significantly to plant breeding.

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AQ1. “Belliard et al. 1978” is cited in the text but is not given in the reference list. Please provide a full reference.

AQ2. Please update the following references: “Bannerot et al. 1974” and “Delourme et al. 1994”.

AQ3. Please provide the volume details for reference “Beal 1880”.