MONITORING OF MENDELIAN GENETICS IN RICE BY DNA MARKERS

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BY DNA MARKERS

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บทคัดย่อ

การวิเคราะห์สารพันธุกรรมดีเอ็นเอ ซึ่งรวมถึง การวิเคราะห์ RFLP และการวิเคราะห์ RAPD เป็นอีกวิธีหนึ่งที่ได้เริ่มมีการนำมาใช้เพื่อการปรับปรุงพันธุ์ข้าว ในการศึกษาครั้งนี้ได้ทดลองใช้การวิเคราะห์ดีเอ็นเอโดยวิธี RFLP เพื่อติดตามการกระจายตัวของลักษณะความหอม และลักษณะความไวแสงในสารพันธุกรรมของข้าวไทย โดยเริ่มจาก การนำสารพันธุกรรมดีเอ็นเอของข้าวพันธุ์ขาวดอกมะลิ๑๐๕ และพันธุ์IR36 มาย่อยด้วยเอ็นไซม์ตัดจำเพาะ ซึ่งในการทดลองนี้ใช้เอ็นไซม์๕ ชนิดคือ BamHI EcoRI EcoRV HindIII และ XbaI แยกชิ้นส่วนดีเอ็นเอด้วยกระแสไฟฟ้าในตัวกลางที่เป็นวุ้น ตรึงดีเอ็นเอบนแผ่นไนลอน แล้วจึงนำมาไฮบริไดซ์กับตัวติดตามดีเอ็นเอที่ติดฉลากด้วยสารกัมมันตภาพรังสี (^{32}P) ตัวตรวจสอบทางพันธุกรรมที่สามารถแสดงความแตกต่างระหว่างดีเอ็นเอของข้าวรุ่นพ่อแม่ทั้งสองได้ จึงถูกคัดเลือกมาเพื่อทดสอบการกระจายตัว ว่าเป็นไปในลักษณะเดียวกันกับลักษณะความหอมหรือลักษณะความไวแสงหรือไม่โดยใช้ดีเอ็นเอของข้าวในประชากรรุ่นลูก F_3

จากการใช้ตัวติดตามดีเอ็นเอทั้งหมด ๓๑ ตัวติดตาม ปรากฏว่ามีตัวติดตามดีเอ็นเอที่สามารถแสดงความแตกต่างทางพันธุกรรมระหว่าง ข้าวพันธุ์ขาวดอกมะลิ๑๐๕

และIR36 อยู่ทั้งหมด ๗ ตัวติดตาม ในบรรดา ๗ ตัวติดตามดีเอ็นเอที่สามารถบอกความแตกต่างทางพันธุกรรมได้นั้น พบว่ามีตัวติดตามดีเอ็นเอ RG28 และ RG445รวมอยู่ด้วย โดยตัวติดตามดีเอ็นเอ RG28ซึ่งมีรายงานว่าเชื่อมโยงอยู่กับตำแหน่งควบคุมความหอม (*fgr*) ในสารพันธุกรรมของข้าวนั้นสามารถบอกความแตกต่างทางพันธุกรรมระหว่างข้าวขาวดอกมะลิ๑๐๕ และIR36ได้ โดยใช้เอ็นไซม์ตัดจำเพาะ EcoRV ในขณะที่ตัวติดตามดีเอ็นเอ RG445 ซึ่งมีรายงานว่าอยู่ใกล้กับตำแหน่งควบคุมความไวแสง (*Se-1*)ในสารพันธุกรรมของข้าวนั้น สามารถบอกความแตกต่างทางพันธุกรรมได้โดยใช้เอ็นไซม์ตัดจำเพาะ XbaI หรือ EcoRI การตรวจสอบความสัมพันธ์ของการกระจายตัวระหว่างตัวติดตามดีเอ็นเอ RG28 และลักษณะความหอมโดยใช้ตัวอย่างดีเอ็นเอจากข้าวในประชากรรุ่น F_2 ที่มีลักษณะความหอมมากหรือไม่มีความหอมจำนวน ๒๔ ตัวอย่าง ผลการทดลองแสดงให้เห็นว่าตัวติดตามดีเอ็นเอ RG28 มีการกระจายตัวที่สัมพันธ์ไปในทำนองเดียวกันกับลักษณะความหอมในข้าวไทย อย่างไรก็ตามสำหรับการตรวจสอบความสัมพันธ์ของการกระจายตัวระหว่างตัวติดตามดีเอ็นเอ RG445 และลักษณะความไวแสงเมื่อใช้เอ็นไซม์ตัดจำเพาะ XbaI ปรากฏว่าไม่สามารถแสดงความแตกต่างของรูปแบบดีเอ็นเอจากข้าวในประชากรรุ่น F_2 ที่มีลักษณะความไวแสงหรือไม่ไวแสงได้ นอกจากนี้ ในการทดลองนี้ได้ทดลองปรับปฏิกิริยาที่เหมาะสมสำหรับการวิเคราะห์สารพันธุกรรมดีเอ็นเอชนิด RAPD และได้ทดลองใช้ปฏิกิริยาดังกล่าว เพื่อทดสอบความสามารถทางปฏิกิริยาของ RAPD primerที่มีอยู่จำนวน ๑๕๓ primersด้วยสารพันธุกรรมของข้าวพันธุ์ขาวดอกมะลิ๑๐๕ ผลการทดสอบแสดงให้เห็นว่า ๔๖% ของ RAPD primers ที่ทดสอบสามารถทำให้เกิดชิ้นส่วนดีเอ็นเอที่ได้รับการเพิ่มจำนวนที่สามารถแยกจากกันได้ดีได้

Thesis title Monitoring of Mendelian genetics in rice by DNA markers

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ABSTRACT

Technology of DNA analysis, including restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD), were explored for use in rice improvement. We also tried to used RFLP assay for detecting genetic loci for fragrance and photoperiod sensitivity in Thai rice genomic DNA. Genomic DNA of KDML105 and IR36 was singly digested by 5 restriction enzymes; BamHI, EcoRI, EcoRV, HindIII, and XbaI, fractionated on agarose gel electrophoresis, blotted and later hybridized to RFLP probes. Polymorphic RFLP found were then tested for co-segregation with our two traits of interest by representative genomic DNAs of F_2 plants.

From 31 DNA probes examined, 7 of them showed RFLPs between KDML105 and IR36 genomic DNA. Among the polymorphic RFLP probes, RG28, which was reportedly linked to *fgr* locus on

the rice chromosome 8, revealed DNA variation in our two parental DNA digested by EcoRV, and RG445, which was mapped near Se-1 locus on the rice chromosome 6, also displayed polymorphism with two parental DNA digested by XbaI or EcoRI. With 25 representative aromatic/non-aromatic F_3 plants, 89% of F_3 genomic DNA from aromatic plants had the same DNA pattern as aromatic KDML105 DNA and all of non-aromatic F_3 genomic DNA possessed a restrction fragment of IR36 DNA. This indicated association between the locus of RG28 and fragrance trait in Thai rice. However, RG445/ XbaI combination could not verify polymorphism between photoperiod sensitive and photoperiod insensitive characters among 14 F_3 DNA samples equally selected from both photoperiod sensitive and photoperiod insensitive F_2 lines. Additionally, RAPD assay was also explored by investigation for reaction conditions. About 193 RAPD primers was examined with KDML105 DNA. After standardization of RAPD reaction, an optimized RAPD condition for rice found was 20 ng DNA template, 0.2 µM primer, 100 nM of each dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin and 2.0 units *Taq* DNA polymerase and a PCR programe of 45 cycles of 94°C DNA denaturation for 1 min, 36 °C primer-DNA annealation for 1 min, and 72°C primer extension for 2 min. By the condition, 46% of 193 random RAPD primers examined could produce discrete amplified fragments with KDML105 genomic DNA.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

cm^2	centimeter square
cpm	count per minute
°C	degree of celcius
gm	gram
hr	hour
kb	kilobase pair
LB	Luria-Bertani medium
µCi	microcurie
µg	microgram
µl	microliter
mCi/mmole	millicurie per millimole
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
ng	nanogram
O.D.	optical density
PCR	polymerase chain reaction
RE	restriction endonuclease
rpm	round per minute
sec	second
RT	room temperature
V/cm	volt per centimeter
w/v	weight by volume

CHAPTER I

INTRODUCTION

Rice is the staple crop of Thailand. It also has the highest agricultural export value (1). With the rapid increase of the World's population, food demands, especially that of rice demand, is thus correspondingly high. Overproduction of rice grain is attained at present by many countries with various advance technologies (2,3). This has caused a lot of cost competition in the World's rice markets. Increasing rice grain yield per planting areas has been an important problem for international and Thai breeders alike.

Increasing rice grain production could be attained by expanding rice growing areas. However, growing areas wolrdwide are currently limitted and becoming less available. Moreover, cultivated areas may be rendered ineffective by environmental effects in some years. Therefore, increasing the rice grain yield is a premium problem for Thailand while its ability of low cost investigations with limitted budgets is still be a perpetual problem for Thai rice in the World's market. For this reason, good high-quality rice varieties which can better responsed to commercial demands and having higher yield is a major key for this solution (2, 4).

Currently, crop and rice improvement programs of most countries have depended heavily on conventional breeding and traditional selections (5, 6). This is not currently regarded as an effective mean for producing better new crop varieties. Crossing and selections of plants is a time-consuming process and this process can not guarantee required results (7). To expedite usage of current technologies to generate more effective rice improvement programs, numerous genetic markers, such as morphological, physiological, or biochemical characters, which can identify desired trait of a desirable rice variety from others have been applied into rice breeding and subsequential selection step (5, 8, 9, 10, 11). Unfortunately, despite the availability of tradition markers, they have reached their limits to benefitial plant selection process. Expression of physiological markers in plants is strongly influenced by environments (8) while morphological and biochemical markers, like recessive characters and isozyme or allozyme assays, is numerically insufficient for crop selection application (8).

Table 1. Some of 14 isozymes surveyed in Asian cultivated rice (J.C.Glaszmann).

Enzyme	No. of polymorphic loci	No. of alleles
Phosphoglucose isomerase	2	2,4
Glutamate oxaloacetate tranaminate	1	2
Shikimate dehydrogenase	1	4
Alcohol dehydrogenase	1	4
Isocitic dehydrogenase	1	4
Phosphogluconate dehydrogenase	2	3,2
Malic enzyme	1	2
Leucine aminopeptidase	2	7,5
Alanine aminopeptidase	1	5
Argenine aminopeptidase	1	3
Esterase	4	2,3,2,3
Acid phosphatase	2	3,2
Catalase	1	3
Peroxidase	1	2

BIOTECHNOLOGY HAVE BEEN USED TO IMPROVE CROP PLANTS

Many modern methods have been searched for appling to the crop improvement process (12). Biotechnologies have evidently been found to powerfully support in plant improvement in many directions (13) such as for varietal identifications, for selection of plants containing interesting trait, for studying of how to improve interested plant products, for plant selection and breeding, or even for creating novel transgenic plants via DNA manipulation.

DNA ANALYSIS IS A USEFUL APPROUCH TO IMPROVE PLANT AND RICE

DNA analysis can be applied as a variety determinator to rice variety identification in the same fashion as traditional assays (5, 6, 7, 14, 15, 16, 17). DNA analysis however, provides more advantages in a short time-frame, and with reliable genetic quality of a novel variety. DNA analysis allows monitoring of many DNA loci during plant breeding, with little environmental and epistatic effects (18).

There are many methods of DNA analysis which can be applied into plant systems (6, 19, 20, 21, 22). Three well established methods are DNA fingerprinting (6, 19), RFLP

analysis (5) and RAPD analysis (23).

1. DNA fingerprinting

DNA analysis method depending on specific digestion with some restriction enzymes, Southern blotted, and hybridization with nonspecific locus DNA probes. In this methods, different genomes, or DNA of different groups of a species, can reveal their unique DNA patterns when certain DNA probes were used to analyse, either singly or in group (15,19).

2. RFLP

RFLP is an acronyme for restriction fragment length polymorphism. Although methodological similar to DNA fingerprinting, RFLP requires fewer locus-specific DNA markers for detection of DNA variations in an individual species. Thus, DNA variations detected by RFLP analysis represent the variable sizes of one or few restriction fragments in the digested DNA that contain the same sequence homologous to the clone sequences which were used as DNA probes (5, 15, 22).

3. RAPD

Randomly amplified polymorphic DNA, on the other hand,

is based on *in vitro* amplifications of genomic DNA with single, or multiple, oligonucleotide primers of arbitrary sequence. After gel electrophoresis, DNA variations detected are in the the form of absence or presence of amplified DNA fragments (23, 24).

DNA MARKERS

1. RFLP markers

A set of DNA clones from a living organism is called a library. Three common series of libraries can be constructed for supplying DNA markers for RFLP analysis. **A genomic DNA library** could be constructed from a total genomic DNA isolated from a plant tissue whereas **an organellar library** was attained from an organellar genome such as mitochondrial or chloroplast genome. On the other hand, **a complementrary DNA (cDNA) library** could be prepared from mRNA isolated from plant tissues. This is such the case for most of the rice DNA clones used as markers in this study (6).

In order to do RFLP mapping, single copy DNA sequences are the most useful (18). A good source for single copy clones are cDNA clones, however, they are limited by their lengths

(they tend to be only few kb in length). In practical term, the length limitation is more serious than the second since the exposure time during autoradiography will diminish in case the clone length increases (the amount of radioactivity that can incorporated in to a clone sequence is proportional to its length). Although genomic DNA can provide a large supply of a long insert library, but, the majority of random genomic clones are likely to carry repeated DNA sequences in most plant species which are unsuitable for RFLP mapping. So, a way to select for clones free of repeated DNA is neccessary. There are two publicized methods had been used for resolving this problem; post-cloning selection via colony hybridization and pre-selection of clones by using restriction endonucleases especially C-methylation sensitive restriction endonucleases such as *Pst I* (18).

2. RAPD markers

DNA markers of RAPD type are short (9-10 mers) oligonucleotide primers which are synthesized chemically (33). Distinct from the other PCR procedures, which required a pair of carefully designed and fairly long oligonucleotide primers to amplify a specific target sequence, RAPD primers used are single, short oligonucleotides of arbitrary sequences which can bind to thousand sites of DNA sequences in a complexed DNA

template, such as a eukaryotic genome. Practically, the length of each of those arbitrary nucleotide sequences is commonly 10-mer, comprising of 50% G+C content, as well as having a non-palindomic sequence (24).

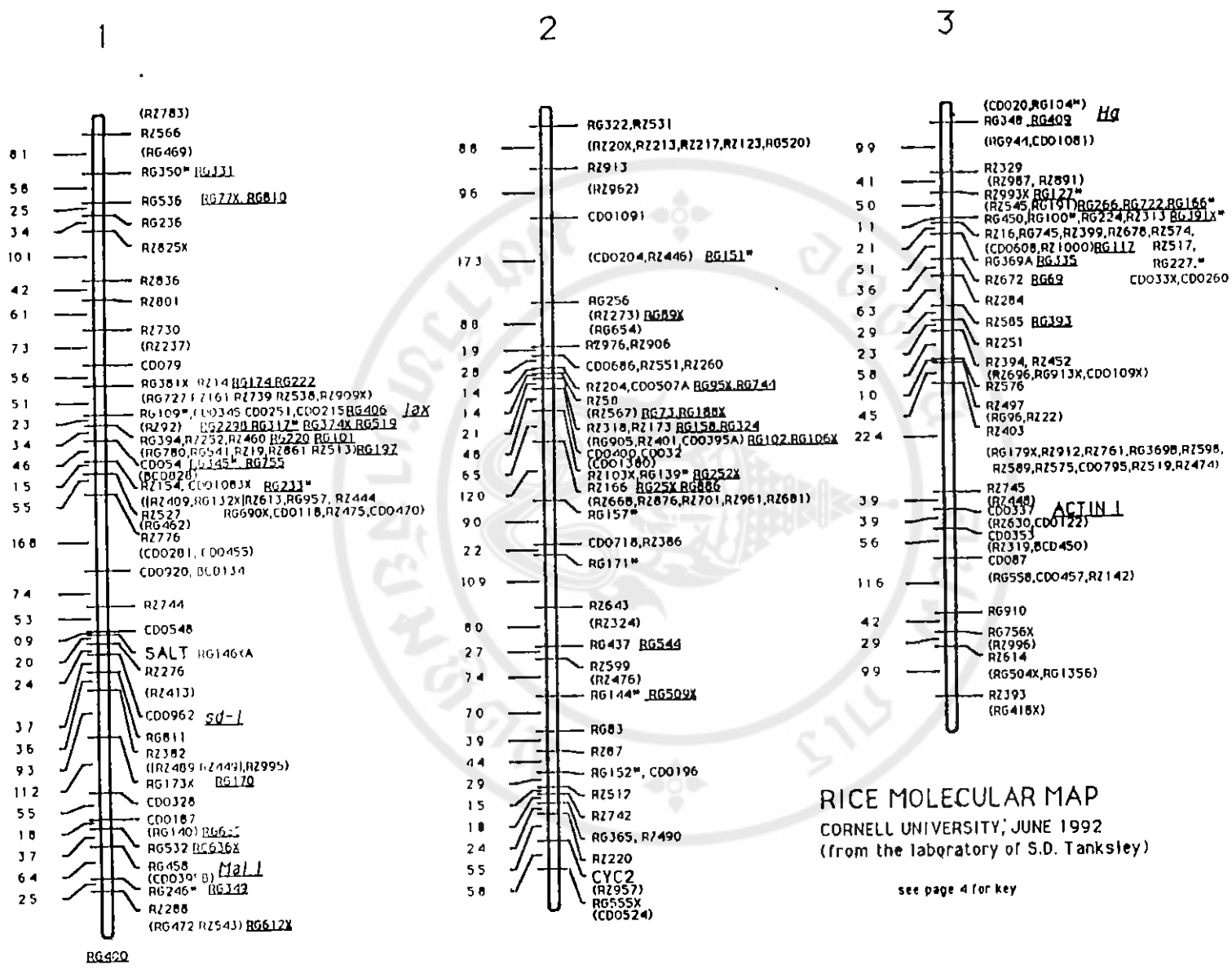
HOW CAN DNA MARKERS ACT AS A POTENTIAL TOOL IN RICE IMPROVEMANT PROGRAM

An important tool in DNA marker-assisted rice improvement and studies of the rice genome is a rice genetic map (18, 25). A map constructed from DNA markers is also called rice molecular genetic map. Information from a genetic map especially a detailed molecular map is very useful for plant breeding in many aspects, including for gene tagging (5, 6, 14). DNA markers such as RFLP probes or RAPD primers have high potential for constructing of the rice genetic map especially a high density rice map (25) due to their unlimitting number for detecting of large number of loci and their ability to probe in either noncoding or coding regions (5, 26, 27).

Figure 1. Rice molecular genetic map

(S.D. Tanksley et al 1992)

Numbers at tops of 12 linkage groups indicate essigned chromosome numbers. This map composed of 571 RFLP markers, 5 isozyme markers, and 27 known genes as of 1992. All genetic markers and known genes are shown on the rigth side of each chromosome map. RFLP markers are shown, such as RZ556 on the chromosome 1. isozyme markers are shown by bold and italic letters, such as ***Mal 1*** on the chromosome 1, while known genes are shown by bold letters, for example **lax** on the chromosome 1. Distance between a pair of two adjacent genetic markers are designated by genetical unit of centiMorgan (cM) and is placed on the left side of an individual chromosome map.

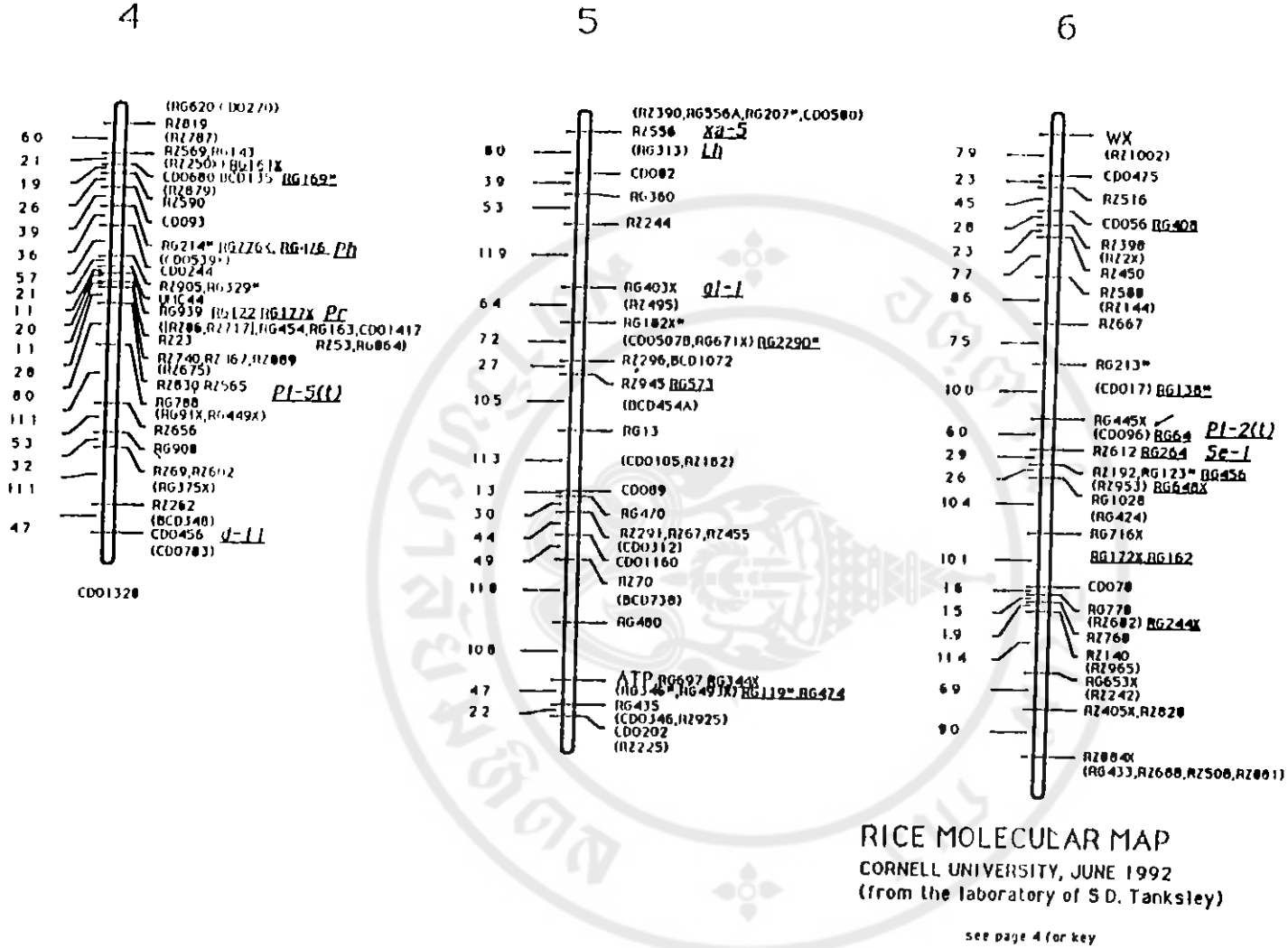


RICE MOLECULAR MAP

CORNELL UNIVERSITY, JUNE 1992

(from the laboratory of S.D. Tanksley)

see page 4 for key



RICE MOLECULAR MAP

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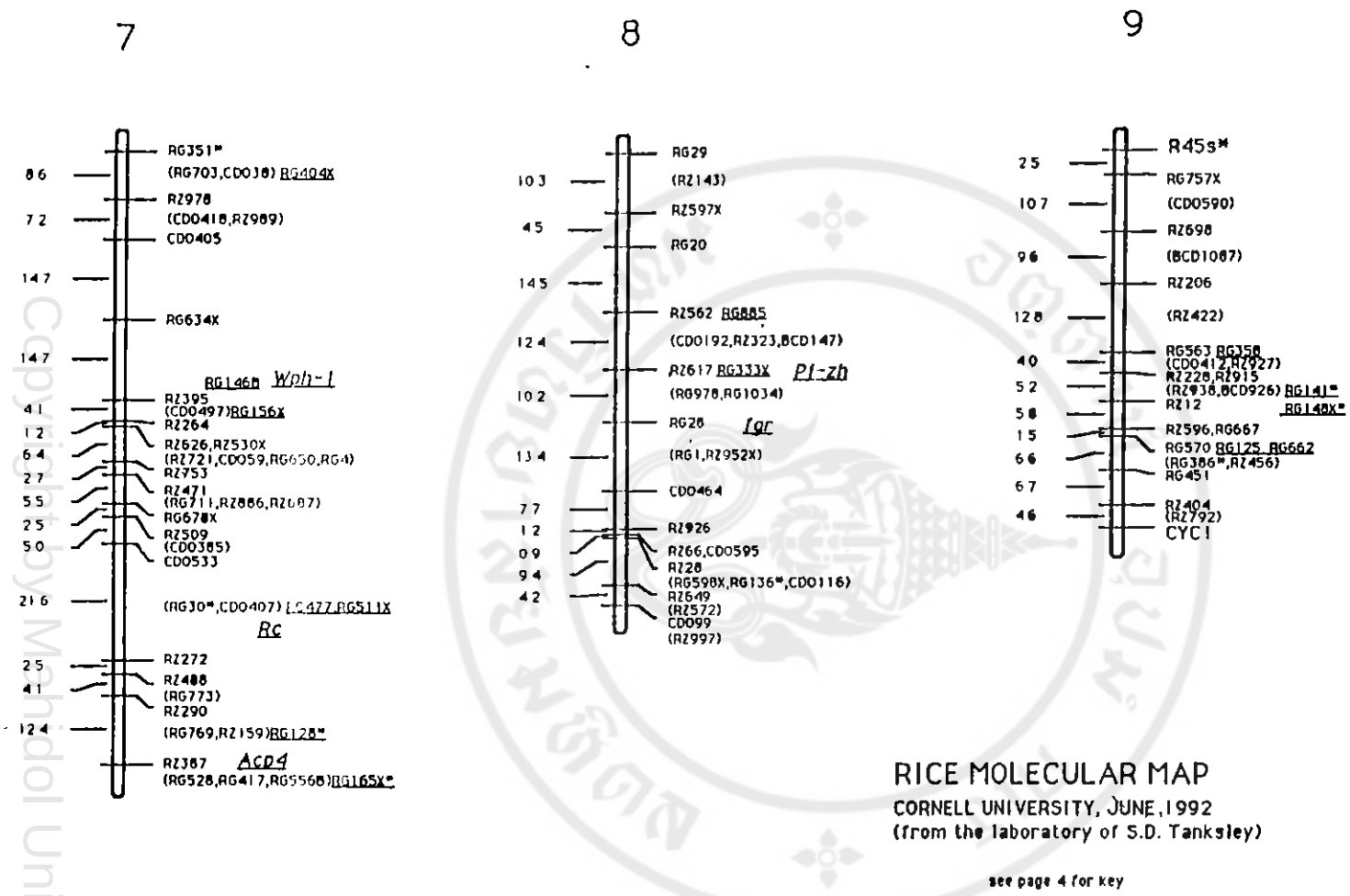


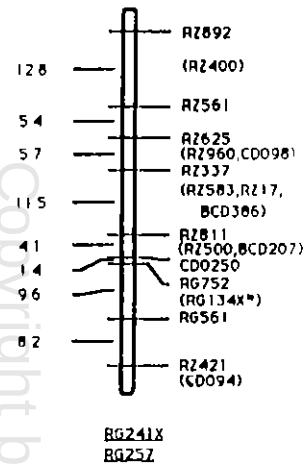
RICE MOLECULAR MAP

CORNELL UNIVERSITY, JUNE, 1992

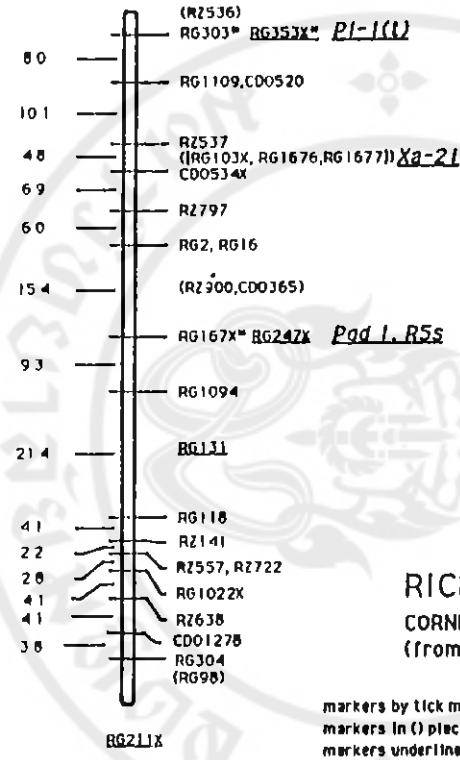
(from the laboratory of S.D. Tanksley)

see page 4 for key

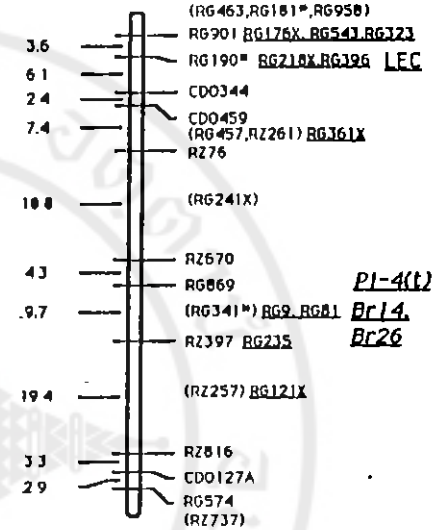
10



11



12



RICE MOLECULAR MAP

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(from the laboratory of S.D. Tanksley)

markers by tick marks ordered with LOD>2 using Mapmaker software
markers in () placed to interval with LOD<2
markers underlined-approximated from previously published maps
markers under chromosome linked to chromosome but not placed to intervals
*indicates located to chromosome w/trisomic analysis
Bold indicates known genes
Bold and Italics indicate isozymes or morphological markers
Loci following commas at markers by tick marks are cosegregants
Markers in [ ] are cosegregants within a LOD<2 interval
Kosambi cM are to the left of the chromosomes
Total map units 1222 cM
Mapping Parents B5125 (O sativa,indica) X (B5125 X WL02(O longistaminata))
Population 113 plants

GENE TAGGING

Gene tagging is a process based on finding a tight linkage between any DNA markers and traits of interest (6). This application has been used a lot for plant breeding, including rice, since such linkages permit breeders to infer the presence of desirable DNA or genotype inherited from one genetic background to another during plant crossing and selection (15, 22). For tagging a gene of interest, there are two essential requirements, sexual reproduction of the organisms and availability of markers (5);

1. Sexual reproduction

Sexual reproduction, obviously a property of higher eukaryotes, is required for producing effective isolines or any versatile segregating populations for genetic studies. Commonly, the availabilty of plant isolines is usefull in tagging single genes, such as those confering disease and insect resistance, when the segregating population that segregate for several traits allow for simultaneously tagging of genes for each of the characters, whether monogenic or polygenic (6).

To detect tight linkages between markers and genes of

interest, sexual reproduction should be produced from the parents which are genetically divergent enough to express sufficient number of RFLP loci, but not so far distant as to be uncrossable or cause sterility of progenies (15). Furthermore, selecting a cross in which some desirable genetic traits are segregated will facilitate a successful RFLP tagging project (6).

2. Availability of abundant DNA markers

Tagging will be more reliable when the genetic distance between a marker or two and the target gene is small because the probability of crossover events separating a marker from the locus governing the trait diminishes with decreasing chromosomal distance. Therefore, copious DNA markers have to be examined to enhance the opportunity for uncovering a useful DNA marker which is closely linking to the loci of the interesting character (15).

KDML105 POSSESSES DESIRABLE GRAIN RICE QUALITY BUT IS PHOTOPERIOD SENSITIVE

As earier mentioned, newly improved rice varieties should carry many desirable features extra from their higher

yield capacity. Those required characters should response for all commercial demands such as pleasant aroma, palatable starch texture or should facilitate other applications for industrial food production, etc. So, rice varieties which possess higher grain yield capacity and having additional desirable features have been used as parents for crossing in recent Thai rice improvement programs. An example of Thai rice variety which has a very good grain quality is Khao Dawk Mali 105.

Khao Dawk Mali 105 (KDML105) is a famous *indica* rice variety of Thailand and worldwide. In 1990, 1991, and 1992, demand of this Thai rice raised up 17.8%, 20.5%, and 22.91%, respectively (28). According to the increasing international demand, KDML105 act as a powerful economic commodity of Thailand. Unluckily, KDML105 production is still inadequate production since KDML105 itself is a tall variety and can not produce high enough grain yield. KDML105 is also photoperiod sensitive which restrict its multiple cropping per year. Moreover, KDML105 is susceptible to many insect pests and diseases. although this variety can resist to several adversed planting conditions such as moderate degree of drought, salted soil, or acid soil (28, 29).

Therefore, in recent Thai rice improvement programs, breeding have been performed between KDML105 and other

varieties of which properties can complement with KDML105's defects, for generating an innovative rice varieties which may possess a number of desirable properties. In our study, IR36, one of the best *indica* rice varieties improved by IRRI, was chosen as the other parent plant in the hope that their anticipated hybrids migth have improved properties over KDML105. Since not only is IR36 a photoperiod insensitive variety, but IR36 itself is also a high yielding variety which can promise breeders for successful required progenies although quality of rice grain of this variety is considered interfere and does not possess any scent phenotype. Furthermore, IR36 variety was chosen as a parent in this breeding due to its ability to resist many insect pests and diseases (4).

Figure 2. Ancestry of rice variety IR36

IR36 is one of the best human and widely planted rice varieties improved by the International Rice Research Institute (IRRI). From this complicated pedegree diagram, it can be seen that there is no Thai rice KDML105 as ancestors involved in this variety construction. This information suggested that they might have quite a genetically divergent background between IR36 and KDML105.

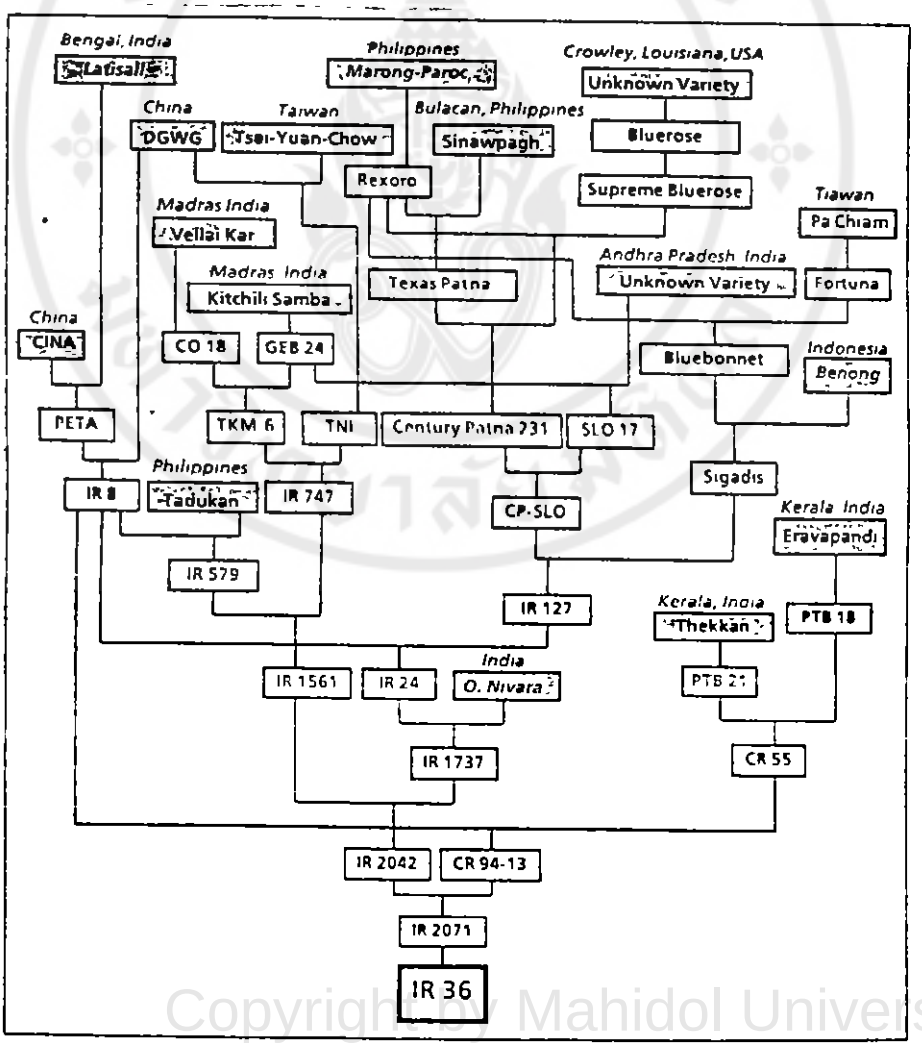


Figure 6 Ancestry of the rice variety IR 36

RESTRICTION FRAGMENT LENGTH POLYMORPHISM AND RICE IMPROVEMENT

Apart from the fact that RFLP essay can directly detect natural variations at DNA level, this technique can be effectively performed at all stages of plant development, no matter those plants are not mature (5, 7). Additionally, this technique can potentially reduce complex or polygenic traits to their individual and combined effects in selected genetic backgrounds (18). Undoubtedly, recent acchivements of the RFLP assay have been continuously publicized to emphasize an elegant conquest of this technology in various plants, for example: tomato (30), barley (31, 32, 33), wheat (34), and cowpea (35), etc.

In case of rice, rice molecular genetic map has been constructed by McCouch et al since 1988 (25) and has been continuously improved by many researchers especially those in the laboratory of S.D. Tanksley (Tanksley et al in 1992; persoal communication) and in japan. The preserve of high resolution molecular map stimulated genetic studies of importance characters of rice. There were various genes reportedly tagged by RFLP analysis, such as blast resistant gene (in 1991) by Yu, Z.H. (40), baterial blight resistant gene (in 1993) by P.K. Ranjekar (41), blast resistant and

semidwarf genes (in 1993) by Linuang Zhu et al (42). However, when RFLP project in our laboratory was commenced in early 1991, there was no one studying about tagging fragrance and photoperiod sensitive characters in *indica* rice.

In our project, KDML105 and IR36 were chosen as parents for studying application of RFLP in *indica* rice breeding as well as to study about fragrance and photoperiod sensitivity in Thai rice (previous mentioned). Prior to the work in this thesis, about 48 RFLP probes has been initially investigated for monitoring polymorphism between the two parental genomes in 1991 by Dr. B. Sonthayanon (unpublished data). The result from that experiment was also included with my result in this thesis.

Table 2. Polymorphism screening between KDML105 and IR36 by 48 RFLP probes

(studied by Dr. B. Sonthayanon, unpublished)

In this table, monomorphism (the same DNA pattern) was recorded as M and polymorphism (dissimilar DNA pattern) was recorded as P. Abbreviation "chr#" was used to designate rice chromosome numbers of the tested RFLP probes.

During the previous survey by Dr. B. Sonthayanon in 1991, total 49 RFLP probe/restriction enzyme combinations were examined. Out of 49 combinations, nine of them could uncover variations between KDML105 and IR36 DNA. Those nine combinations are with RZ154/EcoRV (chr#1), RZ936/EcoRV (chr#1), RZ643/EcoRI (chr#2), RZ403/EcoRI (chr#3), RZ516/EcoRV (chr#6), RZ682/EcoRV(chr#6), RZ12/ScaI (chr#9), RZ206/ScaI (chr#9), and RZ816/XbaI (chr#12).

probes	Restriction Endonucleases				
	EcoRI	EcoRV	HindIII	ScaI	*Xbal*
chr#1					
RZ154		P			
RZ274			M		
RZ288		M			
RZ489				M	
RZ543		M			
RZ783		M			
RZ825				M	
RZ936		P			
chr#2					
RG58		M			
RZ173				M	
RZ643		P			
RG742					M
chr#3					
RZ142			M		
RZ284			M		
RZ393				M	
RZ403	P				
RZ585			M		

Table 2 (continuced)

RZ630			M		
chr#4					
RZ656				M	
chr#5					
RZ70			M		
RZ277					M
RZ556					M
chr#6					
RG64	M				M
RG716					M
RZ140		M			
RZ516		P			
RZ588		M			
RZ612		M			
RZ682		P			
RZ1003		M			
chr#7					
RZ278			M		
RZ387		M			
RZ978	M				
chr#8					
RG1			M		
RG108					M
RG136		M			

Table 2 (continuced).

RZ562			M		
RZ952				M	
CD0464			M		
chr#9					
RZ12				P	
RZ206				P	
chr#10					
RZ561			M		
RZ625					M
chr#11					
RZ141		M			
RZ537		M			
RZ638				M	
chr#12					
RZ261					M
RZ816					P

RAPD markers

Since late 1990s, an innovative procedure called RAPD analysis was developed as another genetic tool based on PCR technique by John G.K. William *et al* (24). This approach is considered a more effective way to screen large number of genetic markers which can be applying for various genome studies as well as using in plant improvement programs (23). Recently, many modifications of the original RAPD procedure have been suggested and used in several plants, such as tomato (43, 44), lettuce (45), *Arabidopsis* (26), and common bean (46). This tecnique is very interesting, simple, and promising a short time consuming of variety selection propose if this technique is really practiced in gene tagging programs. Thus Thus there should attemptly approve this technique consequently in favour of really effective usage (23).

In this thesis, RAPD assay has been preliminarily explored optimization of proper reaction conditions and screening of random primers using one parent DNA: KDML105, was aimed to find RAPD primers which could be used in all species of grass plants.

AIM OF THE THESIS

The fundamental aim of this study was to investigate usefullness of DNA markers in rice breeding. There are three specific aims of this thesis:

(1) to detect RFLP markers which could uncover genetic variations between two supposedly divergent parental genome of *indica* rice, KDML105 and IR36. A numbers of DNA markers had to be investigated for identifying those which were polymorphic RFLP markers.

(2) to monitor co-segregation of putative polymorphic RFLP markers with the two economically important trait in Thai rice; fragance and photoperiod sensitivity.

(3) to standardize a proper reaction condition for studying rice RAPD.

CHAPTER II

MATERIAL AND METHOD

MATERIAL

1. PLANT MATERIAL

Rice plants (*Oryza sativa* L.) were grown from seed stock of Thailand's National Rice Storage Laboratory for genetic reserves. A genetic population used in this study was constructed by S. Chitrakon and B. Sonthayanon during 1989-1992 (personal comm, unpublished) using two varieties of *indica* rice, Khao Dawk Mali 105 (KDML105) and IR36 (I) as parents. The KDML105 is a local Thai variety whereas IR36 is a hybrid line released from IRRI in 1976. The IR36 variety does not contain any Thai rice as ancestors. Plant of F_2 and F_3 lines obtained from a single pair of the above parent plants were grown at Pathum Thani Rice Reserch Center, Thanya Buri, Pathum Thani, Thailand.

Leaves were harvested from mature rice plants and kept frozen at -20°C prior to DNA extraction. Phenotypes of rice offsprings were evaluated at Pathum thani Rice Research Center by S. Chitrakon and N. Kongseree.

2. DNA MARKERS

2.1 RFLP MARKERS

About 90 RFLP probes (DNA clones of defined genetic loci) used in this laboratory were kindly provided by Dr. S.D. Tanksley of Cornell University, USA. Among the DNA probes used, most were short rice genomic DNA clones and some were cDNA clones. Genomic DNA clones were constructed by McCouch (1988) from total rice DNA (cv. "IR36") digested with PstI (25). Size separation of those digested rice DNA was performed on 10-40% sucrose gradient. The 1-2 kb size fraction was then selected for ligating into pUC8 plasmid whose near-restriction-site region contained DNA sequence is compatible for 2 universal primers of M13 phage vecter; 5'GTAAAACGACGGCCAGT3' (down stream primer) and 5'AACAGCTATGACCATG3' (up stream primer). For cDNA clones, complementrary DNAs were cloned from mRNA isolated from rice and oat. Similar to random genomic DNA clones (RG series), those cDNAs were ligated into bacterial plasmid such as pUC8, pGEM. Those DNA clones tried used as RFLP probes in this thesis have already been mapped along the twelve linkage groups of rice genome by Tanksley and his colleagues.

2.2 RAPD PRIMERS

In this study, two hundred arbitrary 10-oligonucleotide primers were purchased from the University of British Columbia Biotechnolgy Laboratory (primer numbers 101-300). Their sequences had been published and were reproduced in the table 2. From the manufacturer, each primer had already been purified by eluted with Tris/ EDTA buffer though NAP-5 drips columns, then, 10 µg of each primer were aliquoted and lyophilized.

Table 3. **DNA sequence and melting temperature of studied 10-Mer RAPD primers (from the University of British Columbia, Canada)**

Of an individual primer, DNA sequence reported in this table was ordered from 5'-phosphate end to 3'-OH end and melting temperature of a given primer (T_m) was obtained from calculation of 2°C for A or T and 4°C for G or C; T_m = (A+T)x2 +(G+C)x4 (51).

primer number	DNA sequence	T_m (°C)	primer number	DNA sequence	T_m (°C)
101	GCGGCTGGAG	36	107	CTGTCCCTTT	30
102	GGTGGGGACT	34	108	GTATTGCCCT	30
103	GTGACGCCGC	34	109	TGTACGTGAC	30
104	GGGCAATGAT	30	110	TAGCCCGCTT	30
105	CTCGGGTGGG	36	111	AGTAGACGGG	30
106	CGTCTGCCCG	36	112	GCTTGTGAAC	30
113	ATCCCAAGAG	30	137	GGTCTCTCCC	34
114	TGACCGAGAC	32	138	GCTTCCCCTT	32
115	TTCCGCGGGC	36	139	CCCAATCTTC	30
116	TACGATGACG	30	140	GTCGCATTTC	30
117	TTAGCGGTCT	30	141	ATCCTGTTCG	30
118	CCCGTTTTGT	30	142	ATCTGTTCGG	30
119	ATTGGGCGAT	30	143	TCGCAGAACG	32
120	GAATTTCCCC	30	144	AGAGGGTTCT	30
121	ATACAGGGAG	30	145	TGTCGGTTGC	32
122	GTAGACGAGC	32	146	ATGTGTTGCG	32
123	GTCTTTCAGG	30	147	GTGCGTCCTC	34
124	ACTCGAAGTC	30	148	TGTCCACCAG	32
125	GCGGTTGAGG	32	149	AGCAGCGTGG	34
126	CTTTCGTGCT	30	150	GAAGGCTCTG	32
127	ATCTGGCAGC	32	151	GCTGTAGTGT	30

Table 3. (continuced)

128	GCATATTCCG	30	152	CGCACCGCAC	36
129	GCGGTATAGT	30	153	GAGTCACGAG	32
130	GGTTATCCTC	30	154	TCCATGCCGT	32
131	GAAACAGCGT	30	155	CTGGCGGCTG	36
132	AGGGATCTCC	32	156	GCCTGGTTGC	34
133	GGAAACCTCT	30	157	CGTGGGCAGG	36
134	AACACACGAG	30	158	TAGCCGTGGC	34
135	AAGCTGCGAG	32	159	GAGCCCGTAG	34
136	TACGTCTTGC	30	160	CGATTCAGAG	30
161	CGTTATCTCG	32	185	GTGTCTTCAC	30
162	AACTTACCGC	30	186	GTGCGTCGCT	34
163	CCCCCCAGAT	34	187	AACGGGGGAG	34
164	CCAAGATGCT	30	188	GCTGGACATC	32
165	GAAGGCACTG	34	189	TGCTAGCCTC	32
166	ACTGCTACAG	30	190	AGAATCCGCC	32
167	CCAATTCACG	30	191	CGATGGCTTT	30
168	CTAGATGTGC	30	192	GCAAGTCACT	30
169	ACGACGTAGG	32	193	TGCTGGCTTT	30
170	ATCTCTCCTG	30	194	AGGACGTGCC	34
171	TGACCCCTCC	34	195	GATCTCAGCG	34
172	ACCGTCGTAG	32	196	CTCCTCCCCC	36
173	CAGGCGGCGT	36	197	TCCCCGTTCC	34
174	AACGGGCAGC	34	198	GCAGGACTGC	34
175	TGGTGCTGAT	30	199	GCTCCCCCAC	36

Table 3. (continued)

176	CAAGGGAGGT	32	200	TCGGGATATG	30
177	TCAGGCAGTC	32	201	CTGGGGATTT	30
178	CCGTCATTGG	32	202	GAGCACTTAC	30
179	TCACTA*TACG	28	203	CACGGCGAGT	34
180	GGGCCACGCT	36	204	TTCGGGCCGT	34
181	ATGACGACGG	32	205	CGGTTTGGAA	30
182	GTTCTCGTGT	30	206	GAGGACGTCC	34
183	CGTGATTGCT	32	207	CATATCAGGG	32
184	CAAACGGCAC	32	208	ACGGCCGACC	36
209	TGCACTGGAG	32	233	CTATGCGCGC	34
210	GCACCGAGAG	34	234	TCCACGGACG	34
211	GAAGCGCGAT	32	235	CTGAGGCAAA	30
212	GCTGCGTGAC	34	236	ATCGTACGTG	30
213	CAGCGAACTA	30	237	CGACCAGAGC	34
214	CATGTGCTTG	30	238	CTGTCCAGCA	32
215	TCACACGTGC	32	239	CTGAAGCGGA	32
216	CATAGACTCC	30	240	ATGTTCCAGG	30
217	ACAGGTAGAC	30	241	GCCCGACGCG	38
218	CTCAGCCCAG	34	242	CACTCTTTGC	30
219	GTGACCTCAG	32	243	GGGTGAACCG	34
220	GTCGATGTCG	32	244	CAGCCAACCG	34
221	CCCGTCAATA	30	245	CGCGTGCCAG	36
222	AAGCCTCCCC	34	246	TATGGTCCGG	32
223	GATCCATTGC	30	247	TACCGACGGA	32

Table 3. (continuced)

224	TCTCCGGTAT	30	248	GAGTAAGCGG	32
225	CGACTCACAG	32	249	GCATCTACCG	32
226	GGGCCTCTAT	32	250	CGACAGTCCC	34
227	CTAGAGGTCC	32	251	CTTGACGGGG	34
228	GCTGGGCCGA	36	252	CTGGTGATGT	30
229	CCACCCAGAG	34	253	CCGTGCAGTA	32
230	CGTGCCCCAT	34	254	CGCCCCCATT	34
231	AGGGAGTTCC	32	255	TTCCTCCGGA	32
232	CGGTGACATC	32	256	TGCAGTCGAA	30
257	CGTCACCGTT	32	279	AGACATTAGA	26
258	CAGGATACCA	30	280	CTGGGAGTGG	34
259	GGTACGTACT	30	281	GAGAGTGGAA	30
260	TCTCAGCTAC	30	282	GGGCCACCGT	30
261	CGCCCCCAGT	34	283	CGGCCACCGT	36
262	TTAGAGACGG	30	284	CAGGCGCACA	34
263	TCCACCGAGC	34	285	GGGCGCCTAG	36
264	CAGCTGTTCA	30	286	CGGAGCCGGC	36
265	CAGCTGTTCA	30	287	CGAACGGCGG	36
266	CCACTCAACC3	34	288	CCTCCTTGAC	32
267	CCATCTTGTG	30	289	ATCAAGCTGC	30
268	AGGCCGCTTA	32	290	CCGCGAGCAC	36
269	CCAGTTCGCC	34	291	AGCTGAAGAG	30
270	TGCGCGCGGG	38	292	AAACAGCCCG	32
271	GCCATCAAGA	30	293	TCGTGTTGCT	30

Table 3. (continuced)

272	AGCGGGCCAA	34	294	TGATTGGCCA	30
273	GTTGTCGCCA	30	295	CGCGTTCCTG	34
274	GTTCCCGAGT	32	296	CCGCTGGGAG	36
275	CCGGGCAAGC	36	297	GCGCATTAGA	30
276	AGGATCAAGC	30	298	CCGTACGGAC	34
277	AGGAAGGTGC	32	299	TGTCAGCGGT	32
278	GGTTCCAGCT	32	300	GGCTAGGGCG	36

3. CHEMICALS

Absolute ethanol	Merck (E. Merch, Darmsladt, F.R. Germany)
Agarose (type V)	Sigma (Sigma chenical Comp., St Louis, U.S.A.)
Ampicillin	Sigma
Bacto agar	Difco (Difco laboratories, Michigan, U.S.A.)
Bacto tryptone	Difco
Boric acid	Merck
Bovine serum albumin (fraction V)	Sigma
Bromphenol blue	Sigma
t-Butanol	Fisher (Fisher Scientieic Comp.,

	New Jersey, U.S.A.)
Cesium chloride	Sigma
Chloroform	Merck
Chloramphenical	Sigma
Deoxyadenosine 5'-triphospate (dATP)	Sigma
(α-^{32}P)deoxyadenosine triphospate 6000uCi/mmol in triethylammonium salt, 20uCi/ul	Amersham, U.K.
Deoxycytosine 5'-triphosphate (dCTP)	Sigma
Deoxyguanidine 5'-triphosoate (dTTP)	Sigma
Developer (Kodak Gbx)	Kodak
Dextran sulphate	Sigma
Dialysis membranes	Union Carbide, Union carbide Comp., Chicago, Illinois
Diethyl ether	Merck
Disodiumhydrogen-phosphate-2-hydrate	Merck
Ethidium bromide	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma
Ficoll 400	Pharmacia
Fixer	Kodak

GF/A filter	Whatman (Whatman International Ltd. Maidstone England)
GeneScreen *Plus*™ membranes	Dupont
Glucose	Merck
HEPES	Sigma
Hexadeoxynucleotides	Phamecia
Hydrochloric acid	Carlo Erba (Farmitalia carlo erba S.P.A., Milano)
Isoamyl alcohol	Carlo Erba
Lambda DNA/*HindIII*-digested	New England Biolabs
N-Lauryl sarcosine	Sigma
Lauryl sulfate (SDS)	Sigma
Low melting point agarose (ultra pure™)	BRL (Bethesda Research laboratory, Gaitherburg West germany)
Lysozyme (grade VI)	Sigma
Magnesium Chloride	Fluka
2-Mercaptoethanol	Sigma
Mineral oil	Sigma
Phenol	Carlo Erba
Polymerases	
DNA polymerase I, Large fragment (Klenow enzyme)	New England Biolabs

Vent™ DNA polymerase	New England Biolabs
Polyvinylpyrolidone (PVP)	Calbiochem (Calbiochem-behring division of american Hoeschst Corp. C.A.)
Restriction Endonucleases (*BamHI*, *EcoRI*, *EcoRV*, *HindIII*, *XbaI*)	Quadrant (Quadrant Holing Cambridge Limited, Marisland, Trumpington, Cambrisge CBR 2SY, England) and New England Biolabs
Ribonuclease A type XII (RNaseA)	Sigma
Salmon sperm DNA (type III)	Sigma
Sephadex, G-50 (fine)	Phamacia
Sigma 7-9	Sigma
Sodium acetate	Sigma
Sodium bisulfite	Mallinckrodt (Mallinckrodt Inc. ST. Louis, Missouri U.S.A.)
Sodium chloride	Carlo Erba
Sodium hydroxide	Merck
Sodium Phosphate	J.T. Baker (J.T. Baker chemical Co., Phillipsburg, N.Y.)
Spermidine	Sigma
Trichloro acetic acid (TCA)	Carlo Erba
Trisodium citrate dihydrate	Merck
Trisma base	Sigma

Urea (Puriss)	Fluka (Switzerland)
Whatman 3 MM papers	Whatman
Yeast extract	Difco

METHODS

1. PREPARATION OF DNA PROBES

1.1 By PCR procedure

For RFLP analysis, sufficient amount of various RFLP probes is a prerequisite. Due to its simplicity and rapidity, PCR technique was also applied to prepare our DNA probes, as modified from the method of Janen and Ladley (52).

Preparation of DNA inserts were performed in 50 µl reactions in capped 0.5 ml polypropylene microcentifuge tubes. Every 50 µl reaction composed of 0.44 µM of two M13 universal primers (see Material), 1xPCR buffer (50 mM KCL, 11.5 mM $MgCl_2$, 10 mM Trist-Cl pH8.0, 0.01% gelatin), 2.5 units of untrade *Taq* DNA polymerase, around 1 ng of plasmid DNA template, and sterile distilled water. The reaction mixture was also overlaid with 30 µl of mineral oil to prevent evaporation. Each reaction mixture was preheated at 100°C for 3-5 min prior to adding of the *Taq* DNA polymerase to produce single stand template for the PCR reaction. A PCR program used for DNA amplification started with 29 repeating cycles of 94°C DNA denaturation for 1 min, 55°C primer-DNA annealation for 1 min, and 72°C primer extension for 3 min. After that, a modified

cycle of a longer 7 minutes extension step was added to increase efficiency of amplification (from *Taq* polymerase protocol; Perkin Elmer Cetus). Finally, amplified inserts were quantified by running in agarose gel electrophoresis and then kept at -20°C refrigeration before use.

1.2 Transformation and rapid alkali lysis

For some DNA probes, transformations were used to prepare large amount of plasmid DNAs followed by rapid alkali extraction (53).

For transformation, 2 µl of plasmids inserting RFLP probe DNA (at least 10 ng) was aliquoted into a 15 ml sterile, round-bottom test tube and was placed on ice. Competent cells (Ecoli JM107) were rapidly thawed by warming between hands and 100 µl were dispended immediately into test tubes containing plasmids with rice inserts. Those tubes were gently swirled to thoroughly mix, then placed on ice for 10 min before transferring into a 42°C waterbath for 2 min for heat-shocking cells. Then, 800 µl LB broth was added into each tube. After that, each tube was left at 37°C for 1 hour. Finally, transformed culture was aliquoted and plated on LB agar containing ampicillin. Plates were incubated at 37°C for 12-16 hours when plates were dry.

One colony of each transformant in E coli JM107 was innoculated into 5 ml LB broth, comprising of 1% tryptone, 0.5% yeast extract, 0.5% NaCL, and 100 µg/ml ampicillin. After incubating overnight at 37°C, each culture was divided into three aliquots in 1.5 ml microtubes and centrifuged at 10,000 rpm for 1 minute. After decanting their supernatant, each pellet was added with 100 µl of lysozyme solution, consisting of 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, and 5 mg/ml lysozyme. Following the mixing of suspensions thoroughly, they were placed on ice for 10 min. Then, 200 µl lysis solution containing 0.2 N NaOH, 1% SDS was added and the tube was gentlely mixed again, before placing on ice for another 5 minutes. After that, 150 µl of 3 M NaOAc pH4.5 was added in order to neutalize the solution. The neutalized solution was mix rapidly and then placed on ice for 1 hour. Consequently, suspensions were centrifuged at 10,000 rpm for 1 min and their pellets were removed. Two volumes of absolute ethanol was added to those supernatants. They were mixed thoroughly and left at -20°C for at least 1 hour. Each suspension was then centrifuged at 10,000 rpm for 10 min. Each final pellet was washed twice with 70% ethanol before dissolving with 20 µl of TE pH 7.4, then storing at -20°C.

Concentration of DNA probes, either preparing by DNA amplification or transformations following by rapid alkali

lysis, were estimated by comparing their EtBr-stained band intensity with a known concentration of standard Lambda phage DNA digested with Hind III restriction enzyme.

2. ISOLATION OF RICE GENOMIC DNA

Genomic DNA was isolated from young rice leaves using extraction buffer with established procedure (54).

Approximately, 10 grems of young leaf tissue was ground to fine powder in liquid nitrogen using mortar and pestle. The powdered tissue was subsequently mixed with 25 ml of extraction buffer, containing 500 mM NaCl, 100 mM Tris HCl pH8.0, 50 mM EDTA, 1.25%(w/v) SDS, and 0.38%(w/v) sodium bisulfite (freshly prepared and added when extraction buffer had been warmed up to 65°C before mingling with rice fine powder). The mixture was then incubated at 65°C and the tube mixed gently at 5-10 minutes interval for 20-30 minutes in a 500 ml Elenmenyer flask. To removed plant pigments, 3/4 volumes of chloroform: isoamyl alcohol (24:1) were added and mixed thoroughly. The mixture was fractionated by a centrifugation at 6,000 rpm for 15 min in a bench top centifuge. Then, upper aqueous phase containing genomic DNA was gently fractioned out through a sterile wide-mouth pasture pipette. If rice pigments still

exsist in the supernatant carrying genomic DNA, the extraction step was repeated. After that, supernatant from each sample was subsequently transferred to a 50 ml polypropylene tube and genomic DNA was precipitated by an addition of 2 volumes of absolute ethanol. The precipitation was allowed to proceed at least 1 hour, or overnight at 4°C. Genomic DNA was carefully scooped out by a sterile bent pasture pipette. The DNA pellet was later washed gently in 70% ethanol, then dissolved in 1 ml of TE buffer. To eliminate RNA, the DNA solution was treated with RNaseA and precipitated with absolute ethanol. Centrifuged and dried DNA pellet was consequently dissolved in TE buffer at 55-65°C, up to 1 hour if neccessary.

For further purification, the DNA solution was added with an equal volume of a 1:1 phenol: chloroform mixture then centrifuged at 1,000 rpm for 1 minute, and the upper aqueous phase with the DNA was transferred into a new tube. This step was repeated once. After that, extracted DNA was extrected with an equal volume of diethyl ether for eliminating any remaining phenol/chloroform. The extracted DNA was left at room temperature for a few minutes to evaporate the remaining diethyl ether before adding with 1/10 volume of 3 mM NaOAc pH4.5, and 2 volume of absolute ethanol. This DNA suspension was kept at 4°C for at least 1 hour and after which precipitated DNA clumps were scooped out and washed with 70%

ethanol. Finally, purified genomic DNA was dissolved in 1 ml of TE buffer.

Concentration of these extracted DNA was estimated by measuring absorbancy at 260 nm and by agarose gel electrophoresis. Purity of these genomic DNA was estimated from the ratio between the absorbance at 260 nm and 280nm (OD_{260}/OD_{280}). By this estimation, pure preparation of DNA have a ratio of approximate 1.8. (54)

Although absorbance measurement at 260 nm can be applied for genomic DNA concentration assay, DNA concentration from this method was found much higher than other one obtained from gel electrophoresis. So, these extracted DNA was stilled estimated their concentration by gel electrophoresis comparing to stadard Hind III-digested Lambda DNA marker. About 10 grams of frozen leave tissues of rice could yield an average of 147 µg genomic DNA by this procedure. A ratio of OD_{260}/OD_{280} of 1.7 for extracted genomic DNA was mostly obtained .

3. DNA POLYMORPHISM SCREENING BETWEEN KDML105 AND IR36 DNA BY USING RFLP PROBES

3.1 Digestion of rice parental genomic DNAs

Genomic DNA of KDML105 and IR36 rice was digested with up to five selected type II restriction endonucleases; BamHI, EcoRI, EcoRV, HindIII, and XbaI.

Lyophilized type II restriction endonuclease (Quadrant) which required only 30 µl reaction for 25 units endonuclease was used in this study.

To minimize pipetting step, one dilution of each parental DNA was prepared for all reactions of the 5 restriction endonuclease digestions. Two reactions of one enzyme were performed for obtaining an adequate DNA amount for Southern Blotting. Since 4 µg of each genomic DNA was sufficient for digesting in one reaction of a preset 25 units of the endonuclease. Each stock of parental DNA was warmed in 65°C waterbath, then diluted with sterile distilled water, to give a 4 µg/30 µl final concentration before being aliquoted to each reaction. Later, these reactions were mixed by slightly pipetting up and down then incubated at 37°C for 3-4 hours. Finally, assembling and precipitating each parental genomic DNA

digested with the same endonuclease was also performed to adjust DNA volume to around 20-30 µl in TE prior to final eletrophoresis.

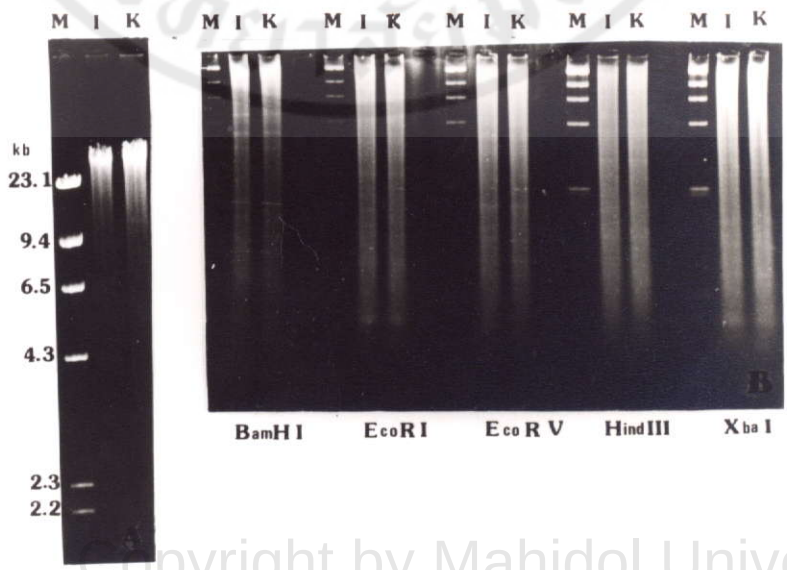
Specific activity of restiction endonuclease for achieving complete digestion depended upon what the kind restriction endonuclease was such as for EcoRV digestion; both two parental DNA and their F_3 DNA had the same maximum specific activity of 5 units enzyme per 1 µg genomic DNA, whereas for *Xba*I digestion; specific activity were in the range of 5 to 10 units enzyme per 1 µg rice genomic DNA.

Figure 3. Rice genomic DNA

(a) Genomic uncut DNA of the two parent DNA; KDML105 (K) and IR36(I)

(b) Genomic DNA of KDML105 and IR36 DNA after cutting with one of five restiction endonucleases; BamHI, EcoRI, ECoRV, HindIII, and XbaI (from left to right).

In this picture, digested DNA were fractionated on 1% agarose in 1x TBE buffer under 30 volts per 11x17.5 cm^2 for 16 hours and HindIII-digested Lambda DNA was used as size markers (M) for each panel. After that, the DNA fragments were trasferred to GreenScreen Plus® membranes by Southern blotting.



3.2 Electrophoresis of digested DNA

Complete digested DNA were fractionated in 1.0% agarose gel electrophoresis, using 44.5 mM Tris, 44.5 mM boric acid and 10 mM EDTA pH8.0 (0.5x TBE buffer). About 25 -30 µl of each digested DNA was loaded into a well of the gel, with a loading dye containing 2% ficoll 400, 10 mM Na_2EDTA pH8.0, 0.1% SDS, 0.025% bromophenol blue, and 0.025% xylene cyanol. To determine sizes of DNA fragments, bacteriophage Lambda DNA digested with HindIII which generating 7 DNA fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5 kb in sizes were also run in the same gel. The usual running time for electrophoresis was 16 hours at 2.7 volt/cm.

For Southern transferring, 9-10 µg genomic DNA was used for obtaining signal of hybridization in this study.

3.3 Southern bloth transfer

Southern blotting technique used in this experiment was alkaline blotting as described in a manual of GeneScreen Plus Membrane (Dupont).

DNA in each gel was partially depurinated with 250 ml of 0.25 N NaCl for 15 minutes. Genomic DNA was denatured with

0.4 N NaOH for 30 minutes with gentle shaking. Then denatured genomic DNA was transferred onto nylon membrane with the same alkali solution; 0.4 N NaOH, for 18-24 hours. After transfering, this membrane was washed in excess 2xSSC solution (0.3 mM NaCl, 0.03 M sodium citrate) for 1-2 min for removing alkaline and some agarose adhering on the membrane. Then, this membrane was baked for 2 hours at 80°C in a hot air oven to ensure complete immobilization of DNA. The baked membranes were stored at 4°C between each blotting. Blotted gel was also checked for efficiency of DNA transferring by staining the gel in 0.1 µg/ml EtBr for 5-10 minutes and visualized with UV light.

3.4 labelling of DNA probes

3.4.1 DNA fragment preparation

To remove some contaminating materials from DNA fragment prepared by both processes, further purification was needed. For RFLP probes prepared by PCR amplification, contaminants were removed by passing through a mini gel column (Sephadex G-50) and separated by centrifugation at 2500 rpm for 1.5 minutes with a bench-top centrifuge. For RFLP probes prepared by transformation following with a rapid alkali lysis, plasmid were digested with PstI and fractionated in 1.0% LM

agarose gel electrophoresis, using 0.5x TBE buffer. Completly-digested insert fragments of DNA clones were then excised and undergone phenol/chloroform extraction to move any accompanying LM agarose.

After passing through mini gel column and were separation by centrifugation, most DNA were eluted out with an approximate 90% efficiency, though their concentration was reduced. An example of purified DNA probes are also present in panel D of figure 4. By restriction digestion of recombinant plasmids, rice inserted DNA and their cloning vector were separated from one another by gel elctrophoresis in LM agarose then by phenol/chloroform extraction for attaining purified products (data not shown).

3.4.2 Labelling by random priming using (α^{32}P)-dATP

About 50-200 ng of each DNA probe was first denatured into single stands by heating in a 100°C waterbath for 5 mintues and then quickly cool in ice. Single standed DNA were incubated for 5 minutes at room temperature with 11.4 ul of a LS mixture, containing HEPES: DTM: OL solution in a ratio 25: 25: 7 (HEPES = 1M HEPES titated to pH6.6 with NaOH, DTM= 100 mM dCTP, dTTP, dGTP each in 250 mM Tris-HCl pH7.0, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol; OL= 90 units per ml hexadeoxynucleotide,

Pharmacia in TE buffer) 15 minutes at 25-55°C for annealing. Reaction mixture was then added with 1 µl of 10 mg/ml BSA, 2.5 units of large fragment DNA polymerase (Klenow enzyme, Biolab) and 50 µCi (α-^{32}P)-dATP (specific activity 6000 mCi/mmole, Amersham), respectively. Distilled water was added to make up volume to 25 µl of reaction mixture. Incubation of the mixture was performed at room temperature for 1-2 hours after which the determination of incorporated ^{32}P nucleotides into the probe was accomplished by using liquid scintilation counting. Specific activity of each labelled probe was found to be around 1-10x10^{8} cpm/µg.

3.5 Prehybridization, Hybridization, and Washing of membranes

(GeneScreen Plus™'s manual, dupont)

Each selected RFLP probes were hybridized to both parent DNAs, KDML105 and IR36, digested by every restriction type II endonuclease utilizing in this experiment (as in previous method). In table 3, a detail of restriction enzyme/ RFLP probe combinations is presented.

For hybridization, nylon membranes were put into a plastic box which could hold more than two membranes. Prehybridization of membrane was carried out at 65°C for 18

hours for new membranes or 2-3 hours for reprobing-membrane in a solution containing 5x SSC (0.75 M NaCl/0.075 M sodium citrate), 50 mM sodium phosphate buffer pH 7.3, 5x Denhardt's solution (100x Denhardts'solution equals to 5 grams of ficoll, 5 grams of polyvinylpyrolidone, 5 grams of bovine serum albumin fraction 5), 2.5 mM EDTA pH7.5, 0.6% SDS, 5% dextran sulfate and 0.1 mg/ml heated denatured salmon sperm DNA. For each prehybridization, about 50 ml of this solution can totally spread over two pieces of 11x17.5 cm^2 of nylon membrane (and if more than two membranes are hybridized in the same box, this solution should be increased proportionally).

Hybridization was continuously performed in the same solution as prehybridization buffer, but with the presence of denatured radio-labelled probes at 65°C for 18-24 hours.

Hybridized membranes were non-stringently washed twice with 2xSSC at room temperature for 15 minutes each. Later, medium stringent wash was performed twice with 2xSSC, 1%SDS at at 65°C for 30 minutes each and carefully monitored with a Geiger counter. Finally, high stringent wash was complete twice with 0.1x SSC at room temperature for 30 minutes each. During all steps of washing, gentle agitaions were neccesary. The membranes were monitored again before being put in sealed plastic bags. Then, these membranes were placed in a X-ray

film cassette (Okamoto), either 14x17 or 8x10 $Inch^2$ in size with Kodax X-O Mat film using two Dupont's Cronex intensifying screens and autoradiographed at-80°C. After 2-4 days, films were developed for 1 minute in Kodax Gbx developer and fixed for 1 minute, according to the recommended process.

3.6 Reprobing of hybridized membranes

For stripping old hybridized DNA probes, membranes were immersed twice in boiling solution of 10 mM Tris-HCl pH 7.5-8.0, 1 mM EDTA, 1%SDS for 10-15 minutes each. Membranes were maintained with a Geiger counter to assure all probes were entirely removed. With this procedure, blotted membranes could be reused up to 6-8 times. (Gene Screen Plus™'s manual.)

Restriction fragment sizes were determined based on mobilities of Lambda/HindIII bands shown in X-ray film and was calculated into sizes (kb) by using a computor program developed in our laboratory.

4. TAGGING GENE BY USING RFLP ANALYSIS

As mentioned in the previous section F_3 population was used as a genetic population for a gene tagging. In studied

segregating population, three distinctive fragrance characters of each F_3 plant; high aroma, little aroma, and non aroma were scored with numerical symbols; 2, 1, and 0. Among 100 F_2 progenies, fragrance character inherited which obeyed 1:3 Mendelian ratio of (nomozygous) recessive genotype: dominant genotype. One F_3 plant of each F_2 line correlating to either high-aromaticity (2) or non-aromaticity (0) was priorily chosen so that their DNA could be genetic representatives for checking linkage fragrance gene and DNA markers. Thus, 10 DNA samples from various high-aromatic F_3 lines and 16 DNA samples from several non-aromatic F_3 lines were chosen for this DNA analysis.

In the same segregating population, each F_3 progeny was also classified into one of the two uncompatible groups; photoperiod sensitive or photoperiod insensitive, depending on its flowering date. F_3 progenies which could flower within four months (130 days) were designated as photoperiod insensitive rice while F_3 progenies which flowered after 130 days were contrastly singled as photoperiod sensitive rice. Within the same F_2 ascendent, each individual F_3 progeny may not express identical character as other progenies. Therefore representative DNA samples were selected from F_2 line of which all F_3 progenies expressed an uniform phenotype; photoperiod sensitive or photoperiod insensitive, by restricion that

standard deviation among these F_3 progenies (13 plants) must not more than 5. This limitation allowed 14 DNA samples which equally optted from both classes of rice to be genetic representatives in this study.

5. INITIAL RAPD INVESTIGATION IN RICE GENOME

In the preliminary study, 200 primers (No. 101-300 of university of British Columbia, Canada) were studied if they could provide RAPD markers for mornitoring of polymorphism between the two parental DNA of KDML105 and IR36. By following an original protocol of John G.K. Willium et al (24) (both for reaction components and PCR program) with a little modification of house-hold made *Taq* DNA polymerase utilization.

5.1 Amplification conditions

(An original RAPD protocol of John G.K. William et al, 1990)

Amplification reactions were performed in volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 µM each of dNTP, 0.2 µM primer, 25 ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Perkin Elmer Cetus). Amplification was performed for 45 cycles of 1 min at

94°C, 1 min at 36°C, 2 min at 72°C.

Then, amplification products were analysed by electrophoresis in 1.2% agarose gel and detected by staining with EtBr.

5.2 Optimization of reaction components for RAPD analysis

5.2.1 Variation of DNA template concentration

Purified parental DNA (method III) was warmed in 65°C waterbath for 30 minutes before successively diluted to 80, 40, and 20 ng/µl with sterile distilled water (HPLC grade). Aliquots of them were stored in 4°C refrigerator for other usage. When an initial observation of this experiment was performed with primer No.127 and KDML105 DNA, another primer No.237 was also used with IR36 DNA simultaneously, with repeated examination of the previous primer No.127 and KDML105 DNA. After that, other 6 RAPD primers; No.149, 155, 156, 157, 233, and 238, were tested for reaction containing 20 ng of a genomic DNA template for considering ability of amplifibility in those representative primers.

For one 25 µl reaction, 1 µl of each 80, 40, or 20 ng/µl stock parental DNA was diluted with sterile distilled

water (amount used depended on the enzyme amount in each reaction). Then, 1 µl of 10x PCR buffer (comprising 100 mM Tris-HCl pH8.3, 500 mM KCl, 20 mM $MgCl_2$, and 0.01% gelatin, 0.4 µM primer, and 100 nM of each dNTP were added continuously before overlaid the reaction mix with 25-30 µl mineral oil (Sigma) to prevent evaporation (In two reactions of the first examination of primer No.127-KDML105 DNA and primerNo.237-IR36 DNA, DNA template was denatured with 100°C for 10 minutes and maintained at 75°C before adding with 1, 2, or 3 unit *Taq* DNA polymerase. Whereas for other examinations, including the secound one of primer No.127-KDML105 DNA, this step was omitted as well as *Taq* DNA polymerase was added before covering the reaction with mineral oil). Each RAPD reaction mixture was was centrifuged at 1500 rpm for 30 sec and then undergone with 45 cycles of 94°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, and 72°C primer extension for 2 min.

5.2.2 Variations of primer and enzyme concentrations

In case of the previous study of DNA template variation, the reaction primer concentration used was 0.4 µM which was expected for primers excess in the reaction (2 times over primer concentration in an original protocol). In this experiment, two primer concentrations, 0.2 and 0.4 µM, were examined to compare for one another. With the same purpose,

variation of three enzyme concentrations, 0.5, 1, and 2 units enzyme per reaction, were simultaneously performed with primer concentration variation. Primarily, four primers, No.127, 155, 156, and 237 were investigated for the study of both parameters. Since a reliable standard RAPD reaction was required for all RAPD primers to be studied, 20 representrative primers No.101, 103, 127, 152, 155, 156, 157, 173, 181, 208, 219, 222, 230, 237, 241, 289, and 295 were later tested, as well as two and three repeating reactions of each condition (different primer-enzyme concentration) was also performed to check reproducibility within the same experiment of an experiment of an individual primer.

In each experiment, 1 µl of 20 ng parental DNA was diluted with appropiate amount of sterile distilled water to adjust reaction volume to 25 µl. Then, 0.2 or 0.4 µM primer and 100 ng of each dNTP were added continuously. After that 0.5, 1.0, or 2.0 units of *Taq* polymerase (from one cocktail of each enzyme concentration prepared with 1x PCR buffer) was allocated to inividual reaction before centrifugation under 1500 rpm for 30 sec. Those reactions were latter amplified under 45 cycles PCR program of 1 min 94°C, 1 min 36°C, 2 min 72°C and their products were finally detected by gel electrophoresis of 1.2% agarose, 0.5x TBE. Distinctively, one dilution of DNA template was preset for all reactions in the last verification of 1 and 2 units enzyme tested with 0.2 mM

primers (table 7).

5.2.3 Variation between 30°C and 36°C annealing temperature in rice RAPD reaction

For common PCR procedures, a pair of carefully designed primers were normally used to amplify at a specific region of a DNA template and increasing annealing temperature will enhance discriminate against incorrectly annealed amplification (55). On the other hand, common RAPD primers can randomly bind to almost anywhere in a DNA template and the chance of getting amplified products depended on the chance of having close distance between each two primers binding. Thus, there would be possible that more two RAPD primers would bind to their DNA template with an appropiate distance to produce amplified fragments if decreasing annealing temperature involved with opportunity of primer-DNA template combination.

In order to test this assumption, RAPD reactions of the 15 representative primers: No.101, 103, 107, 152, 155, 156, 157, 173, 181, 208, 218, 222, 230, 237, 241, 244, 289, and 295, were used under two similar PCR progrems of 30°C and 36°C annealing temperature. All primers had two sets of triplicated reactions composing 20 ng DNA template, 0.2 µM primers, 100 nM of each dNTP, and 2.0 units *Taq* DNA polymerase (from one

cocktail with 1x PCR buffer). Experimentally, each step of this investigation was performed as in the two previous studies. Then, one triplicated reaction set of those primers was undergone through the 45 cycles PCR program of 94°C DNA denaturation for 1 min, 30°C primer-DNA annealation for 1 min, 72°C primer extension for 2 min when another was amplified with the 45 cycles PCR program of 94°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, 72°C primer extension for 2 min. Reaction products were resolved by gel electrophoresis of 1.2% agarose, 0.5x TBE, were stained in 1.0 µg/ml EtBr, and were detected under UV light. Then, the amplifiability and the reproducibility from differrrent triplicated reaction set of individual primers were compared for each other.

6. PRIMARY OBSERVATION FOR PRODUCT AMPLIFYABLE RAPD PRIMERS WITH THAI RICE DNA (KDML105)

From observation that common RAPD primers can usually develope the number of PCR products in each amplification; an average 2-10 amplified products are predicted to generate by 9-10 nucleotide primers, and polymorphisms result from absent or or present of some specific amplified fragment in different

genome investigated (23). Thus, other 178 primers (from two sets of primer No. 101-200 and 201-300) were primarily screened with a sample of rice genomic DNA (of KDML105) to preview a putative product-amplifyable RAPD primer.

With the optimized reaction of 20 ng DNA template, 0.2 μM primer, 100 nM of each dNTP, 10 mM Tris-HCl pH8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, and 2.0 units of *Taq* polymerase, duplicates were performed for measuring reproducibility within each primer. All experimental processes in this examination were succsessively executed as in previous studies and all RAPD reactions were amplified under 45 cycles PCR program of 1 min 94°C, 1 min 36°C, 2 min 72°C. Finally, reaction products were fractioneted by gel electrophoresis of 1.2% agarose, in 0.5x TBE. The positive product-amplifyable primer was determined to RAPD primers whose products could be uncovered in at least one of duplicate reactions.

CHAPTER III

RESULT

1. PREPARATION OF DNA PROBES BY PCR METHOD

Sufficient amount supply of the DNA probes was neccessary for primary polymorphism screening in parental DNAs and the following verification of putative positive RFLP clones. For rapid replication of DNA probe supply, many of them were amplified by polymerase chain reaction with two M13 universal primers (material). After checking on 1% agarose, most rice DNA clones showed that they were amplified at their insert site since their major amplified fragments (the highest intensity band) possessed sizes which coresponded to their reported sizes as documented by Tanksley's laboratory. Such rapidity promoted RFLP probe preparation with PCR to be more practical for routine preparation of DNA probes than the conventional transformation procedure. In this thesis, some of amplified RFLP probes prepared are presented in figure 4 and their amplified sizes comparable to their reported sizes are shown in the table 4.

Figure 4. Some of amplified RFLP probes amplified by PCR and purified by mini-gel columns

Amplified RFLP probes were checked by running in 1.0% agarose, 0.5x TBE gel electrophoresis, then stained by 0.1 µg/ml EtBr. Lambda DNA digested by two restriction enzymes (HindIII and BstEII) were placed at the left and the far right lanes for comparing these amplified fragment sizes. In panel A, B, and C, major amplified products (the highest intensity band) of each used RFLP probes carried the correspoding size to their reported sizes as documented by Tanksley's laboratory (unpublished data supplied). Panel D presented some of amplified RFLP probes purified by passing through centifuged mini-gel column (at 2500 rpm for 1.5 min). In all amplified RFLP probes purified, only one insert fragment was eluted from each mini-gel column. Such eluted fragment was a major amplified product of respective RFLP probe.

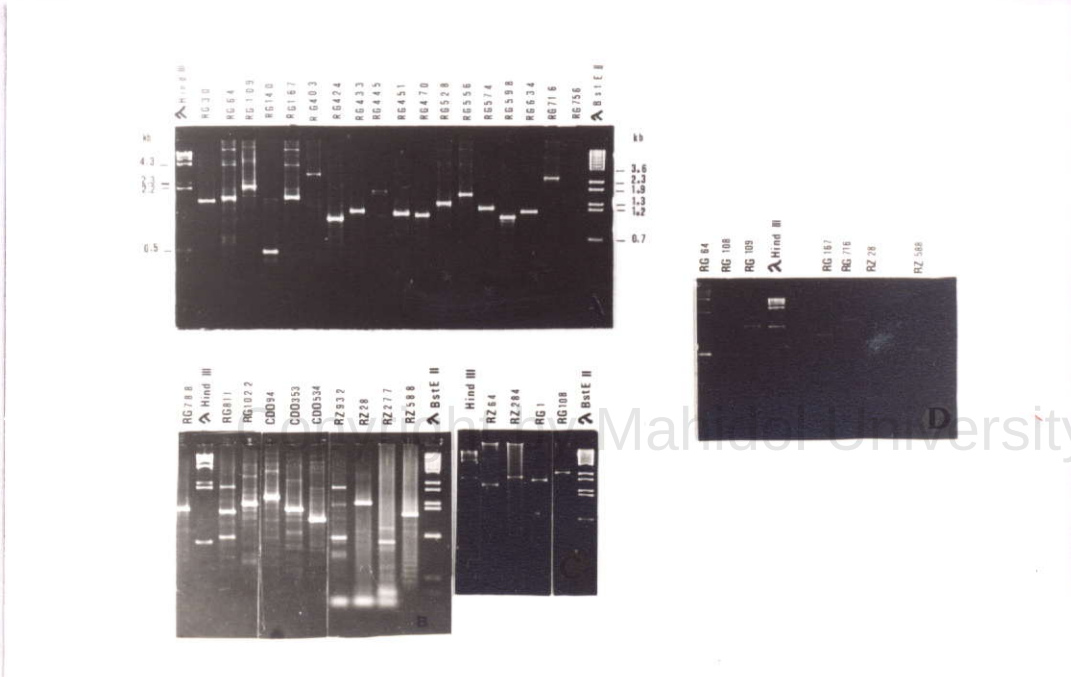


Table 4. Size of selected amplified DNA probe fragments by PCR

For RFLP probes prepared by PCR, the temperature profile used in the PCR programe composed of 2 sets of similar repeating steps; one was 29 cycles of 94°C DNA denaturation for 1 min, 55°C DNA-primer annealation for 1 min, 72°C primer extension for 3 min, and the other was 1 cycle of 94°C DNA denaturation for 1 min, 55°C DNA-primer annealation for 1 min, 72°C primer extension for 7 min. After separating on 1% agarose, sizes of those major amplified products were compared to standard marker sizes of BstEII-digested Lambda DNA and and HindIII-digested Lambda DNA then were calculated by a computer program improved in our laboratory. Those calculated sized of most amplified RFLP inserts (23 of 25 reported amplified RFLP inserts) were correlated with their reported size documented by Tanksley's laboratory. Such result confirmed reliability of this technique for DNA probe preparation.

probes	Size (kb)		probes	Size (kb)	
	reported	amplified		reported	amplified
RG1	1.8	1.73	RG556	1.5	1.64
RG30	1.3	1.35	RG574	1.1	1.26
RG64	1.5	1.48	RG634	1.1	1.17
RG108	1.0	2.3	RG716	2.4	2.52
RG109	2.0	1.88	RG788	1.1	1.29
RG140	1.5	1.42	RG811	1.0	1.21
RG167	1.6	1.49	RG1022	1.1	1.35
RG433	1.1	1.17	RZ28	1.4	1.41
RG445	1.3	1.6	RZ284	1.8	1.92
RG451	1.1	1.12	RZ588	1.0	1.16
RG470	0.9	1.0	CD094	1.6	1.52
RG528	1.3	1.38	CD0353	1.2	1.23
			CD0534	1.0	1.03

2. SCREENING OF POLYMORPHISM BETWEEN KDML105 AND IR36 DNA

Subsequence from a preceeding experiment investigated by Dr. B. Sonthayanon in 1991, RFLP-type DNA probes spreaded about 30 cM or less around the position of fragrance locus (*fgr*) and photoperiod sensitivity locus in the chromosome 8 and 6 were surveyed in order to find RFLP markers which link to the genetic locus. Some example of those RFLP probes were RG28 locus which reportedly linked to a fragrance locus (*fgr*) on the chromosome 8 (Rice molecular map, from the laboratory of S.D. Taksley, Cornell University), RG64, RG123 and RG445 which are close to one photoperiod sensitive locus (*Se-1*) on on the rice chromosome 6 (Rice molecular map in figure 5). Many RFLP probes in other regions were also examined (figure 6 and table 9).

Since the chance for uncovering DNA sequence divergence between the two parental DNAs depended on types of restriction endonucleases used and numbers of RFLP markers utilized for polymorphism screening, 96 RFLP probes restriction enzyme combinations were investigated by 31 additional RFLP probes with nearly all 5 selected restriction enzymes (method). Out of that 31 RFLP probes, 7 probes could uncover polymorphism between KDML 105 and IR36 DNA (22.5% polymorphism). Among such 7 polymorphic RFLP probes, one probe has earlier been located

on the rice chromosome 6 and three probes located on the rice chromosome 8.

Figure 5. Location of *Se-1* and *fgr* loci on a rice molecular genetic map

Photoperiod sensitive locus (*Se-1*) and fragrance locus (*fgr*) of rice genome are reportedly mapped on the chromosome 6 and 8, respectively. From recently report, *Se-1* locus was mapped near to RG64 locus (6.0 cM above the *Se-1* locus) and *fgr* locus was linked to RG33 at a distance of 4.5 cM.

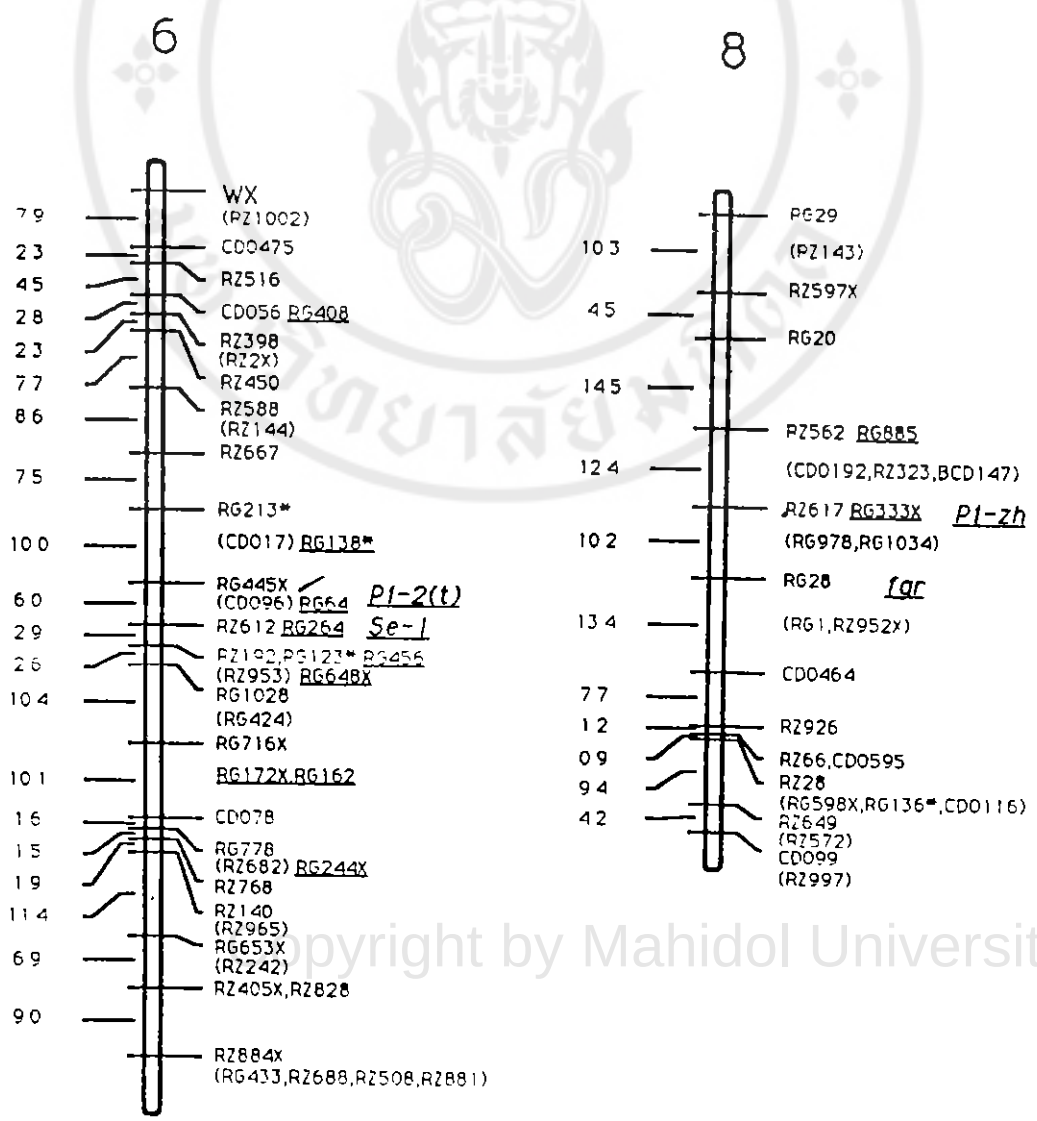


Figure 6. Location of RFLP probes selected for polymorphism screening between KDML105 and IR36 DNAs

All positions of 31 RFLP probes in this picture were correlated with their location in rice linkage map constructed by S.D. Tanksley et al in 1992 in figure 1. From the diagram, other 18 RFLP probes were also surveyed aside from the 13 RFLP probes locating on the 6^{th} and the 8^{th} chromosome.

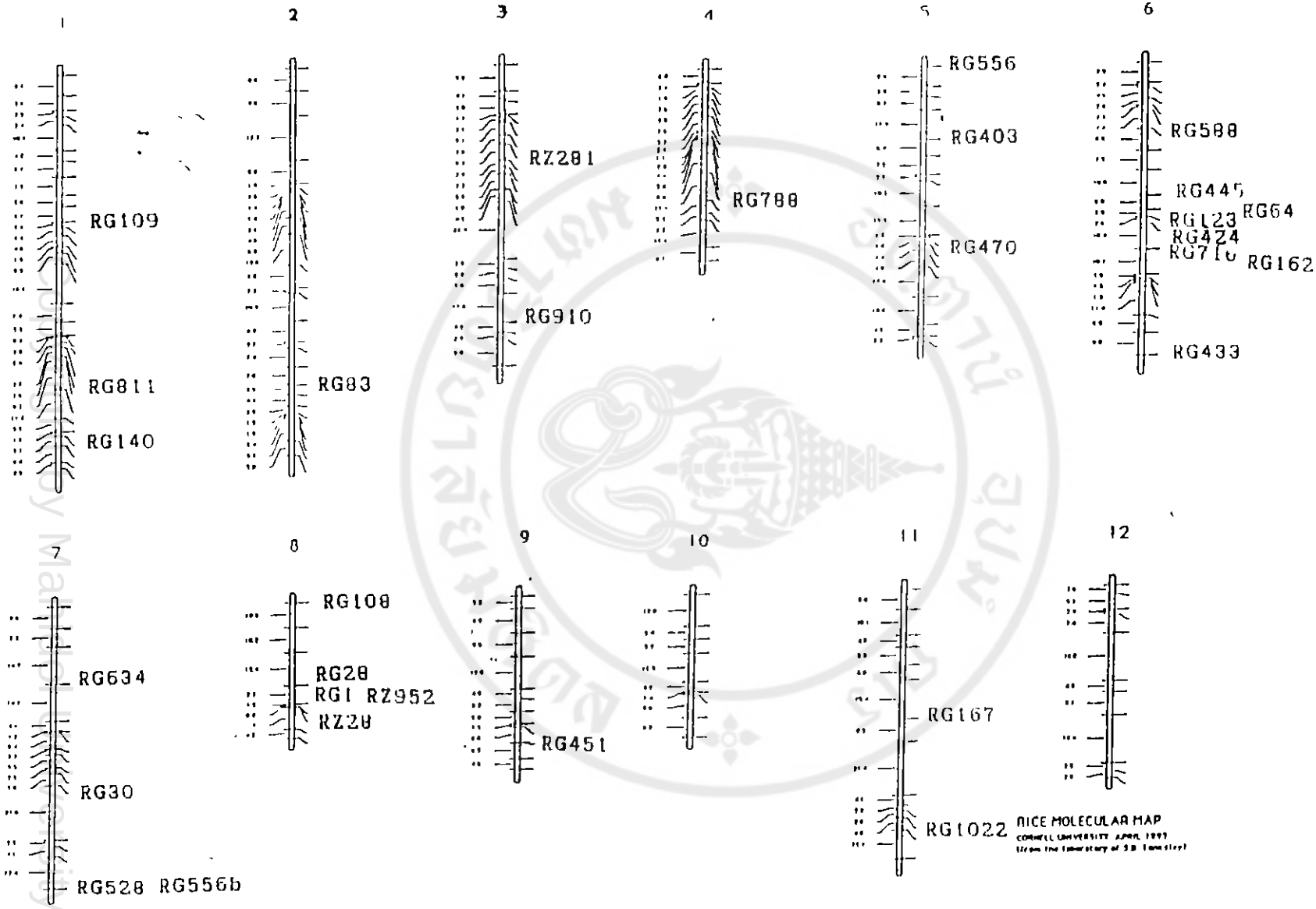

1
RG109
RG811
RG140
2
RG83
3
RZ281
RG910
4
RG788
5
RG556
RG403
RG470
6
RG588
RG445
RG64
RG123
RG424
RG162
RG433
7
RG634
RG30
RG528 RG556b
8
RG108
RG28
RG1 RZ952
RZ20
9
RG451
10
11
RG167
RG1022
12
RICE MOLECULAR MAP

Table 5. List of RFLP probes surveyed between our parental KDML105 and IR36 genomic DNAs

A total of 96 restriction enzyme/ RFLP probe combinations were examined for detecting genetic divergence between KDML105 and IR36 DNA. M or P in the table connotes to the monomorphism (identical DNA patterns) or polymorphism (disimilar DNA patterns) between these two DNAs. From the table, eigth of 96 restriction RFLP probe/enzyme combinations exhibited RFLPs between our parental DNA. In the 8 polymorphic probe/enzyme combinations, two of them belonged to RG445/EcoRI, RG445/XbaI, while other 6 combinations were RG634/HindII, RG1/XbaI, RG28/EcoRV, RZ952/EcoRV, RG451/EcoRV and RG167/EcoRI.

probes	Restriction Endonucleases				
	BamHI	*EcoRI*	*EcoRV*	*HindIII*	*XbaI*
chr#1					
RG109			M		
RG140			M	M	M
RG811	M	M	M	M	M
chr#2					
RG83			M	M	M
chr#3					
RG910				M	M
RZ281			M		M
chr#4					
RG788		M	M	M	
chr#5					
RG403		M	M		
RG470	M	M	M	M	M
RG556				M	M
chr#6					
RG64			M	M	M
RG123	M	M	M	M	M
RG162				M	M
RG424		M		M	M

Table 5. (continuced)

RG433	M	M	M	M	M
RG445	M	P	M	M	P
RG716	M	M	M	M	
RG588		M		M	
chr#7					
RG30	M	M	M	M	
RG529			M	M	
RG556b			M	M	
RG634	M	M	M	P	M
chr#8					
RG1	M	M	M		P
RG28	M	M	P	M	M
RG108	M	M	M	M	
RZ28			M		M
RZ952			P		
chr#9					
RG451		M	P	M	M
chr#11					
RG167		P	M		
RG1022		M	M	M	M

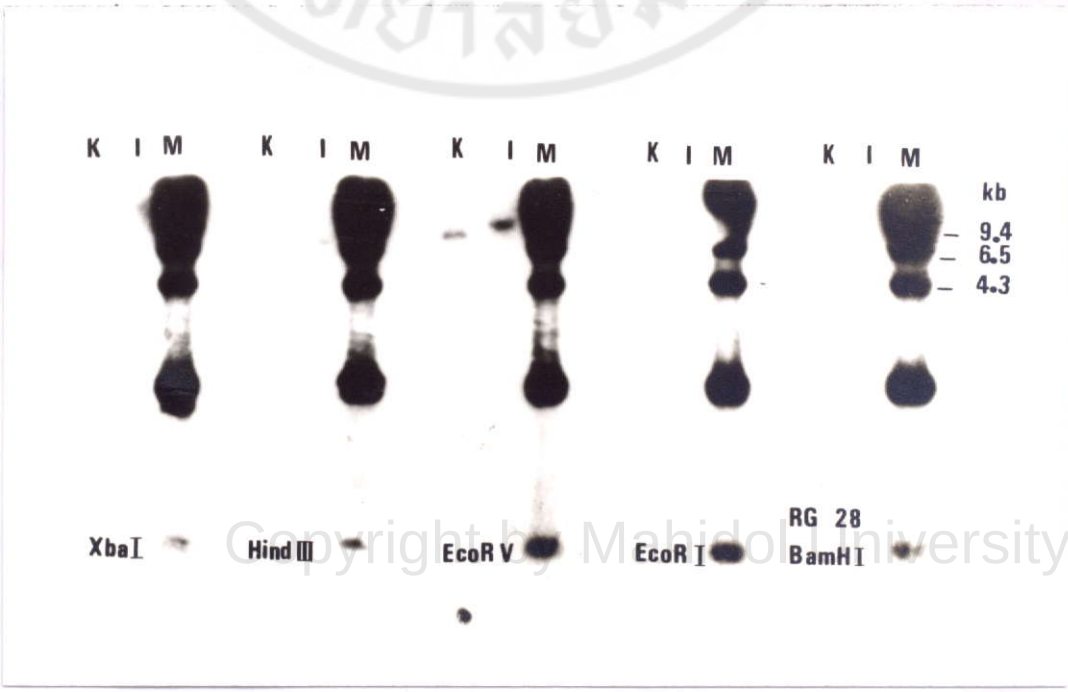
3. POLYMORPHISM SCREENING BETWEEN KDML105 AND IR36 DNA NEAR A FRAGRANCE LOCUS ON RICE CHROMOSOME 8

A total of 20 restriction enzyme/ RFLP probe combinations had been examined during the DNA polymorphism screening. Only 14 restriction enzyme/ RFLP probe combinations, however, were shown on the table 5. Other combinations were conducted by Dr. S. Sonthayanon. These 6 unlisted RE/RFLP probe combinations were HindIII/RG1, EcoRV/ RG136, XbaI/RG108, HindIII/RZ562, ScaI/RZ952, and HindIII/ CD0464. All of them revealed monomorphism between KDML105 and IR36 DNA.

Out of 14 restriction enzyme/ RFLP marker combinations in the table 5, 2 combinations: EcoRV/ RG28 and XbaI/ RG1, were found to reveal polymorphism between the two rice varieties. As mentioned, RG28 was recently reported as a RFLP marker linking to the *fgr* locus on the chromosome 8. Therefore EcoRV/ RG28 combination was selected for further experiment to confirm linkage between the RG28 marker and the aromatic locus in our genetic population.

Figure 7. DNA patterns of the two parental KDML105 and IR36 DNA digested by five restriction endonucleases; BamHI, EcoRI, EcoRV, HindIII, and XbaI (rigth to left) and hybridized with RG28 probe

In this autoradiogram, each panel belonged to only one restriction endonuclease digestion of KDML105 and IR36 DNA and their detectable RFLP sizes were determined by comparing with HindIII-digested Lampda DNA (M). Among the 5 selected restriction endonucleases, only EcoRV could reveal DNA variation of the 8.0 kb restriction fragment from KDML105 DNA and 10.4 kb restriction fragment from IR36 DNA while other 3 restriction endonucleases; EcoRI, HindIII, and XbaI revealed monomorphism which were uninformative.

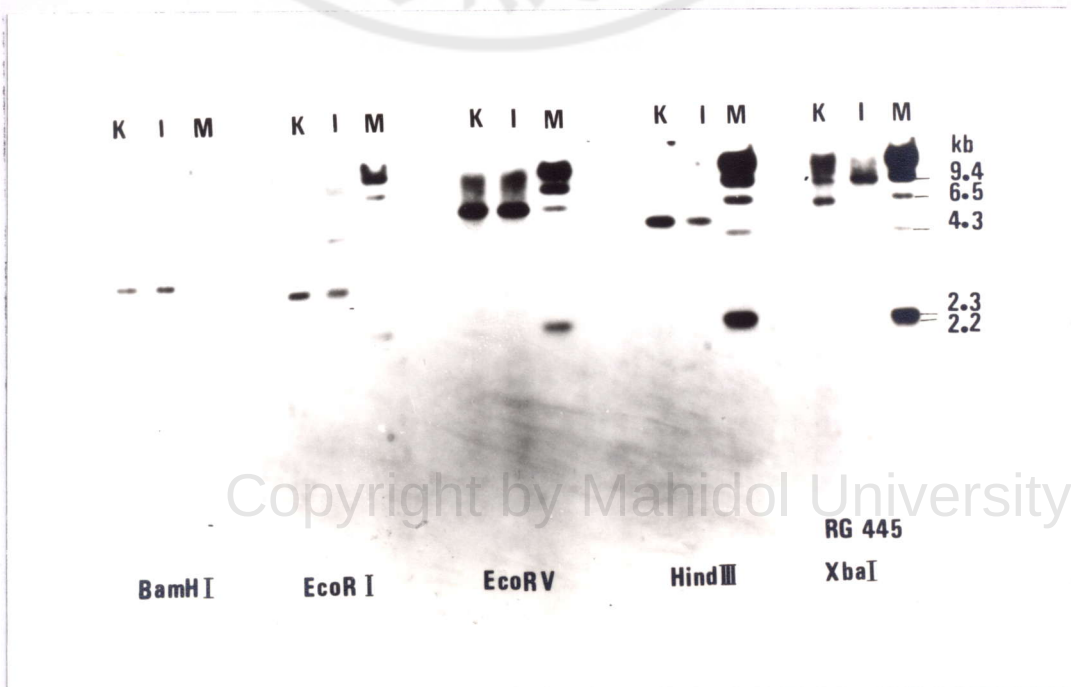


4. POLYMORPHISM SCREENING BETWEEN KDML105 AND IR36 DNA NEAR A PHOTOPERIOD SENSITIVE LOCUS (*Se-1*) ON THE RICE CHROMOSOME 6

Similar to an earier polymorphism screening, 30 RE/RFLP probe combinations were investigated for uncovering DNA level differences between the two parent DNAs but 8 of them are recorded in the table 2. Among those 8 combinations, EcoRV/RZ516 and EcoRV/RZ682 could display DNA variations between the parent DNA while other six combinations; EcoRI/RG64, EcoRV/RG140, EcoRV/RZ588, EcoRV/ RZ612, EcoRV/RZ1003, and XbaI/RG716 revealed monomorphic DNA patterns. For RG64, which reportedly linked to photoperiod sensitive controlling locus (*Se-1*) in rice chromosome 6, could not discriminate between our two parental DNAs, after digested by EcoRI, EcoRV, HindIII, and XbaI. However, a nearby marker RG445, on the chromosome 6 seemed to reveal RFLP with both EcoRI and XbaI. So, RG445 was consequently chosen for further study.

Figure 8. DNA pattern of the two parent DNA; KDML105(K) and IR36(I) DNA, digested by five restriction endonucleases; BamHI, EcoRI, EcoRV, HindIII, and XbaI (left ot right) and hybridized with RG445 rice genomic clone.

This autoradiogram composes of 5 panels of one restriction endonuclease digestion of KDML105 and IR36 genomic DNA. In each panel, Hind III-digested DNA was used as DNA markers (M) for comparing detectable RFLP sizes. For RG445, DNA variation between KDML105 and IR36 could be revealed when digesting with either EcoRI or XbaI; EcoRI digestion reveals distinct of 3.0 kb restriction fragment from KDML105 and 3.0, 4.5 kb restriction fragments from IR36 DNA while XbaI digestion revealed distinct of 5.0 kb fragmemt from KDML105 and 19.2 kb restriction fragment from IR36 DNA.

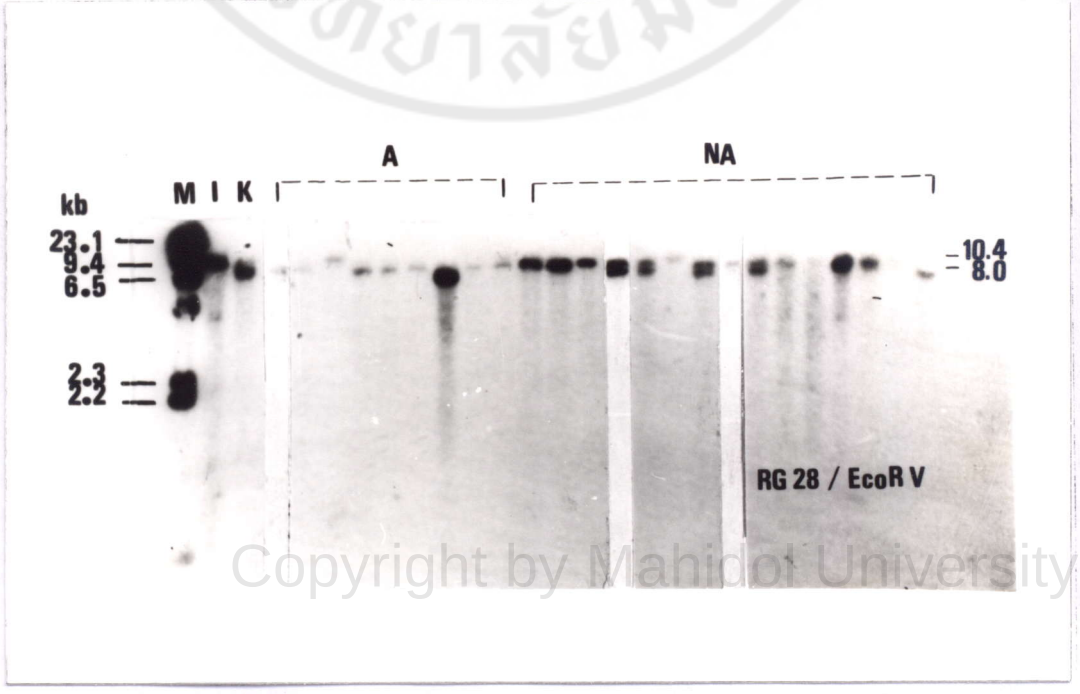


5. CO-SEGREGATION OF THE RG28 LOCUS WITH THE AROMATIC TRAIT LOCUS; *fgr*

As earier mentioned, DNAs for this study were F_3 DNAs derived from F_2 lines whose parental F_2 were also scored for their phenotypes as either highly aromatic (2) or non-aromatic (0). Linkage between the RG28 and the scent phenotype was obtained through usage of 10 highly aromatic and 16 non-aromatic F_3 DNA samples, then following with the second verification of the other 12 non-aromatic F_3 DNA samples (data not shown). In the former experiment, out of 10 aromatic F_3 DNA samples, nine of them possessed an identical restriction fragments as obtaining from their aromatic plant; KDML105 while all of non-aromatic F_3 DNA samples carried a uniform restriction fragment as occuring in their non-aromatic donor; IR36. Moreover, heterozygous DNA pattern could be detected in some non-aromatic F_3 DNA sample as in lane 13, 14, 26, and 18 in figure 9. In later experiment, many non-aromatic F_3 DNA samples expressed heterozygous DNA pattern (when the others could not reveal any visualized restriction fragment). This evidence indicated that there was co-segregation between RG28 and aromatic controlling loci among these tested F_3 DNA samples.

Figure 9. Hybridization of rice genomic clone RG28 to EcoRV digested DNA from the two parent DNA; KDML105 (K) and IR36(I) DNA and their 26 aromatic (A) or non-aromatic (NA) F_3 progenies (2 lanes with too low DNA concentration to detect any signal are not shown).

In this investigation, 89% of aromatic F_3 DNA possessed identical restriction fragment of 8.0 kb as in KDML105 (aromatic parent) while 100% of non-aromatic F_3 DNA exhibit the same restriction fragment of 10.4 kb as in IR36 (non-aromatic parent). Moreover, some non-aromatic F_3 DNA also expressed heterozygous DNA pattern as in lanes 13, 14, 16, and 18. In this picture, molecular weight marker at left hand margin is Hind III-digested Lambda DNA.



6. ANALYSIS OF RG445 LOCUS IN F_3 DNA SAMPLES

In F_2 lines of which all F_3 progenies expressed either photoperiod sensitive or photoperiod insensitive phenotype with standard deviation among these F_3 plant maximizing at 5, only one F_3 plant was chosen as a representative DNA sample for monitoring association between putative positive RFLP marker and the photoperiod sensitive genotype in Thai rice DNA. With this limitation, 14 F_3 DNA samples equally selected from both photoperiod sensitive and photoperiod insensitive F_2 lines have been digested with XbaI then hybridized with putative positive RG445. Unfortunately, although RG445 could uncover DNA variation between KDML105 and IR36 with discrete restriction fragment of 5.0 and 19.02 kb during polymorphism screening as in figure 8, XbaI/RG445 combination could not reveal any polymorphism among investigated F_3 DNA samples in this verification. From this examination, all F_3 DNA samples exhibit uniform fragment of 4.1, 5.4, and 11.6 kb. This observation implied a false positive RFLP marker of RG445 after testing with these representative F_3 DNA.

7. A PRELIMINARY INVESTIGATION ON USING RAPD IN ANALYSIS OF RICE GENOME

From my preliminary RAPD primers screening using 200 RAPD primers, the percentage of primers originating amplified products were too low (10% amplifyable RAPD primers), although most of the 200 primers were recently reported to present a satifying amplification efficiency in other plant genome (65) and later on rice in our laboratory. Thus some reaction components were varied for an only one standard RAPD reaction of rice genome studied by using all 200 primers and *Taq* DNA polymerase (Perkin Elmer Cetus). Informations from these preliminary RAPD experiments such as effective of primer and and template DNA were usefull for the subsequent investigation in our laboratory.

Optimization of RAPD reaction

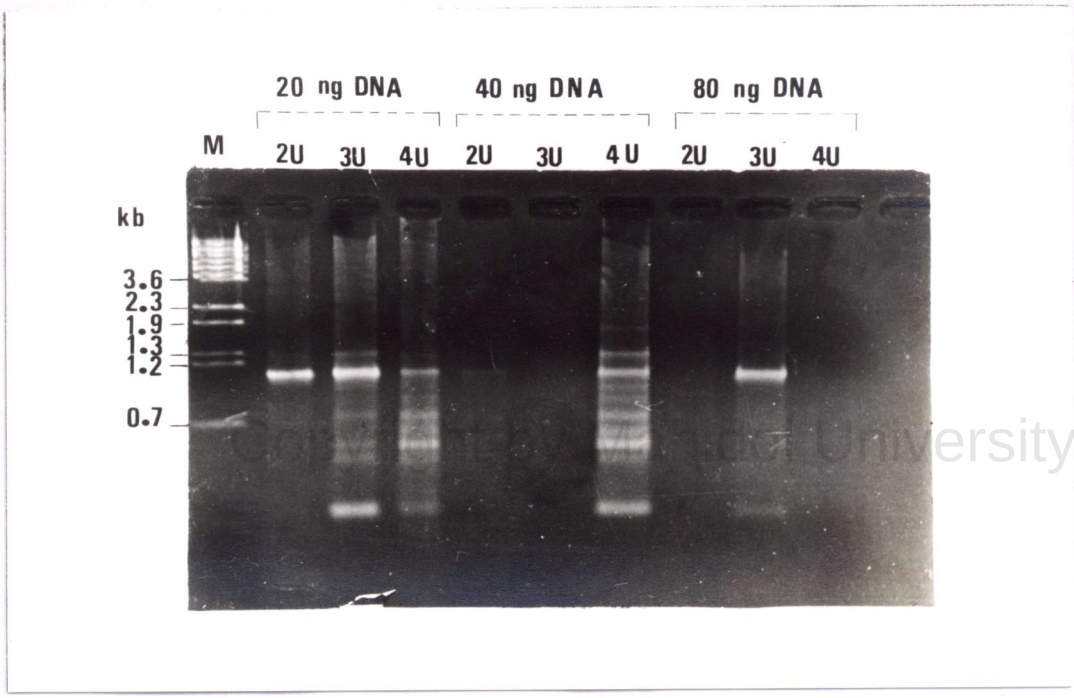
(i) Variation of DNA template concentration

Three concentrations of DNA template were used; 20, 40, or 80 ng. Among them the best that gave products in RAPD reaction was the use of 20 ng DNA template. The result in figure 10 showed an experiment using primer No.127-KDML105 DNA, but a similar examination of primer No.237 using IR36 DNA

was unreported. From result of both experiments, increasing of DNA template concentration tended to reduce amount of amplification products in all enzyme concentrations tested (2, 3, or 4 units per 25 µl reaction) as shown in figure 10, lanes 1-3, 4-6, 7-9. The more *Taq* polymerase used, the more discrete amplification products were observed among reactions which used either 20 and 40 ng DNA template. Whereas this data was not confirmed in 80 ng DNA template; the higher amount of *Taq* DNA polymerase could not improve the amplification ability. Thus, the 20 ng template DNA concentration was then tested with other 6 addition random primers; numbers 149, 155, 156, 157, 230, and 289, in confirm suitable concentration of the 20 ng DNA DNA template usage. All 6 primers examined could present their amplified products with the RAPD reaction containing 1 unit *Taq* polymerase (data unpublished). For this reason, 20 ng DNA template was thus decided upon as an optimal DNA amount for our RAPD reaction. In this report, an example of these result was shown by RAPD reactions of primer No.127-KDML105 DNA in figure 10.

Figure 10. Effect of DNA template concentration on RAPD patterns of rice genome

In an initial experiment, a selected RAPD primer No. 127 and KDML105 DNA was tested for evaluating effect of amount of genomic DNA template. Three different amounts of DNA template, 20, 40, and 80 ng, were examined with varying amount of *Taq* DNA polymerase, from 2, 3, and 4 units. Nine combinations of enzyme-target DNA concentrations were performed in 25 µl reaction each composing of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.4 µM primer, and 100 nM of each dNTP. After 45 cycles of 90°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, and 72°C primer extension for 2 min, amplified products were fractionated by agarose gel electrophoresis of 1.2% agarose, 0.5x TBE. Molecular marker (M) used in this experiment was Hind III-digested Lambda DNA.



(ii) Variation of primer and enzyme concentration

After an optimum amount of DNA template was determined, other two additional parameters; primer and enzyme concentrations, were later optimized to provide a standard reaction condition for both high amplification ability and reproducibility, concurrently.

From a primary examination of 4 primers; No.127, 155, 157, and 234, there was higher chance for four investigated primers to amplify in RAPD reactions containing 0.2 µM primer than in RAPD reactions containing 0.4 µM primer (figure 11). At the same time, when reactions of three enzyme concentrations (method) were compared, reactions containing 2 units of *Taq* DNA polymerase showed the highest amount of amplified products from both 0.2 and 0.4 µM primer reactions. In a confirmatory experiment (table 5), 15 representative RAPD primers, No.101, 103, 127, 149, 152, 155, 156, 157, 208, 218, 222, 230, 237, 241, and 289, were tested to compare their efficiency of amplification. Two additional repeating reactions of the same primer-emzyme concentration were later performed for each individual primer to evaluate their reproducibility as affected by varied primer concentration (0.2 or 0.4 µM) and enzyme (1.0, 2.0, or 3.0 units per reaction) concentration. When the result from this experiment confirmed that usage of 0.2 µM primer in a

RAPD reaction was still more preferable than another of 0.4 µM primer espectially when amplification reaction contained 1.0 and 2.0 units of *Taq* polymerase, usage of 2.0 units enzyme gave highest reproducible result between duplicate reations of an individual primer among three studied enzyme concentrations. In the last experiment (table 6), 12 representative RAPD primers No.101, 103, 127, 149, 152, 155, 156, 157, 208, 218, 222, and 230 were re-examined to compare efficiency of amplification between 0.2 µM primer reactions containing 1.0 or 2.0 units of *Taq* polymerase. This study, triplicate reactions of an individual primer were performed for evaluating their reproducibility. From the last result, usage of 2.0 units enzyme also gave both more productive and more reproducible results than usage of 1.0 unit enzyme. From three correlated results, the selected amount of primer and enzyme for further amplification reactions in this study was, therefore, 0.2 µM primer and 2 unit *Taq* polymerase.

Figure 11. Variation of primer and enzyme concentration in reactions studied by four investigated RAPD primers (No.127, 155, 156, and 237)

Reactions, composing with 20 ng DNA, 0.2 or 0.4 μM primer, 100 ng of each dNTP, and 1.0, 2.0, or 3.0 units of *Taq* DNA polymerase (from one cocktail of each enzyme concentration prepared by 1x PCR buffer), off our investigated RAPD primers (No.127, 155, 156, and 237) were amplified by 45 cycles PCR program of 1 min 94°C, 1 min 36°C, and 2 min 72°C. Then amplified products were separated on agarose gel electrophoresis of 1.2% agrarose, 0.5x TBE by using HindIII-digested Lambda DNA as a marker (M) for size comparision and were detected by staining with 0.1 μg/ml EtBr. Comparing between two studied primer concentrations, there were six of of twelve reactions containing 0.2 μM primer which could produce amplified fragments but there was only one of twelve reactions containing 0.4 μM primer which could amplify (with usage of 3.0 units *Taq* DNA polymerase of IR36-primer No.155). Among six productive reactions of 0.2 μM primer usage, half of them belonged to reactions containing 2.0 units of *Taq* DNA polymerase, two of them belonged to reactions containing 3.0 units of *Taq* DNA polymerase, and the another one belonged to reaction containing 1.0 units of *Taq* DNA polymerase. From this result, reactions comprising with 0.2 μM primer and 2.0 units

of *Taq* DNA polymerase presented more productivity than other primer and enzyme concentrations studied by RAPD primer No.127, 155, 156, and 237.

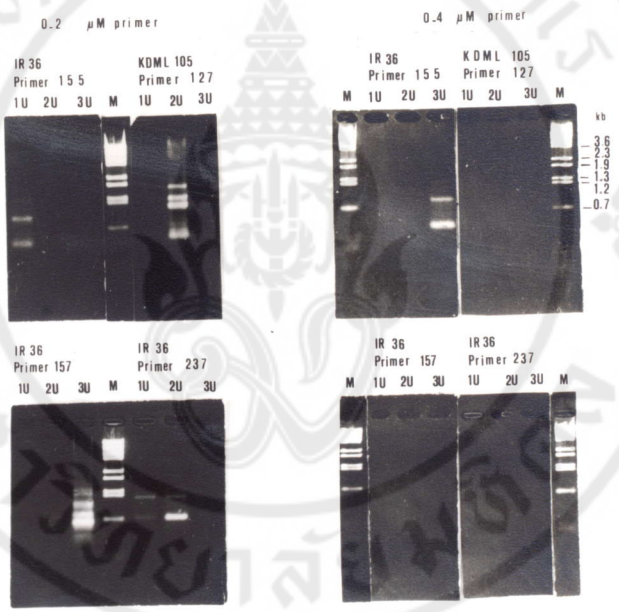


Table 6. **Amplification ability value from 15 RAPD primers studying about the effect of primer and enzyme concentration variation**

In this experiment, 15 representative RAPD primers were selected to study on effect of primer concentration and enzyme concentration to amplify discrete DNA band as well as to check for reproducibility, every primer was done in duplicates. To check effect of *Taq* DNA polymerase concentration, three different enzyme amounts of 0.5, 1.0, and 2.0 units were used. As in the previous result (figure 10), increasing amount of *Taq* DNA polymerase could better produce amplified fragments (bands). In this table, amplification of each primer was ranked in three degree by scale of plus symbol. For the effect of primer concentration, amplification and reproducibility of an individual primer were simultaneously considered within each enzyme concentration. In reaction containing 0.5 units of *Taq* DNA polymerase, though more amplification could be presented at 0.4 µM of primer (4 of 15 tested primers), more reproducibility could be shown at 0.2 µM of primer. Whereas both more amplification and more reproducibility could be presented at 0.2 µM of primer in reactions containing 1.0 unit of *Taq* DNA polymerase; out of 15 investigated primers, 12 were productive and 3 of them were reproducible at reaction of 0.2 µM primer concentration when 5 of 15 investigated primers were productive

and only 1 of them was reproducible at reaction of 0.4 μM primer concentration. For reaction of 2.0 units enzyme, 0.2 μM primer concentration could present fewer amplification and fewer reproducibility than 0.4 μM primer concentration (3 reproducible, 11 productive primers and 5 reproducible, 12 productive primers respectively). However, inconsistent amplified fragments could be more detectable from reaction of 0.4 μM primer concentration than reaction of 0.2 μM primer concentration as in primer No.222 and 230). Thus, 0.2 μM primer concentration was considered to present more reliable reaction (containing 2.0 units enzyme) than 0.4 μM primer concentration. When reaction of 0.2 μM primer concentration were compared between two concentrations for *Taq* polymerase, 1.0 and 2.0 units, although nearly equal amplification and reproducibility were obtained. Not only could more amplified fragments be generated but also more constant products were revealed in the reaction containing 2.0 units enzyme. For this reason, reaction containing 0.2 μM primer and 2.0 units of *Taq* DNA polymerase thus seem to be more advantage reaction than another reaction containing 1.0 unit of *Taq* DNA polymerase.

U of British columbia primer number	*Taq* polymerase											
	0.5 unit				1.0 unit				2.0 unit			
	0.2μM primer		0.4μM primer		0.2μM primer		0.4μM primer		o.2μM primer		0.4μM primer	
101	0	0	+	+	+	++	0	0	0	0	+	+
103	0	0	0	0	0	+	0	0	0	0	0	++
127	0	0	0	+	+	0	0	0	++	++	+	+
149	0	0	0	0	++	0	+++	0	0	+	0	0
152	0	0	0	0	0	0	0	0	+	0	+	0
155	0	0	0	0	+	0	0	0	++	0	++	0
156	++	+	0	0	++	+	+++	0	++	++	++	0
157	0	0	0	0	0	0	+	+	0	++	+++	0
208	+	+	0	0	++	0	0	0	+	+	++	++
218	0	0	0	+	++	++	0	0	0	+	+	+
222	+	0	0	0	+	0	0	0	+++	0	++	+
230	0	0	0	0	++	0	+	0	0	+++	++	+
237	0	0	0	0	++	0	+	0	0	0	0	0
241	0	0	0	+	0	+	0	0	0	0	+	+
289	0	0	0	0	0	0	0	0	0	+	0	0

Table 7. Differentiation between 1.0 and 2.0 units of *Taq* polymerase in the studied reaction containing 0.2 μM of each RAPD primer

To confirm that usage of 2 units *Taq* polymerase provide better result than usage of 1 unit *Taq* polymerase, 12 RAPD primers were tested. Form this study, 1.0 or 2.0 unit of enzyme was used in triplicates. As seen in this table, all of tested primers could amplify in reaction containing 2.0 units of *Taq* DNA polymerase and seven of them could reproduce those amplified fragments among the triplicates of an individual primer. Whereas there were eigth of 12 tested primers which were productive in reaction containing 1.0 unit of *Taq* polymerase and there was none of those 8 productive primers could reproduce their products in all of triplicate reactions. There were 3 of 8 productive primers, No.127, 149, and 222, which could reproduce amplified fragments in only two of triplicate reactions. In addition, when an effective RAPD primer which could amplify in both reactions containing 1.0 unit or 2.0 units *Taq* polymerase was considered, more amount of ampliified fragments could be produced by 5 tested primers No. 157, 208, 218, 222, and 230 in reaction containing 2.0 units of *Taq* DNA polymerase.

U of British columbia primer number	*Taq* polymerase					
	1.0 unit			2.0 unit		
101	0	0	0	0	+	+
103	0	0	+	+	+	+
127	+	+	0	+	0	0
149	++	0	++	++	++	+
152	0	0	0	+	+	+
155	+	0	0	+	0	0
156	0	0	0	+	0	+
157	0	0	+	++	++	++
208	0	+	0	+	++	++
218	0	+	0	+	++	++
222	0	+	+	0	++	++
230	0	0	0	+	+	+

(iii) Variation between 30°C and 36°C annealing temperature in rice RAPD reaction

To study the effect of annealing temperature on amplification of RAPD primer, 18 representative primers No. 101, 103, 127, 152, 155, 156, 157, 173, 181, 208, 218, 222, 230, 241, 244, 289, and 295 were investigated by two PCR programs different from each other only at 30°C or 36°C annealing temperature. Every primer investigated was also performed in triplicate for evaluation of their reproducibility in each PCR program. Amplification used in this experiment composed with 20 ng DNA template, 0.2 μM primers, 100 nM of each dNTP, and 2.0 units *Taq* DNA polymerase (from one cocktail with 1xPCR buffer). In this experiment,

$$\text{average percentage of reproducibility} = \frac{100 \times \Sigma(\text{reproducibility of each primer})}{N}$$

when N = numbers of investigated primers,

reproducibility of primer which could amplify in all triplicated reactions = 100,

reproducibility of primer which could amplify in two of triplicated reactions = 67,

reproducibility of primer which could amplify in one of triplicated reactions = 33.

From this experiment, while all of 18 investigated primers could develope their products with an average of 87% reproducibility by PCR program with 36°C annealing temperature, 13 of the 18 investigated primers could create their products with around 78% reproducibility by another PCR program which primer-DNA annealation occurred at 30°C (table 7). In addition, there were more amplified fragments uncovered by various primers tested (such as primer No.127, 156, 157, and 181 in figure 12) when their reactions were used at a 36°C annealing temperature PCR program. For this reason, a preferable annealing temperature of the PCR program in this study was at 36°C.

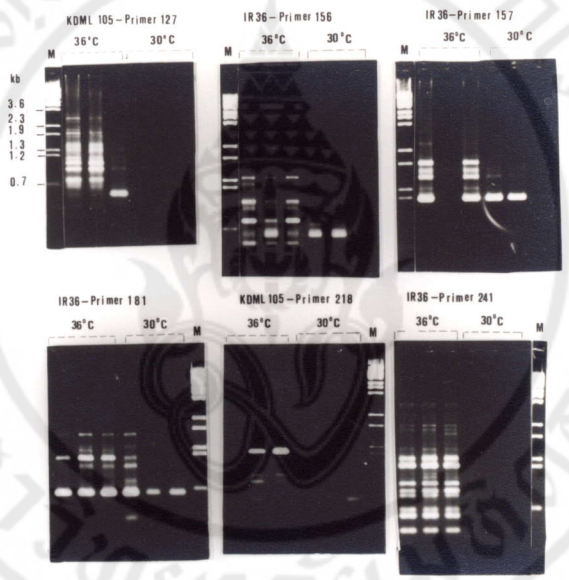
Table 8. **Variation of amplification ability and reproducibility between 30°C and 36°C annealing temperature detected by 18 representative RAPD primers**

In case of annealing temperature effect, though attenuated annealing temperature setting should theoretically promote amplification ability of a 10-mer RAPD primer, after examination not only could amplified products be developed but also some more number of amplified products could be detected at the higer annealing temperature. From this table, all 17 investigated primers which primer-DNA annealation occurred at 36°C were productive and 14 of them could reproduce their products in all triplicate reactions. But 18 investigated primers from 30°C primer-DNA annealation event expressed a lower productity and only little of those productive primers could reproduced their products in all triplicate reactions. Additionally, when the two conditions was compared, some primer displaid a more amplification ability with 36°C annealing event than with 30°C annealing event since some additive products were found in that condition. Among triplicate reactions of each primer, a futile reaction was scored by zero scale (0), on the other hand a productive reaction was recorded by plus scale (+) and additive plus (++ or +++) represent a more number of amplified products detected.

primer number	annealing temperature					
	36 °C			30 °C		
101	+	0	+	0	0	0
103	+	+	+	+	0	+
127	+	0	0	0	0	0
149	+	++	++	0	0	0
152	+	+	+	0	0	0
155	0	0	+	0	+	0
156	+	++	++	+	+	0
157	++	++	++	+	+	0
173	+	++	++	+	+	0
181	++	++	++	+	++	++
208	+	++	++	+	0	0
218	+	++	++	0	0	+
222	+	++	++	0	0	+
230	+	+	+	+	+	0
237	ND	ND	ND	0	+	+
241	+	+	+	0	0	0
244	+	+	+	+	+	+
289	+	+	+	0	0	0
295	++	++	++	+	+	+

Figure 12. **Effect of annealing temperature (36°C vs 30°C) in amplification reactions of rice genome**

This picture presented six samples of 18 representative RAPD primers examined in this study. All of the reactions compose of 20 ng DNA template, 10 mM Tris-Cl pH8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.2 µM primer, 100 ng of each dNTP, and 2 units *Taq* polymerase and were undergone through 45 cycles of either 94°C DNA denaturation for 1 min, 30°C primer-DNA annealation for 1 min, 72°C primer extension for 2 min, or 94°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, 72°C primer extension for 2 min. Their amplified products were identified by gel electrophoresis of 1.2% agarose, 0.5x TBE. In each gel, BstEII—digested Lampda DNA was loaded in the farthest left or right lane as a molecular marker (M). Amplified products of triplicate RAPD reactions amplified by 36°C annealing temperature program were loaded in three lanes of the left hand side and Amplified products of triplicate RAPD reactions amplified by 30°C annealing temperature program were loaded in three lanes of the right hand side.



8. PRIMARY OBSERVATION FOR PRODUC-AMPLIFYABLE RAPD PRIMERS WITH THAI RICE DNA (KDML105)

Finally, an optimized RAPD reaction for rice was set to 20 ng DNA template, 0.2 μM primer, 100 nM of each dNTP, 10 mM Tris-HCl, pH8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin and 2 units *Taq* DNA polymerase and a basic PCR program of 45 cycles of 94°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, and 72°C primer extension for 2 min. In this condition, other 175 primers (from two sets of primer No.101-200 and 201-300) were primarily tested with KDML105 genomic DNA as template. Those were also performed with two repeating reactions to evaluate their reproducibility. Productive primers were recorded when they could produce their amplified products in at least one of duplicate reactions.

Among additional 175 RAPD primers investigated, 70 primers were found productive with an average of 73% reproducibility (formular for calculation was reported earier). Those productive primers could generate around 1-10 amplified fragments from each amplification and such amplified products were in the size range of 0.5-3.6 kb. After adding to the 19 representative primers previously studied during optimization for RAPD condition, therefore, there would be at least about 88 oligonucleotide primers (46%) which could be useful for genetic study in rice.

Table 9. **Primary observation for putative productive RAPD primers with Thai rice DNA (KDML105)**

After a standard RAPD reaction for this study was determined, other 175 RAPD primers were examined for previewing some RAPD primer which could generate PCR products with KDML105 DNA. In this table, there were 70 productive RAPD primers. It should be note that, DNA sequences (Material) of most effective primers which could produce amplified fragments in both duplicate reactions contained about 60-90% G+C content. However, the base percentage were not exactly restricted reproducibility of those all RAPD primers investigated. Since there were some primer whose products could not reproduce between two repeating reactions containing 60% or more G+C content in their DNA sequence.

U of British columbia primer number	PCR product		U of British columbia primer number	PCR product		U of British columbia primer number	PCR product	
102	0	+	123	0	0	144	0	0
104	0	0	124	0	0	145	+	0
105	+	+	125	+	+	146	0	0
106	0	0	126	0	0	147	+	+
107	0	0	128	0	+	148	0	0
108	0	0	129	0	+	150	0	0
109	0	0	130	0	0	151	0	0
110	0	0	131	+	0	153	+	0
111	0	0	132	+	+	154	0	0
112	0	0	133	0	0	158	0	+
113	0	0	134	+	0	159	0	0
114	0	0	135	+	+	160	0	0
115	0	0	136	0	0	161	0	0
116	0	0	137	+	+	162	+	0
117	0	0	138	0	0	163	0	0
118	0	0	139	0	0	164	+	0
119	0	0	140	0	0	165	0	+
120	0	0	141	0	0	166	0	0
121	0	0	142	0	0	167	0	+
122	0	0	143	0	0	168	0	+

Table 9. (continuce).

U of British columbia primer number	PCR product		U of British columbia primer number	PCR product		U of British columbia primer number	PCR product	
169	+	+	191	0	0	212	+	+
170	0	0	192	+	0	213	0	0
171	+	+	193	0	0	214	0	0
172	+	0	194	+	+	215	+	0
174	+	+	195	0	0	216	0	0
175	+	+	196	+	+	217	0	0
176	+	+	197	+	0	219	+	0
177	+	+	198	+	0	220	+	+
178	+	+	199	0	+	221	0	0
179	0	0	200	0	+	223	0	0
180	0	+	201	0	0	224	0	0
182	+	+	202	+	+	225	+	+
183	0	0	203	0	+	226	+	+
184	+	+	204	0	+	227	+	0
185	0	+	205	0	0	228	ND	ND
186	+	0	206	0	0	229	ND	ND
187	0	0	207	0	0	231	ND	ND
188	+	+	209	+	+	232	ND	ND
189	0	+	210	+	+	233	+	+
190	0	+	211	+	+	234	0	0

Table 9. (continuce).

U of British columbia primer number	PCR product		U of British columbia primer number	PCR product		U of British columbia primer number	PCR product	
235	+	0	258	0	0	279	0	0
236	0	0	259	0	0	280	0	0
238	0	0	260	0	0	281	+	+
239	0	0	261	0	0	282	0	0
240	0	0	262	+	0	283	+	+
242	0	0	263	0	0	284	0	0
243	+	+	264	0	0	285	0	0
244	0	+	265	0	0	286	+	+
245	0	0	266	0	0	287	0	0
246	0	0	267	0	+	288	0	0
247	0	0	268	+	+	290	0	0
248	0	0	269	0	0	291	0	0
249	0	0	270	+	+	292	0	0
250	0	0	271	0	0	293	0	0
251	0	0	272	0	0	294	0	0
252	0	0	273	0	0	296	0	0
253	+	+	274	0	0	297	0	+
254	0	0	275	0	0	298	0	+
255	ND	ND	276	0	+	299	+	+
256	0	0	277	0	0	300	ND	ND
257	0	0	278	0	0			

CHAPTER IV

DISCUSSION

In this initial RFLP survey, seven of 31 probes (22.5%) could reveal RFLP between KDML105 (*indica*) and IR36 (*indica*) DNA (table 5). The 22.5 percent polymerase loci was found from this investigation was acceptable for genetic variation within *indica* subspecies of rice though it was much lower to the 58% observed between IR34583 and Bulu Dalam (which one of *indica* and *japonica* subspecies, respectively, (25)) or the 42% observed between IR39352 and Kuang Lu Ai4 (both *indica* subspecies recived from Philippines and China, respectively, (25)). Our lower rate was resonable since the genetic structure of cultivated rice corresponded to their "Continental" and "Insular" dissimilation (58); Thai rice (KDML105) is more closely related with rice from South and and Southeast Asia (IR36) than China rice. The seven RFLP probes detecting polymorphism between KDML105 and IR36 DNA encomposed with one clone located on the chromosome 6, three clones located on the chromosome 6, and three clones located on the other chromosomes.

With 22.5% of RFLP probes detecting DNA polymorphisms in the initial survey, RFLP assay could promise larger extention of varietal determination over traditional means

including isozyme analysis. At present, more than 571 loci in 12 chromosomes of rice had already been mapped by RFLP probes (as in figure 1). According to our percentage between, KDML105 and IR36, we might predict that approximately 128 loci in the 12 chromosomes would be assayable in *indica* rice. That number of expected polymorphic loci were around twice as much over 59 loci detectable with 24 isozymes assayable in rice (table 1). Moreover, not all 59 isozyme loci could be examined, it also depended on genetic diversity between individual varieties of the plants investigated. This is because most isozymes in rice and other plants represent primary products of structural genes which appeared to be well conserved among species, compared to other classes of DNA (60). For this reason, and other advantages of DNA analysis (see introduction), RFLP assay has been considered effective genetic markers in this study.

Table 10. Summary of polymorphism among five selected restriction endonucleases, BamHI, EcoRI, EcoRV, HinDIII, and XbaI, observed in KDML105 and IR36 DNA

Enzymes	Recognition sequence	RFLP probes		% polymorphism
		polymorphic	total	
BamHI	G*GATCC	0	11	0
EcoRI	G*AATTC	2	18	11.1
EcoRV	GAT*ATC	3	25	12.0
HindIII	A*AGCTT	1	23	4.3
XbaI	T*CTAGA	2	19	10.5
Total		8	96	8.3

In table 10, the percentage of RFLP probes detecting polymorphism in our studies was shown with each restriction enzyme investigated. EcoRV was found to give the higest percentage of RFLP among five selected restriction endonucleases when EcoRI, XbaI showned lower percentage whereas HindIII and BamHI could detect little or none polymorphism. This information supported previous finding in rice (10, 25).

tomato (61), which suggested that a probability of detecting genetic variations by RFLP correlated with restriction enzyme used. In contrast, though each restriction endonuclease could still generate averagly large polymorphic restriction fragments, there were no relationship between the number of polymorphism detected by an enzyme (for example polymorphism recived by RG1, RG28, RG451, and RG634). This data slighly was contradicted to an assumption that if RFLPs were arisen largely by base substitutions, as opposed to insertions/ deletions, one would predict no correlation between the amount of polymorphisms detected by an enzyme and the average size of restriction fragments generated by the enzyme (which recognized the same number of base pairs) (25). However, this observation could explain that significant portions of RFLP variations in rice is due to deletions, insertions, or other DNA rearrangements (10), at the same time base substitutions can cause the RFLP variation in closely related rice such as KDML105 and IR36 (the same subspecies and related growing areas).

Co-segregation between RG28 marker and scented genotype among highly aromatic or non-aromatic F_s DNA in this study pointed to a linkage between RG28 marker and fragrance controlling locus in Thai rice genome. This information confirmed an earlier study in rice NILs of Lemnent and aromatic

Lemment by S.N. Ahn *et al* (62) which reported that the aromatic gene (*fgr*) was connected to a sigle-copy DNA clone; RG28, on chromosome 8 at a distance of 4.5 cM. In addition, heterozygous DNA pattern uncovered in many non-aromatic F_3 DNA could be a clue of dominant-recessive relationship of the two alleles at the fragrance locus. Thus, our ability for detecting aromatic gene by linking codominant RG28 marker may provide an effective selection process to Thai rice breeding program .

In the case of photoperiod locus in rice, recent RFLP study had reported tighly linkage of RG64 and this locus on the chromosome 6 between Puang Rai2 and IR26760-27-1-3-2-1 (63, 64). Unfortunately, this marker could not revealed RFLP variation between KDML105 and IR36, in spite of the fact that PR2 was originated from Thailand. This observation might assume to be an multiple allelism affect (34). Though there is no present RFLP document inferring this presumtion in rice, this notion can be advocated by RFLP reports studied in in other plants. For example, in wheat when Xwhs179 marker can detect a restriction fragment of *Pm3* locus conferring powdery mildew resistance, other alleles (*Pm1*, *Pm2*, *Pm3a* and *Pm4*) can express different or null restriction fragment with this marker (34).

AN INITIAL RAPD INVESTIGATION IN RICE GENOME

In our initial study of DNA template concentration, the higher amount of *Taq* DNA polymerase used could improve the amplification ability in reactions containing 20 and 40 ng of DNA. However, this finding was not true at DNA template of 80 ng per reaction. This phenomenon could be explained if one consider interactions between a RAPD primer and target DNA. By random binding of primer molecules to the target sites, too much amount of complex DNA template used could reduce annealing probability of each binding site when a constant primer concentration was maintained in the reaction. Even increasing enzyme amount failed to improve DNA amplification. This notion was experimentally supported when the lower amplified product intensity was detected at the 4 unit *Taq* polymerase reaction of both 20 and 40 ng DNA template (figure 10).

For applicable annealing temperature, it was wildly belived that it should be 5°C below a true T_m of the annealing primer (55). For the RAPD primers used in this study, they had an average T_m of 32°C so the suggested annealing temperature of those primer should be 27°C. In this experiment, a higher annealing temperature (36°C) could develope their products with more reproducibility than the lower temperature (30°C) could. This could be explained by a hypothesis on terminal hairpin

formulation as proposed by Gustovo Caetano-Anolles *et al* in 1992 (65).

From the hypothesis, DNA amplification was postulated to comprise of two levels : (i) the selection of target sites for amplification, which was determined by primer sequence and conditioned by the reaction environment and (ii) the product amplification, which was initiated by a single primer. Following the first template "screening" phase, a set of DNA fragments is synthesized. In the first round amplification products are initially single-stranded and have palindromic termini which allow the formation of hairpin loops. After that, the product can be in the form of template-template and primer-template duplexes, as well as single strand and hairpin loop in the subsequent round of amplification. At this stage, temperature can affect the formation, stability, and equilibium between primer-template duplexes, hairpin loops, and other possible formular DNA molecular species formed during amplification; the increased temperature can unstabilize both duplex constructions. However those rare primer-template duplexes are quickly stabilized by enzyme anchoring and primer extension. Finally, there is thus more accumulaton of amplification product at higher annealing temperature than at the lower annealing temperature Gustovo Caetano-Anolles *et al* in 1992 (65).

Recently, the same set of RAPD primers investigated in this study (primer numbers 101-300 from the University of British Columbia Biotechnology laboratory) have been documentedly tested for amplification ability with genomic DNA of three flowering plants, *Datisca glomerata*, *Yucca baccata*, and *Y. schidigera*, by Peter Fritsch et *al* (66). Such experiment suggested that the ability of a particular primer to produce differential degree of amplified products is not depended the source of DNA examined (according to the degree of amplification of each of the 480 primers investigated was strongly similar among those of three genera and was highly significant). Therefore, this assumption was anticipated to be generally among other plant groups including rice as well.

In this study, our 193 primers examined during screening for productive RAPD primers for rice, a lot of them presented no amplified product (0) in the initial attempts while some 85 productive primers could alway generate discrete products in only one of duplicated reactions (+ and 0). Explanation for dissimilarity seen in amplification ability and reproducibility within an individual primer could be separately considered from two factors; base sequence variation in an individual primer and DNA sequence variation in the genome studied (66).

1. Base sequence variation in an individual primer.

Normally, arbitrary sequence oligonucleotide primers like RAPD primers do not need to perfectly anneal to the target sequence (24). That means amplified products may result from either absolutely or partially binding between primer and target DNA. For promoting amplification, most active arbitrary sequence primers applied for RAPD reaction developing under modulate annealing temperature (ie. 36°C), thus, contain at least 50% G+C content. The result from this study also conformed by the idea; most primers which successfully generated PCR products in duplicated reactions comprised mainly of primers with 60-90% G+C content. Therefore, base sequence variation within individual primer investigated could affect on both amplification ability and reproducibility of RAPD reactions in this study (66).

2. DNA sequence variation in the genome studied.

Similar to base sequence variations in various primers, amplification ability and reproducibility of a RAPD primer can be influenced by both availability and overall distribution of primer complementrary sequences in the genome. Whereas availability of primer binding sequences would allow primers to bind, distribution of the sites would influence probability of each primer to develope PCR products. Thus, a genome containing a lot of binding sites could improve amplification

and genome comprising of many clonly spread binding would increase number of amplified products.

From our preliminary observation for product-amplifiable RAPD primers, experimental objective was served for further application of DNA polymorphism identification although only KDML105 (an *indica* rice) DNA was examined. The result from that experiment shown at least 46% of primers investigated to be available for monitoring genetic variation between DNA of rice and other cereal crops.

Normally, genetic variation detected by DNA markers can caused by either nucleotide sequence alternation of primer binding site for example by point mutation or size differentiation for example by deletion, insertion, or inversion (24). For RAPD markers, nucleotide sequence alteration, even by a single base change in a binding site, may also prevent amplification of certain DNA locus by unstabilizing association between the primer and target DNA site, including a mismatch at just one end of a primer template duplex. Apparently, size differences target DNA regions can also affect on the size of amplified products (deletion, small insertion, or inversion) or absent of specific amplified fragments (large insertion, or inversion) (23, 24).

In the rice genome, recent RFLP report suggested that major RFLPs in cultivated rice were resulted from insertions, or deletions of some nucleotide sequences (25). Comparing this to a general belief that size variations are rarely detected by RAPD primers, since common RAPD reactions tend to produce PCR products of limitted site (normally about <3 kb length). Thus, RAPD reaction migth seem to be less effective than an alternative approch of RFLP probes which cover longer stretch of DNA regions. But if each discrete amplification product of RAPD represented one allele per DNA locus and any 9-10 oligonucleotide primers are predicted to averagly originate 2-10 amplified fragments, RAPD primers will be promising rapid genetic markers to assay DNA variations in genome (23). In another aspects, when a large number RAPD primers can unlimitted survey to whole genome; even to low- or various repeating-copy region, most RFLP probes generally uncover low-copy region of the genome (23). Additionally, while RFLP probes are co-dominant marker for mornitoring Mendelian inheritance, nearly all RAPD primers are dominant dominant markers, as a DNA fragment might be amplified from one individual but not from another). Dominant markers, however, are acceptable for genetic mapping using homozygous parents, so RAPD markers should be usefull to study a natural homozygous (a self-fertilization) plants, including rice. For this above

reasons, both RFLP probes and RAPD primers, thus, can be used an alternative DNA markers to complement each other to study genetics in various eukaryotic organisms.

Recently, G.B. Martin et al (43) had reported used RAPD technique to identify a linkage of a *Pseudomonas* resistance/ sensitive gene in tomato. The co-dominant RAPD primer; R/S 120, could identify genetic variations originated by insertion between two small size amplified fragments; one was 1.2 kb and another was 1.4 kb in length. That result had shown efficiency of RAPD primers to uncover both kind of polymorphism exsisting in an individual organism studied, despite only some size-limitted PCR products could be analysed. In my study, each of 85 candidate primers could reveal around 1-10 discrete PCR products and they were in the size range of 0.5-3.6 kb.

CHAPTER V

SUMMARY

1. In this study on KDML105 and IR36 rice DNA, RFLP probes were shown to be better genetic markers than conventional markers. From 31 investigated DNA probes, seven of them (or 22.5%) could disclose RFLPs between the two two parental DNAs.

2. A RFLP marker, RG28, could uncover RFLPs between KDML105 and IR36 genomic DNA digested by EcoRV during primarily polymorphism screening and was confirmed to be linked to a fragrance locus in Thai rice when studied in representative genomic DNA of aromatic and non-aromatic F_2 plants.

3. RG445/ XbaI combination revealed DNA variation in KDML105 and IR36 genomic DNA during primarily polymorphism screening but this RFLP probe/ enzyme combination could not display RFLPs among 14 genomic DNA samples of F_2 plants equally selected from photoperiod sensitive or photoperiod insensitive F_2 lines.

4. In an initial investigation of RAPD analysis for rice genome, an optimized reaction consisted of 20 ng DNA template, 0.2 µM primer, 2.0 units *Taq* DNA polymerase (Perkin

Elmer Cetus), 100 nM of each dNTP, 10 mM Tris-Cl pH8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin and 45 cycles consisting of 94°C DNA denaturation for 1 min, 36°C primer-DNA annealation for 1 min, and 72°C primer extension for 2 min.

5. From 193 random primers, 85 RAPD primers were found to be of use in analysis of rice DNA. These 85 productive RAPD primers revealed around 1-10 PCR products from each amplification and those products were in the range of 0.5-3.6kb in length.

BIBLIOGRAPHY

1. อัจฉรา วรศิริกุล พืชผลเกษตร ปี ๒๕๓๕/๓๖ วารสารเศรษฐกิจ ธนาคารกรุงเทพ จำกัด ปีที่ ๒๕(มกราคม), ๑๑-๑๓

2. David CC. The World rice economy: Challenges ahead. In: Rice biotecnology. Editted by Khush GS, Toenniessen GH. Oxford: Alden press, 1991:1-18

3. นพดล พิระเสถียร สถานการณ์ข้าวไทย ฤดูการผลิต ๒๕๓๕/๓๖ วารสารเศรษฐกิจ ธนาคารกรุงเทพ จำกัด ปีที่ ๒๕(เมษายน), ๑๖-๑๘

4. Khush GS, Toshiro Kinoshita. Rice karyotype, marker genes, and Linkage groups. In: Rice biotechnology. Editted by Khush GS, Toenniessen GH. Oxford: Alden press, 1991: 83-108.

5. Tanksley SD, Young ND, Paterson AH, et al. RFLP mapping in plant breeding: new tool for an old science. Biotechnology 1989; 7: 257-264.

6. Young ND. Restriction fragment length polymorphisms (RFLPs) and crop improvement. Expl Agric 1992; 28: 385-397

7. Khush GS. Rice breeding: Past, present and future. J. Genet 1987; 66(3): 195-216.

8. Te-Tzu Chang, Bardenas EA, DEl Rosario AC. The morphology and varietal characteristic of the rice plant. The International Rice Research Institute. Los Banos, Laguna. The Philippines. Mail address: Manila Hotel

9. Pham JL, Glaszmann JC, Sano R, *et al.* Isozyme markers in rice: genetic analysis and linkage relationships. Genome 1991; 33: 348-359.

10. Wang ZY, Tanksley SD. Restriction fragment length polymorphism in *Oryza sativa* L. Genome 1989; 32: 1113-1118

11. Toenniessen GH, Khush GS. Prospective for future. In: Rice biotechnology. Editted by Khush GS, Toenniessen GH. Oxford: Alden press, 1991: 309-313.

12. Fraley RT. Genetic engineering for crop improvement. In: Plant biotechnilogy. Editted by Shaine-dow Kung and Artzen CS.

13. Beckmann JS, Soller M. Restriction fragment length polymorphisms and genetic improvement of agricultural species. Euphytica 1986; 111-124

14. Kochert G. Introduction to RFLP mapping and plant breeding applications (The Rockefeller Foundation International Program on Rice Biotechnology) Department of Botany, University of Gorgia Athens GA30602.

15. McCouch SR, Tanksley SD. Development and use of restriction fragment length polymorphism in rice breeding and genetics. In: Rice biotechnology. Editted by Khush GS, Toenniessen GH. Oxford: Alden press, 1991: 109-133.

16. Landry BS, Kesseli RV, Barry F, *et al.* A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics 1987; 116: 331-337

17. Weining S, Langridge P. Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. Theor Appl Genet 1991;82: 209-216.

18. Tanksley SD, Miller J, Paterson A. *et al.* Molecular mapping of plant chromosomes. In: Chromosome structure and function. Editted by Gastafson JP and Appels R. Plenum publishing corporation, 1988: 157-173

19. Davies KE. Genome analysis: a practical approach. Oxford: Oirl press. Washington DC 1988.

20. Robinson C. The genome is on the road. TIBTECH 1992: 10: 1-5

21. Capel J, Montero LM, Martinez-Zapater JM, *et al.* Non-random distribution of transposable elements in the nuclear genome of plants. Nucleic acids research 1993, 21:2369-2373

22. Tanksley SD, Hewitt J. Use of molecular markers in breeding for scluble solid content in tomato: a re-examination. Theor Appl Genet 1988: 75: 811-823

23. Robbie Waung, Powell W. Using RAPD markers for crop improvement. TIBTECH 1992; 10: 186-191

24. Williams John GK, Kubelik AR, Livak KJ, et al. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research 1990, 18(22): 6531-6535

25. McCouch SR, Kochert G, Yu ZH, et al. Molecular mapping of rice chromosomes. Theor Appl Genet 1988; 76: 815-829

26. Reiter RS, Williams Jonh GK, Feldmann KA, et al. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. Proc Natl Acad Sci USA 1992; 89: 1477-1481.

27. Foolad MR, Jones RA, Rodriguez RL. RAPD markers for constructing interspecific tomato genetic maps. Plant cell reports 1993; 12: 293-297.

28. แผนการส่งเสริมข้าวขาวดอกมะลิ การประชุมสัมมนาเชิงปฏิบัติการ เรื่องการจัดแผนประสานความร่วมมือในการพัฒนาข้าวหอมดอกมะลิ กรุงเทพมหานคร ๒๕๓๖ หน้า ๑-๓

29. อรรควุฒิ ทัศน์สองชั้น เรื่องของข้าว (Rice story) พิมพ์ครั้งที่สอง กรุงเทพมหานคร: ภาควิชาพืชไร่นา คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์, ๒๕๒๓: หน้า ๕๕-๗๐

30. Young ND, Zamir D, Ganal MW, et al. Use of isogenic lines and simultaneous probing to identify DNA

markers tightly linked to the *Tm-2a* gene in tomato. Genetic 1988; 120: 579-585.

31. Giese H, Holm-Jensen AG, Jensen HP, *et al*. Localization of the *Laevigatum* mildew resistance gene to barley chromosome 2 by the use of RFLP markers. Theor Appl Genet 1993; 85: 897-900.

32. Graner A, Siedler H, Jahoor A, *et al*. Assessment of the degree and the type of restriction fragment length polymorphism in barley (*Hordeum vulgae*). Theor Appl Genet 1990; 80: 826-832.

33. Graner A, Bauer E. RFLP mapping of the *ym4* virus resistance gene in barley. Theor Appl Genet 1993; 86: 689-693.

34. Hartl L, Weiss H, Zeller FJ, *et al*. Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 1993; 86: 959-963.

35. Menancio-Hautea D, Fatokun CA, Kumar L, *et al*. Comparative genome analysis of mungbean (*Vigna radiata* L. Wilczek) and cowpea (*V. unguiculata* L. Walpers) using RFLP mapping data. Theor Appl Genet 1993; 86: 797-810.

36. Pineda O, Bunierbale MW, Plaisted RL. Identification of RFLP markers linked to the *H1* gene conferring

resistance to the potato cyst nematode *Globodera rostochiensis*. Genome 1993; 85: 921-925.

37. Devine TE, Weisemann JM, Mathews BF. Linkage of the *Fr2* locus controlling soybean root fluorescence and four loci detected by RFLP markers. Theor Appl Genet 1993; 85: 921-925.

38. Zaitlin D, Demars S, Yu Ma. Lingkage of *rhm*, a recessive gene for resistance to southern corn leaf blight, to RFLP marker loci in maize (*Zea mays*) seedings. Genome 1993; 36: 555-564.

39. Hughes MA, Sharif AL, Dunn A, *et al*. Restriction fragment length polymorphism segregation analysis of the *Li* locus in the *Trifolium repens* L. Plant molecular biology 1990; 14: 407-414

40. Yu ZH, Mackill DJ, Bonman JM, Tanksley SD. Tagging gene for blast resistance in rice via linkage to RFLP markers. Theor Appl Genet 1991: 81: 471-476.

41. Ranjeker PK, Gupta VS, Tamhankar SA, *et al*. Rice biotechnology program at NCl, India: RFLP studies on bacterial blight. Sixth annual meeting of the international program in rice biotechnology. Chaing Mai, Thailand, 1993; 22.

42. Lihuang Zhu, Ying Chen, Jichen Xu, *et al*. Tagging genes for resistance and semidwarf in rice. Sixth annual meeting of the international program in

rice biotechnology. Chaing Mai, Thailand, 1993: 14-15.

43. Martin GB, William John GK, Tanksley SD. Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. Proc Natl Acad Sci USA 1991; 88: 2336-2340.

44. Klein-Lankhorst RM, Vermunt A, Weide R. Isolation of molecular marker fortomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). Theor Appl Genet 1991; 83: 108-114.

45. Michelmore RW, Paran I, Kesseli RV. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregation populations. Proc Natl Acad Sci USA 1991: 88: 9828-9832.

46. Haley SD, Miklas PN, Stavely JR, *et al*. Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theor Appl Genet 1993; 86: 505-512.

47. Collins, Synoms. Polymorphisms in grapevine DNA detected by the RAPD PCR technique. Plant Molecular Biology reporter 1993: 11(2): 105-113.

48. Wilkie SE, Issac PG, Slater RJ. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 1993; 86:497-504.

49. Singsit C, Ozias-Akins P. Genetic variation in monoploids of diploid potatoes and detection of clone-specific random amplified polymorphic DNA markers. Plant cell reports 1993; 12: 144-148.

50. Lanham PG, Fennell S, Moss JP, *et al*. Detection of polymorphic loci in *Arachis* germplasm using random amplified polymorphic DNAs. Genome 1992; 35: 855-889.

51. Thein SL, Wallance RB. The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Human genetic diseases: a practical approch. Editted by Davis KE. IRL Press: Herndon Virginia. 1986, p. 33-50

52. Jansen R, Ledley FD. Production of discrete high specific activity DNA probes using the polymerase chain reaction. Gen Anal Techn 1989; 79-83.

53. Maniatis T, Fritsch EE, Sambrook J. Introduction of plasmid and bacteriophage Lambda DNA into *Escherichail coli*. In: Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Lab. 1982; 248-268.

54. Kochert G, Tanksley SD, Prince JP. Laboratory manual for RFLP training course sponsored by the Rockefeller Foundation and held at Cornell University 1989.

55. Innis MA, Gelfand DH. Optimization of PCRs. In: PCR protocols: a guide to methods and applications. Editted by Innis MA, Gelfand DH, Shinsky JJ, and White TJ. Academic press USA, 1990; 3-12.

56. Saiki RK. Amplification of Genomic DNA. In: PCR protocols: a guide to methods and applications. Editted by Innis MA, Gelfand DH, Shinsky JJ, and White TJ. Academic press USA, 1990; 21-27.

57. Compton T. Degenerate primers for DNA amplification. In: PCR protocols: a guide to methods and applications. Editted by Innis MA, Gelfand DH, Shinsky JJ, and White TJ. Academic press USA, 1990;39-45.

58. HI Oka. Genetic Diversity of Wild and cultivated rice. In: Rice biotechnology. Editted by Toenniessen GH, Khush GS. Oxford: Alden press, 1991: 55-81.

59. Glasmann JC. A varietal classification of asian cultivated rice (*Oryza sativa* L.) bases on isozyme polymorphism. In: Rice Genetics: proceeding of international rice genetics symposium. Editted by International Rice Research institute. Manila, Philippines, 1986:

60. Second G. Isozymes and phylogenetic relationship in *Oryza*. In: RiceGenetics: Proceeding of international rice genetics symposium. Editted by International Rice Research Institute, Manila, Philippines, 1986:

61. Miller JC, Tanksley SD. Effect of different restriction enzymes, probe source, and probe length on detecting restriction fragment length polymorphism in tomato. Theor Appl Genet 1990; 80: 385-389.

62. Ahn SN, Bollich CN, Tanksley SD. RFLP tagging of a gene for aroma in rice. Theor Appl Genet 199; 84 825-828.

63. Mackill DJ, Salam MA, Wang ZY, et al. A major photoperiod-sensitivity gene tagged with RFLP and isozyme markers in rice. Theor Appl Genet 1993; 85: 536-540.

64. Monton Poonyarit, Mackill DJ, Vergara BS. Genetics of photoperiod sensitivity and critical daylength in rice. Crop Sci 1989; 29: 647-652.

65. Caetano-Anolles G, Bassam BJ, Gresshoff PM. Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligo-nucleotides. Mol Gen Genet 1992; 335: 157-165.

66. Fritsch P, Hanson MA, Spore CD, et al. Constancy of RAPD primer amplification strength among distantly related taxa of flowering plants. Plant molecular Biology Reporter 1993; 11(1): 10-20.