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ORIGINAL PAPER

Alterations in cytosine methylation and species-specific transcription induced by interspecific hybridization between *Oryza sativa* and *O. officinalis*

Huajun Jin · Wei Hu · Zhe Wei · Linglin Wan · Gang Li · Guangxuan Tan · Lili Zhu · Guangcun He

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Abstract Interspecific hybridization and polyploidization may involve programmed genetic and epigenetic changes. In this study, we used the methylation-sensitive amplified polymorphism (MSAP) method to survey cytosine methylation alterations that occurred in F_1 hybrid and BC_1 progeny following interspecific hybridization between *Oryza sativa* and *O. officinalis*. Across all 316 parental methylated sites, 25 (7.9%) cytosine methylation alterations were detected in the F_1 and/or BC_1 progeny. Thirty additional cytosine methylation alterations were detected at parental non-methylated or novel sites. In total, 55 cytosine methylation alterations (90.9% of all alterations) were detected in the F_1 hybrid, which were maintained in the BC_1 progeny. The alterations in cytosine methylation were biased toward the *O. officinalis* parent and were in some cases repeatable in independent hybridizations between *O. sativa* and *O. officinalis*. Twelve fragments showing cytosine methylation alterations were isolated, sequenced and subsequently validated by methylation-sensitive Southern blot analysis. Where possible, we designed species-specific primers to amplify the polymorphic transcripts from either the *O. sativa* or the *O. officinalis* parent using reverse transcription (RT)-PCR in combination with single-strand conformation polymorphism (SSCP) analysis. In four of five cases, modified gene expression could be correlated with the altered cytosine methylation pattern. Our results demonstrated cytosine methylation alterations induced by interspecific hybridization between a rice cultivar and its wild relative, and indicated a direct relationship between cytosine methylation alteration and gene expression variation.

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H. Jin · W. Hu · Z. Wei · L. Wan · G. Li · G. Tan · L. Zhu · G. He (✉)
Key Laboratory of Ministry of Education for Plant Development Biology, College of Life Sciences, Wuhan University, 430072 Wuhan, China
e-mail: gche@whu.edu.cn

H. Jin
Xin Yuan Institute of Medicine and Biotechnology, School of Life Science, Zhejiang Sci-Tech University, 310018 Hangzhou, Zhejiang, China

Introduction

Hybridization and polyploidization play significant roles in the evolution of higher plants (Rieseberg and Wendel 2004). In recent years, the genetic and functional consequences of hybridization and polyploidization have been extensively investigated in several plant systems, including Brassica (Song et al. 1995; Albertin et al. 2006; Lukens et al. 2006; Gaeta et al. 2007), wheat (Feldman et al. 1997; Liu et al. 1998a, b; Ozkan et al. 2001; Shaked et al. 2001; Kashkush et al. 2002; He et al. 2003; Bottley et al. 2006), Arabidopsis (Comai et al. 2000; Lee and Chen 2001; Madlung et al. 2002; Wang et al. 2004; Madlung et al. 2005; Josefsson et al. 2006; Wang et al. 2006; Zhang et al. 2008), cotton (Wendel et al. 1995; Liu et al. 2001; Adams et al. 2003; Adams et al. 2004) and Spartina (Salmon et al. 2005). In early generations, the formation of interspecific hybrids and polyploids can often result in rapid and biased structural changes (Song et al. 1995; Wendel et al. 1995; Feldman et al. 1997; Liu et al. 1998a, b; Ozkan et al. 2001; Madlung et al. 2005; Lukens et al. 2006; Gaeta et al. 2007), radical alterations in cytosine methylation (Liu et al. 1998a, b;

Shaked et al. 2001; Madlung et al. 2002; Salmon et al. 2005; Lukens et al. 2006; Gaeta et al. 2007; Zhang et al. 2008) and epigenetic gene silencing (Comai et al. 2000; Lee and Chen 2001; Kashkush et al. 2002; Madlung et al. 2002; Adams et al. 2003; He et al. 2003; Adams et al. 2004; Wang et al. 2004; Albertin et al. 2006; Bottley et al. 2006; Josefsson et al. 2006; Wang et al. 2006; Gaeta et al. 2007; Zhang et al. 2008). The impact of cytosine methylation alteration on epigenetic gene silencing, which has been observed in interspecific hybrids and polyploids, has been widely discussed because cytosine methylation can strongly influence gene expression (reviewed in Liu and Wendel 2003; Riddle and Birchler 2003; Rapp and Wendel 2005). However, there is a paucity of evidence to support any direct link between the epigenetic phenomena of cytosine methylation alteration and gene silencing following hybridization and polyploidization.

The genus Oryza comprises two cultivated species and 22 wild species, with genomes designated AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ and HHKK (Vaughan et al. 2003). A series of intraspecific and interspecific F_1 hybrids has been successfully generated (Brar and Khush 1997; Tan et al. 2005), and alterations in cytosine methylation patterns have been reported in intraspecific F_1 hybrids between cultivars (Xiong et al. 1999). However, cytosine methylation alterations induced by interspecific hybridization and polyploidization are less well understood, particularly those between cultivated species and their wild relatives. Previously we reported that a heritable cytosine methylation alteration occurred at one restriction fragment length polymorphism (RFLP) locus in rice lines containing introgressed chromosomal segments from *Oryza officinalis*, a wild relative of *O. sativa*, which is the Asian cultivated rice (Jin et al. 2006).

In this study we analyzed the cytosine methylation patterns of a large number of unbiased loci distributed throughout the genome by applying a global methylation-sensitive amplified polymorphism (MSAP) approach (see Reyna-Lopez et al. 1997; Xiong et al. 1999) to an *O. sativa* species, an *O. officinalis* accession, their interspecific F_1 hybrid, and their BC_1 progeny. We found changes in species-specific gene expression induced by cytosine methylation alterations using reverse transcription (RT)-PCR in combination with single-strand conformation polymorphism (SSCP) analysis.

Materials and methods

Plant material

The plant material used in this study consisted of two sets of BC_1 progenies, F_1 hybrids, and their homozygous diploid parents. The first, used in most of the experiments reported here, was obtained from a cross between *O. sativa* ssp. *japonica* cv Hejiang 19 (genome AA) and the wild species *O. officinalis* accession HY018 (genome CC). The second was obtained from a cross between *O. sativa* ssp. *indica* cv Zhengshan 97B (genome AA) and the wild species *O. officinalis* accession HY018 (genome CC). In both the cases, the hybrid (genome AC) was backcrossed to the recurrent *O. sativa* parent (genome AA) to obtain BC_1 progenies (genome AAC). The chromosome number was determined for the F_1 hybrid and the BC_1 progeny by examining root tips during mitosis. The F_1, BC_1 and parental plants were planted in pots and grown under natural conditions.

MSAP analysis

To explore the possible alterations in cytosine methylation patterns at unbiased, but specific loci in F_1 and BC_1 plants from the first of the crosses, relative to their parents (*O. sativa japonica* cv Hejiang 19 and *O. officinalis* accession HY018), we used the MSAP method described by Reyna-Lopez et al. (1997) and Xiong et al. (1999). Genomic DNA was isolated from fully expanded flag leaves at the day of heading using the CTAB method (see Murray and Thompson 1980) and then purified using phenol extractions. For the parental plants, genomic DNA was extracted from pooled samples of eight plants. In total, one pair of preselective primers and 30 pairs of selective primers were used for the amplifications (Supplementary Table 1). Silver stained sequencing gels were used to resolve and visualize the amplification products. Each MSAP gel analysis was duplicated (using DNA obtained from independent extractions), and only clear and reproducible bands were used for scoring.

Cloning of the fragments subjected to cytosine methylation alterations and validation by methylation-sensitive Southern blot analysis

Fragments of interest were eluted and re-amplified with appropriate selective primer combinations. PCR product sizes were verified by agarose gel electrophoresis, and the MSAP-isolated fragments (MIFs) were cloned into a TA cloning vector (TaKaRa Biotech, Dalian, China). The cloned fragments were labeled with phosphorus-32 and used as hybridization probes for methylation-sensitive Southern blot analysis, as described by Huang et al. (2001), except that either *Msp*I or *Hpa*II were used in the methylation analyses. To ensure complete digestion, excess restriction enzyme (10 U of enzyme per μg DNA) was used and the incubation time was extended to 48 h. The validated fragments were sequenced with vector primers by automated

sequencing. Sequences obtained were analyzed for similarity to known sequences in public rice databases using the BLASTN program on the NCBI (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=4530) and the Gramene (http://www.gramene.org/multi/blastview).

RT-PCR and SSCP analysis

Total RNA was isolated at the day of heading from fully expanded flag leaves of F_1 and BC_1 plants, and pooled samples from their parent plants, using the Trizol reagent method (Invitrogen) in accordance with the manufacturer's protocol. The RNA was treated with DNase I (Invitrogen) and first-strand cDNA was synthesized using the SuperScript™ First-Strand Synthesis System III for RT-PCR (Invitrogen), according to the manufacturer's instructions. Primers for subsequent gene-specific PCR were designed by the online tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of the available cDNA sequences matching the sequenced fragments. For controls, PCR was also performed using genomic DNA from the two parents (Hejiang 19 and HY018), the F_1 hybrid, and the BC_1 progeny as templates. Both RT- and genomic DNA-products were used for SSCP analysis, as described by Adams et al. (2003). Electrophoresis was performed using 6% polyacrylamide gels (49:1 acrylamide to bisacrylamide) at room temperature, and the resulting gels were silver stained.

Results

Banding patterns detected in the parental, F_1 and BC_1 plants

The MSAP technique involves the use of a pair of isoschizomers, *Msp*I and *Hpa*II, which have differential sensitivity to cytosine methylation at CCGG sites. While *Hpa*II is sensitive to methylation of the internal cytosine on both the strands, *Msp*I is sensitive to methylation of the external cytosine. Methylation of the external cytosine (on both the strands) or full methylation of both the cytosines prevents cutting, which makes these two methylation patterns indistinguishable using the MSAP technique. However, comparison of amplification products from *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I digests allows the methylation status to be detected. A fragment present in both the digestions indicates that the corresponding CCGG site (or sites) is unmethylated. The presence of fragments in *Eco*RI + *Msp*I digestions and their absence in *Eco*RI + *Hpa*II digestions is attributed to methylation of the internal cytosine on both the strands. The presence of fragments in *Eco*RI + *Hpa*II digestions and their absence in *Eco*RI + *Msp*I digestions can be attributed to hemimethylation of the external cytosine.

Thirty pairs of selective primers produced 996 clear bands in the two parents, *O. sativa japonica* cv Hejiang 19 (genome AA) and *O. officinalis* accession HY018 (genome CC), of which 235 were common to both the parents, while 351 were unique to Hejiang 19 and 410 were unique to HY018. Each of the bands represented a recognition site cleaved by one or both of the isoschizomers. On the other hand, of the 996 bands, 316 was parental methylated, i.e. one or both parents showed polymorphism between the amplification products from *Eco*RI + *Msp*I and *Eco*RI + *Hpa*II digests; 680 was parental non-methylated, i.e. no parent showed polymorphism between the amplification products from *Eco*RI + *Msp*I and *Eco*RI + *Hpa*II digests. Furthermore, twelve novel bands, which were absent in both the parents, were also detected in both the F_1 hybrid and the BC_1 progeny.

Differential MSAP patterns among parental, F_1 and BC_1 plants

The MSAP patterns were compared between the two parents (Hejiang 19 and HY018), the F_1, and the BC_1 plants (Fig. 1). The banding patterns for the F_1 and BC_1 plants were expected to have the combined methylation pattern of both the parents. Any deviation from this expected pattern (additivity) was considered to be the result of an alteration in methylation pattern related to an unknown molecular aspect in which the F_1 and/or BC_1 plants significantly differed from the parental plants.

Five major groups were identified among the parental methylated bands, as shown in Table 1: (1) 36 bands (16.7% of all 316 methylated sites) were monomorphic, that is they had the same methylation pattern in parents, the F_1 hybrid, and the BC_1 progeny (group M); (2) 255 bands (80.7% of all methylated sites) showed differential cytosine methylation between the parents, but no deviation from additivity in the F_1 hybrid or BC_1 progeny (group A); (3) one band (0.3% of all methylated sites) showed differential cytosine methylation patterns between parents and the F_1 hybrid, but methylation was restored to the parental type in the BC_1 progeny (group B); (4) 23 bands (7.3% of all methylated sites) showed alteration in cytosine methylation in the F_1 hybrid, which was maintained in the BC_1 progeny (group C), and (5) one band (0.3% of all methylated sites) showed methylation alteration in the BC_1 progeny, but not in the F_1 hybrid (group D).

Among the parental non-methylated bands, 655 showed additivity in the F_1 hybrid or BC_1 progeny (data not shown); 25 cytosine methylation alterations were detected in the F_1 hybrid and/or the BC_1 progeny (Table 2): three in the F_1 hybrid only (group E) and 22 in both the F_1 hybrid and the BC_1 progeny (group F). Furthermore, five methylation alterations were also detected among the 12 novel sites in the F_1 hybrid and the BC_1 progeny (group G).

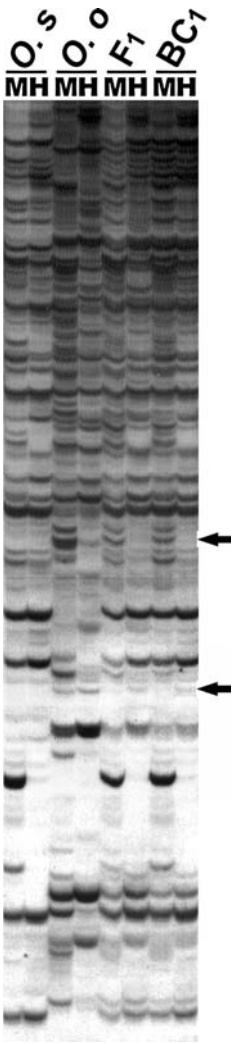

Fig. 1 MSAP patterns detected in the two parents, the F_1 hybrid, and the BC_1 progeny, using the primer combination MH + TCAA/E + GAT. The four samples analyzed in the gel represent *O. sativa* ssp. *japonica* cv Hejiang 19 (*O. s*), *O. officinalis* accession HY018 (*O. o*), the F_1 hybrid (F_1), and the BC_1 progeny (BC_1). *M* and *H* refer to digestion with *Eco*RI + *Msp*I and *Eco*RI + *Hpa*II, respectively. Bands that showed alterations in cytosine methylation patterns in the F_1 hybrid and the BC_1 progeny are indicated by *arrows*

Therefore, 55 methylation alterations in total were detected in the F_1 hybrid and/or the BC_1 progeny (including bands in group B, C, D, E, F, and G). Four of these alterations (7.3%) occurred in the F_1 hybrid only (i.e. bands in group B and E); 50 (90.9%) occurred in both the F_1 hybrid and the BC_1 progeny (i.e. bands in group C and F); and one occurred in only the BC_1 progeny (i.e. band in group pattern D). Interestingly, only 12 (21.8%) were from the *O. sativa* parent, Hejiang 19 (i.e. bands in group B, E, and subgroup F1), while 38 (69.1%) were from the *O. officinalis* parent, HY018 (i.e. bands in group C and D, and subgroup F2 and F3).

Validation of the MSAP-detected methylation alterations by methylation-sensitive Southern blot analysis

Since the MSAP technique involves two sets of PCR amplifications, it is necessary to rule out possible PCR artifacts as a cause of the observed differential methylation patterns in the F_1 hybrid and BC_1 progeny, relative to their parents. Hence, methylation-sensitive Southern blot analysis was performed to validate the cytosine methylation alterations detected by MSAP using all 55 MSAP-isolated fragments (MIFs) as probes. The results revealed that 39 of the 55 fragments had a high-copy number and 13 had a low-copy number; the other three failed to give any hybridization signal. Cytosine methylation alterations in the F_1 hybrid and/or BC_1 progeny were validated for 12 of the 13 low-copy fragments using methylation-sensitive Southern blot analysis. An example in which MIF19 was used as a probe is shown in Fig. 2. The high-copy probes led to smearing in the autoradiograms, preventing their use to assess the cytosine methylation alterations (data not shown).

Validated methylation alterations occurred in other F_1 hybrid and BC_1 progeny

To investigate whether the observed cytosine methylation alterations are stochastic or non-random, genomic DNA from the leaves of another pair of pooled parental plants, *O. sativa indica* ssp. cv Zhenshan 97B and *O. officinalis* accession HY018, and individual plants of their F_1 hybrid (referred to as H22) and BC_1 progeny (referred to as H42) were digested with *Msp*I or *Hpa*II and hybridized against the 12 MIFs that had been found to be involved in cytosine methylation alterations and validated by methylation-sensitive Southern blot analysis. The results showed that eight MIFs detected additive cytosine methylation patterns, while the other four (MIF9, MIF13, MIF21, and MIF24) detected cytosine methylation alterations in H22 and H42 (Fig. 3).

Alteration in gene expression detected by RT-PCR and SSCP analysis

The 12 MIFs that were clearly involved in cytosine methylation alteration were mapped, by sequence analysis, to eight of the 12 rice chromosomes (Table 3), indicating that cytosine methylation alterations in the F_1 hybrid and BC_1 progeny are likely to occur throughout the genome. Furthermore, of the 12 MIFs: eight were located in intragenic regions, showing significant similarities to known-functional genes; three were located in intergenic spaces between two putative genes; and the remaining one was related to transposable elements (Table 3). These results indicate that both cellular genes and transposable elements are targets for cytosine methylation alterations.

Based on the available cDNA sequences of predicted homologous genes matched by the eight MIFs in the intragenic region (Table 3), several pairs of primers were designed. Of those, primers that can generate species-specific products in preliminary RT-PCR and SSCP experiment were selected for further analysis (Supplementary Table 2). Primers for the putative genes downstream of the

Table 1 Patterns of parental methylated bands observed in the parents, the F_1 Hybrid, and the BC_1 progeny

Methylation pattern	Group	Hejiang 19 (AA)		HY018 (CC)		F_1 (AC)		BC_1 (AAC)		Number of sites	Total (%)
		*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II		
Monomorphic	M1	+	–	+	–	+	–	+	–	30	36 (16.7)
	M2	–	+	–	+	–	+	–	+	6	
Polymorphic between parents											
Additivity in F_1 and BC_1	A1[a]	+	+	+	–	+	+	+	+	21	255 (80.7)
	A2	+	–	+	+	+	+	+	+	19	
	A3	+	+	–	+	+	+	+	+	15	
	A4	–	+	+	+	+	+	+	+	8	
	A5	+	–	–	–	+	–	+	–	74	
	A6	–	–	+	–	+	–	+	–	93	
	A7	–	+	–	–	–	+	–	+	10	
	A8	–	–	–	+	–	+	–	+	15	
Methylation alteration in F_1 only	B1	+	–	–	–	–	–	+	–	1	1 (0.3)
Methylation alteration in F_1 and BC_1	C1	–	–	+	–	–	–	–	–	8	23 (7.3)
	C2	–	–	–	+	–	–	–	–	6	
	C3	–	–	+	–	+	+	+	+	8	
	C4	–	–	–	+	+	–	+	–	1	
Methylation alteration in BC_1 only	D1	–	–	+	–	+	–	+	+	1	1 (0.3)
	Total									316	

Bands were considered methylated if one or both parents showed polymorphism between the two isoschizomers

[a] Since MSAPs are dominant markers, it is possible that a band that is non-methylated in one parent and methylated in the other will appear as non-methylated in their F_1 hybrids, even though their methylation status is heterozygous

Table 2 Methylation alterations observed in parental non-methylated and novel bands

	Group	Hejiang 19 (AA)		HY018 (CC)		F_1 (AC)		BC_1 (AAC)		Number of sites
		*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	
Parental non-methylated[a]										
Methylation alteration in F_1 only	E1	+	+	–	–	+	–	+	+	3
Methylation alteration in F_1 and BC_1	F1	+	+	–	–	+	–	+	–	8
	F2	–	–	+	+	+	–	+	–	7
	F3	–	–	+	+	–	+	–	+	7
Novel[b]										
Methylation alteration in F_1 and BC_1	G1	–	–	–	–	+	–	+	–	5
	Total									30

[a] Bands were considered non-methylated if both the parents showed no polymorphism between the two isoschizomers

[b] Novel bands are absent in both the parents, but present in the F_1 and BC_1 progeny

three MIFs in intergenic spaces were also designed, although no RT-PCR products were generated from them. RT-PCR using RNA from the two parents (Hejiang 19 and HY018), the F_1 hybrid, and the BC_1 progeny as templates were conducted using all of these primers and the resulting PCR products were used for SSCP analysis. The results showed that among the five MIFs for which we had appropriate primers: *O. officinalis* alleles of MIF11, MIF21, and MIF24 were activated in the F_1 and BC_1 plants, whereas these alleles were silenced in the *O. officinalis* parent HY018 (Fig. 4); *O. sativa* allele MIF17 was repressed in the F_1 hybrid, whereas this allele was expressed in both the *O. sativa* parent (Hejiang19) and the BC_1 progeny (Fig. 4); both parental alleles MIF23 were equally expressed in the two parents, the F_1, and the BC_1 plants (data not shown). The fragments identified in the SSCP gel were isolated,

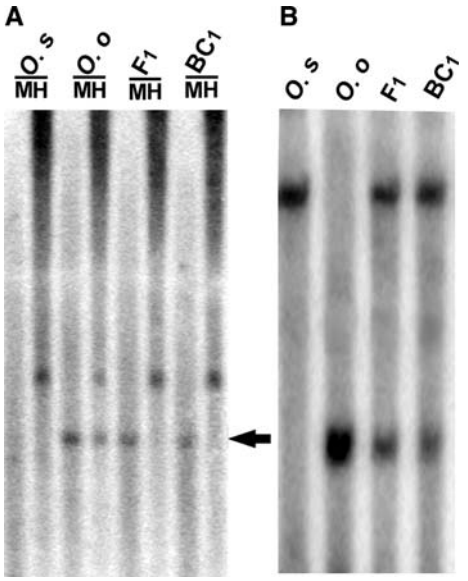

Fig. 2 Validation of cytosine methylation alteration detected by MSAP analysis using methylation-sensitive Southern blot analysis (with MIF19 as a probe). The four samples in the gel represent *O. sativa* ssp. *japonica* cv Hejiang 19 (*O. s*), *O. officinalis* accession HY018 (*O. o*), the F_1 hybrid (F_1), and the BC_1 progeny (BC_1). **a** *M* and *H* represent genomic DNA digested with *Msp*I and *Hpa*II, respectively. The *arrows* indicate the alleles involved in cytosine methylation alteration. **b** DNA samples were digested with *Eco*RV (a restriction enzyme that is not sensitive to cytosine methylation) using MIF19 as a probe

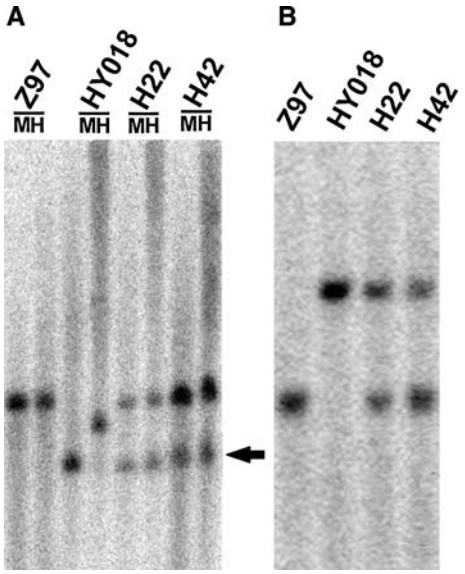

Fig. 3 Repeatable alterations in cytosine methylation patterns detected in *O. sativa* × *O. officinalis* hybridization using the methylation-sensitive Southern blot analysis (with MIF24 as a probe). The four samples represent *O. sativa* ssp. *indica* cv Zhenshan 97B (Z97), *O. officinalis* accession HY018 (HY018), the F_1 hybrid (H22), and the BC_1 progeny (H42). **a** *M* and *H* represent genomic DNA digested with *Msp*I and *Hpa*II, respectively. The *arrows* indicate the alleles involved in cytosine methylation alteration. **b** DNA samples were digested with *Eco*RV (a restriction enzyme that is not sensitive to cytosine methylation) using MIF24 as a probe

sequenced and the sequencing results were consistent with the anticipated primer-specific amplifications (data not shown).

Discussion

By MSAP analysis, 25 cytosine methylation alterations were detected in the F_1 hybrid and/or BC_1 progeny among the parental methylated sites (24 in each; see Table 1). Therefore, cytosine methylation alteration occurred at approximately 7.6% (24 out of 316) of the methylated CCGG sequences amenable to MSAP analyses in the F_1 hybrid and BC_1 progeny. This proportion was higher than the proportion (4.1%) of methylation alterations in F_1 hybrids of intraspecific crosses between rice cultivars found by Xiong et al. (1999) using MSAP. The results suggest that hybridizations between distinct relatives may induce higher rates of cytosine methylation alterations than hybridization between close relatives, in the genus Oryza at least.

We detected 55 cytosine methylation alterations in the F_1 hybrid and/or BC_1 progeny versus the parents. Most (90.9%) of the observed cytosine methylation alterations were initiated in the F_1 (2n = 2x = 24, AC) hybrid and were maintained in the BC_1 (2n = 2x = 36, AAC). This result supports the hypothesis (for which there is increasing evidence) that epigenetic changes are triggered by hybridization rather than by polyploidization (Madlung et al. 2002; Wang et al. 2004; Salmon et al. 2005; Albertin et al. 2006; Lukens et al. 2006). Another interesting observation from our study was that 69.1% of bands showing cytosine methylation alteration in the F_1 hybrid and/or BC_1 progeny were from the genome of the *O. officinalis* parent HY018. This observation is consistent with an investigation on wheat, in which 10 of 11 bands showing cytosine methylation alteration in an F_1 hybrid and an allopolyploid were from the genome of the same parental species (Shaked et al. 2001). One hypothesis that has been proposed to explain this result is that polyploidy may affect genomes' capacity for demethylation and/or hypermethylation (Shaked et al. 2001). A second possibility is that nucleocytoplasmic incompatibility may affect methylation patterns. In support of this hypothesis, the cytoplasm of the interspecific F_1 hybrid was derived from the *O. sativa* parent Hejiang19. Thus, nucleocytoplasmic incompatibilities may be induced between the C genome from HY018 and the cytoplasm from Hejiang19 in the F_1 hybrid that trigger a higher frequency of cytosine methylation alterations in the genome from HY018. A third possible explanation is related to the difference in the sizes of the Hejiang19 and HY018 genomes. The *O. sativa* and *O. officinalis* genomes are approximately 430 and 697 Mb long, respectively (Uozu

Table 3 Sequence analysis of the twelve validated MIFs

MIFs	Group	Size (bp)	*E* value[a]	Chromosome	Location	Locus	*E* value[b]	Putative gene function
MIF4	C3	275	2.5e-10	1	Intergene	–	–	
MIF9	F2	244	9.0e-70	2	Intragene	AK070377	None[c]	Putative Oligoribonuclease
MIF11	C3	481	6.2e-266	1	Intragene	AK069590	6.0e-39	Putative enoyl-CoA hydratase/ isomerase family protein
MIF12	F2	413	7.1e-10	11	Intragene	CB633721	6.0e-30	
MIF13	F2	248	5.1e-106	5	Intragene	AK102665	None	Putative kinase family protein
MIF17	E1	415	2.4e-188	8	Intragene	AK099637	None	Cytochrome P450 71D7
MIF18	F1	215	8.5e-113	4		NM_001058960		Transposable elment-related
MIF19	F2	153	8.0e-44	7	Intergene	–	–	
MIF21	C3	556	2.0e-241	3	Intragene	AK066505	4.0e-31	Putative activating signal cointegrator-related protein
MIF23	E1	420	6.0e-235	2	Intragene	AK241820	4.0e-140	Putative wall-associated serine/threonine kinase
MIF24	C3	237	3.5e-108	11	Intragene	AK066354	2.0e-31	Putative peptide chain release factor
MIF27	F2	275	3.4e-108	7	Intergene	–	–	

[a] The *E* value is the queried MIF sequence to rice genomic sequence on the Gramene website

[b] The *E* value is the queried MIF sequence to the predicted homology EST or full length cDNA sequence on the NCBI website

[c] The queried MIF is located in the intron of the predicted homologous gene

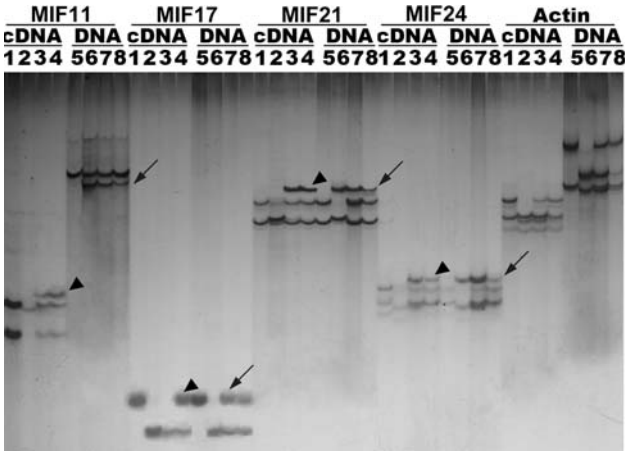

Fig. 4 Alterations in species-specific gene expression detected by RT-PCR in combination with SSCP analysis. PCR products of MIF11, MIF17, MIF21, MIF24 and *Actin* in the SSCP gel are shown, which were amplified from either cDNA (*lanes 1–4*) or genomic DNA (*lanes 5–8*). The four samples we analyzed were from Hejiang 19 (*lanes 1*, *5*) and HY018 (*lanes 2*, *6*), and F_1 hybrid (*lanes 3*, *7*), and BC_1 progeny (*lanes 4*, *8*), respectively. A pair of primers for rice *Actin* amplifying a 341-bp cDNA fragment (or a 424-bp genomic DNA fragment) was used as a control to check that equal quantities of template were used and that there had been no significant genomic DNA contamination. The *arrowheads* indicate altered species-specific transcripts from either parent, and the *arrows* indicate corresponding PCR products obtained using genomic DNA preparations as templates

et al. 1997). Thus, if the rates of cytosine methylation alterations are roughly constant, irrespective of genome origin, more alterations should originate from the *O. officinalis* parent simply because its genome is larger.

We found that four of the 12 validated alterations in cytosine methylation were reproduced in the F_1 hybrid and BC_1 progeny derived from another independent interspecific hybridization. This finding indicates that cytosine methylation alterations may be non-random in some cases. Apparently directed alterations in cytosine methylation have also been found in other hybrid and allopolyploid studies (e.g. Shaked et al. 2001; Salmon et al. 2005; Lukens et al. 2006). These stochastic or non-random alterations in cytosine methylation may partially explain both phenotypic variation and variation in gene expression within allopolyploids (Comai et al. 2000; Wang et al. 2004; Gaeta et al. 2007).

The important role cytosine methylation plays in epigenetic regulation of gene expression has been well documented (Martienssen and Colot 2001; Rangwala and Richards 2004). However, to date links between cytosine methylation alterations and epigenetic gene silencing in response to hybridization and polyploidization have only been demonstrated indirectly. In Arabidopsis, several studies have indicated that gene silencing accompanies hybridization and polyploidization, but this could be reversed by treatment with 5-aza-20-deoxycytidine (aza-dC), a chemical that specifically blocks the action of DNA methyltransferases and thus demethylates the genome. This finding suggested that at least some of the silencing events were associated with alterations in the cytosine methylation status at specific sites (Comai et al. 2000; Lee and Chen 2001; Madlung et al. 2002). Using transgenic technology involving RNA interference, Wang et al. (2004) created lines that

had defects in the expression of two genes involved in cytosine methylation, and showed that the expression of two previously silenced genes was reactivated in these lines. Since other genes that were silenced in polyploid Arabidopsis were not similarly reactivated, cytosine methylation was clearly responsible for only some of the gene silencing in them. Recently, Zhang et al. (2008) found that cytosine methylation alterations immediately upstream or downstream of the gene were inversely correlated with the degree of expression variation for that gene. In contrast, methylation variation within genic region showed weak positive correlation with expression variation. In other plant species, such as wheat and cotton, less evidence is available on the association between alterations in cytosine methylation and gene silencing. In our study, direct and immediate effects of altered cytosine methylation on gene expression were demonstrated in rice. Interestingly, previous work has shown that biased expression and organ-specific gene silencing are prominent features in cotton (Adams et al. 2003), but no cytosine methylation alteration has been observed in allopolyploid cotton (Liu et al. 2001). These findings indicate that other mechanisms such as chromatin compaction, allele-dosage effects and siRNA regulations, rather than cytosine methylation alterations, are involved in modulating gene expression in cotton. The contrasting patterns found in cotton, Arabidopsis and rice suggest that different plant systems may regulate gene expression in different ways during hybridization and polyploidization processes.

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