RESEARCH ARTICLE

Homeotic-like modification of stamens to petals is associated with aberrant mitochondrial gene expression in cytoplasmic male sterile Ogura *Brassica juncea*

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Abstract

We have previously reported correction of severe leaf chlorosis in the cytoplasmic male sterile Ogura (also called Ogu) *Brassica juncea* line carrying Ogura cytoplasm by plastid substitution via protoplast fusion. Two cybrids obtained from the fusion experiment, Og1 and Og2, were green and carried the plastid genome of *B. juncea* cv. RLM198. While Og1 displayed normal flower morphology comparable to that of its euplasmic *B. juncea* counterpart except for sterile anthers, Og2 retained homeotic-like floral modification of stamens to petal-like structures and several other floral deformities observed in the chlorotic (Ogu) *B. juncea* cv. RLM198 (or OgRLM). With respect to the mitochondrial genome, Og1 showed 81% genetic similarity to the fertile cultivar RLM while Og2 showed 93% similarity to OgRLM. In spite of recombination and rearrangements in the mitochondrial genomes in the cybrids, expression patterns of 10 out of 11 mitochondrial genes were similar in all the three CMS lines; the only exception was *atp6*, whose expression was altered. While Og1 showed normal *atp6* transcript similar to that in RLM, in Og2 and OgRLM weak expression of a longer transcript was detected. These results suggest that the homeotic-like changes in floral patterning leading to petaloid stamens in Og2 and OgRLM may be associated with aberrant mitochondrial gene expression.

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Introduction

A spontaneous cytoplasmic male sterile (CMS) mutant was identified in wild *Raphanus sativus* (Ogura 1968), and was later used to construct CMS lines of *Brassica oleracea, B. napus* (Bannerot *et al*. 1974) and *B. juncea* (Kirti *et al*. 1995a). While Ogu cytoplasm caused arrest of microspore development in *R. sativus*, in *B. napus* flower morphology was influenced by temperature. At high temperature (23–28°C) normal anthers were produced but microspore development was affected (Polowick and Sawhney 1987), while at low temperature (15–18°C) stamens were converted into carpelloid structures (Polowick and Sawhney 1991). In *B. juncea* also, Ogu cytoplasm induced numerous floral abnormalities like petaloid anthers, short and stumpy crooked style, reduced nectaries, low female fertility and severe leaf chlorosis (Kirti *et al*. 1995a,b). These abnormalities were corrected through somatic hybridization in *B. napus* (Pelletier *et al*. 1983) and *B. juncea* (Kirti *et al*. 1995a,b). The CMS trait in the (Ogu) *B. napus* cybrids was found to be linked to the presence of a 2.5-kb *Nco*I (Nco2.5) region in the mitochondrial genome encoding three open reading frames; a $tRNA^{fmet}$, a novel transcript (ORF138) coding for a 138-amino-acid polypeptide, and ORF158 (ORFB or *atp8*). The ORF138 and ORFB were transcribed as a single, bicistronic 1400-nucleotide (nt) CMS-specific transcript (Bonhomme *et al*. 1991, 1992). The novel chimeric ORF138 transcript translated into a membrane protein (Grelon *et al*. 1994), which associated with the inner mitochondrial membrane (Duroc *et al*. 2005). Also, CMS in (Ogu) *R. sativus*

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is linked to the ORFB locus, which harboured the novel ORF138 along with ORFB in the male sterile plants. However, ORFB transcript was identified even in normal plants, confirming that ORF138 was responsible for male sterility in wild (Ogu) *R. sativus* (Krishnasamy and Makaroff 1993). Nuclear restoration of fertility was accompanied by a dramatic reduction in the amount of ORF138 protein in mitochondria of flowers and leaves but not of roots of (Ogu) *R. sativus* (Krishnasamy and Makaroff 1994) and in reproductive organs like anthers of (Ogu) *B. napus* (Bellaoui *et al* 1999).

While male sterility in Ogu CMS lines has been linked to expression of ORF138, the molecular basis for other floral abnormalities observed in *B. napus* or *B. juncea* has not been investigated. We have developed two chlorosis-corrected Ogu CMS lines through somatic hybridization between (Ogu) *B. juncea* cv. RLM198 (or OgRLM) and euplasmic *B. juncea* cv. RLM198 (or RLM) (Kirti *et al*. 1995a,b). While one of the CMS cybrid lines, Og1, displayed normal flower morphology comparable to that of its euplasmic *B. juncea* counterpart, except for sterile anthers, the other, Og2, retained homeotic-like floral modification of stamens to petal-like structures and several other floral deformities observed in the chlorotic OgRLM. Availability of these two isonuclear cybrids with contrasting flower morphology prompted us to examine the changes in their mitochondrial genomes, which would presumably reveal the molecular basis of these differences.

In this investigation, we have studied the organization and expression of mitochondrial genomes in the cybrids and their parents through RFLP and Northern blot analyses. Despite several changes detected in mitochondrial genome organization, the transcript pattern differed only for *atp*6 in the two cybrid lines, out of the 11 mitochondrial genes whose expression was analysed. This study points to the possible role of mitochondrial gene expression in floral patterning in *B. juncea*.

Materials and methods

Plant material

Three CMS lines, OgRLM, Og1 and Og2, and one euplasmic *B. juncea* male fertile line cv. RLM198 were used in the present study. The development of the CMS lines via protoplast fusion has been described earlier (Kirti *et al*. 1995a,b). The CMS lines have been backcrossed to euplasmic *B. juncea* cv. RLM198 for at least eight generations and hence all lines are isonuclear.

Molecular studies

Standard molecular methods were followed for Southern and Northern analyses. Mitochondrial DNA and RNA isolation and probe preparation were as described earlier (Pathania *et al*. 2003).

Analysis of Nco2.5 fragment and ORF138 transcript

The Nco2.5 mitochondrial DNA fragment was amplified by PCR using the primers NcoF (5′-GACAATAATCTTAGTCGGAGT-3′) and NcoR (5′-GGATCCTCATCACCATCAGC-3′) from 50 ng of mitochondrial DNA from all the experimental lines. The ORF138 transcript was amplified by RT-PCR using Superscript™ reverse transcriptase and Pfx^{TM} DNA polymerase (Invitrogen, Carlsbad, USA) starting with 2 μg of mitochondrial RNA from all the lines using the primers orf138F (5′-GAAACGGGAAGTGACAATAC-3′) and orf138R (5′-GCATTATTTTCTCGGTCCAT-3′). All the fragments were cloned in pTZ57R/T vector using InsT/A cloning kit (MBI Fermentas Life Sciences, Canada) and sequenced by Sanger's dideoxy nucleotide chain termination method (Sanger *et al*. 1977) using an ABI-Prism automated DNA sequenator and M13 forward and reverse primers.

Calculation of similarity index

To estimate genetic similarity (F) between the parental and the cybrid mitochondrial genomes from the RFLP data, equation 21 of Dice similarity index (Nei and Li 1979) was used: $F = 2n_{XY}/(n_X + n_Y)$, in which n_X and n_Y are the numbers of fragments in populations X and Y, respectively, whereas n_{XY} is the number of fragments shared by the two populations. An F of 1.0 would indicate two populations possess identical fingerprints, while 0.0 would represent two populations with no bands in common.

Results

Floral morphology of the CMS lines

The flowers of euplasmic *B. juncea* and the CMS lines are shown in figure 1. Stamens were replaced by petal-like structures in the original chlorotic CMS OgRLM (figure 1,b). The cybrid Og1 displayed normal stamen development but contained aborted microspores (figure 1,c), while cybrid Og2 exhibited petaloid stamens similar to those in OgRLM (figure 1,d). Other floral deformities present in CMS OgRLM, such as short, stumpy and crooked style, and reduced nectaries, were also observed in Og2, while these deformities were corrected in Og1.

RFLP analysis reveals recombination of mitochondrial genomes in the cybrids

Mitochondrial DNA from the parental lines euplasmic RLM and CMS OgRLM, and the cybrids Og1 and Og2 was digested with different restriction enzymes and probed with 11 mitochondrial genes. The RFLP patterns are summarized in table 1. *atp6* in Og1 was from the fertile *B. juncea*, as it showed a pattern similar to that from RLM. While *atp6* of Og2 was mostly derived from OgRLM, recombination was detected with *Bam*HI and *Eco*RV digestions (figure 2,a &b). Similarly, *atpA* in Og1 was inherited from RLM, while Og2 showed a pattern similar to that from OgRLM

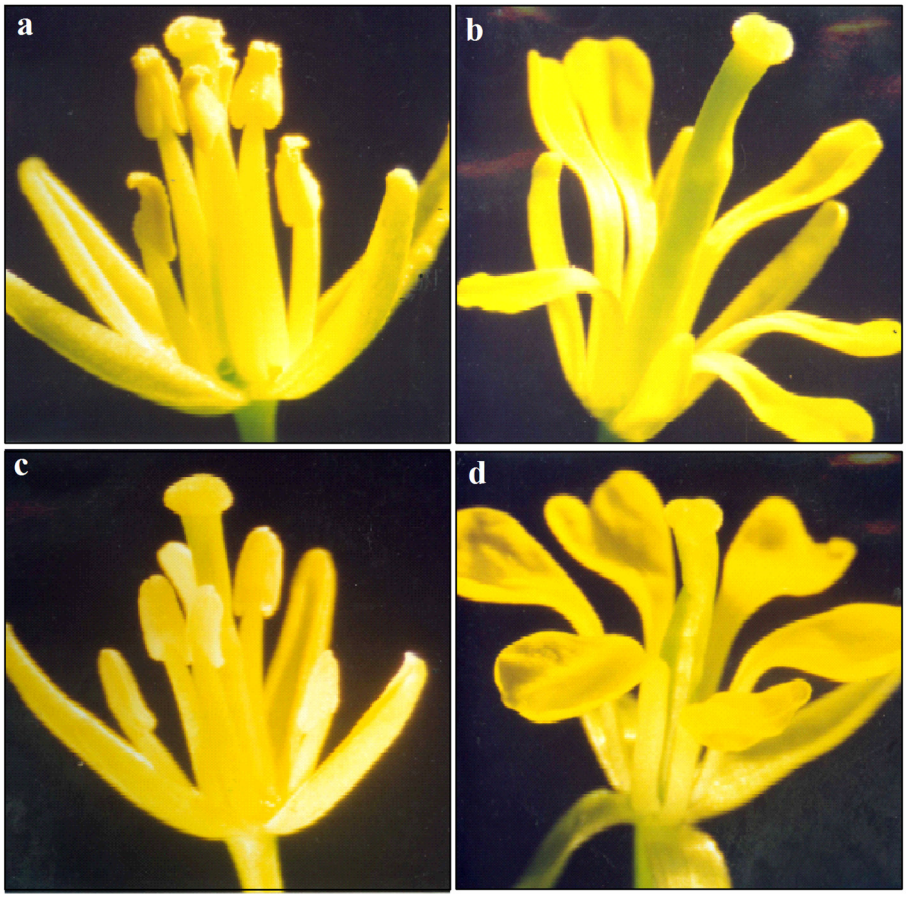

Figure 1. Flower morphology: (a) euplasmic fertile *Brassica juncea*, (b) uncorrected CMS parent OgRLM, (c) cybrid Og1, (d) cybrid Og2. The sepals and petals have been removed to reveal the petaloid stamens and deformed pistil.

Table 1. RFLP pattern and transcript length of mitochondrial genes in the CMS (Ogu) *B. juncea* cybrids Og1 and Og2.

Gene probe	Og1		Og2	
	RFLP pattern	Transcript length	RFLP pattern	Transcript length
atpA	Fertile	Fertile	Recombinant	Fertile
atp6	Fertile	Fertile	Recombinant	CMS
atp9	Fertile	Fertile	CMS	Fertile
coxI	Recombinant	Fertile	CMS	CMS
**coxII*	Fertile	Fertile	Fertile	Fertile
coxIII	Recombinant	Fertile	Recombinant	Fertile
**nad5/rsp12*	Fertile	Fertile	Fertile	Fertile
**cob*	Fertile	Fertile	Fertile	Fertile
18S rRNA	Fertile	Fertile	Fertile	Fertile
26S rRNA	Fertile	Fertile	CMS	Fertile
ORF138	CMS	CMS	CMS	CMS

RFLP pattern was obtained by comparing Southern hybridization patterns of cybrids Og1 and Og2 with those of the parental lines, i.e. euplasmic fertile *B. juncea* cv. RLM and CMS OgRLM. Transcript length for corresponding mitochondrial genes were determined by Northern hybridization. 'Fertile' and 'CMS' indicate that the hybridization pattern is similar to that of fertile RLM and CMS OgRLM, respectively, while modification in RFLP pattern is indicated by 'Recombinant'. When the hybridization pattern with a particular mitochondrial gene probe was similar in all the four lines studied in Southern and Northern analysis, the gene probe is marked with an asterisk.

except for a novel band in Og2 with *Hin*dIII digestion (figure 2,c) indicating mitochondrial genome recombination. RFLP pattern of *coxII* was identical in all the four lines, while *coxI* was recombinant in Og1. For example, a novel band of 2.7 kb was detected for *coxI* in Og1 when digested with *Eco*RV (figure 2,d), and one of 4.2 kb was detected when digested with *Hin*dIII (not shown), indicating difference from both parents. On the other hand, *coxI* of Og2 was derived from OgRLM. For *coxIII*, the RFLP pattern was complex, with presence of novel bands in both the cybrids detected with a number of restriction digestions. The novel pattern for *coxIII* in Og1 and Og2 when restricted with *Eco*RV is shown in figure 2,e. The Nco2.5 fragment, which has been reported to be associated with Ogura male sterility (Bonhomme *et al*. 1991, 1992), was detected with *Nco*I-digested DNA in all the CMS lines, but not in the fertile RLM when probed with ORF138 (figure 2,f). From table 1 it appears that the mitochondrial genomes of both the cybrids were recombinant. However, Og1 mitochondrial genome was largely derived from RLM, whereas that of Og2 was derived mostly from OgRLM. To determine the contribution of each parent to the mitochondrial genome of the cybrids, genetic similarity indices were calculated using the RFLP data (table 2). On the basis of similarity index, Og1 shows significantly high similarity of 81% to euplasmic *B. juncea*, while Og2 shows 93% similarity to OgRLM. The cybrids show only 59% similarity between them. This clearly illustrates that the recombinant mitochondrial genomes of the cybrids had originated by unequal contribution from the two parents, which by themselves are significantly different from each other (similarity value of only 48% between them).

Table 2. Estimation of genetic similarity index.

	Og1	Og2	OgRLM
RLM	0.81	0.50	0.48
Og1		0.59	0.55
Og2			0.93

Values are calculated from the number of bands shared in the RFLP analysis by any two lines among the four lines chosen, i.e. parental fertile RLM and CMS OgRLM, and cybrids Og1 and Og2.

Nco2.5 fragment and ORF138 transcripts are unaltered in the CMS lines

The presence of Nco2.5 fragment in all the CMS lines and its absence in the fertile *B. juncea* were confirmed by Southern analysis. PCR cloning and sequencing of the 2.5-kb *Nco*I fragment also revealed no differences among the CMS lines. Northern hybridization revealed a 1400-nucleotide-long transcript for ORF138 (figure 3,a), which was expected as ORF138 cotranscribed with ORFB/*atp8* on a binary transcript (Bonhomme *et al*. 1992). ORF138 transcript was present at high level in all the male sterile lines, but was absent in euplasmic *B. juncea*. The ORF138 cDNA amplified by RT-PCR from the cybrids and OgRLM showed nucleotide sequence identical to that of ORF138 reported earlier in NCBI GenBank, accession no. Z12626.

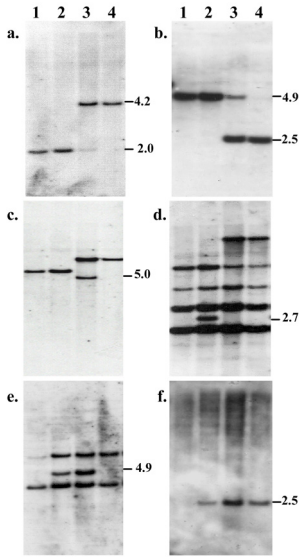

Figure 2. RFLP pattern of mitochondrial DNA restricted with (a) *Eco*RV and (b) *Bam*HI, and probed with *atp6*; (c) *Hin*dIII, and probed with *atpA*; (d) *Eco*RV, and probed with *coxI*; (e) *Eco*RV, and probed with *coxIII*; (f) *Nco*I, and probed with ORF138. Lanes 1, 2, 3 and 4 are euplasmic *B. juncea* cv. RLM198, Og1, Og2 and OgRLM, respectively; band sizes are given in kilobasepair (kb).

Northern analysis reveals altered atp6 transcription pattern between the cybrids

Northern hybridization was carried out using the 11 mitochondrial gene probes mentioned in table 1. No difference was detected among the four lines for transcription pattern of 10 of the genes. They were absolutely of the same length and were present in equal quantity indicating their normal expression, irrespective of their source of inheritance. Thus mitochondrial genome recombination did not appear to have affected expression of these genes. Only *atp6* transcription pattern showed difference between the cybrids. The typical *atp6* transcript in fertile euplasmic *B. juncea* was ~1000 nucleotides long and showed strong expression (figure 3,b, lane 1). This transcript was absent in CMS OgRLM, which possesses the Ogura mitochondrial genome. Instead, a faint signal for a transcript of ~1400 nucleotides was detected (figure 3,b, lane 4), which could have resulted from disruption of the reading frame, altered transcription pattern of *atp6* gene, or even lack of proper post-transcriptional modification of *atp6* transcript in the alien nuclear background. Among the cybrids, Og1 revealed the normal *atp6* transcript length and expression level seen in typical fertile euplasmic *B. juncea* (figure 3,b, lane 2). In Og2 the normal *atp6* transcript was absent and the pattern was similar to that of OgRLM (fig-

ure 3,b, lane 3). In case of *coxI*, the length of the transcript was ~2500 nucleotides in all the lines. However, the CMS lines differed from the fertile *B. juncea* in level of expression; Og1 showed significantly higher expression, while Og2 and OgRLM showed comparatively lower level of expression (figure 3,c). The same Northern blot was hybridized with 18*S* rRNA to show equal loading of RNA (figure 3,d). These results indicated that Og1 and Og2 differed significantly in length as well as level of expression of *atp6*. In case of *coxI*, although there was reduction in transcript level in Og2 and OgRLM, there was no apparent difference in transcript size.

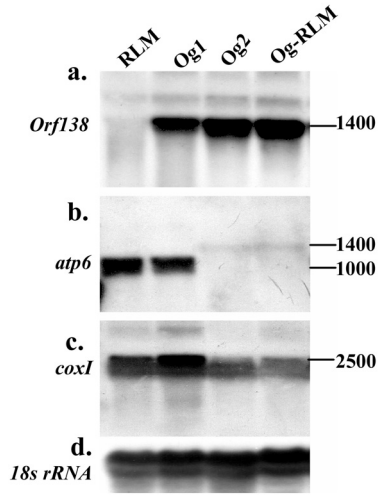

Figure 3. Northern blot hybridization with (a) ORF138, (b) *atp6*, (c) *cox1* mitochondrial gene probes and (d) 18S rRNA to show equal loading of RNA. The numbers are transcript length in nucleotides.

Discussion

Protoplast fusion offers an opportunity for mitochondrial genome recombination leading to appearance of novel CMS floral morphologies. Earlier studies on cybrids produced from protoplast fusion between *Nicotiana bigelovii* and *N. undulata* as well as *N. suaveolens* revealed that the cybrid lines with floral morphological similarity to one parent exhibited mitochondrial-DNA patterns that resembled the corresponding parent (Kofer *et al*. 1991; Fitter *et al*. 2005). A correlation between cybrid floral morphology and mt-DNA patterns after asymmetric fusion in *Nicotiana* spp. was also observed earlier (Belliard *et al*. 1979; Kumashiro *et al*. 1989). Floral morphology was disturbed when cybrids were made between different *Nicotiana* species (Gerstel *et al*. 1978), such as by introducing the *N. repanda* cytoplasmic genomes into a *N. tabacum* nuclear background. Interspecific cybrids between *N. tabacum* and *Hyoscyamus niger* containing recombinant mitochondrial genomes also resulted in individuals with a variety of floral modifications (Zubko *et al*. 2003). Individual cybrid lines containing the *B. napus* nuclear genome and recombinant mt-DNA derived from a combination of *B. napus* and *Arabidopsis* exhibited homeotic transformation of anthers to carpels and some disturbances in vegetative growth (Leino *et al*. 2003). Leino *et al*. (2005) further showed that alloplasmic effects on mitochondrial transcriptional activity and RNA turnover resulted in accumulated transcripts of *Arabidopsis* ORFs in CMS *B. napus*. In carrot, lines containing a CMS cytoplasm may exhibit either a petaloid or a carpelloid phenotype, depending on nuclear background (Nakajima *et al*. 2001). Homeotic conversion of stamens into carpelloid structures has also been described for CMS lines of rapeseed (Leino *et al*. 2003), tobacco (Kofer *et al*. 1991; Farbos *et al*. 2001), carrot (Linke *et al*. 2003), wheat (Murai *et al*. 2002) and *B. juncea* (Kirti *et al*. 1995a,b; Prakash *et al*. 2001).

In the present investigation, the cybrids Og1 and Og2 are examples of the effect of mitochondrial genome recombination and interaction of the recombinant mitochondrial genomes with the nuclear genome on floral morphology. The RFLP patterns of mitochondrial DNA of the fertile parent *B. juncea*, the sterile parent OgRLM and the two cybrids revealed that the cybrids possessed mitochondrial genomes that were recombinant. However, the recombination events were asymmetrical as the parental mitochondrial genomes were represented unequally in the cybrids. The parallel between inheritance of mitochondrial genomic material and floral morphology was apparent in the present study as Og2 had petaloid stamens like those of the sterile OgRLM, while Og1 possessed typical stamens like the fertile parent. Northern analysis revealed that the transcripts of all but one of the genes studied were of similar length and intensity The typical 1000-nucleotide *atp6* transcript of fertile *B. juncea* was present in Og1 but was absent in Og2 and OgRLM. Instead, a very low-level expression of a transcript of 1400 nucleotides was detected in Og2 and OgRLM. Presumably, OgRLM lacked proper post-transcriptional modifications of *atp6*, which was subsequently inherited by Og2. There are examples where a CMS phenotype resulted from absence of RNA editing in sunflower (Howad and Kempken 1997) or loss of proper transcript processing activity in wheat (Song and Hedgecoth 1994). However, it is not *atp6* but ORF138, which encodes a mitochondrial membrane peptide, that has been linked to male sterility in the Ogura system. As both Og1 and Og2 inherited the 2.5-kb *Nco*I locus from OgRLM, strong expression of ORF138, responsible for CMS, was detected in these lines. Earlier studies revealed transcript differences for only *atp6*, *atpA* and *coxI*, although these were not actually involved in causing male sterility in Ogura cytoplasm (Makaroff *et al*. 1989, 1990, 1991). In the present study, we found no differences in length of transcript of *atpA* and *coxI* between Og1 and Og2, although *coxI* expression was slightly higher in Og1. The *atp6* transcript pattern was the most obvious difference between Og1 and Og2. It was also clear that ORF138 was only responsible for the male sterility in the CMS lines, but not for the homeotic-like modification of stamens to petals in Og2 and OgRLM as it was

also expressed prominently in Og1. This study has shown that only *atp6* expression was aberrant in Og2 from among 11 genes whose expression was examined in the present study. It is possible that there are more ORFs in the recombined mitochondrial genome that show aberrant expression.

The idea that there may be mitochondrial influences on floral developmental genes is rather recent. The floral homeotic alterations found in many alloplasmic CMS lines resemble mutants in *Antirrhinum* and *Arabidopsis*, whose analysis resulted in the synthesis of a model for floral patterning (Weigel and Meyerowitz 1993; Theiβen 2001). In carpelloid carrot flowers, reduced expression of homologues of *Antirrhinum GLOBOSA* and *DEFICIENS* was observed (Linke *et al*. 2003). The CMS somatic hybrid *Arabidopsis thaliana + B. napus* showed strongly reduced expression of the *B. napus* homologue of *APETALA3* and *PISTILLATA* during the second and third whorl organ differentiation stages (Teixeira *et al*. 2005). However, the hypothesis that disturbed expression of the known floral homeotic genes could be responsible for CMS-associated abnormal floral morphology was experimentally proved when *N. tabacum* CMS lines were transformed with the tobacco homologue of *Arabidopsis SUPERMAN*. Flowers in the CMS line had severely distorted stamens fused to the carpel, but transgenic plants exhibited flowers with improved morphology (Bereterbide *et al*. 2002). In a rapeseed male sterile line, ORF222 expression might act to alter stamen and petal development by influencing expression of B function genes. The study has shown that mitochondrial dysfunction triggered by ORF222 expression may interfere with downregulation of *AP3* expression, showing that sterility-related genes and their expression may have an effect on expression of nuclear genes affecting floral development (Geddy *et al*. 2005).

A similar situation occurs with aberrant mitochondrial gene expression resulting in homeotic floral abnormalities in Og2. How particular mitochondrial/nuclear gene combinations can produce disturbances in floral development is an intriguing topic. Further work is in progress in the study of expression of *MADS* gene family members in Og2.

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