Gene 537 (2014) 143–148

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short communication

DNA methylation changes in photoperiod-thermo-sensitive male sterile rice PA64S under two different conditions

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ARTICLE INFO

Article history:
Accepted 11 December 2013
Available online 21 December 2013

Keywords:
Two line hybrid rice
PTGMS
DNA methylation
Gene expression
MSAP

ABSTRACT

Epigenetic modification can occur at a high frequency in crop plants and might generate phenotypic variation without changes in DNA sequences. DNA methylation is an important epigenetic modification that may contribute to environmentally-induced phenotypic variations by regulating gene expression. Rice Photoperiod-Thermo-Sensitive Genic Male Sterile (PTGMS) lines can transform from sterility to fertility under lower temperatures and short-day (SD) conditions during anther development. So far, little is known about the DNA methylation variation of PTGMS throughout the genome in rice. In this study, we investigated DNA cytosine methylation alterations in the young panicles of PTGMS line PA64S under two different conditions using methylation sensitive amplified polymorphism (MSAP) method. Compared with the DNA methylation level of PA64S under lower temperatures and SD conditions (fertility), higher methylation was observed in PA64S (sterility). The sequences of 25 differentially amplified fragments were successfully obtained and annotated. Three methylated fragments, which are homologous to D2, NAD7 and psaA, were confirmed by bisulfite sequencing and their expression levels were also evaluated by qPCR. Real time quantitative PCR analysis revealed that five of the six selected methylated genes were downregulated in PA64S (sterility). These results suggested that DNA methylation may be involved in the sterility–fertility transition of PA64S under two different environmental conditions.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important grain crops in the world, and provides a staple food for almost half of the global population (Delseny et al., 2001). Hybrid rice has made a tremendous contribution to food security both in China and in many other countries. Hybrid rice seed production is mainly based on male sterility systems. There are two well-known male sterility systems: CMS (Cytoplasmic Male Sterile) and PTGMS (Photoperiod-Thermo-Sensitive Genic Male Sterile). The former is called the three-line hybrid rice system; the latter is called the two-line hybrid rice system. The three line system includes CMS line, maintainer line and restorer line, in which CMS line and restorer line are used to produce hybrid rice seed (Xu et al., 2011; Zhou et al., 2012).

Compared with the three-line system, the two-line system, or PTGMS, has many advantages for hybrid seed production in rice (Yuan, 1990). Firstly, since the male sterility of PTGMS line is induced by environmental factors (such as temperature and photoperiod), the line can be used to produce hybrid seed for male sterile line under long-day conditions (critical photoperiod between 13.50 and 13.38 h) and when the environmental temperature is above a critical point (≥23.3 °C, photoperiod-sensitive temperature ranging 23–28 °C) (Liao and Yuan, 2000; Xu et al., 2011). While under short-day conditions or below a critical point during sensitive growth stages, the plants can also be used as maintainer lines by self propagation. Secondly, the two-line hybrid rice system has a wide range of germplasm resources for hybrid combinations due to any normal fertile variety that can be used as a parental line to restore fertility. Therefore, these advantages make the two-line system beneficial for simpler procedures for breeding, better grain quality and higher yields, and widely employed in hybrid rice seed production (Xu et al., 2011; Yang et al., 2007).

In China, Pei'ai 64S (PA64S) is one of the most important *indica* nuclear male sterile lines in practice, which has a complex genetic background contributed by three cultivated rice subspecies which are *indica*, *japonica*, and *javanica* (Wang et al., 2008). Its paternal line is Pei'ai 64 and its maternal line is Nongken 58S that is the first photoperiod-sensitive genic male sterile (PSMS) line discovered by Mingsong Shi in Hubei, China in 1973 (Shi, 1985). Some studies reported that PA64S is certainly sensitive to photoperiod and day length has positive compensation effect (Zeng et al., 2000). In addition, based on self-fertility and pollen fertility of PA64S in four regions of China

Abbreviation: PTGMS, Photoperiod-Thermo-Sensitive Genic Male Sterile; SD, short-day; LD, long-day; MSAP, methylation sensitive amplified polymorphism; PA64S, Pei'ai 64S; PA64S (S), PA64S (sterility); PA64S (F), PA64S (fertility); CMS, Cytoplasmic Male Sterile; PSMS, photoperiod-sensitive genic male sterile; LDMAR, long-day-specific male-fertility-associated RNA; DM, differentially methylated; psaA, photosystem I P700 chlorophyll A apoprotein A1; Cytf, cytochrome f; D2, photosystem II protein D2; NAD7, NADH dehydrogenase subunit 7; VIP2, putative VIP2 protein; TE, transposable element.

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http://dx.doi.org/10.1016/j.gene.2013.12.015

under different temperatures and day length, Zou et al. (2003) found that PA64S has lower fertility transition temperature and obvious short-length compensation effect. Therefore, the PA64S sterile–fertile conversion was controlled by photoperiod and temperature: sterility at temperatures higher than 23.5 °C during the anther development and long-day (14 h) conditions can suppress the sterility–fertility conversion. Under lower temperatures and short-day (SD) conditions during anther development, PA64S can transform from sterility to fertility (Xu et al., 1999; Zhou et al., 2012).

Epigenetic changes can occur at a high frequency in crop plants and might generate phenotypic variation that is not correlated with DNA sequence variations (Lukens and Zhan, 2007). DNA methylation is an important epigenetic modification that may contribute to environmentally-induced phenotypic variations by regulating gene expression in a tissue-specific or a developmental stage-dependent manner (Angers et al., 2010; Zhang et al., 2010). Recently, some studies reported that a substitution of C-to-G in the male-fertility-associated noncoding RNA *p/tms12-1* in PA64S produces a mutant small RNA, namely osa-smR5864m. This mutated noncoding small RNA gene may lead to PTGMS in PA64S (Zhou et al., 2012). Furthermore, another noncoding RNA called LDMAR was required for sterility–fertility conversion in PSMS rice Nongken 58S. In addition, increased methylation in the promoter of LDMAR in Nongken 58S reduced the expression of LDMAR, leading to male sterility (Ding et al., 2012). Therefore, DNA methylation might be involved in regulating the PTGMS. In this study, we investigated DNA cytosine methylation alterations in the young panicles of PTGMS line PA64S under two different environmental conditions. The aim of this work was to explore the characterization and regulation of DNA methylation during the conversion of sterility to fertility in PA64S.

2. Materials and methods

2.1. Plant materials and DNA extraction

The rice photo-thermo-sensitive genic male sterile cultivar, PA64S, which was maintained at Wuhan University, was used in this study. The seeds of PA64S were sown under two different natural ecological conditions: in Hainan (18°48′ N, 110°02′ E) from December, 2010 to March, 2011 (fertility, F), and in Wuhan (30°30′ N, 114°18′ E) from May 2011 to August 2011 (sterility, S). Young panicles of PA64S of the two phenotypes at meiosis stages were collected and total genomic DNA was extracted using Genomic DNA Miniprep Kit (Axygen) following the manufacture's instruction.

2.2. Methylation-sensitive amplified polymorphism analysis

Methylation-sensitive amplified polymorphism (MSAP) analysis was performed as described by Xiong et al. (1999) with minor modifications. Briefly, 500 ng of genomic DNA was digested with 10 U *EcoR* I and 20 U *Hpa* II or 10 U *Msp* I (New England Biolabs) at 37 °C for 8 h. The digested fragments were ligated to 5 pmol of *EcoR* I adaptors and 50 pmol of *Hpa*II–*Msp*I adaptors using 3 U T4 DNA ligase (New England Biolabs) at 16 °C for 12 h.

The pre-amplification was carried out in a final volume of 20 μl, containing 50 ng of the ligation products as templates, 0.2 μM *Eco*RI and *Hpa* II–*Mse*I preselection primers (Table 1), 1 × PCR buffer, 0.2 mM dNTP, and 2 U *Taq* polymerase (Tiangen Biotech, Beijing, China). The PCR reactions were performed with the following program: 5 min at 94 °C, 30 cycles consisting of 30 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. The pre-amplification PCR product was diluted 1:20 with ddH_2O, and 1 μl was used as template for selective amplification with 0.2 μM of the primers "*EcoR* I + 3" (containing three selective bases) and "*Hpa* II/*Msp*I + 3" (three selective bases) (Table 1). The other components were the same as those in the pre-amplification reactions. The selective amplification was performed using a 'touch-down' PCR program: 94 °C for 5 min; 12 cycles of 94 °C for 30 s, 65 °C (with a −0.7 °C/cycle fall) for 30 s, 72 °C for 1 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; 72 °C for 10 min. Finally, formamide dye was added to the products of selective amplification and then separated on 5% denaturing polyacrylamide gels. The gels were stained with silver nitrate and the sizes were determined in comparison with the 1 kb markers. The scored MSAP bands were transformed into a binary character matrix, using "1" and "0" to indicate the presence and absence of a band at a particular position, respectively.

Table 1
Sequences of adaptors and primers used for pre-amplification and selective amplification.

	Sequence (5′–3′)
Adaptors/primers	
*Eco*R I adaptor 1	CTCGTAGACTGCGTACC
*Eco*R I adaptor 2	AATTGGTACGCAGTCTAC
*Hpa*II/*Msp*I adaptor 1	GATCATGAGTCCTGCT
*Hpa*II/*Msp*I adaptor 2	CGAGCAGGACTCATGA
*Eco*RI pre-selective primer	GAATTGGTACGCAGTC
*Hpa*II/*Msp*I pre-selective primer	ATCATGAGTCCTGCTCGG
Selective primers	
E1	GACTGCGTACCAATTCAAC
E2	GACTGCGTACCAATTCAAG
E3	GACTGCGTACCAATTCATG
E4	GACTGCGTACCAATTCACA
E5	GACTGCGTACCAATTCAGA
E6	GACTGCGTACCAATTCAGT
E7	GACTGCGTACCAATTCTAG
E8	GACTGCGTACCAATTCTCA
E9	GACTGCGTACCAATTCCAA
E10	GACTGCGTACCAATTCCTG
E11	GACTGCGTACCAATTCCGT
E12	GACTGCGTACCAATTCCGC
E13	GACTGCGTACCAATTCGAT
E14	GACTGCGTACCAATTCGAG
HM1	ATCATGAGTCCTGCTCGGAGT
HM2	ATCATGAGTCCTGCTCGGACT
HM3	ATCATGAGTCCTGCTCGGATC
HM4	ATCATGAGTCCTGCTCGGTAA
HM5	ATCATGAGTCCTGCTCGGTCAA
HM6	ATCATGAGTCCTGCTCGGTGC
HM7	ATCATGAGTCCTGCTCGGCAT
HM8	ATCATGAGTCCTGCTCGGCTG
HM9	ATCATGAGTCCTGCTCGGCGA
HM10	ATCATGAGTCCTGCTCGGGAT
HM11	ATCATGAGTCCTGCTCGGGTC
HM12	ATCATGAGTCCTGCTCGGGCA
E15	GACTGCGTACCAATTCGTC
E16	GACTGCGTACCAATTCGCA

The MSAP pattern for displaying the DNA fragments resulting from digestions with the isoschizomers was divided into the following four types (Table 2): Type I bands, present for both enzyme combinations; Type II bands, present only for *EcoR* I/*Hpa* II; Type III bands, present for *EcoR* I/*Msp* I; and Type IV bands, absent from both enzyme combinations. Here, Type II represents semi-methylated bands, whereas Type III and IV bands represent semi- or full methylation, respectively (Lu et al., 2008).

2.3. Cloning and characterization of the differentially amplified DNA fragments

The polymorphic bands were selected and excised from the gel using a sterilized surgical blade, hydrated in 20 ml of TE buffer (pH 8.0) in an Eppendorf tube and incubated at 100 °C for 10 min. The supernatant was recovered by centrifugation and used for the re-amplification. Sizes of the PCR products were verified by agarose gel electrophoresis, then cloned into the vector PMD18-T (TaKaRa, Dalian, China) and transformed into an *Escherichia coli* TOP10 for sequencing, and the sequences obtained were analyzed by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EMBL BLAST (http://www.ebi.ac.uk/Tools/blast/).