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Analysis of mitochondrial DNA using amplified fragment length polymorphism markers of isonuclear allocytoplasmic male sterile wheat accessions and their maintainer lines

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ABSTRACT. To produce a good F1 hybrid variety wheat crop, it is necessary to explore novel cytoplasmic male sterility (CMS) lines and their maintainer line. This study aimed to identify cytoplasmic variation in three isonuclear-alloplasmic male sterile lines *Aegilops kotschyi* (*Ae. kots*) -90-110, *Aegilops ventricosa* (*Ae.ven*) -90-110, and *Triticum spelta* (*T.spelta*) -90-110 and their maintainer line, A-90-110, at the molecular level. Mitochondrial DNA (mtDNA) was isolated using a combination of centrifugation and density gradient ultracentrifugation, sucrose sedimentation, lysis with sodium dodecyl sulfate (SDS), potassium proteinase, and phenol/chloroform extraction methods. To detect mtDNA purity, specific primers were designed for nuclear (β-actin) and mitochondrial (COXIII) genes. Results indicated that the mtDNA was pure, and therefore suitable for polymerase chain reaction (PCR) and genetic analysis. Comparative analysis of mtDNA was conducted using amplified fragment length polymorphism (AFLP) markers. Reproducible polymorphisms were detected between the *Aegilops* and *Triticum* species and the male sterile lines. Four specific primers were screened from 64

AFLP marker primers, which provided the molecular basis for further studies investigating specific cytoplasmic male sterility characteristics.

Key words: Wheat; Cytoplasmic male sterility; mtDNA; AFLP; Molecular marker

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a staple food and one of the most important agricultural crops in the world. Wheat constitutes the basis for human nutrition and is of enormous economic importance worldwide. Wheat is used mainly for human consumption and supports nearly 35% of the world's population (Schuster et al., 2009). Cytoplasmic male sterility (CMS) is a very important trait for improving yield and quality of crops to meet the needs of increasing global population. In some well-studied species, the trait appears to be associated with mitochondrial DNA (mtDNA) mutations that disrupt mitochondrial function at a critical stage of anther development, thus causing male sterility (Breiman and Galun, 1990; Hanson, 1991; Mackenzie et al., 1994). Molecular studies involving sterile and fertile plants have revealed variations in the restriction pattern of mtDNA transcripts and demonstrated differences at the molecular level (Levings and Pring, 1976; Song and Hedgcoth, 1994). Alterations in specific mitochondrial genes and inserts have been identified (Song and Hedgcoth, 1994). Moreover, nuclear and cytoplasmic combinations from different plant species often leads to CMS, indicating that sterility is likely caused by functional incompatibilities between the nuclear and mitochondrial genomes (Kofer et al., 1991). The contribution of mtDNA to the expression of CMS has been demonstrated in a variety of plant species (Braun et al., 1992). Extensive rearrangements in mitochondrial genomes between CMS and fertile males are considered as a general source of CMS (Braun et al., 1992; Mackenzie et al., 1994). It is generally believed that plant CMS results from mtDNA rearrangements, such as insertions and deletions generated by mutations. These mutations can express the open reading frame, leading to changes in transcription and translation of the product, which ultimately result in male plant sterility (Lorenz et al., 1997; Budar et al., 2003; McDermott et al., 2008; Chen et al., 2009; Han et al., 2010). CMS of the wheat mitochondrial atp6 gene transcript editing sites edit inadequate transcripts, which affect the normal functioning of mitochondria, thereby affecting the generation of receptor male sterility (Li et al., 2007). Wheat mitochondrial genome variability is likely related to changes in the nature of the sterile line; however, it is difficult to determine whether the sterility is due to nuclear genes, cytoplasmic genes, or interactions between nuclear and cytoplasmic elements. To solve this problem, in this study, continuous backcrossing was performed in up to a dozen generations in order to establish nuclear male sterile wheat lines with the same nuclear genetic background. These lines are considered to have the same nuclear genome and different cytoplasmic genomes. Amplified fragment length polymorphisms (AFLP) markers were used to analyze wheat mtDNA at the molecular level in order to identify the different types of sterile cytoplasm variations, as well as to determine specific fertility. Together, these results should help to improve the performance and actual utilization of cytoplasmic infertility (Vos et al., 1995).

MATERIAL AND METHODS

Four wheat accessions, *Aegilops kotschyi*, *Aegilops ventricosa*, *Triticum spelta*, and one

fertile common wheat variety, 90-110, were used in the present investigation. These lines were derived from recurrent backcrosses of *Aegilops kotschyi*-Chris, *Aegilops ventricosa*-Chris, and *Triticum spelta*-Chris. Yellow etiolated wheat seedlings were grown in the dark over a period of 7 to 10 days at 30°C in growth chambers, and their growth needs were met by nutrients supplied by sterile water only. The plants were strictly protected from light. All of the cultural procedures were performed in the dark to prevent plants from becoming green, and to obtain high quality mtDNA free of any chloroplast, genomic DNA, or other impurities. This study was carried out with emphasis on mtDNA variation among *Aegilops* to identify the different types of sterile cytoplasm as well as specific fertility in order to improve the performance of the actual utilization of cytoplasmic infertility and to produce a good hybrid variety at the Key Laboratory of Crop Heterosis of Shaanxi Province, Northwest Agricultural & Forest University, Yangling, China.

DNA isolation

mtDNA was isolated from etiolated shoots as described by Li et al. (2007). mtDNA was isolated with differential centrifugation, DNase treatment, lysis with sodium dodecyl sulfate (SDS), and potassium proteinase, removing proteins by TE-saturated phenol/chloroform extraction, and a final RNase treatment (Figure 1). The mtDNA samples were tested for purity using spectrophotometry and agarose gel electrophoresis. We analyzed the samples through amplification of the nuclear β subunit, that is, β-actin genes, and the mitochondrial Coxidase subunit (COXIII) gene. Results demonstrated that the mtDNA was not contaminated by nuclear DNA, plastid DNA, RNA, or protein, and could successfully be used for polymerase chain reaction (PCR), cloning, and Southern blot analyses.

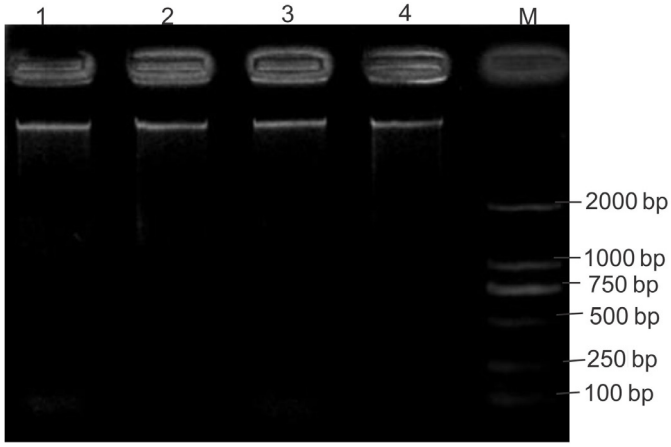

Figure 1. Results of mtDNA. *Lane 1* = (A)-90-110; *lane 2* = ms(Kots)-90-110; *lane 3* = ms(Ven)-90-110; *lane 4* = ms(S)-90-110. *Lane M* = marker DL 2000.

AFLP analysis

AFLP analysis was performed using commercially available kits following manufacturer instructions (Fermentas). Sixty-four primer combinations (Table 1) were tested for poly-

morphism analysis between the varieties. Two hundred fifty nano grams mtDNA was restricted with 5 U each of *Eco*RI and *Mse*I enzymes for 180 min at 37°C and for 150 min at 65°C, respectively, in 20 µL reaction volumes, and the enzymes were heat-inactivated by incubating tubes at 70°C for 15 min. The DNA fragments were ligated to appropriate adapters using 1 U T_4 DNA ligase with 1 µL *Eco*RI and *Mse*I (primer sequences for EAF, EAR, MAF, and MAR are listed in Table 2, which were prepared according to manufacturer instructions) adapter ligation mixture by incubating at 16°C for 12 h in 20 µL reaction volumes. The ligated DNA mixture was pre-amplified using adapter primers with *Eco*RI (E00) and *Mse*I (M00) selective nucleotides (Table 2), 1.6 µL 2.25 mM dNTPs, 0.1 µL 5 U/µL rTaq, and 2 µL PCR buffer in a final volume of 20 µL. The PCR cycling parameters were as follows: 1 cycle of 94°C for 30 s; 32 cycles each of 94°C for 30 s, 56°C for 45 s, 72°C for 120 s; and one cycle of 72°C for 10 min. For selective amplification, 1 µL each *Eco*RI and *Mse*I primers were mixed with 5 µL 30-fold diluted preamplifier DNA, 1.6 µL dNTPs, 0.2 µL rTaq, and 2 µL PCR buffer in a final volume of 20 µL. The following PCR cycling parameters were used for selective amplification: the 1st cycle was at 94°C for 60 s and the 2nd cycle was at 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s. During the next 15 cycles, the annealing temperature was lowered by 1°C per cycle. The temperature conditions for the next 25 cycles were 94°C for 30 s, 45°C for 45 s, and 72°C for 60 s. The amplified products were analyzed on a 6% denaturing polyacrylamide gel.

Table 1. Primer combinations of *Eco*RI and *Mse*I.

E1/M1	E1/M2	E1/M3	E1/M4	E1/M5	E1/M6	E1/M7	E1/M8
E2/M1	E2/M2	E2/M3	E2/M4	E2/M5	E2/M6	E2/M7	E2/M8
E3/M1	E3/M2	E3/M3	E3/M4	E3/M5	E3/M6	E3/M7	E3/M8
E4/M1	E4/M2	E4/M3	E4/M4	E4/M5	E4/M6	E4/M7	E4/M8
E5/M1	E5/M2	E5/M3	E5/M4	E5/M5	E5/M6	E5/M7	E5/M8
E6/M1	E6/M2	E6/M3	E6/M4	E6/M5	E6/M6	E6/M7	E6/M8
E7/M1	E7/M2	E7/M3	E7/M4	E7/M5	E7/M6	E7/M7	E7/M8
E8/M1	E8/M2	E8/M3	E8/M4	E8/M5	E8/M6	E8/M7	E8/M8

Table 2. Amplified fragment length polymorphism (AFLP) primer sequence.

Code	Primer sequence
EAF	5'-GTA GAC TGC GTA CC-3'
EAR	5'-AAT TGG TAC GCA GTC TAC-3'
MAF	5'-GAC GAT GAG TCC TGA G-3'
MAR	5'-TAC TCA GGA CTC AT-3'
E00 (Pre-amplifier primer)	5'-GTA GAC TGC GTA CCA ATC A-3'
M00 (Pre-amplifier primer)	5'-GAC GAT GAG TCC TGA GTAA C-3'
E1	5'-GTA GAC TGC GTA CCA ATC AAC-3'
E2	5'-GTA GAC TGC GTA CCA ATC AAG-3'
E3	5'-GTA GAC TGC GTA CCA ATC ACA-3'
E4	5'-GTA GAC TGC GTA CCA ATC ACT-3'
E5	5'-GTA GAC TGC GTA CCA ATC ACC-3'
E6	5'-GTA GAC TGC GTA CCA ATC ACG-3'
E7	5'-GTA GAC TGC GTA CCA ATC AGC-3'
E8	5'-GTA GAC TGC GTA CCA ATC AGG-3'
M1	5'-GAC GAT GAG TCC TGA GTAA CAA-3'
M2	5'-GAC GAT GAG TCC TGA GTAA CAC-3'
M3	5'-GAC GAT GAG TCC TGA GTAA CAG-3'
M4	5'-GAC GAT GAG TCC TGA GTAA CAT-3'
M5	5'-GAC GAT GAG TCC TGA GTAA CTA-3'
M6	5'-GAC GAT GAG TCC TGA GTAA CTC-3'
M7	5'-GAC GAT GAG TCC TGA GTAA CTG-3'
M8	5'-GAC GAT GAG TCC TGA GTAA CTT-3'

mtDNA purity identification

We analyzed the samples through the amplification of the nuclear β subunit, that is, β-actin genes, and the mitochondrial Coxidase subunit (COXIII) gene. This entailed the use of two separate 20 µL volumes containing 1 µL mtDNA, 6 µL ddH_2O, 1 µL forward and reverse gene-specific primers, and 10 µL master mix. The PCR conditions were as follows: 5 min at 94°C; 5 cycles each of 1 min at 94°C, 30 s at 60°C, and 1 min at 70°C; and 35 cycles each of 50 s at 94°C, 1 min at 60°C, 50 s at 72°C, and 6 min at 72°C. The products were separated on 1% (w/v) agarose gel by electrophoresis. The 400-bp mitochondrial COXIII gene was amplified and the β-actin gene was not amplified, which demonstrated that the mtDNA was not contaminated by nuclear DNA, plastid DNA, RNA or protein, and could be successfully used for PCR, cloning, and Southern blot analyses (Figure 2).

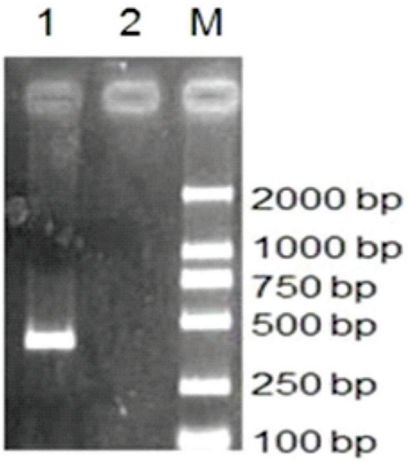

Figure 2. *Lane 1* = COXIII; *lane 2* = β-actin; *lane M* = marker DL 2000.

RESULTS

For AFLP analyses, all 64 primer pairs (Table 1) were used to examine polymorphisms between the three alloplasmic nuclear male sterile lines and their maintainer line of wheat crop. mtDNA of these lines was digested with the *Eco*RI and *Mse*I enzymes. Dozens of clear bright bands were obtained by polyacrylamide gel electrophoresis. From the 64 primer pairs, four primer combinations were screened out (E1/M7, E4/M2, E6/M4, and E7/M6) that showed better polymorphism among these lines (Figures 3 and 4). Primer E1/M7 amplified three specific bands of approximately 200, 400, and 750 bp in the *A. kotschyi* CMS line (*Ae. kots*-90-110), while the *T. spelta* sterile line (*T.spelta*-90-110) amplified a 500 bp specific band, which could be associated with *A. kotschyi* and *T. spelta* cytoplasmic infertility (Figure 3). The primer pair E4/M2 amplified a 200 bp specific band in the *Ae.kots*-90-110 sterile line, where as it amplified two specific bands of approximately 1000 and 600 bp in the *A. ventricosa* sterile line (*Ae.ven*-90-110) (Figure 3). The primer E6/M4 amplified a fragment of approximately 200 bp and a specific gene of 250 bp in the *Ae.kots*-90-110 sterile line (Figure 4). The primer pair

E7/M6 amplified two fragments of approximately 300 and 400 bp in *T.spelta*-90-110, which represented the genes responsible for infertility (Figure 4). From these results, mitochondrial male sterility could be identified in the CMS lines.

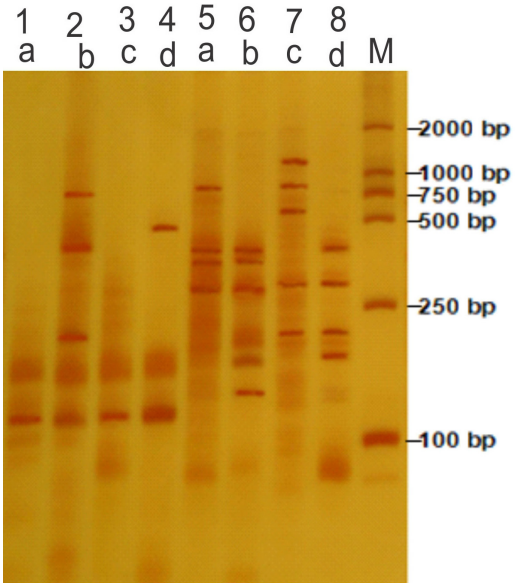

Figure 3. AFLP amplification profiles of three CMS lines and maintainer line. Primer E1/M7: *lane 1a* = (A)-90-110; *lane 2b* = ms(Kots)-90-110; *lane 3c* = ms(Ven)-90-110; *lane 4d* = ms(S)-90-110. Primer E4/M2: *lane 5a* = (A)-90-110; *lane 6b* = ms(Kots)-90-110; *lane 7c* = ms(Ven)-90-110; *lane 8d* = ms(S)-90-110; *lane M* = marker DL 2000.

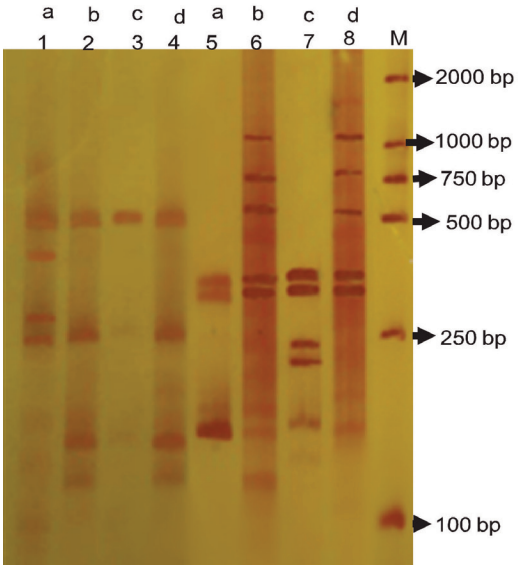

Figure 4. AFLP amplification profiles of three CMS lines and maintainer line. Primer E7/M6: *lane 1a* = ms(S)-90-110; *lane 2b* = ms(Ven)-90-110; *lane 3c* = ms(Kots)-90-110; *lane 4d* = (A)-90-110. Primer E6/M4: *lane 5a* = ms(S)-90-110; *lane 6b* = ms(Ven)-90-110; *lane 7c* = ms(Kots)-90-110; *lane 8d* = (A)-90-110; *lane M* = marker DL 2000.

DISCUSSION

Mitochondria contain genes that cause male sterility. Inter- or intramolecular rearrangements at specific sites of the mitochondrial genome result in CMS in plants (Dewey et al., 1986). Using molecular marker technology, differences in specific sites related to CMS were identified in the mitochondrial genome of male sterile lines compared with their maintainer line. Previously, wheat genetic diversity was compared with a rapid amplified polymorphic DNA (RAPD) molecular marker (Gao et al., 2011); however, this marker was not efficient due to its poor reproducibility.

AFLP molecular markers show good reproducibility, stability, and heterogeneity, and results are therefore more reliable. This study was based on three nuclear male sterile lines of wheat crop and their maintainer line. Four pairs of primers were screened out, which showed clear differences between the nuclear male sterile lines and the maintainer fertile line. These stable differences will prove to be helpful for assessing CMS in K, Ven, and S-type wheat crops. Under normal circumstances, male sterility and fertility restoration are caused by cytoplasmic genes (Li et al., 1998). However, it is generally very difficult to assess whether this phenomenon is due to nuclear or cytoplasmic elements. The three lines used in the present study were developed after back crossing of a male sterile line of the homo-nucleus for up to 15 generations. The results showed that these differences were due to the cytoplasm. The cloning, sequencing, and expression of these specific fragments will provide a foundation for more in depth investigations and further discovery and excavation of new wheat CMS-related genes.

Mitochondria was isolated with differential centrifugation, DNaseI enzyme treatment, lysis with SDS, and potassium proteinase, removing proteins by TE-saturated phenol/chloroform extraction and a final RNase enzyme treatment for obtaining pure and good quality mtDNA that was suitable for PCR and genetic analysis. Cytoplasmic variations were identified in three isonuclear, alloplasmic male sterile lines, *Ae.kots*-90-110, *Ae.ven*-90-110, and *T.spelta*-90-110, and their maintainer line, A-90-110, at the molecular level. AFLP analysis revealed stable polymorphisms; four pair of primers were screened out to detect the different fragments responsible for CMS in these lines, which will provide a foundation for further study.

ACKNOWLEDGMENTS

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