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Hd1, *Hd3a*, and *Hd6* Genes : Possible DNA Methylation Roles in Photoperiod Sensitive Gene Regulation of Rice KDML 105 (*Oryza sativa* L.)

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ABSTRACT

The study was aimed to investigate DNA methylation in rice after treatment with different photoperiods and to determine allelic differences of *Hd1*, *Hd3a*, and *Hd6* genes, involved in photoperiod sensitivity, in KDML 105, a photoperiod-sensitive cultivar, and in Suphan Buri 1, a photoperiod-insensitive cultivar. Detection of DNA methylation was performed by Southern hybridization. The genomic DNA of KDML 105 was digested by *Hpa*II or *Msp*I restriction enzymes, separated by electrophoresis, Southern blotted and hybridized with probes amplified from the 5' region of *Hd1*, *Hd3a*, and *Hd6* genes, respectively. The results showed no difference in band patterns of long-day treatment and short-day treatment of either *Hpa*II or *Msp*I digested groups indicating that the target sequences were not methylated. Primers were designed to amplify the overlapping fragments of *Hd1*, *Hd3a*, and *Hd6* alleles from rice KDML 105 and Suphan Buri 1 to make a contig sequence covering the whole genes. Nucleotide and deduced amino acid sequences data obtained from this experiment indicated that *Hd1*, *Hd3a*, and *Hd6* genes of KDML 105 and Suphan Buri 1 encoded normal Hd1, Hd3a, and Hd6 proteins, respectively.

Taken together, the data suggested that DNA methylation and single amino acid polymorphisms (SAPs) might not be the mechanisms underlying the expression and function of photoperiod genes. However, the differences of the nucleotide in the 5' control region might be the cause of the photoperiod sensitivity difference between KDML 105 and Suphan Buri 1.

Key words: *Hd1*, *Hd3a*, *Hd6*, DNA methylation, rice, photoperiod

INTRODUCTION

Rice is one of the principal cereals in the human diet. In the past decades, researches had been focused on improving rice cultivars to meet the world's increasing demand for rice.

There are a lot of aromatic rice varieties in Thailand but the most famous variety in the world market is KDML 105 due to its pleasant characteristics such as good taste, softness, and aromatic fragrance. The major disadvantage of KDML 105 is the low productivity, about 2,412.5

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kilograms per hectare (386 kilograms per rai), and photoperiod sensitivity (Rice Research Institute, 2003; 2005).

Photoperiodic genes in rice have been studied extensively (Sirigayon, 2000; Takahashi *et al.*, 2001; Yano *et al.*, 2001; Hayama *et al.*, 2002; Hayama and Coupland, 2004). DNA methylation has been reported to regulate gene expression in plants i.e., oil palm (Jaligot *et al.*, 2000), winter wheat (Horv·th *et al.*, 2002) and *Arabidopsis* (Lindroth *et al.*, 2001; Madlung *et al.*, 2002; Kinoshita *et al.*, 2004). Moreover, Finnegan *et al.* (1998) found that DNA methylation level was reduced by vernalization.

KDML 105 is classified as a photoperiod-sensitive cultivar, Khao Jow Hawm Khlong Luang 1 as a weakly photoperiod-sensitive cultivar and Suphan Buri 1 and Pathum Thani 1 as photoperiod-insensitive cultivars (Thacharoen, 1992; Prasertsak *et al.*, 2001; Thongsima, 2001). Up to now there have been few reports on photoperiodic genes regulated by DNA methylation, particularly in rice KDML 105. Thus in this research, DNA methylation level of *Hd1*, *Hd3a*, and *Hd6*, candidate genes for photoperiod sensitivity (Yano *et al.* 2000; Kojima *et al.*, 2002; Takahashi *et al.*, 2001), was determined by exposure of KDML 105 rice to short-day length (11 hours per day). This research would provide valuable information not only for rice improvement but also for other plants.

MATERIALS AND METHODS

Plant materials

Rice KDML 105, a photoperiod-sensitive cultivar, and Suphan Buri 1, a photoperiod-insensitive cultivar, were used in this experiment.

Rice cultivation and photoperiodic control

Rice seeds, KDML 105 and Suphan Buri 1, were soaked in water for 1 day, and grown in the tray containing soil for 7 days then transferred the rice seedlings into the plastic pots. The rice pots were placed in the long-day light (13 hours per day) for vegetative growth for 50 days, and then separated into 2 groups. The first group was exposed to short-day light (11 hours per day) for 15 days, and the other group was maintained in the long-day condition for 15 days (Pipatpongpinyo, 2005).

Genomic DNA extraction

The last inner leaf of rice KDML 105 and Suphan Buri 1 from the short-day treatment at days 0, 5, 6, 7, 8, 9, 10, 12, and 15, and from the long-day condition at days 5, 7, 8, 10, and 15 were excised. Total DNAs were extracted using the method modified from Agrawal *et al.* (1992).

Amplification of *Hd1*, *Hd3a*, and *Hd6* alleles

Nucleotide sequences of *Hd1* alleles of rice cultivars, Nipponbare, Kasalath, Ginbouzu, HS66, and HS110 (GenBank accession numbers: AB041837, AB041839, AB041840, AB041841, and AB041842 respectively) were aligned using ClustalX 1.8 program (available at: http://www-igbmc.u-strasbg.fr/BioInfo/ [August 16, 2004]) to generate a consensus. The primers covering the whole length of the gene were designed based on FastPCR program, version 3.6.17 (available at: http://www.biocenter.helsinki.fi/bi/bare-1_html/download.htm [August 16, 2004]). Other two sets of primers were designed in the same way as *Hd1* from nucleotide sequences of *Hd3a* alleles of rice cultivars, Nipponbare, and Kasalath (GenBank accession numbers: AB052942 and AB052941, respectively) and *Hd6* alleles of the same rice cultivars (GenBank accession numbers: AB036785 and AB036786, respectively). The names and sequences of the primers are shown in Tables 1-3. DNA from KDML 105 and Suphan Buri 1 were amplified using the above primers for *Hd1*, *Hd3a*, or *Hd6*. *Taq* DNA polymerase (QIAGEN®) was used with Q-solution.

Table 1 Names and sequences of primers for amplification of *Hd1* gene.

Primer	Sequences	Ta (°C)
Hd1.03F	5′ GGA CAA AAA CAC CGT GAC TTT CCC CTC CCT 3′	59
Hd1.03R	5′ TAC CAC ACA CTC GCT CCC TTC CTT CTC TGC 3′	
Hd1.04F	5′ AGA TGA ACA GAG GTG GAC TG 3′	52
Hd1.04R	5′ TTA GCT TGC ACC ACA TGG AC 3′	
Hd1.05F	5′ TGG GAT GCA TGA ACA GCA AGA GC 3′	54
Hd1.05R	5′ TAC TGT CAG ATA GAG CTG CAG TGG 3′	
Hd1.06F	5′ AAG GCG TAT GCA GAG GCA CG 3′	55
Hd1.06R	5′ TCC TGT ATA TGC GGT TGG TGC 3′	
Hd1.07F	5′ CAC TAA ACC ATG GGC TAT CG 3′	54
Hd1.07R	5′ TGA TGT GCT AGG AGC TCC CTC C 3′	

Table 2 Names and sequences of primers for amplification of *Hd3a* gene.

Primer	Sequences	Ta (°C)
Hd3.01aF	5′ CCT CGT CCA CAC GTA CAG GAA GAC GAT GC 3′	70
Hd3.01aR	5′ TCA TTG CCG CCG ACC TCG ACC CTA GGC TG 3′	
Hd3.02aF	5′ TCT GCC GGG TGC GTG CAT GAT 3′	65
Hd3.02aR	5′ ACT GTG TGT GGT GCA GGG CTG CTG GAT CG 3′	
Hd3.03aF	5′ AGG TCA CCT ATG GCT CCA AGA CCG TGT C 3′	65
Hd3.03aR	5′ ACA TAT CAT GGC AGC TGG CTG ATC G 3′	
Hd3.04aF	5′ TGC CAC ACC GCG AGC AAT CCA CG 3′	55
Hd3.04aR	5′ TGC TGG AAC AGC ACG AAC ACC AGC CGG 3′	
Hd3.05aF	5′ AGC TAG CTG TTG CAC ATG CTC 3′	54
Hd3.05aR	5′ GCC TGA ATC TTC GAC GAT TGG 3′	

Table 3 Names and sequences of primers for amplification of *Hd6* gene.

Primer	Sequences	Ta (°C)
Hd6.01F	5′ CTC AAT GAT CGG GAC ACA TTC 3′	60
Hd6.01R	5′ GCC ATT CAC AAT CAA ACA AAG G 3′	
Hd6.02F	5′ ACA CTA TTG GCC TGG CCT TAG TGC 3′	65
Hd6.02R	5′ TGG GAC GGC GAC TCT GGA ATC TGC 3′	
Hd6.03F	5′ CAA TGG CCG CAT GAC CGA TG 3′	60
Hd6.03R	5′ GGC ACC ATA TTA CTC AGC ATG 3′	
Hd6.04F	5′ AGT ATA GTG CTT TGG GCC TA 3′	55
Hd6.04R	5′ AAT CTG TCA ACG TGG GGT AC 3′	
Hd6.05F	5′ TGG TGC ATC TTT AAC AGT GAG C 3′	55
Hd6.05R	5′ TCC CTG GAT GAT AGA ACT CAG C 3′	
Hd6.06F	5′ TGC ATC GAG ATG TCA AGC CC 3′	55
Hd6.06R	5′ GAC CAT AGA AGA ATG GCT CC 3′	
Hd6.07R	5′ ACC ATA GCT TCA CGT GCA GTG AGC 3′	58
Hd6.07F	5′ TGC TGG GAT GGT ATG TGT GGC TG 3′	
Hd6.08F	5′ AGC TCT TGT TGG GAG GTA CGT TG 3′	63
Hd6.08R	5′ TGT GCA TGT CAC GGC ATT CA 3′	
Hd6.09F	5′ GAA TAC TTG CAG GCA CAT CC 3′	63
Hd6.09R	5′ AGA ACA CGG TAC CTT CAC CGC 3′	

Sequencing of *Hd1, Hd3a,* and *Hd6* alleles

DNA from KDML 105 and Suphan Buri 1 were amplified and PCR products were detected on 1% agarose gel. DNA fragments were extracted from the gel by QIAquick® Gel Extraction Kit (QIAGEN®) and directly sequenced. The whole sequences of each gene were assembled.

Methylation analysis

DNA samples of KDML 105 from the short-day treatment at days 0, 5, 6, 7, 8, 9, 10, 12, and 15, and from the long-day treatment at days 5, 7, 8, 10, and 15 were used. All DNA samples were divided into 2 groups. Ten micrograms of genomic DNAs were separately digested with *Hpa*II and *Msp*I. Southern hybridization was performed by a method modified from Sambrook and Russell (2001). PCR products amplified using Hd1.03F/Hd1.03R, Hd3.01aF/Hd3.01aR and Hd6.01F/Hd6.01R primers were used as probes for *Hd1*, *Hd3a* and *Hd6* genes, respectively.

RESULTS AND DISCUSSION

PCR amplification and sequencing of *Hd1, Hd3a* and *Hd6* genes of KDML105 and Suphan Buri 1

Primers were designed to amplify the overlapping fragments of *Hd1, Hd3a,* and *Hd6* alleles from rice KDML 105 and Suphan Buri 1 to make a contig sequence covering the whole genes. The sizes and positions of the amplified fragments of KDML 105 are shown in Figure 1. Nucleotide sequences were submitted to the GenBank as the accession numbers DQ 157459 and DQ 157460 for *Hd1* alleles of KDML 105 and Suphan Buri 1, DQ 157461 and DQ 157462 for *Hd3a* alleles of KDML 105 and Suphan Buri 1 and DQ 157463 and DQ 157464 for *Hd6* alleles of KDML 105 and Suphan Buri 1, respectively.

Determination of DNA methylation by restriction enzymes and Southern analysis

DNA methylation level around the 5′ control region of *Hd1*, *Hd3a*, and *Hd6* genes was investigated by Southern hybridization using PCR products amplified from *Hd1*, *Hd3a* and *Hd6* genes as probes. DNA samples from the short-day (SD) and long-day (LD) treatments were digested with either *Hpa*II or *Msp*I restriction enzyme. The Southern hybridization results are shown in Figures 2 - 4.

For *Hd1* gene (Hd1.03F/Hd1.03R probe), DNA bands of 442 and 544 bp were found in both DNA digested with *Hpa*II and *Msp*I (Figures 2A and 2B). Actually, DNA bands of 15 and 117 bp should be found but the small sizes of these DNA bands were difficult to detect by Southern hybridization because of the low efficiency of this technique. This result indicated that all restriction sites inside the probe region were not methylated. Such DNA bands were found in both SD and LD treatments indicating that DNA methylation level did not change throughout the treatments and was not affected by daylength.

For *Hd3a* gene (Hd3.01aF/Hd3.01aR probe), DNA bands of 337, 361, and 1898 bp were found in both DNA digested with *Hpa*II and *Msp*I (Figures 3A and 3B). Again, DNA band of 70 bp was not detected due to its small size. This result also indicated the unmethylated restriction sites in probe. Such DNA bands were found in all days and in both SD and LD treatments indicating that DNA methylation level did not change throughout the treatments and was not affected by daylength.

For *Hd6* gene (Hd6.01F/Hd6.01R probe), DNA bands of 250 bp and a large fragment near the position of genomic DNA were found in DNA digested with *Hpa*II (Figure 4A), and DNA bands of 250 and more than 8000 bp were found in DNA digested with *Msp*I (Figure 4B). DNA bands of 10 and 142 bp were not found as expected because of their small sizes. *Msp*I cut the CCGG sequence for all types of internal cytosine, while *Hpa*II only cut the unmethylated internal cytosine. If the outer C was methylated, neither of these two enzymes would cut the CCGG sequence (Oakeley, 1999). This result indicated that all restriction sites

inside the probe region were not methylated and methylation at the inner C occurred outside *Hd6* gene resulted in the larger upper fragment in *Hpa*II digested DNA. However, DNA methylation level did not change throughout the treatments in both SD and LD conditions. These results indicated that DNA around the 5′ regions of *Hd1*, *Hd3a*, and *Hd6* genes (at the CCGG motifs) were maintained as unmethylated sequences during transition from vegetative to reproductive phase and did not depend on photoperiod *per se*.

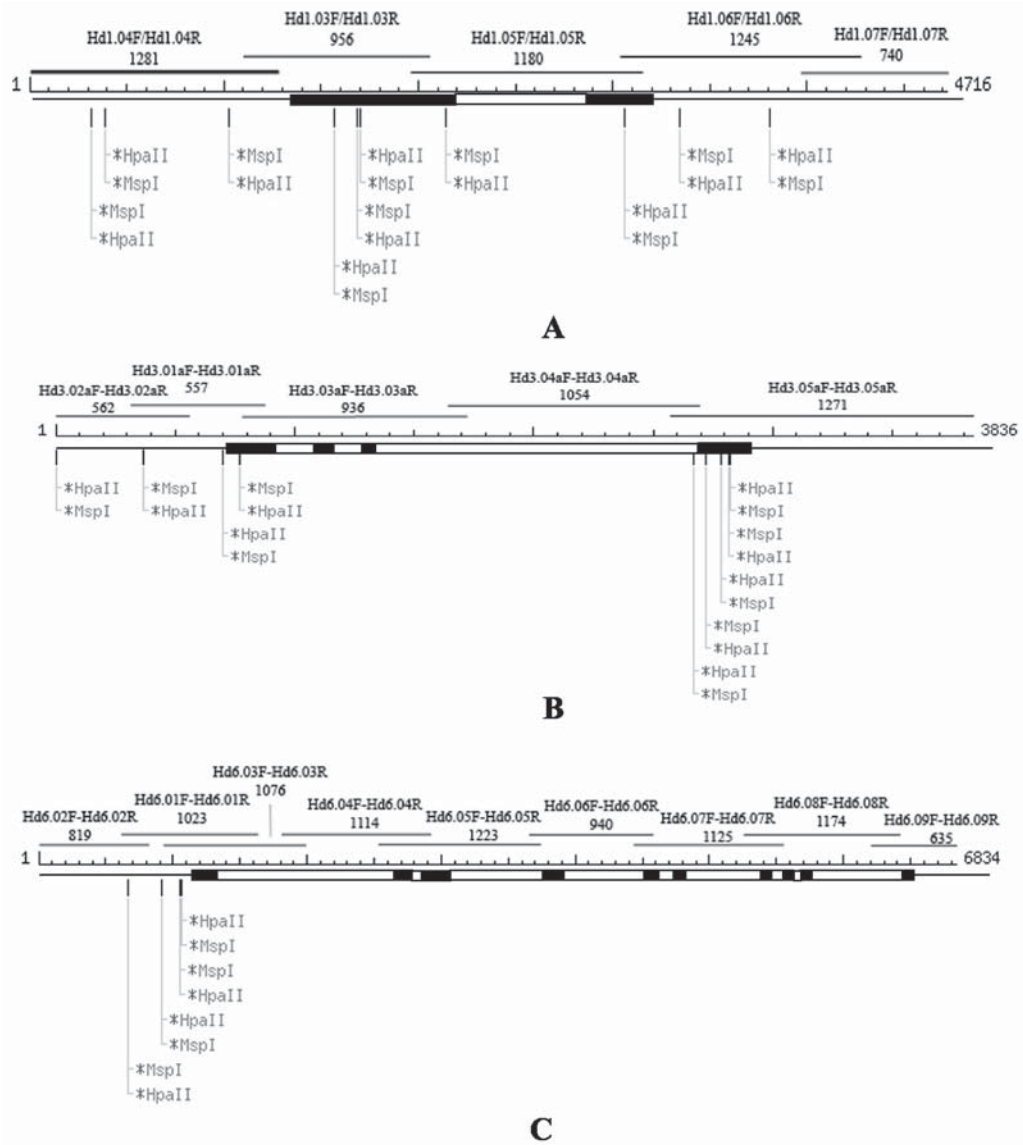

Figure 1 *Msp*I and *Hpa*II restriction maps of *Hd1* (A), *Hd3a* (B) and *Hd6* (C) of KDML 105 cultivar. Black and white boxes represented the exon and intron, respectively. The lines above the diagram showed the location and the length of the expected PCR fragments after amplification with the indicated primer pairs.

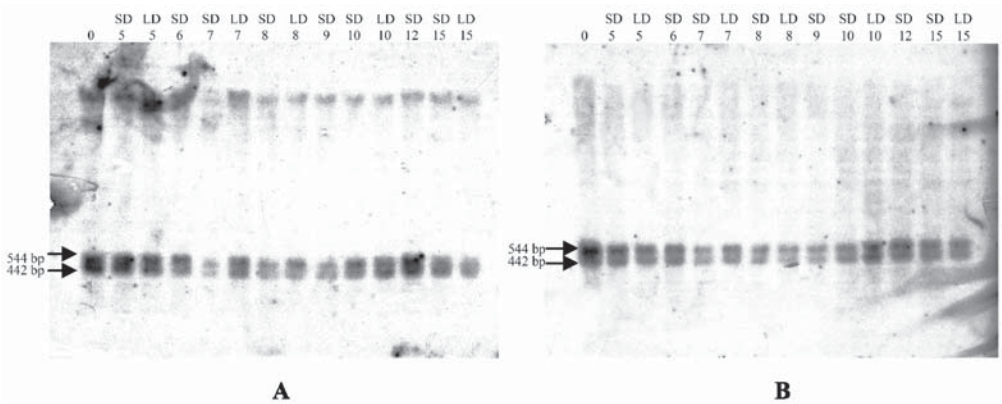

Figure 2 Southern hybridization of the DNA samples from rice KDML 105 treated under the short-day (SD) or long-day (LD) conditions for the specified days using a PCR product of *Hd1* gene (Hd1.03F/Hd1.03R fragment) as a probe. (A) digested with *Hpa*II; (B) digested with *Msp*I. Numbers indicated days after treatment.

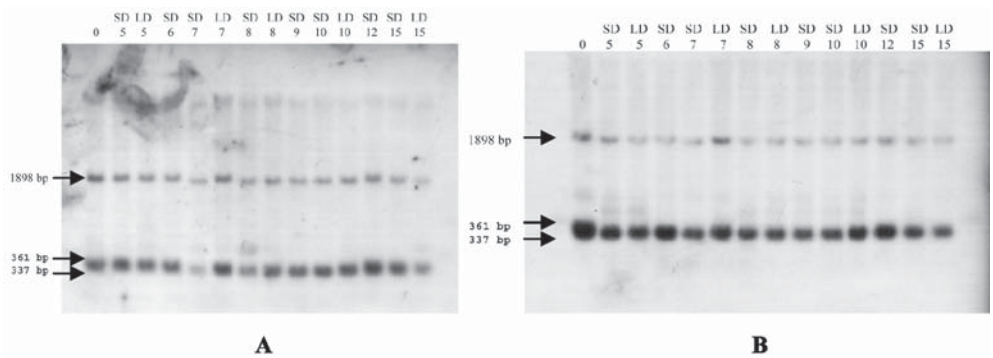

Figure 3 Southern hybridization of the DNA samples from rice KDML 105 treated under the short-day (SD) or long-day (LD) conditions for the specified days using a PCR product of *Hd3a* gene (Hd3.01aF/Hd3.01aR fragment) as a probe. (A) digested with *Hpa*II; (B) digested with *Msp*I. Numbers indicated days after treatment.

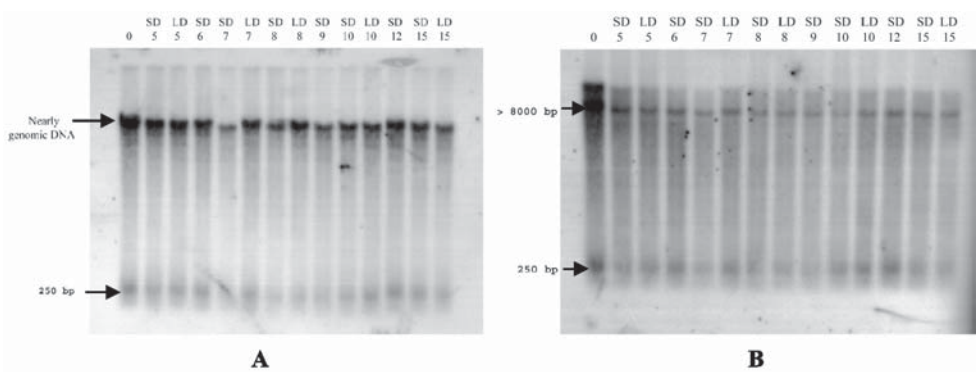

Figure 4 Southern hybridization of the DNA samples from rice KDML 105 treated under the short-day (SD) or long-day (LD) conditions for the specified days using a PCR product of *Hd6* gene (Hd6.01F/Hd6.01R fragment) as a probe. (A) digested with *Hpa*II; (B) digested with *Msp*I. Numbers indicated days after treatment.

Single nucleotide polymorphism (SNP) and single amino acid polymorphism (SAP) of *Hd1*, *Hd3a*, and *Hd6* genes and possible functions of their products

To determine the sequence variation in *Hd1*, *Hd3a*, and *Hd6* genes responsible for photoperiod sensitivity (PS), the whole gene sequences from two rice cultivars (KDML 105 and Suphan Buri 1) were compared. The genomic structure of *Hd1* was composed of two exons and one intron (Figure 1A). The average size of coding regions was 1.2 kb. From conserved domain searching with rpsblast, Hd1 protein was similar to transcription factor having two B-box (zinc-finger motifs) in the exon1. CCT domain, the nuclear localization signal, was found in the exon 2. Types of sequence variation found in those alleles were compared to those of Nipponbare and Kasalath. The 36-bp insertion (more likely, 36-bp deletion in Nipponbare) was found in exon1 of KDML 105 and Suphan Buri 1. This deletion caused 12 amino acids lost in Nipponbare but did not affect the B-boxes and CCT domains. The *Hd1* from Nipponbare was shown to be transcriptionally and functionally active while *Hd1* from Kasalath was shown to be non-functional because of 2-bp deletion in the 5′ region of exon 2. This deletion caused frame-shift mutation, resulting in drastic change in amino acid composition of the carboxy terminal region of the protein, and in premature stop codon (Yano *et al.*, 2000). The 2-bp insertion (more likely, 2-bp deletion in Kasalath) was found in exon 2 of KDML 105 and Suphan Buri 1. Along those sequences, there were three types of sequence variation: substitution, insertion, and deletion. Base substitution was the most frequent. Insertion and deletion were also found in some sequences. Because *Hd1* genes of KDML 105 and Suphan Buri 1 encoded proteins that had complete B-box 1, B-box 2, and CCT domain, it could be concluded that *Hd1* genes of both cultivars encoded for normal Hd1 proteins. *Hd1* was a bifunctional gene which promoted flowering in the SD and inhibited it in the LD condition (Yano *et al.*, 2000). Nipponbare, a photoperiod-sensitive rice cultivar, carried a normal *Hd1* gene so flowering was inhibited under LD condition while Kasalath, a photoperiod-insensitive rice, carried a mutated allele. *Hd1* allele of KDML 105 was found to be function as in Nipponbare so this might be the cause of photoperiod sensitivity in KDML 105 as well. However, the functional *Hd1* allele in Suphan Buri 1 must be further clarified.

Kojima *et al.* (2002) reported that *Hd3a* gene promoted flowering of rice under short-day conditions. The genomic structure of *Hd3a* was composed of four exons and three introns (Figure 1B). From conserved domain searching with rpsblast, Hd3a sequence was similar to phosphatidylethanolamine-binding protein. The average size of coding regions was 550 bp. Types of sequence variation found in those alleles were compared to those of Nipponbare and Kasalath. Along those sequences, there were three types of sequence variation: substitution, insertion, and deletion. Base substitution was the most frequent. Sequences of *Hd3a* alleles of the two cultivars revealed that KDML 105 and Suphan Buri 1 differed from Nipponbare and Kasalath mostly in the intron regions. Only one base substitution occurred in exon 4 of Nipponbare that caused the last amino acid to change from asparagine to proline. Both KDML 105 and Suphan Buri 1 alleles of *Hd3a* were functional because of the 100% amino acid sequence identity to the functional one of Kasalath. The amino acid substitution occurring at the carboxyl end of the predicted protein in Nipponbare reduced the functionality in this cultivar (Kojima *et al.*, 2002).

Takahashi *et al.* (2001) reported that *Hd6* gene inhibited flowering of rice under long-day conditions. The genomic structure of *Hd6* was composed of ten exons and nine introns (Figure 1C). From conserved domain searching with rpsblast, Hd6 was similar to protein kinase (serine/threonine protein kinase and threonine kinase), however, Takahashi *et al.* (2001) reported that *Hd6*

encoded an α-subunit of CK2 (casein kinase II). The average size of coding regions was 1 kb. Types of sequence variation found in those alleles were compared to those of Nipponbare and Kasalath. Base substitution in exon 3 caused premature stop codon which changed lysine to stop codon in Nipponbare, resulting in a drastic change of amino acid composition. Amino acid sequences deduced from the nucleotide sequences of KDML 105 and Suphan Buri 1 alleles were identical to Hd6 protein of Kasalath. Therefore, *Hd6* gene of KDML 105 and Suphan Buri 1 must encode for normal Hd6 protein.

Since Nipponbare and KDML 105 are photoperiod-sensitive (Nakagawa *et al.*, 2005; Rice Research Institute, 2003; 2005) while Kasalath and Suphan Buri 1 are photoperiod-insensitive cultivars (Anonymous, 2005; Rice Research Institute, 2003; 2005). Their photoperiodic genes should correspond to their transcriptional proteins. However, the results of encoded proteins revealed that both KDML 105 and Suphan Buri 1 encoded for normal protein. Hd1 proteins of KDML 105 and Suphan Buri 1 were similar to Nipponbare. Hd3a and Hd6 proteins of KDML 105 and Suphan Buri 1 were identical to Kasalath. Thus it could be concluded that *Hd1* gene might be involved in photoperiod sensitivity in rice or photoperiod sensitivity in KDML 105 and Nipponbare might be controlled by a different mechanism.

Results of sequencing of the *Hd1*, *Hd3a*, and *Hd6* genes of KDML 105 and Suphan Buri 1showed some differences in the 5′ control regions, thus such differences might cause the photoperiodic sensitivity difference between these two cultivars.

CONCLUSION

In this experiment, the level of DNA methylation was determined using Southern hybridization method. The results showed that there were no differences of KDML 105 DNA in either *Hpa*II or *Msp*I-digested groups in terms of the pattern of DNA bands. Moreover, there were no differences between long-day treatment and short-day treatment. The data suggested that DNA methylation levels at the CCGG sequences of the target sites of *Hd1*, *Hd3a*, and *Hd6* genes were not altered by photoperiod. Further analysis of Nucleotide and deduced amino acid sequences obtained from this experiment indicated that *Hd1*, *Hd3a*, and *Hd6* genes of KDML 105 and Suphan Buri 1 encoded normal Hd1, Hd3a, and Hd6 proteins, respectively.

Amino acid sequences of the proteins subjected to functional domains prediction using rpsblast based on CDD database showed that Hd1 protein was similar to transcription factor protein with zinc finger domain (B-box 1 and B-box 2) and nuclear localization signal (CCT domain). Hd3a protein was similar to phosphatidylethanolamine-binding protein while Hd6 protein was similar to serine/threonine protein kinase. The results also indicated that DNA methylation and single amino acid polymorphisms (SAPs) might not be the mechanisms controlling gene function in *Hd1*, *Hd3a*, and *Hd6* genes of rice KDML 105. The differences in the 5′ control region of those genes, however, might cause the photoperiodic sensitivity difference between KDML 105 and Suphan Buri 1.

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