

Detection of Photoperiod Responsive Gene in KDML 105 Rice (*Oryza sativa* L.) using cDNA-SRAP Technique

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ABSTRACT

Sequence-related amplified polymorphism (SRAP) was used for detecting photoperiod responsive gene of KDML 105 rice (*Oryza sativa* L.). Ten out of 16 primer combinations gave 42 different bands from the cDNA of KDML 105 rice after exposure to short day (SD) photoperiod. The DNA bands larger than 200 bp were collectively selected for cloning and sequencing. Fourteen from 16 cDNA-SRAP markers showed 87–100% similarity to sequences in the GenBank database, but most nucleotide sequences were unknown genes. Gene expression was further elucidated by RT-PCR. The results, however, were rather contradicted to the obtained cDNA-SRAP pattern. Considering DNA band intensity, there were two candidate genes having higher level of expression in SD treatment. Relative qRT-PCR was used for checking the level of gene expression. The result showed that the expression level of candidate gene from KM2-3 marker increased up to 4.63 folds at 6 days after treatment with SD photoperiod. This indicated its high possibility of being a photoperiod responsive gene which most likely corresponds to another class of gene regulating the flowering time in rice. Cloning of this gene was performed by RT-PCR with a primer pair covering the whole open reading frame (ORF). The 546 bp DNA fragment was cloned, sequenced and analyzed. This fragment had one ORF and deduced a polypeptide of 134 amino acid residues. The nucleotide sequence of this gene was submitted to the GenBank database as gil83972339.

Key words: rice, KDML 105, photoperiod, cDNA-SRAP

INTRODUCTION

Thai jasmine rice, Khao Dok Mali 105 (KDML 105) is the most famous rice variety in the world market having both pleasant and exotic flavour, i.e., soft and tender texture with aromatic fragrance. However, the major disadvantage of KDML 105 rice is the low productivity and photoperiod sensitivity. Sirigayon (2000) reported that the minimum induction cycle of 9 days (exposure to SD) is needed for KDML105 to

change from vegetative to reproductive phase. Therefore, the growing period is limited to only one season a year.

Molecular genetic studies revealed several factors and pathways, i.e., photoperiod, autonomous, vernalization, light quality and gibberellins control the floral transition (Ausín *et al.*, 2005). In *Arabidopsis*, the photoperiod pathway plays a major role in floral promotion to the induction of long day (LD) photoperiod. This pathway includes genes encoding photoreceptors,

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components of the circadian clock and clock associated genes (Hayama and Coupland, 2003; Yanovsky and Kay, 2003). In rice (SD plant), 23 flowering time or heading-time loci were found in the genome, but their physiological and biochemical functions in genetic pathways leading to flowering are still unknown (Kojima *et al.*, 2002; Nishida *et al.*, 2002).

Several PCR marker systems are widely used for isolation of preferentially expressed mRNAs such as differential display (Liang and Pardee, 1992) and cDNA AFLP (Bachem *et al.*, 1996). There are advantages and disadvantages for each system. For example, differential display is a simple method but it has poor consistency (Ledakis *et al.*, 1998) and is difficult to amplify the coding region. cDNA AFLP is more consistent but the main disadvantage of this method is its complexity, involving several steps, i.e., reverse transcription, digestion, ligation and amplification, making it difficult to optimize the conditions. SRAP, on the other hand, is a new PCR marker system which combines simplicity and reliability. This technique can be adapted to use in different crops to work on genome mapping, gene tagging, genomic and cDNA fingerprinting (Li and Quiros, 2001). cDNA-SRAP technique could, therefore, be helpful in elucidating the differential gene expression in KDML 105 rice during transition from vegetative to reproductive phase as reported in this article.

MATERIALS AND METHODS

Plant materials and photoperiod treatment

KDML 105 rice plants were grown under long day (LD) photoperiod (13 h light/11 h dark) for 50 days, and then separated into two groups. The first group was exposed to short day (SD) photoperiod (11 h light/13 h dark) for 15 days while the other was maintained in the long day condition (Peyachoknagul *et al.*, 2005; Roongsattham *et al.*, 2006).

RNA extraction

Total RNA was extracted from the young leaves of KDML 105 rice on days 0, 5, 7, 8, 10 and 15 for the control plants (LD) and days 5, 6, 7, 8, 9, 10, 12 and 15 for the SD exposed plants using TRIzol[®] Reagent (Invitrogen[™] Life Technologies, USA).

cDNA synthesis

mRNA was isolated from 20 µg of total RNA of KDML 105 rice using Dynabeads[®] mRNA DIRECT[™] Micro Kit (DYNAL[®], Norway). M-MLV reverse transcriptase (Test User Friendly[™] USB, USA) was used to synthesize single-strand cDNA following the manufacturer's protocol.

Examination of gene expression using cDNA-SRAP technique

SRAP protocol was applied to fingerprint 14 cDNA samples using primer combinations following the procedure of Li and Quiros (2001). The PCR products were separated on a 6% denaturing polyacrylamide gel and detected by silver staining as described by Echt *et al.* (1996).

DNA fingerprinting patterns of SD and LD exposed rice samples were compared and the DNA fragments unique for SD condition were cloned and sequenced for further analysis.

Cloning and sequencing of cDNA-SRAP fragments

Only the DNA bands unique for SD exposed plants were removed from polyacrylamide gel following the procedure described by Thanananta *et al.* (2006) and cloned into pGEM-T plasmid (Promega, USA). They were then sequenced and compared to the database in the GenBank (<http://www.ncbi.nlm.nih.gov>).

Determination of gene expression by RT-PCR

PCR primers were designed to amplify the candidate genes from cDNA-Dynabeads

complex of KDML105 rice. PCR products were separated on a 1.5% agarose gel. For the control of mRNA quantity and quality, the primer pair for amplification of a part of putative actin gene (350 bp) in rice was added in the same reaction mixture as a duplex PCR (Thanananta *et al.*, 2006).

Analysis of gene expression by real-time PCR

Real-time RT-PCR was performed using Mastercycler® ep *realplex* (Eppendorf, Germany). Total RNA was treated with RQ1 DNase (Promega, USA) to remove genomic DNA contamination following the manufacturer's protocol and each treated RNA sample (500 ng) was used as a template for relative qRT-PCR using SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR (Invitrogen™ Life Technologies, USA) with specific primers. The amplification of

actin gene was performed in the same condition to normalize the concentration of each RNA sample. The data were analyzed by realplex software version 1.0 using $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Differences in cDNA-SRAP markers of SD and LD plants

cDNA-SRAP fingerprints of LD and SD exposed rice were developed using 16 primer combinations. Out of these, ten primer combinations gave 42 different bands from the total of 158 bands (Table 1 and Figure 1). Since the sizes of some bands were too small to be informative, only 16 bands having the size larger than 200 bp were cloned and sequenced. These 16

Table 1 Primer combinations, sequences and number of bands of cDNA-SRAP markers.

Primer combination	Primer sequences	Total no. of bands	No. of different bands
1. A1B7	5'-TGAGTCCAAACCGGAAT-3' 5'-GACTGCGTACGAATTCAT-3'	16	8
2. A3B1	5'-TGAGTCCAAACCGGAAG-3' 5'-GACTGCGTACGAATTAAT-3'	10	3
3. A3B5	5'-TGAGTCCAAACCGGAAG-3' 5'-GACTGCGTACGAATTAGT-3'	18	5
4. A5B2	5'-TGAGTCCAAACCGGAGC-3' 5'-GACTGCGTACGAATTTGC-3'	16	3
5. A7B2	5'-TGAGTCCAAACCGGATC-3' 5'-GACTGCGTACGAATTTGC-3'	25	4
6. A7B4	5'-TGAGTCCAAACCGGATC-3' 5'-GACTGCGTACGAATTCAC-3'	25	4
7. A9B2	5'-TGAGTCCAAACCGGATG-3' 5'-GACTGCGTACGAATTTGC-3'	14	3
8. A11B2	5'-TGAGTCCAAACCGGATT-3' 5'-GACTGCGTACGAATTTGC-3'	5	3
9. A11B5	5'-TGAGTCCAAACCGGATT-3' 5'-GACTGCGTACGAATTAGT-3'	18	5
10. A11B12	5'-TGAGTCCAAACCGGATT-3' 5'-GACTGCGTACGAATTGTT-3'	11	4
Total		158	42

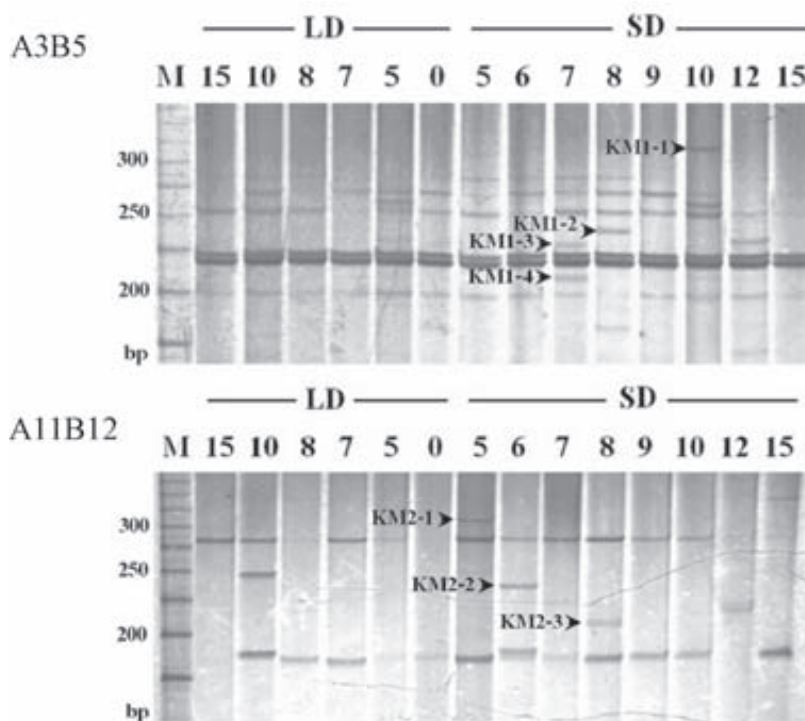


Figure 1 cDNA-SRAP patterns of KDML105 rice showing the KM1-1, KM1-2, KM1-3, KM1-4 markers from primers A3B5 and KM2-1, KM2-2, KM2-3 markers from primers A11B12 (M= standard DNA of 25 bp DNA ladder).

bands were cDNA-SRAP markers responding to SD photoperiod: primer combinations A3B5 showing 4 different bands (KM1-1, KM1-2, KM1-3 and KM1-4), A11B12 showing 3 different bands (KM2-1, KM2-2 and KM2-3), A5B2 showing 3 different bands (KM3-1, KM3-2 and KM3-3), A7B2 showing one different band (KM4-1), A7B4 showing 3 different bands (KM5-1, KM5-2 and KM5-3) and primer combination A1B7 showing 2 different bands (KM6-1 and KM6-2) (Figure 1 and Table 2). It is interesting to find that these cDNA-SRAP markers gave the distinct bands on days 5 - 12 after SD treatment (Table 2) which is in the same range of time needed for flowering induction of KDML 105 rice as reported by Sirigayon (2000). Comparing these 16 cDNA-SRAP markers, 14 showed 87 - 100 % similarity to sequences in the GenBank database, but most

nucleotide sequences were unknown genes (Table 3). For the two known genes, KM1-4 showed 98 % similarity to *GF14-c* gene, which encodes for phosphorylation and signal transduction protein (Ikeda *et al.*, 2000), whereas KM2-2 showed 98 % similarity to *OsBP-73* gene, which encodes a transcription regulator SAP-like protein (Chen *et al.*, 2003).

Apparently, most nucleotide sequences shared homology to genes (cDNA or mRNA) in *japonica* rice in the database, whereas KM2-3 and KM 5-1 showed high similarity to the sequences of genomic DNA in *japonica* rice chromosomes 5 and 2 which are not yet clearly distinguished as specific genes (Table 3). These indicated that gene database of rice is not complete and there should be some different genes in KDML 105 which is an *indica* rice.

Table 2 Sixteen cDNA-SRAP markers revealed by six primer combinations.

Primer combination	cDNA-SRAP marker	Length (bp)	Present after SD treatment (days)
A3B5	KM1-1	323	10
	KM1-2	240	8
	KM1-3	231	7
	KM1-4	221	7
A11B12	KM2-1	310	5
	KM2-2	235	6
	KM2-3	207	7
A5B2	KM3-1	258	6
	KM3-2	229	8 and 12
	KM3-3	215	8 and 9
A7B2	KM4-1	270	9
A7B4	KM5-1	334	7
	KM5-2	266	10
A1B7	KM5-3	205	6
	KM6-1	356	9
	KM6-2	326	5

Analysis of candidate gene expression by RT-PCR

RT-PCR primers for the amplification of candidate genes from cDNA-SRAP markers were designed using nucleotide sequences of each marker and similar nucleotide sequences from the database. The results, however, were rather contradicted to the cDNA-SRAP pattern. RT-PCR products of candidate genes from KM1-1, KM2-3, KM3-1 and KM3-2 markers appeared in both

LD and SD treatments (Figure 2). It might be the limitation of the SRAP technique that was not quite efficient in identifying genes with low expression. Using the specific primers, RT-PCR could amplify both low and high expression genes in different time points (LD and SD). Considering DNA band intensity, there were two candidate genes (KM1-1 and KM2-3) having higher level of expression in the SD treatment (Figure 2). The relative qRT-PCR was used for checking the level of gene

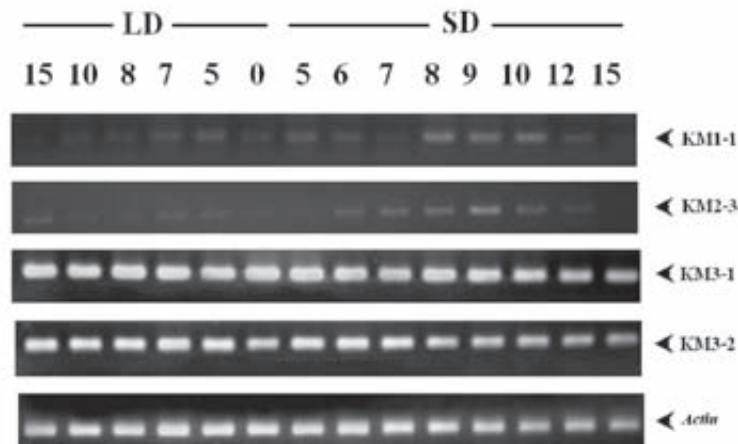


Figure 2 RT-PCR products showing the expression of the candidate genes from KM1-1, KM2-3, KM3-1, KM3-2 markers and *Actin* gene in LD and SD treatments.

Table 3 The sequences similarity between cDNA-SRAP markers and those of GenBank database.

cDNA-SRAP marker	Sequences in the GenBank database similar to cDNA-SRAP marker	% similarity
KM1-1	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J033028F04, gil32983321	98
KM1-2	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J033061I03, gil37991276	99
KM1-3	<i>O. sativa (japonica</i> cultivar-group) predicted mRNA, gil50922284	49.5
KM1-4	<i>O. sativa</i> GF14-c protein mRNA, gil1519250	98
KM2-1	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:006-211-D09, gil32971263	98
KM2-2	<i>O. sativa</i> mRNA for SAP-like protein (<i>BP-73</i> gene), gil21727821	98
KM2-3	<i>O. sativa (japonica</i> cultivar-group) genomic DNA, chromosome 5, clone:OJ1362_G11, gil51339042	98
KM3-1	<i>O. sativa (japonica</i> cultivar-group) unknown protein (OSJNBa0068N06.4), mRNA gil37534939	98
KM3-2	<i>O. sativa (japonica</i> cultivar-group) mRNA, gil50906424	45
KM3-3	<i>O. sativa (japonica</i> cultivar-group) mRNA, gil55775451	100
KM4-1	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J033117I03, gil32988261	98
KM5-1	<i>O. sativa (japonica</i> cultivar-group) genomic DNA, chromosome 2, gil58530788	87
KM5-2	<i>O. sativa (japonica</i> cultivar-group), predicted mRNA, gil34902003	98
KM5-3	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J013124A16, gil37990137	98
KM6-1	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J033050N17, gil32986789	99
KM6-2	<i>O. sativa (japonica</i> cultivar-group) cDNA clone:J033050N17, gil32986789	98

expression in triplicate. Interestingly, the results showed that the expression level of candidate gene from KM2-3 marker increased up to 4.63 folds at 6 days after treatment with SD photoperiod (Figure 3) which strongly suggested it to be a photoperiod responsive gene and most likely corresponds to another class of gene regulating the flowering time in rice.

Cloning of candidate gene from KM2-3 marker

KM2-3 was found to be a new cDNA sequence (gene) corresponding to SD photoperiod. Not only this sequence showed high similarity to the sequence of gil51339042, the ORF finder program in the GenBank also indicated that gil51339042 had an open reading frame overlapping the 5' region of KM2-3. Primers

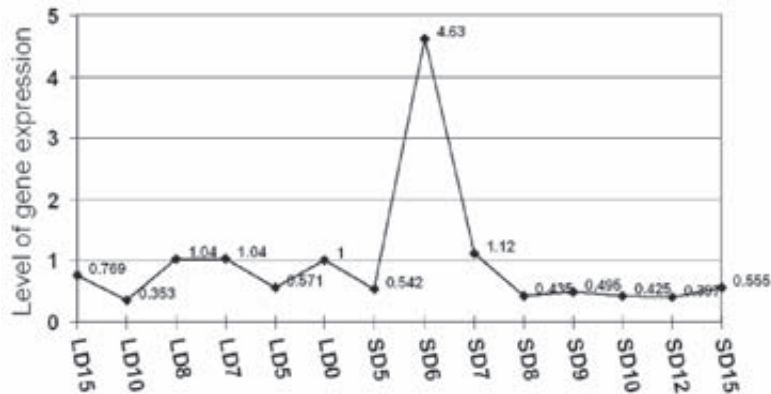


Figure 3 Relative qRT-PCR products showing the expression of candidate gene from KM2-3 marker in LD and SD treatments. The expression level increased up to 4.63 folds at 6 days after treatment with SD photoperiod.

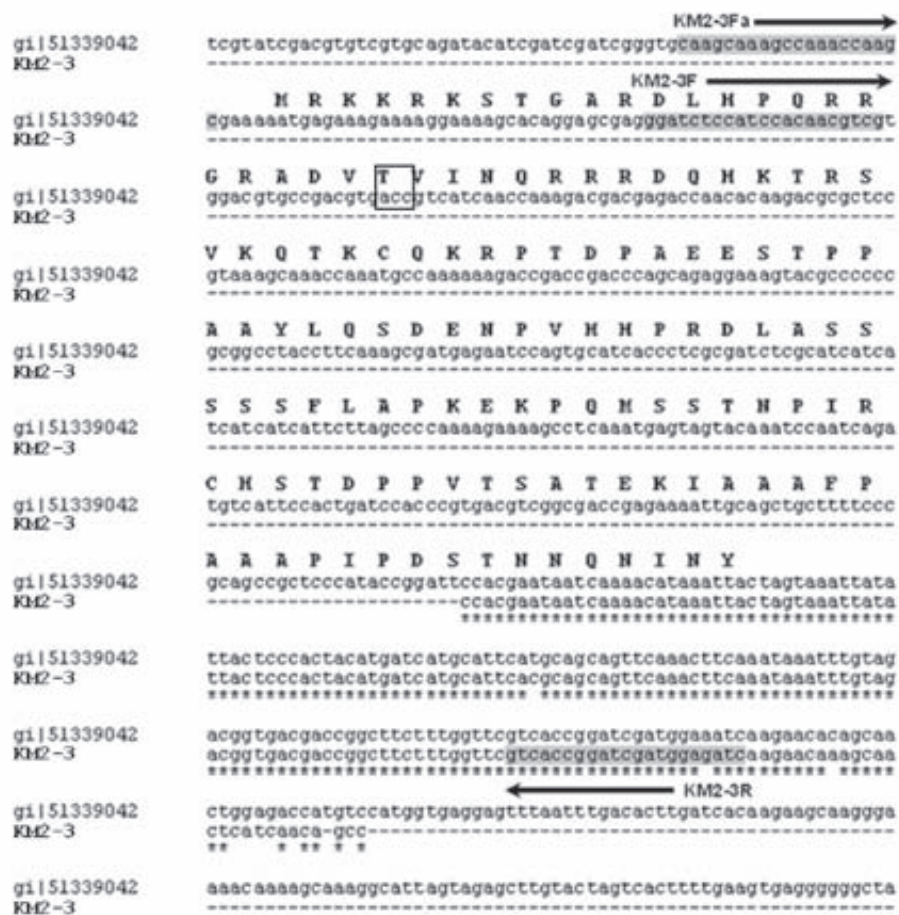


Figure 4 Alignment of gil51339042 and KM2-3. Nucleotide sequence of gil51339042 had an open reading frame overlapping the 5' region of KM2-3 (→ primer direction from 5' to 3'). □ showed the site of one base substitution in KM2-3.

KM2-3F and KM2-3R were, therefore, designed for checking the gene expression and the obtained RT-PCR product showed the expected band (490 bp) of the candidate gene in KDML 105 rice. Primer KM2-3Fa was then designed for amplification of the whole ORF (546 bp) (Figures 4 and 5). After the 546 bp fragment was cloned, sequenced and analyzed, the results showed that this fragment had one ORF and deduced a polypeptide of 134 amino acid residues. The nucleotide sequence of the whole ORF from KM2-3 also showed 99% similarity to the nucleotide sequence of gil51339042. However, there was one base substitution in the ORF (codon 24: acc (T) to gcc (A)) (Figure 6). This clearly indicated that the candidate gene from KM2-3 of KDML 105 rice is located on the same locus as gil51339042. The nucleotide sequence of this gene was, therefore, submitted as a new gene to the GenBank database as gil83972339.

ACKNOWLEDGEMENTS

This research was supported by the grant from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. It was also

partially supported by the Development and Promotion of Science and Technology Talents Project (DPST), Thailand.

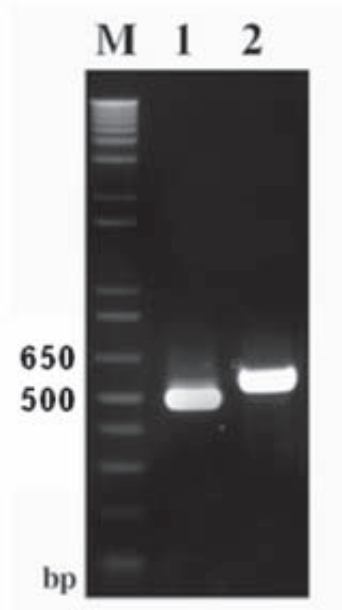


Figure 5 RT-PCR products showing a fragment of candidate gene from KM2-3 (490 bp) and a fragment of the whole ORF (546 bp) (M= standard DNA of 1 Kb plus DNA Ladder).



Figure 6 Nucleotide and deduced amino acid sequence of candidate gene from KM2-3 in KDML 105 rice. □ showed one base substitution in the ORF (codon 24: acc (T) to gcc (A)).

LITERATURE CITED

- Ausín, I., C. Alonso-Blanco and J. Martínez-Zapater. 2005. Environmental regulation of flowering. **Int. J. Dev. Biol.** 49: 689-705.
- Bachem, C.W.B., R.S. Van der Hoeven, S.M. de Bruijn, D. Vreugdenhil, M. Zabeau and R.G.F. Visser. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. **Plant J.** 9:745-753.
- Chen, J., W.H. Tang, M.M. Hong and Z.Y. Wang. 2003. *OsBP-73*, a rice gene, encodes a novel DNA-binding protein with a SAP-like domain and its genetic interference by double-stranded RNA inhibits rice growth. **Plant Mol. Biol.** 52: 579-590.
- Echt, C.S., P. May-Marquardt, M. Hseih and R. Zahorchak. 1996. Characterization of microsatellite markers in eastern white pine. **Genome** 39:1102-1108.
- Hayama, R. and G. Coupland. 2003. Shedding light on the circadian clock and the photoperiodic control of flowering. **Curr. Opin. Plant Biol.** 6:13-19.
- Ikeda, Y., N. Koizumi, T. Kusano and H. Sano. 2000. Specific binding of a 14-3-3 protein to autophosphorylated WPK4, an SNF1-related wheat protein kinase, and to WPK4-phosphorylated nitrate reductase. **J. Biol. Chem.** 275: 31695 - 31700.
- Kojima, S., Y. Takahashi, Y. Kobayashi, L. Monna, T. Sasaki, T. Araki and M. Yano. 2002. *Hd3a*, a rice ortholog of the *Arabidopsis FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. **Plant Cell Physiol.** 43:1096-1105.
- Li, G. and C.F. Quiros. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. **Theor. Appl. Genet.** 103: 455 - 461.
- Ledakis, P., H. Tanimura and T. Fojo. 1998. Limitations of differential display. **Biochem. Biophys. Res. Commun.** 251: 653-656.
- Liang, P. and A.B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. **Science** 257: 967-971.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. **Methods** 25: 402-408.
- Nishida, H., H. Inoue, Y. Okumoto and T. Tanisaka. 2002. A novel gene *efl-h* conferring an extremely long basic vegetative growth period in rice. **Crop Sci.** 42: 348-354.
- Peyachoknagul, S., W. Pipatpongpinoy, P. Pongtongkam, A. Thongpan, L. Kaveeta and P. Harinasut. 2005. DNA methylation differences in the genes responded to photoperiod in rice cultivar KDML 105. **Kasetsart J. (Nat. Sci.)** 39: 689- 694.
- Roongsattham, P., P. Pongtongkam, A. Thongpan, L. Kaveeta, P. Harinasut and S. Peyachoknagul. 2006. *Hd1*, *Hd3a*, and *Hd6* genes: possible DNA methylation roles in photoperiod sensitive gene regulation of rice KDML 105 (*Oryza sativa* L.). **Kasetsart J. (Nat. Sci.)** 40: 462- 471.
- Sirigayon, U. 2000. **Detection of Photoperiod Responsive Genes in Rice Using Differential Display Method**. M. S. Thesis. Kasetsart University, Bangkok.
- Thanananta, T., P. Pongtongkam, A. Thongpan, L. Kaveeta, S. Peyachoknagul. 2006. Effect of short day photoperiod on DNA methylation and expression of a gene in rice KDML105. **Afr. J. Biotechnol.** 5: 1375-1382.
- Yanovsky, M.J. and S.A. Kay. 2003. Living by the calendar: how plants know when to flower. **Nat. Rev. Mol. Cell Biol.** 4: 265-275.