R Region of S Type of Cytoplasmic Male Sterility in Maize
Mitochondrial DNA is Transcribed in Both Directions
and May Be Associated with Male Sterility

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Abstract: It is well accepted that S type of cytoplasmic male sterility (CMS) in maize (Zea mays L.) is associated with R region in mitochondrial genome. R region includes two open reading frames — orf355 and orf77 and it is speculated that orf77 is an important candidate gene of CMS. RT-PCR showed that both DNA strands of R region are transcribed. Nuclear background or developing stages of plant do not influence the transcription of R region in the strand which also acts as template strand of coxI / coxII locating just upstream of R region, but they do influence the transcription of R region in the other strand (template strand of orf355-orf77). In tassel with nuclear background of rf3rf3, transcription of the template strand of orf355-orf77 is different from that in etiolated shoots with nuclear background of Rf3- or rf3rf3 and tassel with nuclear background of Rf3-. Compared to the same other three, it is truncated at about 238 base from the 5’ end of R region. Rabbit antiserum against putative ORF77 expressed in Escherichia coli was prepared but Western blot did not detect ORF77 in plant materials. It seems that double-strand transcription of R region inhibits the translation of orf77 and the transcribing mode of R region may be associated with male sterility.

Key words: maize; S type of CMS; mitochondrial DNA; R region; double-strand transcription
CGTCTAGAGACACGATTCCATTATT-3')；P4(5'-GCTCATGACCTAATCAATCCACTCATCG-3')。The target sites of the primers are shown in Fig.1.

1.4 Preparation of mitochondrial RNA and RT-PCR analysis

Mitochondria were isolated as above, then Trizol (Gibco, USA) was applied to extract RNA. Prior to the synthesis of first-strand cDNA, total RNA was treated with DNaseⅠ. First-strand cDNA is synthesized with Revertaid™H Mius First Strand cDNA Synthesis kit #K1631 (MBI, Fermentas). PCR was performed after digesting the first-strand cDNA with RNase H and RNase A.

1.5 Sequence analysis of products from RT-PCR

Amplified fragments were reclaimed and constructed to pGEM®-T Easy Vector (Promega, USA). Sequence analysis was then finished in Shangon Biotechnology Company (Shanghai, China).

2 Results

2.1 Western blotting analysis of plant mitochondrial protein

To investigate the expression of orf77 at translation level under different nuclear backgrounds, rabbit antiserum against putative ORF77 was prepared. The specificity of the antiserum was tested in Western blot on protein extracted from *E. coli* expressing the 26 kD glutathione S-transferase (GST) fused to 9 kD putative ORF77 and a 35 kD protein was detected. However, when 150 μg of mitochondrial proteins were analyzed in Western blot, no specific bands could be identified.

2.2 RT-PCR analysis of the transcription of R region

RT-PCR experiments with primer oligo(dT)18 on all four samples showed the same amplified cDNA fragment of 1.6 kb with P1 and P2 (Fig.2A, lanes 5-8). When using P2 which is located in the 3' terminal of R region for RT, different PCR results with P1 and P2 appeared, only sample 3 had none (Fig.2A, lanes 1-4). Two pairs of primers P3 and P4, P1 and P2 were then used as PCR primers respectively and the same amplified fragments of 1.1 kb (Fig.2C, lanes 1-4), 1.4 kb (Fig.2C, lanes 5-7) were obtained accordingly in all four samples. When using P1 anchored in the IR sequence of R region for RT and then P1 and P2 for PCR, the two same fragments of 1.6 kb, 1.0 kb appeared in all samples (Fig.2B, lanes 9-12). All the results are shown in Table 2.

2.3 Sequence analysis result of RT-PCR products

Sequence analysis showed that all the amplified fragments from RT-PCR of 1.6 kb, 1.4 kb and 1.1 kb above were almost the same as the DNA sequence of R region. But the fragments of all 1.0 kb amplified by P1 and P2 were totally different from the sequence in R region and sequence BLAST showed that they were almost the same as the DNA sequence in episome S1.

Because there is no similar sequence to P2 in the amplified product of 1.0 kb and a pair of invert repeat sequence of P1 locates on its 5' terminal and 3' terminals, it can be deduced that the 1.0 kb fragment actually amplified from mRNA of episome S1 only by P1. Furthermore PCR by P1 confirmed this speculation (Fig.2D).

2.4 Based on all the data above, several conclusions can be made as follows

2.4.1 Reverse transcription succeeded with both specific primers P1 and P2 showed that there are two opposite transcription directions of R. One is the same as that of coxⅡ (defined as positive direction), the other is opposite (defined as negative direction).
2.4.2 Reverse transcription with oligo(dT)$_{18}$ is in the same direction as that of P1 and the mRNA template is in positive direction. 

2.4.3 Nuclear background or developing period of plant do not influence the transcription of R region in positive direction, but they do influence the transcription of R region in the negative direction.

2.4.4 The transcription product of R region in negative direction in tassel of nuclear background of $rf3fr3$ is different from that in etiolated shoots with nuclear background of $Rf3$- or $rf3fr3$ and that in tassel with nuclear background of $Rf3$-. RT-PCR result implied that the difference is limited to the sequence between P1 and P3 (from IR sequence to +238) of negative mRNA strand.

2.4.5 Perhaps R region can not be translated to proteins because its mRNA exists in double strand. Western blot result of ORF77 just coincide with this hypothesis.

3 Discussion

Novel open reading frames (ORFs), which are often chimeric in structure, have been found to be associated with CMS in different plants (Hanson, 1991). Fertility restorer
gene (Rf) can restore pollen fertility by regulating the transcription, post-transcription editing, translation or post-translation of these ORFs (Wise et al., 1987; Abad et al., 1995; Singh et al., 1996; Tang et al., 1996; Bergman et al., 2000; Bentolila et al., 2002).

In this experiment, RT-PCR revealed the double-strand transcribing mode and the lengths of transcriptions of R region (including orf77). It was found that mRNA of R region in tassel with sterile nuclear background is different from that in tassel with fertile nuclear background or etiolated shoot with either of the two types of the nuclear background. It can be concluded that this transcriptional difference of R region is associated with the sterility of tassel.

Among the sequence of orf77, there are three stretches similar to atp9 of mitochondria, so it is presumed that S-type male sterility could result from a partial impairment of the mitochondrial function as a result of the expression of orf77. The ORF77 antisera did react with ORF77 expressed in E. coli in Western analysis, but we did not detect ORF77 in plant mitochondrial proteins under the same condition for Western blot. So we can deduce that orf77 does not translate in sterile or fertile plant in different developing stages. Perhaps double strand orf77 mRNA inhibits the translation of orf77 and the sterility of S-type CMS of maize is only associated with the transcription of R region.

Both coxl and coxII are reported locating just upstream of R region. From RT-PCR results in this experiment and bioinformation analysis, we can deduce that polyA belongs to mRNA of coxl or coxII and one strand in R region co-transcribe with coxl or coxII. Although RT-PCR analysis in this experiment can not show the initiation site of transcription in the DNA strand including orf355-orf77 in sterile tassel, it does show that the transcribed product is longer in sterile tassel than that in fertility restore tassels, so perhaps coxl or coxII has part or whole double-strand mRNA in sterile tassel and results in abnormally transcription of itself and sterile phenotype; but in fertile tassel or etiolated shoots, mRNA of coxl or coxII is in single-strand and can translate normally.

Double-strand mRNA of CMS-related segment is also found in Vicia faba (Pfeiffer, 1998) and the analysis of orf274 in mtDNA associated with male-sterile tobacco showed a similar Western blot result (Bergman et al., 2000) as that of orf77 in this experiment. These results may mean something about the same mechanism of plant CMS.

Northern blotting analysis with single-strand cDNA probe of R region and construction of S type CMS maize mitochondrial cDNA library progressing in our laboratory will contribute to test the hypothesis above and explain the CMS mechanism to further degree.

References:
