orf260<sup>era</sup>, a Novel Mitochondrial Gene, is Associated with the Homeotic Transformation of Stamens into Pistil-Like Structures (Pistillody) in Alloplasmic Wheat

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Homeotic transformation of stamens into pistil-like structures (pistillody) can occur in cytoplasmic substitution (alloplasmic) lines of bread wheat (Triticum aestivum) that have the cytoplasm of the related species, Aegilops crassa. Previously we showed that pistillody results from altered patterns of expression of class B MADS-box genes mediated by mitochondrial gene(s) in the Ae. crassa cytoplasm. The wheat cultivar Chinese Spring does not show pistillody when Ae. crassa cytoplasm is introduced. The absence of an effect is due to a single dominant gene (designated Rfd1) located on the long arm of chromosome 7B. To identify the mitochondrial gene involved in pistillody induction, we performed a subtraction analysis using cDNAs derived from young spikes of a pistillody line and a normal line. We found that mitochondrial cDNA clone R04 was abundant in the young spikes of the pistillody line but was down-regulated in the normal line that carried nuclear Rfd1. Sequencing of the full-length cDNA corresponding to clone R04 showed that two genes were present, cox I (cytochrome c oxidase subunit I) and orf260<sup>era</sup>. orf260<sup>era</sup> shows high sequence similarity to orf256, the T. timopheevii mitochondrial gene responsible for cytoplasmic male sterility (CMS). orf260<sup>era</sup> was also present in the cytoplasms of Ae. jarenalis and Ae. ravelovii, which induce pistillody, but not in the cytoplasms of other species not associated with pistillody. Furthermore, Western blot analysis revealed that the ORF260<sup>era</sup> protein was more abundant in the pistillody line than in the normal line. We suggest therefore that orf260<sup>era</sup> is associated with pistillody induction.

**Keywords:** Alloplasmic wheat — Cytoplasmic homeosis — Cytoplasmic male sterility — Mitochondrial gene — Pistillody.

**Abbreviations:** AG, AGAMOUS; AP3, APETALA3; CMS, cytoplasmic male sterility; CS, Chinese Spring; N26, Norin 26; ORF, open reading frame; PCMS, photoperiod-sensitive cytoplasmic male sterility; PI, PISTILLATA; Rf, restorer of fertility; RT–PCR, reverse transcription–PCR; SEP, SEPALLATA; WAP3, wheat APETALA3; WPI, wheat PISTILLATA.

The nucleotide sequences of orf260<sup>era</sup> and orf113<sup>era</sup> in the Aegilops crassa mitochondrial genome have been submitted to the DDBJ/EMBL/GenBank under the accession numbers AB438956 and AB438957, respectively.

**Introduction**

Cytoplasmic male sterility (CMS) is characterized by failure to produce viable pollen and is the most commonly observed effect in higher plants of the introduction of alien cytoplasm (Kaul 1988). In many plant species, CMS is associated with chimeric or unusual mitochondrial open reading frames (ORFs) (Hanson and Bentolila 2004, Chase 2007). These unique ORFs are transcribed and translated into proteins that appear to interfere with mitochondrial function and pollen development. A number of plant species possess nuclear fertility restorer (Rf) genes that can suppress CMS mediated by mitochondrial genes. In several species, such as petunia, radish and rice, the Rf genes encode pentatricopeptide repeat proteins, which are thought to be RNA-binding proteins involved in post-transcriptional processing in mitochondria (Hanson and Bentolila 2004, Chase 2007).

The effects of cytoplasm from wild relatives of common wheat (Triticum aestivum) have been comprehensively investigated using cytoplasmic substitution (alloplasmic) lines produced by recurrent backcrossing (Tsunewaki et al. 1996, Tsunewaki et al. 2002). In an alloplasmic line in which Aegilops crassa cytoplasm was introduced into the wheat cultivar (cv.) Norin 26 (N26), we found that male sterility occurred under long-day conditions (>15 h light period) due to pistillody, the homeotic transformation of stamens into
pistil-like structures (Murai and Tsunewaki 1993). This phenomenon was named photoperiod-sensitive cytoplasmic male sterility (PCMS), and intensive studies were carried out to assess its value for hybrid wheat breeding (Murai 2001). In contrast to N26, the wheat cv. Chinese Spring (CS) did not show pistillody when *Ae. crassa* cytoplasm was introduced; the lack of an effect was due to a single dominant *Rf* gene (designated *Rf1l*) located on the long arm of chromosome 7B (Murai and Tsunewaki 1994). We carried out a loss-of-function analysis to investigate the function of *Rf1l* using an alloplasmic line of CS with ditelosomy of chromosome 7BS, i.e. lacking the long arm of chromosome 7B, and with *Ae. crassa* cytoplasm [(cr)-CSdt7BS]. In the absence of *Rf1l*, the (cr)-CSdt7BS line exhibited pistillody irrespective of photoperiod, whereas CS with ditelosomy of 7BS but with a normal cytoplasm (CSt7BS) formed normal stamens (Murai et al. 2002). These results indicate that pistillody is induced by factor(s), presumably of mitochondrial origin, in the *Ae. crassa* cytoplasm and that the nuclear *Rf1l* gene can block the deleterious effects of the cytoplasm. PCMS in alloplasmic N26 lines might involve an *Rf* gene that functions under short-day conditions.

According to the ABCDE model for flower development, floral organ identity is defined by five classes of homeotic genes, called A, B, C, D and E (Zahn et al. 2006). The class B, C and E genes specify stamens in the third floral whorl, and class C and E genes specify carpels in the inner fourth whorl. In *Arabidopsis*, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are class B genes and *AGAMOUS* (*AG*) is a class C gene that encodes a MADS-box transcription factor (Riechmann and Meyerowitz 1997). The class E genes *SEPALLATA1* (*SEPI*), *SEP2*, *SEP3* and *SEP4* are also members of the MADS-box gene family and redundantly specify petals, stamens and carpels. Loss-of-function of class B MADS-box genes results in the homeotic transformation of stamens into carpel- or pistil-like structures in *Arabidopsis* (Jack et al. 1992, Goto and Meyerowitz 1994) and *Antirrhinum* (Sommer et al. 1990, Tröbner et al. 1992). In monocots, the class B gene mutants *silky1* in maize and *superwoman1* in rice also show male sterility due to homeotic conversion of stamens into carpels (Ambrose et al. 2000, Nagasawa et al. 2003). These findings suggest that wheat class B MADS-box genes could be associated with the induction of pistillody in alloplasmic wheat. In previous studies, we identified a wheat *AP3* ortholog, *WAP3* (wheat *APETALA3*) (Murai et al. 1998), and two wheat *PI* orthologs, *WPI-1* (wheat *PISTILLATA-1*) and *WPI-2* (Hama et al. 2004). *WPI* and *WAP3* are wheat class B genes and are down-regulated in the primordia of pistil-like stamens during pistillody. These results clearly indicate that the induction of pistillody is caused by alterations in the expression patterns of class B MADS-box genes in alloplasmic wheat, which is triggered by mitochondrial gene(s) (Hama et al. 2004).

Although the mitochondrial gene of *Ae. crassa* that is responsible for pistillody in alloplasmic wheat has not been identified, we showed previously that *orf25* is a possible candidate (Ogihara et al. 1997, Ogihara et al. 1999). Of 12 mitochondrial genes investigated, *atpA*, *atpB*, *atp9*, *cox I*, *cox II*, *cox III*, *cob*, *rrn26S*, *rrn18S-5S*, *nad3-rps12*, *nad5ab* and *orf25*, only *orf25* showed any difference between wheat and *Ae. crassa* in a Southern blot analysis (Ogihara et al. 1997). Furthermore, northern blot analysis and in vitro capping and RNase protection assays of alloplasmic lines that have *Ae. crassa* cytoplasm revealed that the *orf25* transcripts differed in size from those of euplasmic lines, including *Ae. crassa* itself (Ogihara et al. 1997, Ogihara et al. 1999). These results suggest that *orf25* is involved in the induction of pistillody. However, the *orf25* transcript patterns did not differ between CMS lines and restorer lines with the *Rf1l* gene.

In this study, we sought to identify the mitochondrial gene causing pistillody in alloplasmic wheat using a cDNA subtraction analysis of cDNAs derived from young spikes from pistillody and normal lines. By means of this strategy, we identified an ORF, *orf260*iso*, that was up-regulated in the pistillody line and down-regulated by *Rf1l*. Furthermore, the ORF260*ora* protein was more abundant in the pistillody line compared with the normal line, suggesting that *orf260*iso* was required for pistillody induction.

**Results**

The clones R04 and R01 are up-regulated in pistillody lines

We previously produced a wheat cv. CS line that is ditelosomic 7BS and has *Ae. crassa* cytoplasm [(cr)-CSdt7BS] (Murai et al. 2002). This cytoplasmic substitution (alloplasmic) line does not contain the *Rf1l* gene as it lacks the long arm of chromosome 7B. The ditelosomic euplasmic line (CSdt7BS) developed normal stamens, but the alloplasmic line, (cr)-CSdt7BS, showed pistillody in all florets (Fig. 1). Despite the pistillody of the stamens, the lodicules remained unchanged in (cr)-CSdt7BS, indicating that the homeotic alteration was restricted to the staminate portion (Murai et al. 2002).

To identify mitochondrial genes associated with pistillody induction, we performed a cDNA subtraction analysis to screen for genes with altered transcript levels between the pistillody and normal lines. The CSdt7BS line was used as the tester and the (cr)-CSdt7BS line was used as the driver in the forward hybridization, and vice versa in the reverse hybridization. From the forward hybridization, we obtained 33 clones from mitochondrial genes that showed increased transcript levels in the normal line, CSdt7BS.
In the reverse hybridization, 27 clones that showed up-regulated expression in the pistillody line (cr)-CSdt7BS were obtained (Table 1).

To confirm the specificity of these expression patterns, we performed reverse transcription–PCR (RT–PCR) analyses using clone-specific primers and cDNAs derived from young spikes of plants of the normal (CSdt7BS) and pistillody [(cr)-CSdt7BS] lines. The alloplasmic line (cr)-CS, which shows normal fertility due to the \( Rfd1 \) gene located on the long arm of chromosome 7B, was also used in the RT–PCR analysis. Clone F01 was expressed in both alloplasmic lines [(cr)-CS and (cr)-CSdt7BS] and in the euplasmic line (CSdt7BS) (Fig. 2). Clone F03 was expressed only in the euplasmic line, indicating that it is derived from a wheat mitochondrial (mt) gene (Fig. 2). In contrast, clones F02, R04 and R01 were derived from \( Ae. \) crassa mitochondrial genes. Clone F02 showed no difference in expression level between the alloplasmic line displaying pistillody [(cr)-CSdt7BS] and that showing a normal phenotype [(cr)-CS] (Fig. 2). The expression levels of clones R04 and R01 were much higher in the young spikes of (cr)-CSdt7BS than in those of (cr)-CS plants (Fig. 2). This suggests that expression of R04 or R01 is associated with pistillody induction in the alloplasmic line.

Clones R04 and R01 identify the novel mitochondrial genes, orf260\(^{ras} \) and orf113\(^{ras} \), respectively

Clone R04 shows high sequence similarity to the predicted protein of orf256, the mitochondrial gene suggested to be responsible for CMS in \( T. \) timopheevii (Song and Hedgoth 1994a). Sequencing of the homologous region in \( Ae. \) crassa indicated that it contained two ORFs, namely cox I and orf256 and cox II, pseudogene for NADH dehydrogenase subunit 3, ribosomal protein S12 and an unknown membrane-bound protein (Fig. 3A). The alignment of the predicted amino acid sequences of orf256 of \( T. \) timopheevii (ORF256tim) and orf260\(^{ras} \) of \( Ae. \) crassa (ORF260cra) showed that a putative transmembrane domain had low sequence similarity compared with other regions, and that a four amino acid insertion/deletion was present in a second putative transmembrane domain (Fig. 4A). Putative transmembrane domains were identified by the computer program SOSUI.

### Table 1 Annotation of clones obtained by cDNA subtraction

<table>
<thead>
<tr>
<th>Annotation by BLAST</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>33 27 60</td>
</tr>
<tr>
<td>cox I for cytochrome c oxidase subunit I</td>
<td>26 7 33</td>
</tr>
<tr>
<td>cox I and hypothetical protein ORF256 gene</td>
<td>0 4 4</td>
</tr>
<tr>
<td>Mitochondrial nad 1 gene</td>
<td>2 2 4</td>
</tr>
<tr>
<td>Mitochondrial gene for apocytochrome b</td>
<td>1 1 2</td>
</tr>
<tr>
<td>atp6 for subunit 6 of ATP synthase complex</td>
<td>2 0 2</td>
</tr>
<tr>
<td>Mitochondrial nad3, rps12, orf156 and cox II, pseudogene for NADH dehydrogenase subunit 3, ribosomal protein S12 and an unknown membrane-bound protein ndb gene for NADH-ubiquinone oxidoreductase</td>
<td>1 5 6</td>
</tr>
<tr>
<td>Cytochrome c maturation subunit Fc gene</td>
<td>1 0 8</td>
</tr>
</tbody>
</table>

\( ^{a} \) Up-regulated in the normal line CSdt7BS.

\( ^{b} \) Up-regulated in the pistillody line (cr)-CSdt7BS.
orf260<sup>ra</sup>, pistillody-related mitochondrial gene

Fig. 2 RT–PCR analysis of mitochondrial clones obtained by cDNA subtraction from the pistillody line (cr)-CSdt7BS and the normal line CSdt7BS. The alloplasmic line (cr)-CS, fertile because of the effect of Rfd1, was also used. Total RNAs were isolated from young spikes at the floret differentiation stage. RT–PCR was performed in the exponential range of amplification, and the Ubi-1 gene was used as the internal control.

Analysis of clone R01 showed that it had high sequence similarity to the NADH-ubiquinone oxidoreductase (ndh) gene (Fig. 3B). The 5′ region of clone R01 had high sequence similarity to the intron sequence of nadl of *T. aestivum*, while the 3′ region showed high sequence similarity to nd6 of *T. aestivum*. Analysis of the 5′-flanking region of clone R01 identified a novel ORF with 113 amino acids, designated here orf113<sup>ra</sup>. This new ORF resulted from a base substitution in which a T was changed to a G, thereby altering a stop codon to an amino acid codon.

orf260<sup>ra</sup>, but not orf113<sup>ra</sup>, is a pistillody-related cytoplasmic factor

orf260<sup>ra</sup> and orf113<sup>ra</sup> also exist in species related to *Ae. crassa*, we first performed a database search using DDBJ (DNA Data Bank of Japan). We found that *T. boeoticum*, *Ae. tauschii* and *Secale cereale* also have orf260 in their mitochondrial genomes. However, an amino acid sequence alignment showed that the products of the orf260 genes of *T. boeoticum*, *Ae. tauschii* and *S. cereale* contain amino acid substitutions compared with that of orf260<sup>ra</sup> of *Ae. crassa*, and thus we named them orf260<sup>boe</sup>, orf260<sup>tau</sup> and orf260<sup>sec</sup>, respectively (Fig. 4B). Next, we performed a PCR analysis using total DNAs from *Ae. juvenalis*, *Ae. vavilovii*, *Ae. tauschii*, *Ae. cylindrica*, *Ae. speltoides*, *T. timopheevii*, *T. aestivum*, *T. dicoccum* and *T. dicoccoides*, and a sequence analysis of the PCR products. We found that orf260<sup>ra</sup> was also present in *Ae. juvenalis* and *Ae. vavilovii* (Fig. 5), species containing a D<sup>2</sup>-type mitochondrial genome that induces pistillody in alloplasmic lines (Murai and Tsunewaki 1993). orf260<sup>ra</sup> was not found in the other species tested. In contrast to orf260<sup>ra</sup>, orf113<sup>ra</sup> was identified in *Ae. tauschii* and *Ae. cylindrica*, species that do not have pistillody-inducing cytoplasm (Fig. 5). This result clearly indicates that orf113<sup>ra</sup> is not associated with pistillody.

Analysis of orf260<sup>ra</sup> expression

To confirm that higher levels of expression of orf260<sup>ra</sup> are related to pistillody induction, we performed RT–PCR analyses using primer sets specific for orf260<sup>ra</sup>, cox I and orf260<sup>ra</sup>-cox I (Fig. 6A). cDNAs were obtained from young spikes at the floret differentiation stage of plants of the (cr)-CS (normal) and (cr)-CSdt7BS (pistillody) lines. The expression levels of orf260<sup>ra</sup>, cox I and the whole orf260<sup>ra</sup>-cox I region were much higher in the pistillody line (cr)-CSdt7BS than in the normal line (cr)-CS line (Fig. 6B). All primer sets amplified a single transcript of the same molecular size in both (cr)-CS and (cr)-CSdt7BS, indicating that orf260<sup>ra</sup> and cox I were co-transcribed and that the processing of transcripts was not altered by the presence of nuclear Rfd1. Real-time PCR analysis confirmed that orf260<sup>ra</sup> was up-regulated in the pistillody line [(cr)-CSdt7BS] and down-regulated in the normal line [(cr)-CS] (Fig. 6C).

The orf260<sup>ra</sup> protein (ORF260cra) accumulates at high levels in the pistillody line

Our previous study indicated that the (cr)-CSdt7BS line shows complete female and male sterility (Murai et al. 2002). To produce this line, the monotelodisomic 7BS line (cr)-CSmd7BS, which has *Ae. crassa* cytoplasm and a single chromosome 7B along with a telosome 7BS, was crossed with CSdt7BS. In addition, we produced the (cr)-CSmd7BS line by crossing (cr)-CS with CSdt7BS. The (cr)-CSmd7BS line showed reduced frequency of pistillody, but was female.
fertile due to hemizygosity for \( Rfd1 \) (Murai et al. 2002). Normal stamens in the florets of (cr)-CSmd7BS plants produce normal pollen in normal quantities. The pistillody line (cr)-CSdt7BS could not be obtained from selfed progeny of (cr)-CSmd7BS, because the telosome was transmitted through female but not male gametes (Murai et al. 2002). As a consequence, it was difficult to obtain a large amount of young spikes from (cr)-CSdt7BS plants for protein analysis.

In the alloplasmic wheat line N26, which has \( Ae. \ crassa \) cytoplasm, we found that male sterility occurred under long-day conditions (light period >15 h) due to pistillody (Murai and Tsunewaki 1993). We used western blotting to examine the accumulation of the ORF260cra gene product in young spikes of the PCMS line (cr)-N26 grown under either short-day (normal) or long-day conditions (pistillody). ORF260cra protein was detected in all samples tested, i.e. (cr)-N26 under short-day conditions, (cr)-N26 under long-day conditions and (cr)-CS under short-day conditions (Fig. 7). However, the highest level of ORF260cra protein accumulation was observed in plants showing pistillody, i.e. (cr)-N26 under long-day conditions, indicating that the protein is associated with pistillody induction.

Discussion

CMS phenotypes have a wide range of reproductive abnormalities and are associated with novel or chimeric mitochondrial ORFs (Chase 2007). In some cases, CMS results from the failure of the pollen to develop normally, leading to pollen sterility. This phenotype is seen in the CMS-S type of \( Zea mays \) (Laughnan and Gabay-Laughnan 1983). CMS can also result from the degeneration of stamens or stamen components, such as tapetal cells in the anthers, preventing dehiscence. The degeneration of stamens or stamen components is presumably controlled by such processes as programmed cell death. This phenotype has been extensively studied in alloplasmic sunflower plants, \( Helianthus annuus \), that carry \( H. \ petiolaris \) cytoplasm (Balk and Leaver 2001). The pistillody phenotype of alloplasmic wheat represents a third type of CMS (Fig. 1).

Murai and Tsunewaki (1993) originally reported that alloplasmic wheat carrying \( Ae. \ crassa \) cytoplasm showed pistillody. Subsequently, it was found that pistillody could be induced by alien mitochondrial gene(s) whose function was suppressed by a nuclear \( Rfd1 \) gene (Murai and Tsunewaki 1994, Murai et al. 2002). Hama et al. (2004)
further demonstrated that pistillody induction is caused by an alteration in expression of class B MADS-box genes in stamen primordia at floral whorl 3. In addition to wheat, pistillody has been reported in various other species: tobacco (Nicotiana tabacum) (Zubko et al. 1996); carrot (Daucus carota) (Linke et al. 2003); and Brassica napus (Leino et al. 2003). In these species, pistillody is again associated with down-regulation of class B genes (Zubko et al. 2001, Linke et al. 2003, Teixeira et al. 2005).

**Fig. 4** Alignment of the deduced amino acid sequence of orf260cra of Ae. crassa (ORF260cra) with orf256 of T. timopheevii (ORF256tim) (A), and with the orf260 genes of T. boeoticum (ORF260boe), Ae. tauschii (ORF260tau) and S. cereale (ORF260sec) (B). The sequence of orf256 was taken from the DDBJ database (accession No. X56186). The accession numbers of the orf260 genes of T. boeoticum, Ae. tauschii and S. cereale are U93503, U93500 and U93502, respectively. The sequences were aligned using the computer program CLUSTAL W. Amino acid residues that are identical or similar are indicated by asterisks and dots, respectively. Hyphens indicate gaps introduced to facilitate alignment. The hypothetical transmembrane domains are expected from using the computer program SOSUI, and are shown in red.
indicate that a common mechanism is involved in homeotic transformation in alloplasmic plants. This phenomenon was recently named 'cytoplasmic homeosis' (Zubko 2004). What is the mechanism of cytoplasmic homeosis, and what differentiates cytoplasmic homeosis from other types of CMS?

In alloplasmic plants, CMS has been suggested to involve a chimeric or unusual mitochondrial ORF in the CMS cytoplasm that affects general metabolic rates (Chase 2007). As pollen production in stamens has a high energy demand compared with other developmental processes in other plant organs, any reduction in the metabolic rate would be expected to impair pollen production. It is unclear whether a similar energy demand hypothesis could explain cytoplasmic homeosis. Chimeric and unusual mitochondrial ORFs can lead to drastic changes in the cellular redox state as well as influence metabolic rate. In yeast, changes in the cellular redox state caused by a mitochondrial respiratory

![Fig. 5](https://academic.oup.com/pcp/article-abstract/49/11/1723/1877115)

**Fig. 5** Phylogenetic relationship of the mitochondrial (mt) genomes of *Triticum* and *Aegilops* species (after Tsunewaki et al. 2002), and the presence (+) or absence (–) of orf260*^{cra}* or orf113*^{cra}* sequences in these species. The mitochondrial genome of *S. cereale* is also indicated. *Ae. juanalis* and *Ae. vavilovii* fell into the same group as *Ae. crassa*, with a D2-type mitochondrial genome and a pistillody-inducing cytoplasm (Murai and Tsunewaki 1993). The D-type mitochondrial genome in *Ae. tauschii* and *Ae. cylindrica* does not induce pistillody. orf260*^{sec*}, orf260*^{boe*}, orf260*^{lim*} and orf260*^{spe*} have amino acid substitutions compared with orf260*^{cra*} in 10, three, four and two amino acids, respectively.

![Fig. 6](https://academic.oup.com/pcp/article-abstract/49/11/1723/1877115)

**Fig. 6** Expression analysis of the orf260*^{cra*}–cox I region. (A) Positions of the primers used for RT–PCR analysis. (B) RT–PCR analysis of orf260*^{cra*}, cox I and orf260*^{cra*}–cox I in the pistillody line (cr)-CSdt7BS compared with (cr)-CS. The (cr)-CSdt7BS line is an alloplasmic line carrying *Ae. crassa* cytoplasm and shows pistillody. The alloplasmic line (cr)-CS does not show pistillody due to the action of Rfd1. Total RNAs were isolated from young spikes at the floret differentiation stage. RT–PCR was performed in the exponential range of amplification, and the Ubi-1 gene was used as the internal control. (C) Expression analysis of orf260*^{cra*} by real-time PCR with the Ubi-1 gene as control. Total RNA was isolated from young spikes at the floret differentiation stage from the (cr)-CS and (cr)-CSdt7BS lines. Young spikes were obtained from a minimum of 10 plants, and the total RNA was isolated from all of them together.
deficiency trigger can alter the expression of nuclear genes, a phenomenon known as ‘retrograde signaling’ (Liu and Butow 2006). It is possible that a similar type of retrograde signaling from mitochondria to the nucleus may regulate the expression of many nuclear genes in higher plants (Yu et al. 2001). Therefore, it may be that mitochondrial dysfunction affects the expression pattern of homeotic genes through retrograde signaling. We recently reported that a protein kinase gene might play a role in the interaction of mitochondrial and nuclear homeotic genes during the development of pistill-like stamens (Saraike et al. 2007).

In order to understand the mechanism of the cytoplasmic homeosis, it will be necessary to identify the mitochondrial gene(s) involved in this phenomenon. Here, we showed that $orf260^{sa}$ is a pistillody-related mitochondrial gene. The function of this gene was suppressed by the nuclear gene $Rfd1$, as evidenced by the fact that the level of transcript and the accumulation of the protein decreased in the line carrying restorer loci (Figs. 2, 6, 7). $orf260^{sa}$ shows high sequence similarity to $orf256$ of $T. timopheevii$, a mitochondrial gene that has been reported to induce CMS when present in an alloplasmic substitution line of wheat (Figs. 3, 4) (Song and Hedgcoth 1994a, Song and Hedgcoth 1994b). The alloplasmic line produced using wheat cv. CS and $T. timopheevii$ [(tm)-CS] displays pistillody, but, unlike the (cr)-CSd7BS line, the frequency is not 100%. In florets of (tm)-CS plants, the incidence of apparently normal stamens was significantly higher in the central position compared with the lateral positions, i.e. stamens located laterally had a greater predisposition to change homeotically (data not shown). Similarly, a reduced frequency of pistillody is seen in (cr)-CSmd7BS, a monotelodisomic 7BS CS line with $Ae. crassa$ cytoplasm that is hemizygous for $Rfd1$ (Murai et al. 2002). In (cr)-CSmd7BS, the central stamens often show a normal developmental process and produce normal pollen. However, the normal stamens in the florets of (tm)-CS fail to produce fertile pollen. In summary, $Ae. crassa$ cytoplasm induces pistillody that is suppressed by CS nuclear $Rfd1$, and the $Rfd1$ gene functions in a gene dosage-dependent manner (Murai et al. 2002). Normal stamens in the florets of (cr)-CSmd7BS produce normal pollen in normal quantities, indicating that the influence of the $Ae. crassa$ cytoplasm is restricted to the homeotic transformation of stamens and has no effect on pollen development when the stamens develop normally. $T. timopheevii$ cytoplasm induces pollen sterility as well as pistillody, and $Rfd1$ has no effect on the phenotype. These facts indicate that the $Ae. crassa$ and $T. timopheevii$ systems have different mechanisms and that different CMS-related mitochondrial genes are involved. Our analysis of the predicted amino acid sequences of $orf260^{sa}$ and $orf256$ indicated that both have two transmembrane domains. In the second domain, $orf260^{sa}$ has a four amino acid insertion that is not present in $orf256$; this insertion may be responsible for the phenotypic differences mediated by the two ORFs. $T. boeoticum$, $Ae. tauschii$ and $Ae. cylindrica$ have $orf260$ genes which show high sequence similarity with $orf260^{sa}$ of $Ae. crassa$ (Figs. 4, 5). However, it is known that their cytoplasmics do not induce pistillody in the alloplasmic wheats (Tsunewaki et al. 2002). $orf260^{boe}$, $orf260^{tau}$ and $orf260^{cy}$ contain substitutions of three, four and two amino acids compared with $orf260^{sa}$ of $Ae. crassa$, respectively (Figs. 4, 5). Most of the amino acid substitution was in the putative transmembrane domains. The amino acid difference may result in the phenotypic difference of the alloplasmic lines.

Our RT–PCR analysis of the $orf260^{sa}$–cox I region indicated that $orf260^{sa}$ is co-transcribed with $cox I$ and that nuclear $Rfd1$ is not involved in processing of the transcripts (Fig. 6). In contrast, it has been reported that processing of the transcript of the $orf256$–cox I region in $T. timopheevii$ does vary on different nuclear backgrounds, i.e. $T. aestivum$ and $T. timopheevii$ (Song and Hedgcoth 1994a). This difference supports our conclusion that different
mechanisms are involved in the CMS mediated by Ae. crassa and T. timopheevii cytoplastms.
orf256-like sequences are present in barley, rye and several species of Aegilops and Triticum (Hedgeoth et al. 2002). Only T. timopheevii and Ae. speltoides have orf256 transcripts; the ORF256 protein is only produced by CMS plants that have one or other of these two species as the maternal donor. We found that Ae. juvenalis and Ae. vavilovii have an ORF identical to orf256. Alloplasmic lines constructed using these two species also show pistillody, a property that is consistent with the results of lines constructed using these two species also show pistillody when their mitochondrial genome was present together with a T. aestivum nucleus. These results reinforce the interpretation that orf256 is the gene responsible for pistillody induction.

CMS lines with T. timopheevii cytoplastm have a mitochondrial protein of about 7 kDa derived from orf256 (Song and Hedgeoth 1994b). A western blot using an ORF260cra antibody was carried out here to identify the protein product of orf260 (Fig. 7). A protein of approximately 20 kDa was identified in the total protein preparations from young spikes of normal and pistillody lines. The amount of protein in the pistillody line was much greater than in the normal line. This suggests that when the ORF260cra protein reaches a threshold concentration, greater than in the normal line. This suggests that when the ORF260cra protein reaches a threshold concentration, pistillody is induced. The function of the encoded protein remains unclear. Our next step will be to study the biochemical and structural properties of the ORF260cra protein.

Materials and Methods

Plant materials

A cytoplasmic substitution (alloplasmic) line of Chinese Spring (CS) that has Ae. crassa cytoplastm [(cr)-CS] was used for RT-PCR analyses. An alloplasmic line of CS with Ae. crassa cytoplastm and a ditelosomic chromosome 7BS [(cr)-CSdt7BS], which thus lacks a pair of chromosome 7B long arms, was used for the cDNA subtraction, isolation of the full-length cDNA of orf256 and RT-PCR analyses. This line exhibits pistillody due to the absence of the R allele, which prevents the effect of the Ae. crassa cytoplastm on stamen formation (Murai et al. 2002). The euplasric CS ditelosomic 7BS line with normal cytoplasm (CSdt7BS), which shows a normal phenotype, was used as the control for cDNA subtraction and RT-PCR analyses.

An alloplasmic line of Norin 26 (N26) with Ae. crassa cytoplastm [(cr)-N26] was used for the western blot analyses. In a previous study, we reported that (cr)-N26 exhibited PCMS caused by Ae. crassa cytoplastm (Murai and Tsenewaki 1993). PCMS is induced by long-day conditions of 15 h or longer light periods during the floret differentiation stage. PCMS is expressed in the form of homeotic transformation of stamens into pistil-like structures, i.e. pistillody (Murai and Tsenewaki 1993, Murai et al. 2002). Plants of the (cr)-N26 line grown under either long-day conditions (>15 h light, the natural light period for spring-sown wheat in Hokkaido, Japan) or short-day conditions (<14.5 h light, the natural light period for autumn-sown wheat in Fukuji) were used for protein analysis.

Total DNAs of Ae. tauschii, Ae. cylindrica, Ae. speltoides, Ae. crassa, Ae. juvenalis, Ae. vavilovii, T. timopheevii, T. aestivum, T. dicoccum and T. dicoccoides were used for identification of the orf256 and orf113 genes.

cDNA subtraction analysis

Total RNA was isolated from young spikes at the floret differentiation stages in the (cr)-CSdt7BS and CSdt7BS lines using ISOGEN reagent (Nippon-gene, Tokyo, Japan). First-strand cDNA synthesis was carried out using the SMART PCR cDNA synthesis kit (BD Biosciences Clontech, San Jose, CA, USA). cDNA subtraction was performed using the PCR-Select cDNA subtraction kit (BD Biosciences Clontech, San Jose, CA, USA) according to the protocol recommended by the manufacturer. The CSdt7BS line was used as the tester while the (cr)-CSdt7BS line was used as the driver in the forward hybridization, and vice versa in the reverse hybridization. Screening for the subtraction cDNA library was performed using the PCR-Select differential screening kit (BD Biosciences Clontech, San Jose, CA, USA).

Identification of the genomic DNA regions containing the clone R04 or R01

Identification of the genomic DNA regions containing R04 or R01 was performed using the Universal Genome Walker Kit (BD Biosciences Clontech, San Jose, CA, USA). The adaptor primer and gene-specific primer sets for clone R04 were as follows: for 5’ sequencing, R04-5’-L (adaptor 2) (5’-ACTATAGGCGCAGCTGTTG-3’) and R04-5’-R (5’-CAGTTGCTGTAATGTCGATAT-3’); for 3’ sequencing, R04-3’-L (5’-CTCTATTTCCATCTTTCCGTTGCACTAT-3’) and R04-3’-R (adaptor 1) (5’-GCCCTATAGTGAGTGATGTGATTAC-3’). The adaptor primer and gene-specific primer sets for clone R01 were as follows: for 5’ sequencing, R01-5’-L (adaptor 2) (5’-ACTATA GGCCACGCGTTGAGT-3’) and R01-5’-R (5’-GTCGACTCAATTGGCTGGCTCC-3’); for 3’ sequencing, R01-3’-L (5’-TTCGACTCATTTGGGACAGG-3’) and R01-3’-R (adaptor 2) (5’-ACCACCGCGCTGGCTATAGT-3’).

Expression analysis

Total RNA was isolated from young spikes at the floret differentiation stage of the (cr)-CS, (cr)-CSdt7BS and CSdt7BS lines. Young spikes were obtained from a minimum of 10 plants, and total RNA was isolated from all these together. DNase-digested total RNA (4.5 μg) was reverse-transcribed with an oligo(dT) primer, and first-strand cDNA was obtained using a first-strand synthesis for RT-PCR kit (GE Healthcare Biosciences, Buckinghamshire, UK). The clone-specific primer sets and annealing conditions for RT-PCR were as follows: for clone F01, F01-L (5’-GCTTACGCCCTTACCCATTA-3’) and F01-R (5’-TAGTCTTTCCAAATCCGCCCTC-3’) at 52°C; for clone F01, F01-L (5’-GGGAGGTGATAATATTAAGTGG AATG-3’) and F01-R (5’-GCTTTAGATTTACGGTCTTCC-3’) at 50°C; for clone R04, R04-L (5’-GACATGCACCAGA GTGACTCAATTTC-3’) and R04-R (5’-TTGGTCTATTTCCAGGCGGTCCTCTC-3’) at 50°C; and for clone R01, R01-L (5’-CTTTTCATTAGGCGCAGGTTG-3’) and R01-3’-R (adaptor 2) (5’-ACCACCGCGCTGGCTATAGT-3’).
orf260(\textsuperscript{m}), pistillody-related mitochondrial gene

TCGTCCTCCCTTGA-3') at 55°C. To examine the transcripts of the orf260(\textsuperscript{m})--cox I region, the following primer sets were used: for orf260(\textsuperscript{m}), 40L (5'-CCTCCAGCTTATGCATTCC-3') and 945R (5'-GAAGAGAAAAGAGGGG-3') at 52°C; for cox I, 1155L (5'-CCGCGTGCCTAGTCTCGC-3') and 2419R (5'-AGAACCGAAACGTCTCAG-3') at 53°C; for orf260(\textsuperscript{m})--cox I, 286L (5'-TTTTTATTCGCGCTGT-3') and 2048R (5'-TGTAGTTGATCGAACC-3') at 52°C, and 40L and 2419R (5'-GAACCGAAACGTCTCAG-3') at 53°C. As a control, a fragment from the wheat ubiquitin gene (Ubi-I) was amplified using the primers Ubi-1L (5'-GCAATGCAAGATTTGTGAA-3') and Ubi-1R (5'-GAGACCTTCTGTGCCAC-3') at 55°C (Murai et al. 2002). RT-PCR analysis was performed in the exponential range of amplification. PCR fragments were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Quantitative real-time PCR experiments of orf260(\textsuperscript{m}) were performed using the thermal cycler, Lightcycler 2.0 (Roche Diagnostics GmbH, Basel, Switzerland). Quantitation was performed using SYBR Green I fluorescence with the wheat ubiquitin gene (Ubi-I) as the control.

**Western blot analysis**

Mitochondrial proteins were isolated from 50–100 g of young spikes of the (cr)-N26 and (cr)-CS lines grown in Fukui or Hokkaido. As we have long-day conditions in Hokkaido, the (cr)-N26 line exhibits pistillody. In contrast, the (cr)-CS line grown in Fukui or Hokkaido. As we have long-day conditions in Hokkaido, the (cr)-CS line exhibits pistillody. In contrast, the (cr)-CS line grown in Fukui.

For SDS–PAGE, equal volumes of extract dissolved in SDS–PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue and 0.1 M dithiothreitol) were loaded onto an SDS–polyacrylamide gel with 12% acrylamide, 4% SDS, 20% glycerol, 0.002% bromophenol blue and 0.1 M dithiothreitol, followed by electrophoresis and photographs. The proteins were transferred to nitrocellulose membranes using the Compact Page system (ATTO Corporation, Tokyo, Japan) and separated by Western blotting. Proteins were transferred to nitrocellulose membranes using the Compact Page system (ATTO Corporation, Tokyo, Japan) and separated by Western blotting. Immunodetection of proteins was carried out using rabbit anti-ORF260cra polyonal antibodies. An anti-rabbit antibody (ECL plus\textsuperscript{TM} Western Blotting Reagent Pack, GE Healthcare Biosciences, Buckinghamshire, UK) was used as the secondary antibody and was visualized using the ECL plus Western Blotting Detection system (GE Healthcare Biosciences, Buckinghamshire, UK). An anti-ORF260cra polyonal antibody was raised against the peptide KSEDLRREELEKRRAG. The specificity of this antibody was confirmed using recombinant antigens that express ORF260cra protein fused with a 220 amino acid Tag protein from the pET-41a-c(+) vector (Novagen, Darmstadt, Germany). The antibody was purified on an affinity column, and its antigen specificity was checked by protein gel blotting.

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**References**


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