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Short Communication

Cytoplasmic effects on DNA methylation between male sterile lines and the maintainer in wheat (*Triticum aestivum* L.)

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ABSTRACT

Male sterile cytoplasm plays an important role in hybrid wheat, and three-line system including male sterile (A line), its maintainer (B line) and restoring (R line) has played a major role in wheat hybrid production. It is well known that DNA methylation plays an important role in gene expression regulation during biological development in wheat. However, no reports are available on DNA methylation affected by different male sterile cytoplasms in hybrid wheat. We employed a methylation-sensitive amplified polymorphism technique to characterize nuclear DNA methylation in three male sterile cytoplasms. A and B lines share the same nucleus, but have different cytoplasms which is male sterile for the A and fertile for the B. The results revealed a relationship of DNA methylation at these sites specifically with male sterile cytoplasms, as well as male sterility, since the only difference between the A lines and B line was the cytoplasm. The DNA methylation was markedly affected by male sterile cytoplasms. K-type cytoplasm affected the methylation to a much greater degree than T-type and S-type cytoplasms, as indicated by the ratio of methylated sites, ratio of fully methylated sites, and polymorphism between A lines and B line for these cytoplasms. The genetic distance between the cytoplasm and nucleus for the K-type is much greater than for the T- and S-types because the former is between *Aegilops* genus and *Triticum* genus and the latter is within *Triticum* genus between *Triticum spelta* and *Triticum timopheevii* species. Thus, this difference in genetic distance may be responsible for the variation in methylation that we observed.

1. Introduction

Hybrid wheat (*Triticum aestivum* L.) has advantages over traditional cultivars primarily due to its increased yield potential, which is important for sustaining global food production and supporting an increasing world population. Hybrid wheat has attracted considerable research effort spanning several decades, and wheat hybrids have shown significantly higher yields and better adaptation to adverse environments than the best homozygous genotypes (Pickett and Galwey, 1997; Singh et al., 2010; Zhang et al., 2001). Since the autogamous nature of wheat makes hybrid seed production a challenging task, it continues to be a major constraint in a wider application of hybrids. In hybrid wheat production, cytoplasmic male sterility (CMS) system plays an important role in hybrid wheat, and three-line system including male sterile (A line), its maintainer (B line) and restoring (R line), has played a major role in wheat heterosis utilization. CMS observed in over 150 plant species (Mackenzie et al., 1994), is realized by a genetic interaction of nuclear gene(s) with cytoplasmic gene(s) for sterility and fertility. Thus, the incompatibility caused by genome barriers between a nucleus and foreign cytoplasm destines the fate of pollen to be dead in cytoplasmic male sterility (Fujii and Toriyama, 2008).

It has been well documented that signals emitted from cytoplasm regulate nuclear gene expression, whose process is known as retrograde signaling, and it is confirmed that pollen fertility is associated with retrograde signaling (Traven et al., 2001). Involvement of retrograde signaling in CMS has been implicated from alloplasmic type CMS, a type of CMS that is derived from the inter-species or inter-genera crosses. Currently, many alloplasmic types have been reported in wheat. Among them, three major male sterile cytoplasms (*Aegilops kotschyi*, *Triticum spelta* and *Triticum timopheevii*), play a major role in hybrid wheat production (Bojnanska and Francakova, 2002; Farooq and Azam, 2006; Laikova et al., 2004; Leonova et al., 2002). The A line is bred from crossing a CMS donor, such as relative genera and species of wheat, as the female parent with a nucleus donor such as common wheat as the male parent, and continuously backcrossing with the nucleus donor for many

Abbreviations: AFLP, amplified fragment length polymorphism; A line, male sterile line; B line, maintainer line; bp, base pair; CMS, cytoplasmic male sterility; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylene diamine tetraacetic acid; K-type and ms(K), *Aegilops kotschyi* cytoplasmic male sterility; mM, millimoles per liter; MSAP, methylation-sensitive amplified polymorphism; ng, nanogram; R line, restoring lines; S-type and ms(S), *Triticum spelta* cytoplasmic male sterility; T-type and ms(T), *Triticum timopheevii* cytoplasmic male sterility; μl, microlitre.

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generations until genetically stable. Consequently, the nucleus donor becomes the B line which can maintain sterility of the A line. The continuous backcrossing completely substitutes the nucleus of the CMS donor. Therefore, A and B lines have exactly the same nucleus, but different cytoplasms, and any differences between A and B lines are due to the maternal cytoplasms. CMS lines and their corresponding maintainers have identical nuclear genome but different cytoplasms, which provide us a good system for dissecting the nucleus–cytoplasm interactions of plant cells.

DNA methylation, which mostly occurs in CpG dinucleotides, is an epigenetic event that alters gene expression and affects cell function. The gene expression which is altered by gene transcription, gene silencing, or mobile element, regulates plant development through a number of biological processes. DNA methylation widely exists in plant genomes and methylation-sensitive amplified polymorphism (MSAP) has been employed to estimate DNA methylation in many plants including pepper (*Capsicum annuum* L.) (Portis et al., 2004), barley species (*Hordeum brevisubulatum*) (Li et al., 2008), rapeseed (*Brassica oleracea*) (Guzy-Wrobelska et al., 2013), *Cymbidium hybridium* (Chen et al., 2009), ginseng (*Eleuterococcus senticosus*) (Chakrabarty et al., 2003), banana (*Musa acuminata*) (Peraza-Echeverria et al., 2001), cotton (*Gossypium hirsutum* L.) (Cao et al., 2011), *Arabidopsis* (Daxinger et al., 2007), rice (*Oryza sativa* L.) (Sha et al., 2005), and wheat (Sha et al., 2005). In wheat, DNA methylation has been investigated for genetic diversity (Tok et al., 2011), development and differentiation (Cantu et al., 2010; Horvath et al., 2003; Meng et al., 2012; Sherman and Talbert, 2002), biotic and abiotic stresses (Kravets et al., 2010; Migiwa et al., 2005; Zhong et al., 2009), allopolyploid formation (Dong et al., 2005) and effect of alien germplasm on genomic DNA (Zhang et al., 2008). However, little information is available on DNA methylation as affected by different male sterile cytoplasms in hybrid wheat. In this study, our objective was to analyze the extent and polymorphism of DNA methylation by comparing the cytoplasms of male sterile lines (A) and the maintainer line (B) using the MSAP technique.

1.1. Materials and methods

1.1.1. Plant materials

Three isonuclear alloplasmic cytoplasmic male sterility lines (A), ms(K)-90–110, ms(S)-90–110 and ms(T)-90–110, representing three major male sterile cytoplasms (*A. kotschyi*, *T. spelta* and *T. timopheevii*), and the corresponding maintaining line, (B)-90–110 (*T. aestivum* L.), were selected for methylation analysis. To secure a complete substitution of the nucleus, the three A lines were backcrossed with their corresponding B line more than 20 generations.

1.2. Seedling culture and DNA isolation

Since genomic methylation is sensitive to the environment and plant development stages (Wang et al., 2011; Xiong et al., 1999), a series of considerations were employed to avoid these sources of errors. All samples were collected from the plants with the same ecologic environment, cultivated condition and growth period. Seeds of three A lines and the maintainer B line were planted in a flat tray filled with sand for germination and growth. The trays were placed in an artificial climate chamber at 25/20 °C (day/night), 80% relative humidity, and artificial light with a 16/8 h day/night photoperiod and about 300 μmol m^{-2} s^{-1} light intensity. At the four-leaf stage, the third leaf from the top was collected as a bulk of six plants in each line for DNA extraction. Genomic DNA was isolated from each sample according to the published methods (Kuleung et al., 2004).

1.3. Methylation-sensitive amplification polymorphism assay

The methylation-sensitive amplification polymorphism (MSAP) technique was modified from published methods (Xiong et al., 1999). The protocol involved the use of the isoschizomers *Hpa*II and *Msp*I instead of *Mse*I as 'frequent cutter' enzymes. The adapter and primer for the 'rare-cutter' enzyme *Eco*RI was the same as that used in standard AFLP analysis, while the *Hpa*II/*Msp*I adapter was designed as in Table 1.

To detect MSAP, restriction and ligation were done concurrently and two sets of digestion/ligation reactions were carried out simultaneously. In the first reaction, 2 μl of the extracted DNA (120 ng DNA) was added to an 18 μl buffer (10 mM Tris–HCl pH 7.5, 10 mM MgAc, 50 mM KAc) containing 6 units *Eco*RI, 5 units *Hpa*II (Takara), 4 units T4 DNA ligase (Takara), 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter and 10 mM ATP. The mixture was then incubated at 37 °C for 6 h. The reaction was stopped by incubating at 65 °C for 10 min and diluting 10 times in 0.1 × TE (1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) for PCR amplification. The second digestion/ligation reaction was carried out in the same way, except that *Msp*I was used in place of *Hpa*II.

We used two consecutive PCRs to selectively amplify the *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I DNA fragments. The pre-selective amplification (first PCR) was performed using 5 μl of the abovementioned diluted mixture added to a 15 μl mixture giving a final concentration of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 ng of *Eco*RI and *Hpa*II/*Msp*I adapter-directed primer, each possessing a single selective base (E + 1; HM + 1) and 1 unit of Taq polymerase (Takara). PCR reactions were performed with the following profile: 94 °C for 60 s, 25 cycles of 30 s denaturing at 94 °C, 30 s annealing at 55 °C and 60 s extension at 72 °C, ending with 10 min at 72 °C to complete extension. After checking for the presence of a smear of fragments (100–1000 bp in length) by agarose electrophoresis, the amplification product was diluted 10 times in 0.1 × TE.

Selective amplification (second PCR) of the diluted pre-amplification products was carried out using a total of 30 primer combinations obtained with four *Eco*RI primers in combination with three *Hpa*II/*Msp*I primers with three selective bases each (E + 3, HM + 3). The *Eco*RI and *Hpa*II/*Msp*I adapters and primers were synthesized by Invitrogen Life Technologies. Selective PCR reactions were performed with the following procedure: 94 °C for 60 s, 36 cycles of 30 s denaturing at 94 °C, 30 s annealing and 60 s extension at 72 °C, ending with 10 min at 72 °C to complete extension. Annealing was initiated at a temperature of 65 °C, which was then reduced by 0.7 °C for the next 13 cycles and maintained at 56 °C for the subsequent 23 cycles.

The second PCR products were mixed with 15 μl of formamide dye (98% formamide, 10 mM EDTA, 0.01% w/v Bromophenol Blue and 0.01% w/v Xylene Cyanol), denatured at 95 °C for 5 min and separated by electrophoresis on 6% denaturing polyacrylamide sequencing gels (6% acrylamide 19:1, 7 M urea) in 1 × TBE buffer. The gels were prerun at 150 W for about 30 min before 10 μl of the mix was loaded.

Gels were run at 250 V for about 2.5 h, and silver stained. The gel was fixed in 10% acetic acid for 10 min, washed twice with a large quantity of ultrapure water for 1 min, transferred to a silver impregnation solution (1 g/L $AgNO_3$, 0.75% formaldehyde) for 8 min and then rinsed with ultrapure water for 20 s. All of the above steps were performed with

Table 1
Sequences of MSAP primers and adapters.

Primers/adapters	Sequences (5′–3′)
*Eco*RI adapter	5′-CTCGTAGACTGCGTACC-3
	5′-AATTGGTACGCAGTCTAC-3
E + 1 primer	5′-GACTGCGTACCAATTCA-3
E + 3 primers	5′-GACTGCGTACCAATTCAAG-3′ (E1)
	5′-GACTGCGTACCAATTCAAC-3′ (E2)
	5′-GACTGCGTACCAATTCACG-3′ (E3)
	5′-GACTGCGTACCAATTCACT-3′ (E4)
*Hpa*II/*Msp*I adapter	5′-GATCATGAGTCCTGCT-3′
	5′-CGAGCAGGACTCATGA-3′
HM + 1 primer	5′-ATCATGAGTCCTGCTCGGT-3
HM + 3 primers	5′-ATCATGAGTCCTGCTCGGTCA-3 (HM1)
	5′-ATCATGAGTCCTGCTCGGTCC-3 (HM2)
	5′-ATCATGAGTCCTGCTCGGTTC-3 (HM3)