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Effect of short day photoperiod on DNA methylation and expression of a gene in rice KDML105

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Methylation-sensitive amplified polymorphism (MSAP) technique was adapted to screen a photoperiod-responsive gene of rice KDML105. Six out of thirty-two primer combinations gave twelve *EcoRI/HpaII*-generated MSAP markers from the genomic DNA of KDML105 after exposure to short-day (SD) photoperiod. These MSAP fragments were cloned and used as probes to hybridize to MSAP fingerprints. Positive fragments generating the signals correlated to the MSAP fingerprints were sequenced and aligned to the database. Ten out of twelve MSAP markers showed 93-100% homology to database sequences and the best homology fragment (F1) was chosen for complete gene cloning, sequencing, and alignment. This candidate gene fragment showed 98% homology to the sequences of rice genomic DNA gi|23237916 and rice cDNA gi|32986083. It also has an open reading frame (ORF) of 402 bp and deduced a polypeptide of 134 amino acid residues, while those of gi|23237916 and gi|32986083 have a deduced polypeptide of 137 amino acid residues resulting in the overall homology of 97%. In addition, RT-PCR products and the Southern blot hybridization of the genomic DNA of KDML105 digested with *HpaII* or *MspI* indicated that the expression of this candidate gene depends on DNA demethylation, which is induced by 10 days of SD photoperiod. It is speculated that this gene might belong to another class of regulatory gene for the flowering time or heading-time loci in rice or it is a downstream product of flower initiation.

Key words: DNA methylation, photoperiod, rice, *Oryza sativa* L., MSAP, methylation sensitive AFLP.

INTRODUCTION

Rice (*Oryza sativa* L.) is a main cereal for more than half of the world population. There are three subspecies *japonica*, *indica*, and *javanica*. Thai jasmine rice (Khao Dok Mali 105, KDML105) cultivar, an *indica*, is the most popular due to its pleasant aroma, having soft and tender texture after cooking. KDML105 is a short-day (SD) and photoperiod-sensitive cultivar generally grown in the rainfed lowland of the North and Northeast of Thailand (Oka, 1958; Poonyarit et al., 1989). However, it also could be grown at long-day (LD) photoperiod in the rainy

season and having a floral transition in winter after exposure to SD photoperiod.

Flowering time is regulated by both endogenous and environmental factors. Some of the genes involved in the photoperiodic control of flowering have been reported specially in LD (Goto et al., 1991; Johnson et al., 1994; Guo et al., 1998; Lin 2000; Hayama et al., 2002) but less is known in the SD plant (Childs et al., 1997). However, as high as twenty-three flowering time or heading-time loci were found in the genome of SD rice, but their physiological and biochemical functions in genetic pathways leading to flowering are still unknown (Kojima et al., 2002; Nishida et al., 2002) except some speculation on the conservation of these genes in both LD and SD plants (Samach and Gover, 2001).

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Similar to what is known about photoperiod, thermoinduction of flowering was reported to be an epigenetic process (Burn et al., 1993; Finnegan et al., 1998). Although epigenetic controls of gene expression are not well understood, there is increasing evidence that the DNA methylation pattern is an important factor. It plays a critical role in regulating gene expression and genome stability, and it is essential for proper growth and development (Gonzalzo and Jones, 1997; Malagnac et al., 2002). So, DNA methylation pattern is maintained with high fidelity through each DNA replication cycle and is transmitted to both daughter cells. In higher plant genomes, 5'-methylcytosine residues occur preferentially in CG dinucleotides and CNG trinucleotides when N represents any of the four common nucleotide base (Gruenbaum et al., 1981; Papa et al., 2001). The levels of cytosine methylation have been observed with significant difference among various tissues in rice (Dhar et al., 1990), tomato (Messeguer et al., 1991), maize (Lund et al., 1995), banana (Peraza-Echeverria et al., 2001) and pepper (Portis et al., 2004). Promoter regions of silent genes have been found to be more methylated than actively transcribed sequences (Pikarard, 1999), but how methylation regulates gene expression is still not clear (Xiong et al., 1999).

Amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995) was modified to detect 5'-methylcytosine in DNA of dimorphic fungi (Reyna-López et al., 1997), and this approach has proven to be useful for determining DNA methylation status. Later on, methylation-sensitive amplified polymorphism (MSAP) technique was adapted from AFLP technique to determine DNA methylation pattern in a rice hybrid (Xiong et al., 1999). In this study, we made an attempt to detect the light responsive gene via DNA methylation mechanism in SD rice that is possibly involved in the photoperiodic control of flowering. The MSAP technique was adapted to investigate the DNA methylation changes in rice KDML105 under SD photoperiod.

MATERIALS AND METHODS

Plant materials and growth conditions

A photoperiod-sensitive rice KDML105 and a photoperiod-insensitive rice RD7 were grown under LD photoperiod. After growing for 60 days, half of the plant samples were transferred to SD photoperiod (8 h light/16 h dark) for 15 days.

DNA and RNA extraction

Genomic DNA and total RNA were extracted from the base of the last leaves of KDML105 and RD7 plants at 0, 5, 10 and 15 days after exposure to either SD or LD photoperiods. Genomic DNA was extracted using a modified CTAB (cetyl trimethyl ammonium bromide) procedure described by Agrawal et al. (1992), and total

RNA was extracted using TRIzol[®] Reagent (GibcoBRL[™] Life Technologies, USA).

Methylation-sensitive amplified polymorphism (MSAP) analysis

Rice genomic DNA (250 ng) was digested with 2.5 U of *EcoRI* and 2.5 U of *HpaII* (New England Biolabs[®], Inc., USA) in 25 µl reaction mixture for 2 h at 37°C. The digested DNA fragments were ligated with an equal volume of the adapter/ligation solution containing 1 U of T4 DNA ligase (New England Biolabs[®], Inc., USA), 5 pmole *EcoRI* adapter (5'-CTCGTAGACTGCGTACC-3'/3'-CATCTGACGCATGGTTAA-5') and 50 pmole *HpaII/MspI* adapter (5'-GATCATGAGTCCTGCT-3'/3'-AGTACTCAGGACGAGC5'), and incubated for 1 h at 37°C and 2 h at 25°C. The ligation mixture was used as template DNA in pre-selective amplification with E+A primer (5'-GACTGCGTACCAATTC+A-3') and HM+1 primer (5'-ATCATGAGTCCTGCTCGG+N-3', when N consisted of A, C, G or T). The PCR reaction was performed for 20 cycles with 30 s denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The pre-selective product from pre-amplification was diluted 20 folds with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and further used as template DNA for selective amplification. E+3 primer (5'-GACTGCGTACCAATTC+ANN-3', when NN consisted of AC, AG, CA, CT, CC, CG, GC or GG) and HM+1 primer were used in selective amplification following the protocol of Vos et al. (1995). The selective amplification products were separated on 6% denaturing polyacrylamide gel and the bands were detected by silver staining as described by Echt et al. (1996). The adapters and primers for *EcoRI* were the same as those used in standard AFLP analysis (Vos et al., 1995), while the *HpaII/MspI* adapters and primers were designed according to Xiong et al. (1999).

Cloning of MSAP fragments

The different MSAP bands were cut from the stained polyacrylamide gel and incubated in ultra pure water for 1 h at 60°C. The DNA bands were reamplified with the same MSAP primer pairs. The PCR reaction was performed for 35 cycles with 30 s denaturation at 94°C, 30 s annealing at 56°C, and 1 min extension at 72°C. Reproducibility of each fragment was detected on 1% agarose gel. DNA bands were cut from the agarose gel and purified using QIAquick[®] Gel Extraction Kit (QIAGEN, USA), then ligated with pGEM[®]-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* XL1-Blue. The inserted DNA from the selected clones were labelled with fluorescein-11-dUTP using Gene Images[™] Random Prime Labelling Module (Amersham Biosciences, UK), and used as probes to hybridize to MSAP fingerprints on nylon membrane. The positive clones were sequenced and compared to database sequences from the GenBank (<http://www.ncbi.nlm.nih.gov>) and the Beijing Genomics Institute (<http://bgi.genomics.org.cn>).

Blotting and hybridization

MSAP fingerprints on polyacrylamide gel were capillary transferred onto nylon membrane (Nytran[®] N, Schleicher&Schuel, USA), pre-hybridized and hybridized with the related probes for 12 h at 65°C. Washing of probe was done at high stringency with 0.5x SSC (1x SSC = 15 mM sodium citrate and 150 mM NaCl, pH 7.0) and 0.1% SDS (sodium dodecyl sulphate) for 15 min at 65°C, then washed with 0.1x SSC and 0.1% SDS at the same condition. The signals on

Table 1 Twelve different MSAP bands revealed by six primer combinations.

Primer combinations	MSAP bands		
	Name	Length* (bp)	Days of present or absent
E+AAC/HM+G	A1	208	Present at 5, 10 and 15 days
E+AAG/HM+G	B1	276	Present at 10 and 15 days
	B2	165	Present at 15 days
	B3	142	Present at 5, 10 and 15 days
E+ACA/HM+G	C1	233	Absent at 5 days
	C2	110	Present at 10 and 15 days
E+ACT/HM+G	D1	232	Present at 10 and 15 days
E+AGC/HM+G	E1	248	Present at 10 and 15 days
	E2	217	Present at 10 and 15 days
E+AGG/HM+G	F1	372	Present at 10 and 15 days
	F2	216	Present at 5, 10 and 15 days
	F3	142	Present at 5, 10 and 15 days

* Length of MSAP bands including adapter primer sequence.

nylon membrane were detected using CDP-Star™ Detection Module (Amersham Biosciences, UK) and exposed to Kodak® Diagnostic Film (Eastman Kodak Company, USA).

Open reading frame (ORF) finding and cloning

Sequences of the cloned MSAP fragments were aligned and compared to sequences in the GenBank. PCR primers were designed to amplify the candidate gene from genomic DNA of rice KDML105. The forward and reverse primers were 5'-GATGCGCTCTCTTACC-3', and 5'-ACTCTGTATCAGGAATTCAGG-3', respectively. The PCR products were cloned and sequenced. The sequences of the candidate gene were aligned to the previous sequences and their ORFs were analyzed using ORF Finder program in the GenBank. The new primers were designed to amplify the cDNA of the candidate gene. The forward and reverse primers were 5'-CACCGCCTTCGAGTCTGA-3', and 5'-GAGTTCAGAGTTTCGCTCCA-3', respectively.

Analysis of gene expression by RT-PCR

mRNA was isolated from 20 µg of total RNA of rice KDML105 using Dynabeads® mRNA DIRECT™ Micro Kit (DYNAL®, Norway) and reverse transcription polymerase chain reaction (RT-PCR) was performed using SuperScript™ One-Step RT-PCR with Platinum® Taq (Life Technologies, USA). RT-PCR products were separated on 2.5% agarose gel. For control of mRNA concentration, the primer pair for amplification of a part of putative actin gene (350 bp) in rice was added in the same reaction. They were designed from database in the GenBank (gi|34905849; mRNA for putative actin of *Oryza sativa japonica*) included a forward primer as 5'-CGAGCGGAAATTGTGAGG-3', and a reverse primer of 5'-AGGGCAGTGATCTCCTTGC-3'. Both genes were co-amplified using multiplex titration RT-PCR (Nebenführ and Lomax, 1998).

Detection of DNA methylation level by Southern hybridization

20 U of either *Hpa*II or *Msp*I (New England Biolabs®, Inc., USA) was used to digest 10 µg of genomic DNA of rice KDML105 for 12 h at 37°C. The DNA fragments were separated on 0.8% agarose gel, transferred onto nylon membrane for 12 h using capillary transfer method as described by Wu et al. (1997), and hybridized with the candidate gene as previously described.

RESULTS

MSAP fingerprints using thirty-two primer combinations were generated from genomic DNA of both KDML105 and RD7, which had been exposed to either SD or LD photoperiods at 0, 5, 10 and 15 days. Out of these, six primer combinations showed one to three different bands in KDML105 under SD photoperiod: primer combinations E+AAC/HM+G and E+ACT/HM+G showed one different band (fragments A1 and D1), primer combinations E+ACA/HM+G and E+AGC/HM+G showed two different bands (fragments C1, C2 and E1, E2), and primer combinations E+AAG/HM+G and E+AGG/HM+G showed three different bands (fragments B1, B2, B3 and F1, F2, F3) (Table 1 and Figure 1). As for the genomic DNA of RD7 after exposure to both SD and LD photoperiod, it could generate twelve MSAP bands having the same sizes as those unique different bands in KDML105. Among these, four from twelve different bands (A1, B3, F2 and F3) were present at days 5, 10 and 15, but six from twelve different bands (B1, C2, D1, E1, E2 and F1) were present at days 10 and 15, one different band (B2) was present at day 15, while fragment C1 was present in all samples except day 5 (Figure 1).

These twelve different MSAP bands from KDML105 were cloned and used as probes to hybridize to MSAP fingerprints. Positive fragments that generated the signals correlated to the MSAP fingerprints were then sequenced. The result showed that nucleotide sequence of B3 was identical to that of F3. So, the whole eleven nucleotide sequences were submitted to the GenBank with the accession numbers of AY562108 to AY562118 (Table 2), and were aligned to the database. Eight from eleven positive fragments (A1, B1, B2, B3, C1, D1, E1 and F1) showed 93-99% homology to database sequences in the GenBank (Table 2). Five from eleven positive fragments (C1, D1, E1, F1, and F2) showed 97-100% homology to contig 1526, contig 2637, contig 896, contig 9305 and contig 10370 in the Beijing Genomics

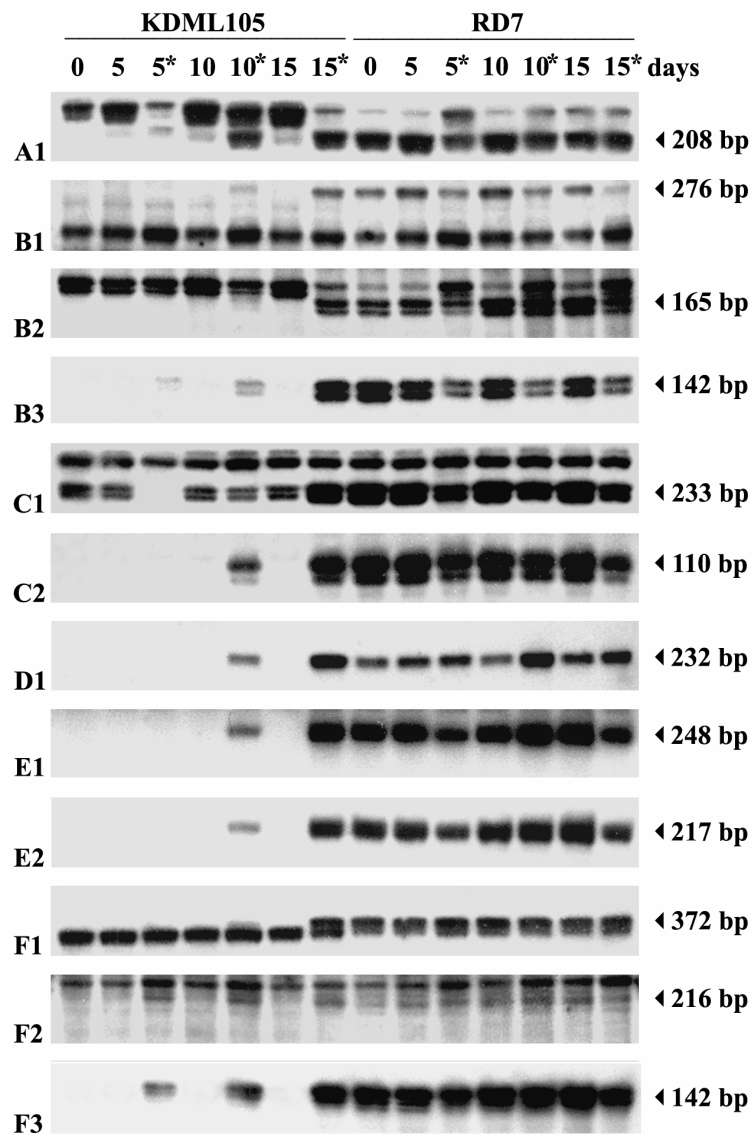


Figure 1. Twelve different MSAP bands generated by hybridization of each MSAP fingerprints with their cloned positive fragment. The number with the asterisk represented the days after exposure to SD photoperiod. A1, B1, B2, B3, C1, C2, D1, E1, E2, F1, F2 and F3 are MSAP selected markers. The sizes of these fragments are indicated on the right hand side.

Institute, respectively, whereas C2 and E2 showed less than 25% homology to both sources of database. From this result, it was found that two from eleven positive fragments (B3 and F1) were located within the cDNA sequences. Since F1 was found to contain higher shared homology to database sequence i.e., 99% homology to gi|23237916 (*Oryza sativa japonica* genomic DNA, chromosome 7) and gi|32986083 (*Oryza sativa japonica* cDNA) and also have longer base pairs (348 bp) than B3 (118 bp), it was chosen for complete gene cloning, sequencing, and alignment. PCR primers for the amplification of a candidate gene from F1 and its flanking region were designed using both sequences. The

sequenced gene was found to contain 599 nucleotides and showed 98% homology to the sequences of gi|23237916 and gi|32986083. This nucleotide sequence was then submitted to the GenBank as the accession number of AY566228. The ORF Finder program in the GenBank indicated that this candidate gene has an open reading frame extending from nucleotides 181 to 582, and it deduced a polypeptide of 134 amino acid residues (Figure 2a), while the gi|23237916 and gi|32986083 deduced a polypeptide of 137 amino acid residues. Both proteins shared the overall homology of 97% with each other (Figure 2b).

Inner primers were further designed for amplification of

Table 2. GenBank accession numbers of the MSAP fragments and the accession number of the best homology ones.

MSAP fragment	Length* (bp)	GenBank accession number	Best homology: GenBank accession number
A1	184	AY562108	<i>O. sativa japonica</i> genomic DNA, chromosome 1: gi 8096463
B1	252	AY562109	<i>O. sativa japonica</i> genomic DNA, chromosome 3: gi 28316619
B2	141	AY562110	<i>O. sativa japonica</i> genomic DNA, chromosome 4: gi 38605939
B3	118	AY562111	<i>O. sativa japonica</i> cDNA: gi 32976919 and gi 32987574
C1	209	AY562112	<i>O. sativa japonica</i> genomic DNA, chromosome 4: gi 38605759
C2	86	AY562113	-
D1	208	AY562114	<i>O. sativa japonica</i> genomic DNA, chromosome 1: gi 19571107 and gi 20146488
E1	224	AY562115	<i>O. sativa japonica</i> genomic DNA, chromosome 4: gi 32488072
E2	193	AY562116	-
F1	348	AY562117	<i>O. sativa japonica</i> genomic DNA, chromosome 7: gi 23237916 and <i>O. sativa japonica</i> cDNA: gi 32986083
F2	192	AY562118	-

* Length of MSAP fragments excluding adapter primer.

a

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TACTCTGTATCAGGAATTCAGGATGAATCTCTGAAATGTTGTTTGGAGTCAGGAGTTCAGA 60
GTTTCGCTCCATTGATTTGAGGATATTCAGTTAACCTGTCATTTTCATTTTGCTGTAACCG 120
AAATGAGAAGAACATGTAACAACATATCATAATTCGATCAGGTGCAGATCATCATCATGTA 180
ATGAAGGAAGATTTTGTACACGAAATGGAGATCAATCACCTTGTGGAGAGAGAGAGAGAG 240
M K E D F V H E M E I N H L V E R E R E 20
ACGAAGAACAGTACAACACTGCTGCCTTATTGGTTCATGGAATCATGTGACTCTTGTGT 300
T K N S T T L L P Y W F M E S C D S C C 40
CCTCGCGGTGTTGAATTGCTGATAGGAAGAGGAGGACGATGGGCATCTTCTTCCCGGGA 360
P R G V E L L I G R G G R W A S S S P G 60
CGCGCGGCCCGTGGTACGCCGCCGCCGGAGGGAAGCGCGCGGATACGTGGGCGCGCGC 420
A A A P W Y A A G R R E A R G Y V G G G 80
GCGTCATCTCCCCTTCTCTTCTCGCTCCGCCGCCGCCGGAGGCCGCACTCGACG 480
A S S P L L S S R S A A A G R R P H S T 100
GGTCAGACTCGAAGGCGGTGGGTTAGAGCGGAGACGGTGCAGAGGAGGATGGGGGAGCCC 540
G Q T R R R R W V R A E T V Q R R M G E P 120
CCTCGCCGCCGCCGCCGCCGCCGTGGGCGCGCGGCCATGGTGAAGAGAGAGCGCATC 599
P R R P P A P P W A A A A W 134
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b

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gi|32986083 MKEDFVHEMEINHLVEREREREETKNSTLLPYWFMESCDSCCPRGVELL 50
AY566228 MKEDFVHEMEINHLVERERE---TKNSTLLPYWFMESCDSCCPRGVELL 47
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gi|32986083 IGRGGRWASSSPGAAAPWYAAGRREARGYVGGGASSPLLSSRSAAAGRRP 100
AY566228 IGRGGRWASSSPGAAAPWYAAGRREARGYVGGGASSPLLSSRSAAAGRRP 97
*****

gi|32986083 HSTGQTRRRWVRAETVQRRMGEP RRRPPAPPPAAAAW 137
AY566228 HSTGQTRRRWVRAETVQRRMGEP RRRPPAPPWAAAAW 134
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Figure 2. Sequence of a candidate gene homologous to F1 fragment. (a) Nucleotide and deduced amino acid sequence. (b) Comparison of deduced amino acid sequence of the candidate gene and gi|32986083.

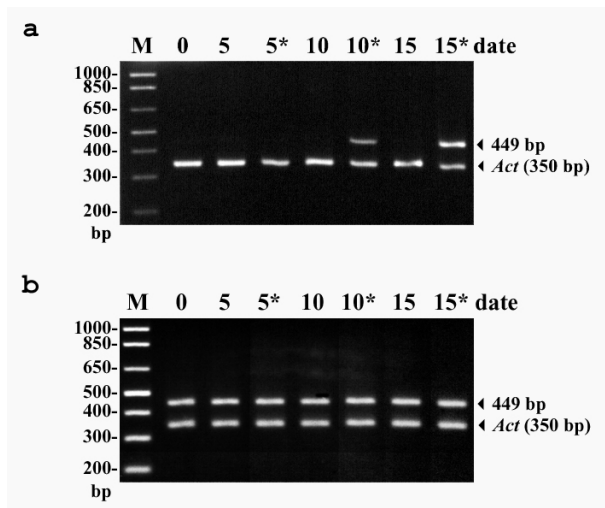


Figure 3. RT-PCR product showing the expected bands (449 bp) of the candidate gene in KDML105 samples at 10 and 15 days under SD photoperiod (a) and in all samples of RD7 (b).

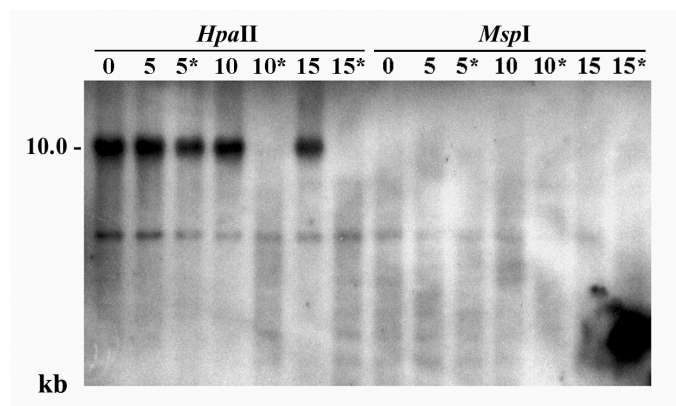


Figure 4. Southern analysis of either *HpaII* or *MspI* digested genomic DNA of KDML105 with the candidate gene probe showing very strong signals of about 10 kb in the genomic DNA digested with *HpaII* of 0, 5, 10 and 15 days under LD photoperiod, and 5 days under SD photoperiod. No fragment was found in the genomic DNA digested with *HpaII* of 10 and 15 days under SD photoperiod, or in the genomic DNA digested with *MspI*.

a cDNA fragment of the candidate gene (449 bp). RT-PCR products showed the expected bands of the candidate gene in KDML105 samples at 10 and 15 days under SD photoperiod which was consistent with the MSAP fingerprints, whereas the amplification of the putative actin gene was similar in all samples (Figure 3a). The expected bands of 449 bp were found in all samples of rice RD7 both in SD and LD conditions (Figure 3b). Southern blot hybridization of genomic DNA of KDML105 with the 499 bp candidate gene probe showed no band in the genomic DNA digested with either *HpaII* in 10 and 15

days under SD photoperiod or those digested with *MspI* in any samples (Figure 4). However, there were very strong signals of about 10 kb in the genomic DNA digested with *HpaII* in 0, 5, 10 and 15 days under LD photoperiod, and 5 days under SD photoperiod. The results were correlated to those of MSAP fingerprints and RT-PCR amplification.

DISCUSSION

Since the MSAP technique for rice genome (Xiong et al., 1999) was adapted from AFLP technique (Vos et al., 1995), there is no requirement for prior genome information while the high number of methylation events could also be detected using a relatively small number of primer combinations (Peraza-Echeverria et al., 2001). We have further adapted the MSAP technique to detect the light responsive gene via DNA methylation mechanism in KDML105, a photoperiod-sensitive rice. *EcoRI/HpaII*-generated MSAP markers were detected in rice KDML105 after various days of exposure to either SD or LD photoperiods. Rice RD7, a photoperiod-insensitive cultivar was used as a control. Eleven MSAP markers were generated from thirty-two primer combinations. The result confirmed that MSAP is an efficient technique for investigating 5'-methylcytosine in genomic DNA of KDML105 while also agreeing with DNA methylation in rice genome by Xiong et al. (1999) and Ashikawa (2001).

The photoperiod-sensitive rice plants do not respond to photoperiod during the entire vegetative period from sowing to flowering. They are sensitive to photoperiod only during the inductive phase, but do not respond during pre-inductive and post-inductive phases (Yin and Kropff, 1998). After growing for 60 days, the vegetative phase of KDML105 was supposed to change to flowering stage by exposure to SD photoperiod. The duration of exposure to SD photoperiod was also necessary to induce the growth phase shift in photoperiod sensitive rice. During SD (8 h light/16 h dark) photoperiod, irreversible process of flower initiation has been microscopically detected after 9 days of exposure to SD light (Sirikayon, 2000). In this study, DNA samples were extracted from rice after growing under SD photoperiod for 5, 10 and 15 days, which is the time spanned over the flowering period. The methylation of DNA is important for regulation of genome stability and gene expression during growth and development (Finnegan et al., 1996). Using MSAP technique, the differences in DNA methylation pattern were found during transition from vegetative to reproductive phase in rice KDML105. Eleven MSAP markers were detected in KDML105 after exposure to SD photoperiod for 5, 10 and 15 days. These markers were not found in KDML105 after exposure to LD photoperiod or in RD7 after exposure to either SD or LD photoperiods. These results indicated that the methy-

lation change in the genomic DNA of KDML105 was stimulated by exposing to SD photoperiod and hence the methylation control of flowering.

Although MSAP is a very effective tool to reveal restriction fragment polymorphism (Xiong et al., 1999), we found the nucleotide sequence of B3 shared 100% identity with F3. This B3 was generated by primer combination of E+AAG/HM+G, while F3 was generated by primer combination of E+AGG/HM+G. They were different at the second selective nucleotide of E+3 primers. This result showed a limitation of PCR reaction even though touch down PCR was used for amplification reaction as has been commonly used in AFLP technique (Vos et al., 1995). However, the repetitiveness of these two fragments was also possible.

Eleven markers present or absent at the given days in Table 1 were cloned and sequenced. The sequence data were submitted and compared to the sequences in GenBank. B3 and F1 markers were highly similar to the cDNA sequences (Table 2) indicating them to be the candidate genes with unknown function. B3 was present at days 5, 10 and 15 while F1 was present only at days 10 and 15 under SD photoperiod. Subsequently, a candidate gene homolog of F1 was isolated from genomic DNA of KDML105. It has an open reading frame of 402 bp encoded a polypeptide of 134 amino acid residues. The expression of this gene was confirmed by multiplex titration RT-PCR (Nebenführ and Lomax, 1998) using a putative actin as a control of mRNA concentration. Our result indicated that this candidate gene was expressed in KDML105 at days 10 and 15 of exposure to SD photoperiod while the expression of this gene was constitutive in all days in RD7 both under SD and LD photoperiods.

The isoschizomers *HpaII* and *MspI* recognize the same tetranucleotide sequence (5'-CCGG-3'), but display differential sensitivity to DNA methylation. *HpaII* is inactive when one or both cytosines are fully methylated (both DNA strand methylated), but cleaves the hemimethylated sequence (only one DNA strand methylated) at a lower rate compared to the unmethylated sequence, whereas *MspI* cleaves 5'-C^{5m}CGG-3', but not 5'-_{3m}CCGG-3' (McClelland et al., 1994).

DNA methylation change in the candidate gene was confirmed by Southern hybridization. The hybridization pattern of DNA samples in both SD and LD were identical when digested the genomic DNA of KDML105 with *MspI*. There was no band detected in any of these samples because many sites for the enzyme (CCGG sequence) existed within the target sequence producing very small fragments below 300 bp which made them less efficient in membrane binding and hybridization. On the contrary, DNA digested with *HpaII* remained large fragments in LD and at 5 days of SD photoperiod indicating that demethy-

lation of the candidate gene occurred after exposure to SD photoperiod no later than 10 days. Demethylation and expression of this gene are well correlated, supporting the role of DNA demethylation as a regulatory factor of gene expression reported in the transgenes of transgenic plants (Kumpatla et al., 1997).

The genes involved in flowering in *Arabidopsis thaliana*, a LD plant, have been divided into 2 groups, photoreceptor encoded genes and circadian clock controlled genes (Hayama et al., 2002). The genes homologous to both two groups of *Arabidopsis* genes were also found in rice with a little difference in controlling flowering mechanism (Childs et al., 1997; Izawa et al., 2000; Yano et al., 2001). However, their definite function in regulation of flowering time is still unclear. Different rice varieties respond to photoperiod in different ways. Therefore, control of flowering pathway may involve various mechanisms. Flowering in KDML105 occurred after exposure to SD for a period of 9 days (Sirikayon 2000). During that period DNA methylation patterns of some genes have been changed and one of these corresponded well with its expression. The candidate gene found in this experiment is an example of the genes expressed by demethylation of DNA induced by SD photoperiod. It might be another class of gene regulating flowering time in rice or it is a downstream product of flower initiation. Additional information and further study should be carried out to clarify this speculation.

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