Development and Application of SCAR Markers for Discriminating between CMS lines and Their Maintainer Lines in Indica Rice (*Oryza sativa* L.)

LU Chao¹, LIU Jian¹, JIANG Jian-hua¹, Caleb Manamik BRERIA¹, TAN He-lin¹, Masahiko ICHII², HONG De-lin¹

¹State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China; ²Faculty of Agriculture, Kagawa University, Miki, Kagawa, 761-0795, Japan

Abstract: The DNA fragments about 1 600 bp were amplified using random amplified polymorphism DNA (RAPD) primer OPA12 with the templates of mitochondrial DNA of Zhenshan 97A and Zhenshan 97B, and were sequenced. The nucleotide sequences and lengths of the fragments from Zhenshan 97A and Zhenshan 97B showed no difference. The precise length of the fragment was 1 588 bp. Sequence characterized amplification region (SCAR) primers were then developed to discriminate the cytoplasmic male sterile (CMS) lines and their maintainer lines. A specific 1 588 bp fragment could be amplified with SCAR primers, CHI19F2/CHI19R2 and CHI20F3/CHI23R3, in the mitochondrial DNA of Zhenshan 97A, but not Zhenshan 97B. Furthermore, the specific fragment could be also amplified from the total DNA from green leaf tissue of Zhenshan 97A with SCAR primers, but not Zhenshan 97B. With the corresponding primers, the specific fragment could be also amplified from the total DNA of green leaf of other two CMS-WA lines, namely Zhenpin A and Tianfeng A, but not in their maintainer lines. Moreover, using total DNA as template, each of the four pairs of SCAR primers could also be used to amplify the 1 588 bp fragment in CMS-ID line II-32 A, but not in II-32 B, and the specific fragment was amplified from DNA of both F₁ and F₂ seedlings of Shanyou 63. The results of detecting genetic purity of a man-made mixture seed lot of Zhenshan 97A using CHI20F3/CHI23R3 were completely consistent with the phenotypes. In all, these results indicated that the specific 1 588 bp-fragment amplified by CHI20F3/CHI23R3 was the unique amplification products of CMS mitochondrial DNA, and could be used to distinguish CMS-WA and CMS-ID lines from their corresponding maintainer lines at seedling stage.

Keywords: *Oryza sativa*, CMS line, maintainer line, SCAR marker, genetic purity

Rice (*Oryza sativa* L.) is one of the most important crops in the world and it provides the main resource of food for more than half of the world population, especially in Asian. It is estimated that the world population will reach 9.3 billion by 2050 (Nguyen N V, 2009), which necessitates an increase of the rice production by 23% to keep pace with the population growth. One of the available strategies to deal with the great challenge is commercial exploitation of heterosis through large scale cultivation of hybrid rice. Hybrid rice has been cultivated commercially in China since 1976, and has occupied more than 50% of total rice planting area in China since 1988 (Lu and Hong, 1999; Deng et al, 2006; Mao et al, 2009). Production of high-quality and high-purity seed is critical for the production and development of hybrid rice. Various admixtures in hybrid rice seeds may result in yield reduction. Genetic purity of hybrid seeds supplied to farmers must surpass 96% in China (National Standard Bureau of PRC, 1983). The India seed act prescribes that the purity for hybrid rice should be 98% (Vermma, 1996). To guarantee the required level of purity in hybrid seed, the genetic purity of parental lines that will be utilized in hybrid seed production must reach a 99% level. CMS plants and its maintainer plants were the majority of off-type plants in indica hybrid rice (Lu et al, 1982, 1983; Yashitola et al, 2004). These off-type plants originated from the contamination of the CMS population by maintainer seeds in F₁ seeds production field in the previous year.

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Corresponding author: HONG De-lin (delinhong@njau.edu.cn)
(Lu and Hong, 1989). Owing to cognate isonuclear situation between CMS lines and maintainer lines, it is difficult to differentiate the two lines from each other before flowering. Therefore, development and application of practical molecular markers would greatly facilitate the detecting of CMS seed lots contaminated with the maintainer’s. Although polymorphism was observed in mitochondrial DNA between normal and CMS lines (Yamaguchi et al., 1983; Nawa et al., 1985; Kadowaki et al., 1986, 1989) as well as among the CMS lines (Kadowaki et al., 1988), the discrimination is very expensive for routine inspection purpose. Restriction fragment length polymorphism (RFLP) makers have been identified to accurately distinguish CMS-WA lines from their maintainer lines (Narayanan et al., 1996; Sane et al., 1997). However, RFLP markers are unfit for routine screening on a large scale. In our previous study, we found a RAPD primer OPA12 could be used to distinguish an indica CMS-WA Zhenshan 97A from Zhenshan 97B according to the existence of a specific amplification product under a given annealing temperature (Hong et al., 2002; Ichii et al., 2003). However, many investigations indicated that RAPD markers were low reproducibility, and thus were practically unfeasible to discriminate CMS between maintainer lines in a routine manner (Sane et al., 1997; Jena and Pandey, 1999). In this study, therefore, we tried to develop stably reproducible SCAR markers through DNA sequencing and analysis of the amplicons (about 1 600 bp) with the RAPD primer OPA12 and the thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The SCAR markers developed from the amplified sequence were applied to distinguish two other CMS-WA lines and their maintainer lines, and a CMS-ID line and its maintainer line. The SCAR primers were also used to test the mitochondrial origination with the total DNA from seedlings leaf of Shanyou 63 grown in paddy field as template. The purity assessment of Zhenshan 97A seeds was performed by using the SCAR markers individually. Therefore, development and use of molecular markers described in this study will be of great significance in hybrid seed production and seed purity testing of CMS lines at early stages of plant growth.

MATERIALS AND METHODS

Rice materials

Rice materials used in this study were four pairs of CMS lines and their corresponding maintainer lines, one restorer line Minghui 63, F1 and F2 populations of a famous hybrid Shanyou 63 (Zhenshan 97A/Minghui 63), and a predetermined mixture of seeds of CMS line Zhenshan 97A with its maintainer line Zhenshan 97B. The four pairs of CMS lines and corresponding maintainer line were WA-type Zhenshan 97A and its maintainer line Zhenshan 97B, WA-type Zhenpin A and its maintainer line Zhenpin B, WA-type Tianfeng A and its maintainer line Tianfeng B, ID-type II-32 A and its maintainer line II-32 B. Seeds of Zhenpin A and Zhenpin B were introduced from Wenzhou Academy of Agricultural Sciences, Zhejiang Province, China. Seeds of remaining materials were stored and propagated by our laboratory. All rice materials were grown in the paddy fields at Jiangpu Experimental Station of Nanjing Agricultural University, China in 2010 and 2011.

DNA isolation and primer design

Total DNA was isolated following cetyltrimethylammonium bromide (CTAB) method using 0.5–0.8 g green leaves for each sample (Murray et al., 1980). Mitochondrial DNA was extracted according to the procedure described by Kadowaki et al. (1986) using 15-30 g etiolated shoots for each sample. The OPA12 primer was purchased from Operon Co., Ltd. Four primers, i.e. CHI18F1, CHI18R1, CHI19F2 and CHI19R2 containing the sequence of OPA12 were designed based on sequence information of DNA fragment amplified using mitochondrial DNA of Zhenshan 97A and Zhenshan 97B as templates. The primers were designed using the software Primer3 (White Head Institute) and synthesized by Life Table 1. Primers used in the study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotides sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD1</td>
<td>NGTCGASGWANANGAA</td>
</tr>
<tr>
<td>AD2</td>
<td>GTNCGASWCANAWGTT</td>
</tr>
<tr>
<td>AD3</td>
<td>WGTGNAWANCANAGA</td>
</tr>
<tr>
<td>CHI18F1</td>
<td>TCGGGCAGTAGAGATGCTG</td>
</tr>
<tr>
<td>CHI18R1</td>
<td>TCGGGCAGTAGGCACATAA</td>
</tr>
<tr>
<td>CHI19F2</td>
<td>TCGGGCAGTAGAGCTGTA</td>
</tr>
<tr>
<td>CHI19R2</td>
<td>TCGGGCAGTAGGCACATAAT</td>
</tr>
<tr>
<td>CHI20F3</td>
<td>GTGGCCGATAGAGCTGAGG</td>
</tr>
<tr>
<td>CHI23R3</td>
<td>TCGCAATAGGGCCATAATTAGA</td>
</tr>
<tr>
<td>OPA12</td>
<td>TCGCCGATAG</td>
</tr>
<tr>
<td>TF1</td>
<td>CCAGAACCAGAGCAGGGCAAGTTA</td>
</tr>
<tr>
<td>TF2</td>
<td>GATTTCTCGGTCGCTCAGCA</td>
</tr>
<tr>
<td>TF3</td>
<td>GTGGCCGAAAGTCAAGACAGGGAA</td>
</tr>
<tr>
<td>TR1</td>
<td>GGTCCGGTATCTGGCTCTACA</td>
</tr>
<tr>
<td>TR2</td>
<td>TGCTTACATTTAAAGGGCGGCG</td>
</tr>
<tr>
<td>TR3</td>
<td>CCTACCGGTTCCTCTACCC</td>
</tr>
</tbody>
</table>
Biotec Co., Ltd. The short arbitrary degenerate (AD) primers (Table 1) used for TAIL-PCR were the same as those described by Liu and Whittier (1995).

**PCR amplification**

For the PCR-RFLP reactions, 20 ng of mitochondrial DNA were used as template in a final volume of 40 μL reaction mixture (10 mmol/L Tris- HCl pH 8.3, 1.5 mmol/L MgCl2, 50 mmol/L KCl, gelatin 0.001%, 0.2 mmol/L dNTPs, 0.163 μmol/L primer (Operon 10 mer), 0.5 U Taq DNA polymerase). The PCR cycling was performed in a Thermal Cycler 2720 (Applied Biosystems, USA) and consisted of an initial denaturation at 95 °C for 12 min, followed by 45 cycles of 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C, and terminated a final extension step for 7 min at 72 °C. PCR-RFLP assay was carried out to find out polymorphism in the monomorphic PCR products at 72 °C. All PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and visualized under a ultraviolet (UV) transilluminator. Fragment length was estimated by comparison with standard size markers.

**DNA sequencing and analysis**

The fragment about 1 600 bp amplified by the OPA12 in Zhenshan 97A and Zhenshan 97B were gel eluted and purified with QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany), cloned in TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA), and sequenced in both forward and reverse directions using an Aroca LIC-4200L2 Automated DNA Sequencer according to the Manufacturer’s instruction. Homology search was performed by BLASTn algorithm (Altschul et al, 1997) through the NCBI website (www.ncbi.nlm.nih.gov).

Thus, the SCAR primers were designed based on sequence information. In addition, TAIL-PCR was performed to investigate the precise sequence matching with OPA12 in Zhenshan 97A and Zhenshan 97B. The reaction conditions for TAIL-PCR were as follows.

For the primary PCR reaction, 30 ng mitochondrial DNA were used as template in a final volume of 20 μL reaction mixture (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, gelatin 0.001%, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs, 0.25 U Ampli Taq Gold, 4.0 μmol/L degenerate primer and 0.2 μmol/L specific primer); for the second PCR reaction, 1 μL 50-fold dilution of the first PCR products were used as template in a final volume of 100 μL reaction mixture (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, gelatin 0.001%, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs, 0.25 U Ampli Taq Gold, 4.0 μmol/L degenerate primer and 0.2 μmol/L specific primer). The reaction procedures for TAIL-PCR were as follows. For the primary reaction, 1 cycle of 12 min at 95 °C; 5 cycles of 1 min at 94 °C, 1 min at 65 °C, 3 min at 72 °C; 1 cycle of 1 min at 94 °C, 5 min 28 s at 65 °C, ramping to 72°C over 329 s, 3 min at 72 °C; 15 cycles of 30 s at 94 °C, 1 min at 66 °C (on the occasion using TF1 and AD1, AD2, AD3) or 68 °C (on the occasion using TR1 and AD1, AD2, AD3), 3 min at 72 °C, 30 s at 94 °C, 1 min at 66 °C (on the occasion using TF1 and AD1, AD2, AD3) or 68 °C (on the occasion using TR1 and AD1, AD2, AD3), 3 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, 3 min at 72 °C (These are nine-segment super cycles each consisting of two high-stringency and one reduced- stringency cycle); 1 cycle of 7 min at 72 °C. For the secondary reaction, 1 cycle of 12 min at 95 °C; 12 cycles of 30 s at 94 °C, 1 min at 64 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 64 °C, 2 min at 72 °C, 30 s at 94 °C, 1 min at 44 °C, 3 min at 72 °C (These are nine-segment super cycles each consisting of two high-stringency and one reduced-stringency cycle); 1 cycle of 7 min at 72 °C. For the tertiary reaction, 1
cycle of 12 min at 95 °C; 15 cycles of 30 s at 94 °C, 1 min at 64 °C (on the occasion using TF3 and AD1, AD2, AD3) or 66 °C (on the occasion using TR3 and AD1, AD2, AD3), 2 min at 72 °C, 30 s at 94 °C, 1 min at 40 °C (on the occasion using TR3 and AD1, AD2, AD3) or 44 °C (on the occasion using TF3 and AD1, AD2, AD3), 2 min at 72 °C (These are nine-segment super cycles each consisting of two high-stringency and one reduced-stringency cycle); 1 cycle of 7 min at 72 °C. The program files in each reaction were linked automatically. Thermo cycling was carried out using a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer).

Assessment of Zhenshan 97A seeds

A predetermined mixture of 100 seeds of CMS-WA line Zhenshan 97A was made with its maintainer line Zhenshan 97B and the seeds were sown on May 20 and transplanted on June 20 individually in paddy rice field of experimental farm of Nanjing Agricultural University, Nanjing, Jiangsu Province, China. Each plant was given a code number and the leaf blade was collected from 40-day-old seedlings for total DNA extraction. The DNA from individual seedling was then used for PCR amplification using the SCAR markers CHI20F3/CHI23R3. After resolving the amplified fragments on agarose gel, off-type plants among the 100 coded plants were identified based on amplification results.

RESULTS

DNA polymorphism analysis of Zhenshan 97A and Zhenshan 97B based on PCR-RFLP

We amplified the DNA fragment from the mitochondrial DNA samples of Zhenshan 97A and Zhenshan 97B with OPA12 primer. The length of amplified products was about 1600 bp. The amplified products were digested by the seven restriction enzymes (Hap II, Hae III, Acc II, Hha I, Alu I, Taq I). The nucleotide sequences and digestion sites of restriction enzymes of the DNA fragments amplified from Zhenshan 97A and Zhenshan 97B using mitochondrial DNA as templates with primer OPA12 are shown in Fig. 1.
and *Rsa*I, the cutting sites in sequence were shown in Fig. 1), and then electrophoresed. No DNA polymorphism was found between Zhenshan 97A and Zhenshan 97B.

**Sequence analysis of DNA fragments amplified by OPA12**

The fragments amplified by OPA12 with the mitochondrial DNA of Zhanshan 97A and Zhanshan 97B were sequenced. The full-lengths of the fragments amplified from Zhenshan 97A and Zhenshan 97B were both 1,588 bp, and their sequences shared identical base pairs (Fig. 1). Through BLASTn search of the 1,588 bp against GenBank (www.ncbi.nlm.nih.gov), the sequence aligned with a mitochondrial DNAs region of rice (nucleotide sequence identity 99%, the accession numbers were AP011077, DQ167400, DQ167807, AP011076, BA000029).

**Discriminating Zhenshan 97A and Zhenshan 97B based on SCAR markers**

Two pairs of SCAR primers, designated as CHI18F1/CHI18R1 (18 bp) and CHI19F2/CHI19R2 (19 bp), were designed, which contain the sequence of OPA12 at the 5′ terminal. By combination of the two pairs of primers, four pairs of primers, viz. CHI18F1/CHI18R1, CHI18F1/CHI19R2, CHI19F2/CHI18R1, and CHI19F2/CHI19R2 were obtained. PCRs were performed with the four primer pairs using mitochondrial DNA templates isolated from Zhenshan 97A and Zhenshan 97B. The expected 1,588 bp DNA fragments were amplified by all the primer pairs except CHI18F1/CHI18R1 in Zhenshan 97A. A clearest 1,588 bp band was amplified using the primer pair CHI19F2/CHI19R2. In Zhenshan 97B, however, no 1,588 bp band was amplified with all the four primer pairs. These results indicated that the 1,588 bp specific band derived from CMS mitochondrial DNA can be used as a SCAR marker to distinguish Zhenshan 97A and Zhenshan 97B plants.

**Determination of annealing sites of OPA12 by TAIL-PCR analysis**

Using mitochondrial DNA from Zhenshan 97A as template, an amplification segment about 500 bp flanking the 1,588 bp sequence at 5′ terminal and a 300 bp segment at the 3′ terminal were obtained by TAIL-PCR, respectively. Sequencing showed that both of the amplification fragments contained annealing sites of OPA12 (data not shown). By comparison of the two full-length sequences, speculated precise sequence of template matching with OPA12 (Fig. 1) showed that the exact sequence of 5′ terminal of the template matching with OPA12 was 5'-GTGGCGATA G-3′ and the accurate sequence of 3′ terminal was 5'-TGCGCAGTAG-3′. The first and second bases at 5′ terminal were ‘GT’ instead of ‘TC’, and the sixth base at 3′ terminal was ‘A’ rather than ‘G’, compared to the sequence of OPA12 (5'-TGCGCAGTAG-3′). However, no amplification product was obtained by TAIL-PCR using mitochondrial DNA from Zhenshan 97B. The exact sequence pairing with OPA12 in Zhenshan 97B could not be determined by TAIL-PCR method.

**Differentiating Zhenshan 97A and Zhenshan 97B based on primers designed according to results of TAIL-PCR**

A new pair of SCAR primer, designated as CHI20F3/CHI23R3, was designed based on the results of TAIL-PCR mentioned above. The expected 1,588 bp DNA fragment was amplified by CHI20F3/CHI23R3 using mitochondrial DNA from Zhenshan 97A as template, whereas no fragment was obtained using mitochondrial DNA from Zhenshan 97B as template. By combining CHI20F3/CHI23R3 with CHI19F2/CHI19R2, four pairs of primers, viz. CHI20F3/CHI23R3, CHI20F3/CHI19R2, CHI19F2/CHI23R3 and CHI19F2/CHI19R2 were developed. Using total DNA from Zhenshan 97A as template, the 1,588 bp DNA fragment was amplified by all the four pairs of primers. However, no band was obtained using total DNA from Zhenshan 97B as template.

**Application of SCAR marker CHI20F3/CHI23R3 for differentiating A and B plants other than Zhenshan 97**

Using the total DNA of individual seedling of Zhenpin A and Zhenpin B, Tianfeng A and Tianfeng B, II-32A and II-32B as templates, the 1,588 bp DNA fragment was amplified in Zhenpin A, Tianfeng A and II-32A with the SCAR marker CHI20F3/CHI23R3, but not in Zhenpin B, Tianfeng B and II-32 B (Fig. 2). This result indicates that the 1,588 bp fragment amplified by CHI20F3/CHI23R3 was not affected by the nuclear background, and could be used as a SCAR marker to discriminate the four pairs of CMS and their maintainer plants of indica rice at seedling stage.

**Stability of 1,588 bp fragment amplified by CHI20F3/CHI23R3 in segregating nuclear background**
The 1588 bp band was absent when the total DNAs of Zhenshan 97B and Minghui 63 were used as template, but was presented when the total DNAs of Zhenshan 97A, Shanyou 63 F1 and F2 plants were used as template (Fig. 3). This result revealed that the 1588 bp-fragment amplified by CHI20F3/CHI23R3 was not affected by nuclear genome.

Application of SCAR marker CHI20F3/CHI23R3 in purity assessment of Zhenshan 97A seeds

The total DNA of 40-day-old seedlings from the predetermined mixture sample was extracted and PCR amplification was conducted using the SCAR marker CHI20F3/CHI23R3. After resolving the amplified fragments in agarose gel, off-type plants among the 100 coded plants were identified based on amplification results. The genotypes of the plants analyzed are shown in Fig. 4. All the 100 plants were grown to maturity and data relevant to pollen and spikelet fertility were recorded. About 24 of 100 plants were identified as off-type (i.e., Zhenshan 97B) on the basis of DNA marker-based genotyping, and at maturity stage, these plants could set seed (i.e., pollen fertile), indicating a perfect correlation between results based on marker genotype and phenotype. These results confirmed that impurities among CMS-WA plants could be accurately detected using seed or seedling based on SCAR marker analysis.

DISCUSSION

The SCAR marker developed in this study can be used to distinguish CMS-WA and its maintainer plants at seedling stage. WA cytoplasm originated from a pollen abortive wild rice plant grown at Nanhong Farm (latitude 18° N), Hainan Province, China in 1970 (Yuan, 1977). CMS-WA is the first male sterile type which has been commercially used in hybrid rice production in China. The WA male sterility is extremely stable in various environments or regions and remained the predominant type in three-line hybrid rice production over the past four decades (Cheng et al, 2009). Therefore, development and use of molecular markers described in this study will be of great significance in hybrid seed production and seed purity testing of CMS lines at early stages of rice plants. In addition, this marker can also distinguish CMS-ID derived from Indonesia paddy rice from its corresponding maintainer line at seedling stage. CMS-ID is the second male sterile cytoplasm source in hybrid rice on a commercial scale in China. Thus, the SCAR marker was also very important for CMS-ID.

Interestingly, II-32A and II-32B showed an amplification pattern identical to CMS-WA and maintainer line. This result may indicate that the sequence was considerably conserved between WA-type and ID-type cytoplasms in the evolution course of rice mitochondrial genome. Furthermore, the specific 1588 bp band was amplified in all plants of F1 and F2 of Shanyou 63 investigated by SCAR marker CHI20F3/CHI23R3. This indicates that the specific 1588 bp DNA fragment which comes from mitochondrial genome is not disturbed by nuclear genome in this study. A high consistency was
confirmed between the marker genotypic data and phenotypic data (seed setting characters) in an inspection experiment with a predetermined mixture of Zhenshan 97A and its maintainer line Zhenshan 97B. It indicated that the SCAR marker CHI20F3/CHI23R3 described here can be used in a PCR assay to detect accurately contamination of maintainer in seed lots of CMS-WA and CMS-ID lines at seed or seedling stage. Therefore, we believe that this marker could be extensively used in seed purity assessment of hybrid rice with WA and ID cytoplasm.

Apart from the WA and ID cytoplasm, there are three kinds of male sterile inducing cytoplasm, namely, CMS-HL (Honglian), CMS-BT (Boro II) (Lin and Yuan, 1980; Li and Zhu, 1988; Rao, 1988; Shinjyo, 1969) and CMS-D (Dian), being used for hybrid rice seed production in China and other Asian countries (Li and Yuan, 2000; Virmani, 2003). CMS-WA, CMS-ID and CMS-HL were mainly applied to indica hybrid rice, while CMS-BT and CMS-D were predominantly employed for japonica hybrid rice. Up to now, there is no effective and efficient DNA marker to discriminate seeds or seedlings between male sterile line with BT type cytoplasm and its maintainer line, and neither with Dian and HL type cytoplasm. Gene targeted markers (GTMs) for differentiating CMS-WA line from its maintainer line have been reported (Yashitola et al, 2002, 2004; Rajendran et al, 2007; Rajendrakumar et al, 2007; Ngangkham et al, 2010). However, few reports on distinguishing CMS-BT and CMS-HL from their maintainer lines have been described in rice. Along with the elucidation of molecular mechanism for BT-type, D-type and HL-type male sterility, and cloning of mitochondrial chimeric genes (Kadowaki et al, 1990; Iwabuchi et al, 1993; Akagi et al, 1994, 1995, 2004; Yi et al, 2002; Kazama et al, 2003; Komori et al, 2004; Wang et al, 2006; Kazama et al, 2008; Itabashi et al, 2009; Peng et al, 2010; Ohta et al, 2010; Das et al, 2010), it should be possible to develop functional markers (Andersen and Lübberstedt, 2003) to discriminate the male sterility cytoplasm from fertility cytoplasm said, and it is worthwhile to do the research further.

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