"IDENTIFICATION OF FUNCTIONAL MARKERS FOR THERMOSENSITIVE GENIC MALE STERILE RICE (*Oryza sativa* L.)"

By

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DECLARATION

I hereby declare that this thesis entitled "**Identification of functional markers for thermosensitive genic male sterile rice (*Oryza sativa* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Identification of functional markers for thermosensitive genic male sterile rice (*Oryza sativa* L.)"** is a record of research work done by **Mr. Vipin Mohan (2011-09-112)** under my guidance and supervision and this is not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µl	Micro litre
µM	Micro Molar
AFLP	Amplified Fragment Length Polymorphism
bp	base pair
CAPS	Cleaved Amplified Polymorphic Sequence
cm	Centi Metre
cM	Centi Morgan
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
et al.	And other co workers
etc	Etcetera
g	Gram
g-1	Per gram
hrs	Hours
kb	Kilo base

M	Molar
MAS	Marker Assisted Selection
mg	Milli gram
min	Minutes
ml	Millilitre
mM	Milli molar
nm	Nanometre
ºC	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
PAGE	Poly Acrylamide Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
sec	Seconds
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeats
STS	Sequence Tagged Site
UV	Ultra Violet
V	Voltage

Introduction

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the major cereal crop grown worldwide and is the staple food for about a half of the world's population. It occupies 154 million hectares annually with a total production of 600 million tons all over the world which accounts for about 35%–75% of the total calories consumed by more than 3 billion Asians (Khush, 2005). FAO's latest projection indicates that total rice consumption would increase from the present 472 million metric tons to 533 million metric tons in 2030. India annually plant rice on a total area of 43.9 million hectares, which produces an average of 130 million metric tons of rice. Under current consumption rate of rice, the production needs to reach 158 million metric tons in next 10 years, with an annual growth rate of 2.4%, to satisfy the population, which will grow at 1.9% (Robin *et al*., 2010).

Oryza sativa is a grass belonging to Poaceae family with a genome consisting of 430Mb across 12 chromosomes. It includes two major subspecies: non-sticky, *indica* variety (long grained) and sticky, *japonica* or *sinica* variety (short grained). Tropical japonica is another subspecies, which is broad grained and thrives under tropical conditions.

The technology of hybrid rice production makes a feasible method to break the yield reduction and can meet the demands of growing population very effectively. As rice flower is very small, the emasculation procedure is very difficult for hybrid rice production. Majority of the people in Kerala prefer red rice variety. So the availability of a male sterile lines can be used for the production of hybrids of red rice varieties. Two types of male sterile systems are present viz; cytoplasmic male sterile (CMS) and environment sensitive genetic male sterile system (EGMS). Cytoplasmic male sterile (CMS) system has a major drawback of limiting the use of rice germplasm to only 20-30% for which it needs an effective maintainer lines. Also CMS system hybrids have only 15–20% yield as compared to high yielding varieties. To overcome these

constraints, two novel genetic male sterility system have been identified viz., thermosensitive genic male sterility (TGMS) and photoperiod sensitive genic male sterility (PGMS). TGMS can be used for the production of two-line hybrids in tropical regions like India, where significant temperature variations are available between kharif and rabi seasons. Moreover, it has got higher magnitude of heterosis.

It is difficult to select the plants at an early stage based on phenotype while developing hybrids of red rice using TGMS. With the use of molecular markers, it is possible to select desirable traits more directly at seedling stage. Several thermosensitive male sterility markers have been identified in different chromosomes of rice plants and most of them are SSR markers.

EC 720903, is a male sterile rice variety imported from IRRI Philippines which is similar in morphology and physiology to the high yielding varieties Uma and Jyothy, and are extensively grown in Kerala. These similarities helps to genetically transfer genes between each other. So far there is no reported genetic information available about the sterility gene involved in EC 720903. So the present study was carried out to identify the functional marker associated with thermosensitive genic male sterility in rice (EC 720903) with the help of SSR marker, so that this identified gene can be easily transferred to Uma and Jyothy rice varieties so as to use it for further breeding programs.

Review of literature

2. REVIEW OF LITERATURE

2.1 THERMOSENSITIVE GENEIC MALE STERILITY

Male sterility in rice is classified into four major groups: male sterility caused by cytoplasmic male sterility (CMS), photoperiod-sensitive genic male sterility (PGMS), thermo-sensitive genic male sterility (TGMS) and other genic male sterilities. CMS, PGMS and TGMS have been used for practical hybrid production in rice. In TGMS, the sterility and fertility is controlled by environmental temperature changes (Wang *et al.,* 2003). Several TGMS genes in rice have been reported from China (Sun *et al.,* 1989), Japan (Maruyama *et al.,* 1991), and the International Rice Research Institute (IRRI) in the Philippines (Virmani and Voc 1991). The TGMS trait is controlled by a single recessive gene (Yang *et al.,* 1992; Borkakati and Virmani 1996; Reddy *et al.,* 2000) and can be introduced into any local rice variety. The pollen donor parent can be any rice variety thus giving a wider choice for producing hybrids of desired traits.

The pollen abortion in male sterile rice plants are of five types: stainable abortion, spherical abortion, nuclear proliferous type, typical abortion, stainable abortion and non-pollen type, (Li, 1999). Typical pollen abortion is seen in most of the TGMS lines because of insufficient starch accumulation (Peng *et al*., 2006). Normally TGMS plants shows sterility at higher temperature and fertility at lower temperature however in rTGMS (reverse TGMS) low temperature triggers the development of sterile plants (Peng *et al.,* 2009).

The discovery of TGMS system made a breakthrough in hybrid rice breeding and the production of hybrid rice seeds. The use of the TGMS in breeding of rice reduce man power, less expensive, time-saving, effective, simple and can also overcome the limitations of the three line breeding system (Rongbai *et al.,* 2005).

However introduction of such TGMS gene through conventional breeding is very complicated. It involves, the selection of TGMS plants in each segregating generations and subsequent induction of fertility by maintaining the suitable temperature. In order to accelerate the production of TGMS lines in desired genetic background, scientists have identified a new method of selection from a wide segregating population that is Marker Assisted Selection (MAS).

2.2 MARKER ASSISTED SELECTION

Marker assisted selection uses unique DNA sequences (markers) which offers an alternative to normal selection difficulties. Traditionally it takes at least two months for flowering, and there after staining the pollen grains will give the fertility/sterility information which is a time consuming process whereas in MAS, male sterile plants can be identified even at the seedling stage. Its application in hybrid rice production will greatly reduce the time consumption, resources involved and labour cost (Wang *et al.,* 1995).

DNA markers or molecular markers are typically derived from a small region of DNA that show sequence polymorphism. The use of DNA marker increases the speed of selection process and permits the genetic screening of the progeny at every generation (Babu *et al*., 2004). The closely linked DNA markers in the target genome region can be used to accelerate fixation of favorable alleles in the next selection.

The idea for marker-assisted selection dates back to 1923. Sax (1923) reported that, there is an association with seed weight (polygenic, quantitatively inherited trait) and seed colour (monogenic trait) in beans (*Phaseolus vulgaris* L.). He concluded that gene controlling the seed colour and genes controlling seed size are linked. In 1961, Thoday made a first attempt to map and characterize all polygenes controlling a trait with the help of monogenic markers. Working with morphological markers, the main limitation of his work was the fact that only a small number of suitable markers were available. During early 1980s, protein based allozyme markers were used as a tool for

distinguishing genotypes. Allozyme markers are allelic forms of enzymes and can be separated on PAGE and detected by staining. This method has the advantages such as technical simplicity, the codominant nature and low costs. Co-dominance means homozygous and heterozygous genotypes can be distinguished.

With the advance of DNA based markers the genetic differences can be observed at the DNA level. Hence more polymorphisms can be identified and breeders could find out large numbers of markers distributed throughout the genome of any species. Markers can be used to identify associations with desired traits, independent of their stage specific expression (Ruane and Sonnino, 2007). Restriction fragment length polymorphisms (RFLPs) was the first reported DNA-based genetic markers (Botstein *et al.,* 1980). In 1986, Bernatzky and Tanksley constructed the RFLP based linkage map in tomato plant. Later, Paterson *et al.* (1991) resolved quantitative traits into discrete Mendelian factors using a complete RFLP linkage map.

Other than the hybridization-based markers (RFLPs), various PCR based DNA markers such SSRs (Powell *et al.,* 1996), SNPs (Gupta *et al.,* 2001), RAPDs (Williams *et al.,* 1990) and AFLPs (Vos *et al*., 1995) were also developed. Currently genome research is focusing on generating functional markers that can help in identifying genes associated with certain traits. This enables the use of genetic markers in crop improvement programmes. Hence the breeders will be able to design genotypes *in silico* and practice whole genome selection. So there is a gradual movement from marker assisted breeding to genomics assisted breeding. (Varshney *et al.,* 2005).

2.3 PGMS/TGMS GENES

Numerous TGMS/PGMS genes have been identified and mapped on different rice chromosomes. Four PGMS genes, *pms/tms12-1, pms1*, *pms3* and *pms2* are mapped to chromosomes 12, 7, 12 and 3, respectively (Zhou *et al.,* 2011; Mei *et al.,* 1999; Zhang *et al.,* 1994; Lu *et al.,* 2005). In chromosomes 9, 1 and 8, three reverse PGMS

genes, *rpms2, CSA,* and *rpms1* respectively have been mapped. (Zhang *et al.,* 2013; Peng *et al.,* 2008). The thirteen TGMS genes identified in rice are shown in Table 1.

2.4 TGMS GENES AND THEIR LINKED MOLECULAR MARKERS

Wang *et al*. (1995), were the first reporters of tagging and mapping the TGMS gene in rice (*Oryza sativa*). They identified a RAPD marker associated with *tmsl* gene. For this study, they used bulked segregant analysis of the F_2 population obtained from the cross between 5460S × Hong Wan 52. They found that one single-copy fragment (TGMS 1.2) of size, 1.2 kb was amplified by primer OPB-19 and it was co-segregated with the TGMS gene *tmsl.* Then it was mapped on chromosome 8 with a genetic distance of about 19.3 cM from the marker RZ562 on one side and 5.0 cM from the marker RG978 on the other side. Molecular mapping of a TGMS gene in rice using bulked segregant analysis was then reported by Subudhi *et al.* (1997). TGMS 1.2 has been sequenced and converted to a sequence-tagged site (STS) marker, which is available for public use. Zhou *et al.* (2011) found out a candidate gene of the photoperiod and thermo-sensitive genic male sterile gene *pms1(t)* in rice. For this study they have used Peiai64S as PTGMS (photoperiod and thermo-sensitive genic male sterile) plant. With simple sequence repeat (SSR) and, insert and deletion (In-Del) markers, they mapped the gene *pms1(t),* by a strategy of bulked-extreme and recessive-class approach. The mapping population for this experiment was the F_2 population obtained from the cross between Peiai64S and 93-11 (*indica* restorer). Finally they identified the location of *pms1(t)* gene as the region between the RM21242 (0.2 cM) and YF11 (0.2 cM) markers on the short arm of chromosome 7. RM6776 (101.1 kb region) was the functional marker associated with *pms1(t)*gene. Fourteen predicted loci were found in this region by The Institute for Genomic Research (TIGR). Based on the function of locus, LOC_Os07g12130 identified by bioinformatics analysis, it is predicted to encode a protein containing a Myb-like DNA binding domain. The RT-PCR (Reverse Transcription Polymerase Chain Reaction) results revealed that the

Table 1. TGMS genes with SSR primers

Sl. No.	TGMS gene	Linked Marker	Chromosome location	References
1.	*tms1*	OPB-19(RAPD), RM 239	8	Wang *et al*., 1995
2.	*tms2*	RM11, RM2	7	Yamaguchi *et al*., 1997
3.	*tms3*	OPF-18,OPAC-3 (RAPD), SCAR	6	Subudhi *et al.,* 1997
4.	*tms4*	RM27, AFLP, RFLP	2	Dong *et al*., 2000
5.	*tms5*	RM174,RM5862, RM5897	2	Wang *et al*., 2003
6.	*tms6*	RM3351, RM2020	5	Lee *et al*., 2005
7.	*tms7(t)*	-	7	Rongbai *et al*., 2005
8.	*tms8*	RM21,RM224,RAPD, SCAR	11	Hussain *et al*., 2012
9.	*tms9*	Indel37,Indel57	2	Sheng *et al.,* 2013
10.	*Ms-h*	-	9	Koh *et al*., 1999
11.	*rtms-1*	-	10	Jia *et al*., 2001
12	*tmsX*	-	2	Peng *et al*., 2006
13	*Ptgms2-1*	-	2	Xu *et al*., 2011

mRNA levels of LOC_Os07g12130 were altered in different photoperiod and temperature treatments with thermosensory response. Thus they concluded that the locus LOC_Os07g12130 is a candidate gene for *pms1(t)*.

The *tms2* gene encodes indole acetic acid hydrolase (IAAH) which converts naphthalene acetamide (NAM) to the potent auxin naphthalene acetic acid, a phytotoxic derivative. This gene, under the control of the manopine synthase gene 2 promoters from *Agrobacterium tumefaciens* and exogenously applied NAM, have been used effectively as a negative selector in insertional mutagenesis of *Arabidopsis thaliana* (Sundaresan *et al*., 1995). Yamaguchi *et al.* (1997) reported the linkage of molecular markers with the TGMS gene, *tms2*. They estimated the locus of *tms2* using Restriction Fragment Length Polymorphic (RFLP) markers in an F_2 population derived from a cross between Norin PL12 and Aus variety Dular. They concluded that *tms2* gene was located between the markers R643A and R1440, with a distance of 0.2 cM from R643A on chromosome 7. Lopez *et al.* (2000), identified two SSR markers RM2 and RM11 flanked to *tms2* gene. These two markers showed 96.6% accuracy in TGMS plants selection. Alcochete *et al.* (2005) mapped the gene *tms2* in chromosome 7. For this study they used F_2 population, derived from the cross between fertile and sterile *indica* (TGMS) lines. On the basis of microsatellite markers, they constructed the genetic map. Based on the continuous variation in phenotypic selection of progenies, they found that the zygotic selection and TGMS genes were directly related. The constructed linkage map distance was 1,213.3 cM, based on the segregation data of the F_2 population. Out of 116 microsatellite polymorphic markers 95 of them showed polymorphism between individuals and they identified three quantitative trait loci (QTL) for thermosensitive genic male sterility in *indica* rice. Two of the quantitative trait loci were mapped on chromosome 1 and chromosome 12 but they have no association with the genetic control of the TGMS trait. The third QTL was mapped on chromosome 7, where a TGMS locus (*tms2*) was present.

Dong *et al.* (2000), identified four AFLP markers for distinguishing TGMS plants from none TGMS plants. The F_2 plants obtained from the cross between rice lines, TGMS-VN1 and CH1 were the mapping population. Out of 200 amplified fragment length polymorphism (AFLP) primer combinations, four AFLP markers

(E2/M5-600, E3/M16-400, E5/M12-600, and E5/M12-200) showed polymorphism. On the basis of identified molecular markers they found that the linked gene is *tgms-vn1* and subsequently identified its chromosomal location on the linkage map of rice. The marker E5/M12-600 showed polymorphism in RFLP analysis and was closely linked to the TGMS gene at a distance of 3.3 cM. This marker was subsequently mapped on chromosome 2. The results confirmed that SSR marker RM27 was linked to the gene *tgms-vn1*. Then they designed a new PCR based marker from the closest AFLP marker, E5/M12–600.

Subudhi *et al.* (1997) mapped TGMS gene *tms3(t)* in the F_2 population obtained from the cross between TGMS mutant line (IR32364TGMS) and IR68. They have reported that out of 389 arbitrary primers in bulked segregant analysis, four of them were identified as linked RAPD markers. While three primers (OPF182600, OPB19750, and OPAA7550) were linked to *tms3(t)* in repulsion phase and one primer (OPAC3640) was linked to *tms3(t)* in coupling phase. When the markers were used to probe the genomic blots, all four markers were low-copy number and two of them (OPF182600 and OPAC3640) detected polymorphism. By using a mapping population available at IRRI, the RAPD marker OPAC3640 was mapped to the short arm of chromosome 6. They also tried some RFLP markers for linkage analysis, but RFLP markers did not show any polymorphism between parents. Thus they partially sequenced both the ends of polymorphic markers and designed primers. Latter, Lang *et al.* (1999), developed polymerase chain reaction based markers for TGMS gene *tms3(t)* in rice (*Oryza sativa* L.). Two codominant STS markers developed from the RAPD marker, OPF 182600 by Subudhi *et al.* (1997). The STS primer combinations, F18FM, F18RM and F18FM/F18RM were found to be tightly linked to the *tms3(t)* gene with a genetic distance of 2.7 cM. The sizes of the different alleles in case of F18FM/F18RM combinations were 2300 bp, 1050 bp, and 1900 bp, 1000 bp respectively. And they have reported the efficiency of that particular STS markers as 84.6% and so these markers could be used for further marker assisted selection programmes.

Dong *et al.* (2000) reported that TGMS gene, *tms4(t)* was located on chromosome 2 of the rice variety TGMS *indica* mutant line. The mapping population for this particular experiment was the F_2 population developed from a cross between a TGMS *indica* mutant (TGMS–VN1) and a fertile *indica* line, CH1. Bulk segregant analysis was performed using the AFLP technique. Out of 200 AFLP primer combinations, four of them (E2/M5–600, E3/M16–400, E5/M12–600, and E5/M12–200) showed linkage to that TGMS gene. All the identified markers showed coupling phase linkage. All of them showed low copy number except E2/M5–200. E5/M12–600 showed polymorphism in RFLP analysis and identified that it was closely linked to the TGMS gene at a distance of 3.3 cM. With the help of doubled-haploid mapping populations obtained from the crosses IR64´Azucena and CT9993´IR62666 were available at IRRI, Philippines, and Texas Tech University, respectively and this marker was mapped on chromosome 2. The chromosome location of this particular gene was confirmed with the presence of linked microsatellite marker RM27. Then the closest marker E5/M12–600 was sequenced.

Deng *et al*. (1999) was the first inventor of *tms5* gene in AnnongS which is a spontaneous mutant in the AnnongS (*Oryza sativa* ssp. *indica*) variety. Jia *et al.* (2001) constructed a genetic linkage map with an F_2 population obtained from the cross between AnnongS-1 and Nanjing11. And they identified a new TGMS gene, known as *tms5* which was located on the short arm of chromosome 2. On the basis of linkage map they concluded that, *tms5* gene was located between the markers RM349 and RM71 and was closely linked to the marker RM174. Lee *et al.* (2005), identified that *tms6* was also situated on chromosome 2 in an *Oryza sativa* ssp. *Japonica* mutant line, Sokcho-MS. In China derivatives of TGMS AnnongS rice line, such as AnnongS-1, 810S, Xiang125S, SanxiangS and Y58S, play an important role in hybrid rice production, by providing TGMS lines with desirable genetic background. The identification of TGMS rice lines containing *tms5* gene, has an important contribution to hybrid rice production by improving both yield and quality of rice (Deng *et al*., 1996, 1999; Jia *et al.,* 2000; Xiang

et al., 2002; Li *et al.,* 2003; Wang *et al.,* 2003; Liang *et al*., 2004). Hang *et al.* (2003) created a linkage map by AFLP technique and mapped the gene *tms5*. The mapping population for this experiment was the F_2 population obtained from the cross between Annong S-1 and Nanjing 11. They mapped 145 AFLP markers, 25 SSR markers and 5 RFLP markers on 12 different chromosomes covering 1537.4 cM of rice genome. The average interval between these markers was 9.0 cM and this was the first plant AFLP map created in China. During the development of linkage map they mapped and tagged the gene *tms5* on chromosome 2 and they took 3 month for finishing this work. Yang *et al.* (2007) identified a candidate gene of *tms5* in rice by genetic mapping. Previous studies of Lander and Botstein (1989) have suggested that AnnongS-1 and Y58S, two derivative TGMS lines of AnnongS, were both controlled by a single recessive gene named *tms5*, which was genetically mapped on chromosome 2. In this study for constructing molecular map of *tms5* gene, they used three different populations obtained from the crosses between AnnongS-1 and Nanjing11, Y58S and Q611, and Y58S and Guanghui122 rice varieties. They utilised 125 probes, which cover *tms5* locus for analyzing the recombination events of sterile samples. Finally *tms5* gene was physically mapped to a 19 kb DNA fragment between two markers, 4039-1 and 4039-2, located on the BAC clone AP004039. From the physical map obtained from the two markers, ONAC023, a member of the NAC (NAM-*ATAFCUC*- related) gene family, was identified as the candidate of the *tms5* gene. Dagang *et al*. (2006) reported that twelve SSR markers namely RM5780, RMAN13, RMAN7, RAN54, RM4702, RMAN81, RMAN43, RMAN42, RMAN20, RMAN2, C365 and RMAN8 are polymorphic for identifying plants containing *tms5* gene, in the population obtained from the cross between the lines (Annong S-1 X IR36) and (Xiang125S X BAR68).

Lee *et al*. (2005) created a molecular map for genetic analysis with SSR, STS and EST markers. On the basis of this analysis they identified that a single recessive gene locus was involved in the control of genic male sterility in Sokcho-MS. The mapping population

for this particular experiment was an F_2 mapping population derived from a cross between Sokcho-MS and a fertile *indica* variety Neda. And they identified a new TGMS gene *tms*6, which was mapped primarily to the long arm of chromosome 5 of *Oryza sativa* at the interval between markers E60663 (2.0 cM) and RM440 (5.8 cM). By using RM3351 (0.1 cM) and E60663 (1.9 cM) markers, they finely mapped the new TGMS gene *tms6*. Liu *et al.* (2010), mapped *tms6(t)* gene in thermosensitive genic male-sterile (TGMS) rice (*Oryza sativa* L.). A newly discovered rice line, G20S was the TGMS line used for this particular experiment. It showed a special phenotype compared with normal TGMS lines. It was sterile under low temperature conditions (below 29.5 ^{0}C) and fertile under higher temperatures. Through genetic analysis of G20S they identified that the TGMS is controlled by a single recessive gene that was named as *tms6(t)*. The F_2 population obtained from cross between G20S and Jing226 was used as the mapping population for this experiment. They identified four functional microsatellite linkage markers such as RM216, RM2504, RM3152, and RM4455 for distinguishing TGMS plants from non TGMS plants. The above markers were located on chromosome 10. Out of these four markers two of them were closely linked to the gene *tms6(t)* at distances of 3.00 cM and 1.10 cM, respectively. Additionally, integration of the genetic and physical maps showed that there were 15 BAC/PAC clones overlapping between RM3152 and RM4455, and *tms6(t)* was mapped to an interval of 1455 kb.

Rongbai *et al.* (2005) found that three pairs of independent recessive (*tms*) genes with additive effects were involved in TGMS (thermosensitive genic male sterility) expression in UPRI 95-140 TGMS. They crossed 44 pollen donor rice varieties with the TGMS line, at which four from the crosses of UPRI 95 140TGMS with normal fertile genotypes UPRI 95-117, UPRI 95-141, RL 253-3 and IR 58025B were taken for inheritance study and other crosses were used for the confirmation of the genetic segregation of the TGMS gene(s) and to analyze the frequencies of different segregation patterns. Expression of the trait in F_2 generation involved 44 different genetic backgrounds and indicated monogenic (3F:1S), digenic (15:1S) and trigenic (63F:1S)

inheritance with frequencies of 18.2%, 52.3% and 29.5% respectively. It showed that no single pair of genes was capable of causing complete male sterility. The location of unknown TGMS gene(s) of UPRI 95-140 TGMS was analyzed with the help of a set of primary trisomics in the genetic background of IR 36 by three-way cross method. Based on this study they concluded that, two pairs of major TGMS genes in UPRI 95-140TGMS, non-allelic to any of the known TGMS genes, were located on chromosomes 3 and 7, and tentatively designated as *tms6*(*t*) and *tms7*(*t*) respectively.

TGMS gene, *tms8* was mapped on chromosome 11 in *Oryza sativa* by Hussain *et al*. (2012). For this study, F61 was the female parent and IR 58025B was the pollen donor plant. They tagged the TGMS gene by using polymorphic randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers through bulked segregant analysis. They identified two randomly amplified polymorphic DNA markers (UBC 345830, UBC 313927) and two simple sequence repeat markers (RM224 and RM21) for distinguishing fertile lines from sterile lines. The RAPD marker, UBC345830 showed the perfect co-segregation with TGMS trait and have length of 830-bp and 1.33 cM, distantly located from the TGMS locus. So they eluted and cloned the fragment for designing a SCAR primer, which co-segregated with TGMS locus at a distance of 0.8 cM.

Sheng *et al.* (2013) identified a new TGMS gene, *tms9* on the short arm of chromosome 2 in Zhu1S TGMS rice line and it is widely exploited as a female parent in Chinese two-line hybrid rice breeding programme. In chromosome 2 a new TGMS gene, *tmsX* with stable male sterility was identified by Peng *et al*. (2010). Later Matthayatthaworn *et al*. (2011) identified another TGMS gene located on chromosome 2 with 0.0 cM distance from T2 SSR marker (located in the position of 6,396 kb). This newly identified TGMS gene was located in the same region of previously identified *tmsX* gene by Peng *et al*. (2010). They already identified two SSR markers, RM154 and RM300 for *tmsX* gene. So they concluded that, the new gene might be the same allele of *tmsX* gene of two different lines. For this study they used F_2 population obtained from

the cross between TGMS line (female parent) and normal male fertile varieties; CNT1 and PTT1 as pollen donors.

Jia *et al*. (2001) identified a new TGMS gene, *rtms1* in J207S rice variety. J207S was completely sterile when the temperature is lower than 31°C. The AFLP (amplified fragment length polymorphism) technique, combined with BSA (bulked segregant analysis), was used to screen markers linked to the target gene. For this study they used F_2 population obtained from the cross between J207S and E921. Initially they selected eight polymorphic AFLP markers for screening. Out of eight markers two of them showed very tight linkage to *rtms1* gene. The identified AFLP markers were Rev1 and Rev7. Based on rice genetic maps, *rtms1* was identified between RM239 and RG257 with genetic distance of 3.6 cM and 4.0 cM, respectively. Then they designed a SCAR (sequence characterized amplified region) marker from the most closely linked AFLP marker, Rev1, 4.2 cM from the *rtms1* gene.

Qi *et al.* (2014) reported that, in HengnongS-1 they located the TGMS locus on chromosome 9 and named the gene as *tms9-1,* using SSR markers and bulked segregant analysis. The TGMS gene in HengnongS-1 was controlled by a single recessive gene that was non-allelic with the other TGMS loci identified, including C815S, Zhu1S and Y58S. Fine mapping further narrowed the *tms9-1* loci to a 162 kb interval between two dCAPS markers. Sequence analysis showed that a T to C substitution results in an amino acid change in the *tms9-1* candidate gene (Os09g27620) in HengnongS-1 as compared to another rice variety Minghui63.

Recently Hien and Yoshimura (2015) identified map location and markers linked to thermosensitive genic male sterility gene in 103s line. For this study they used F_1 and F_2 populations obtained from the cross between TGMS103S and R20 (These are popular varieties in North Viet Nam). By using SSR analysis they found that the gene controlling male sterility is situated in chromosome 2 and it is having the flanking SSR

markers RM3294, RM6378, RM7575 and RM71. These identified markers can be used for marker assisted selection programmes.

Materials and methods

3. MATERIALS AND METHODS

This study entitled "Identification of functional markers for thermosensitive genic male sterile rice (*Oryza sativa* L)" was conducted in Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram. The stable TGMS line-IR75589-31-27-8-33 (EC 720903) imported from International Rice Research Institute (IRRI), Phillippines was used as the female parent. Two ruling red rice varieties, Uma and Jyothi, were taken as the pollen donors.

3.1 GENERATION OF MAPPING POPULATION

For generating F_1 seeds, we used proximal hybridization method using Uma X 03(TGMS) and Jyothi X 03(TGMS). Both male and female parents were grown side by side with reference to the crop calendar for synchronous flowering and the panicles were watched closely. For anther extrusion, emerged panicles were observed for flowering. Pollen sterility of TGMS plants were assured before hybridization. The plants of female parent (TGMS lines) and male parent (red rice lines) were brought together and shaken for hybridization in the morning just after flower opening. It is important to work quickly to take advantage of the period of maximum pollination since hybridization efficiency is high at the time of anthesis. After pollination the panicle was covered with butter paper and the date was marked on the bag with a wax pencil. The bag was placed over the panicle and the bottom edge was folded over and the paper clip was placed on the fold against the stem to hold it securely in place. The string of the identification tag was attached to the stem and tag was placed under the paper clip at the bottom of the glassine bag with the side showing the parents of the cross inward and the pollination date outward. The pot with female plants is placed in an area protected from wind, rain, and pests but with good exposure to sunlight.

The F_1 plants were selfed to produce F_2 seeds. The mapping population for this experiment was these F_1 and F_2 plants generated from the above seeds.

For assessing the spikelet and pollen sterility of F_1 and F_2 progenies, 1% iodo potassium iodide staining was performed. The fertile pollen grain appeared as blue and sterile pollen grain appeared as yellow.

3.2 PHENOTYPIC EVALUATION

Sterility was assessed in all the parents and progenies. Fresh anthers were collected from field grown rice varieties of Uma, Jyothi, and TGMS plant, during flowering period the anthers were treated with 0.5 ml 70% (v/v) ethanol for fixation and stained with 1% Iodo-potassium iodide and examined under a high resolution microscope (Motic BA310).

3.3 DEVELOPMENT OF MOLECULAR MARKERS

3.3.1 DNA Extraction

Leaf samples of parents, F_1 and F_2 progenies were collected separately in labeled cover. Then the leaf samples were subjected to genomic DNA extraction by a method standardized by Dellaporta *et al.* (1983). Two-three grams of rice leaf bits were transferred into pre-chilled mortar, frozen using liquid nitrogen and ground to a fine powder. The powdered sample was mixed with 15 ml of extraction buffer containing 20µl of ß-mercaptoethanol and then 50 mg of PVP (Polyvinyl pyrollidine) and kept at 4ºC. To the mixture 1ml of 20% SDS was added, mixed thoroughly and incubated at 65ºC for 1 hour in a water bath (Kemi). 5ml of 5M potassium acetate was then added to it and kept on ice for 20 minutes. Centrifugation (Centrifuge 5430R Eppendorf) was performed at 12,000 rpm for 20 minutes and the clear aqueous phase was transferred to a fresh sterile tube. Equal volume of ice cold isopropanol was added and mixed gently by inversion and kept in a -20^0C freezer until DNA was precipitated out. Centrifugation was performed at 12,000 rpm for 10 minutes and then the pellet obtained was dissolved in 500 µl sterile double distilled water. To this DNA solution 3 µl of RNase was added and incubated at 37^0C for 1 hour. To the mixture 500 µl of chloroform: isoamylalcohol

mixture was added and mixed well for 15 minutes. Centrifuged at 12,000 rpm for 15 minutes and aqueous phase was transferred to another microfuge tube without disturbing the inter phase. Two volumes of ice-cold absolute alcohol and 1/10 volume of sodium acetate were added and kept overnight incubation in -20^0C. Then it was centrifuged (12,000 rpm for 5 minutes) and the supernatant was discarded. To this 500 µl of 70% ethanol was used subsequently to wash the DNA. Alcohol was discarded and DNA was air-dried completely. Then the DNA pellet was dissolved in 120µl of TE buffer or sterile double distilled water and stored at -20^0C.

3.3.2 DNA Quality Confirmation

For electrophoresis, 0.8 % agarose gel (melting 0.8 g of agarose in 100 ml of 1X TAE buffer) was made by adding ethidium bromide 10µl(1µg/ml). The following samples were loaded into separate wells.

1.0 µl 1kb ladder, 5 µl sample + 1 µl 6x Loading Buffer

Electrophoresis was done for 30 min at 70 V. The gel was exposed to UV light and photographed in gel documentation system (Syngene G box Documentation System). Presence of a highly resolved high molecular weight band indicates good quality DNA.

3.3.3 Quantification of DNA

The isolated DNA samples are then subjected to quantification procedure by using spectrophotometer (ELICO, SL 218 UV-Vis Spectrophotometer). TE buffer was used as blank. The Optical Density (OD) values were taken at both 260nm and 280nm. The purity of the DNA was checked by calculating the ratio of absorbance at A_{260}/A_{280} and a ratio is 1.8 was indicates good quality DNA. (If the ratio is 2 or above 2, it indicates high RNA contamination in the sample and if the ration is less than 1.8, protein contamination or phenol in the sample). Concentration of the DNA was calculated by the formula shown below.

Concentration of the DNA µg /ml of sample = Optical density at 260nm X 50 X Dilution factor (i.e. 1 OD =50 µg/ml).

3.3.4 Screening of Primers by Polymerase Chain Reaction

All samples were subjected to different PCR reaction (Eppendorf Mastercycler) with different primers. Depending upon the quantity of isolated DNA in each sample the template concentration varied. Depending up on the primer sequence annealing temperature for each reaction varied. The products were confirmed by visualizing (Syngene G box Documentation System) the bands after Agarose Gel Electrophoresis.

Table 2. The General PCR components

PCR components	Stock Conc.	units	Per Reaction (µl)	For 100 Reactions (µl)
Water			11.9	1280
PCR buffer with salt	10	X	2.00	200
dNTPs	2.5	mM	1.00	100
Primer-F	10	µM	1.00	100
Primer-R	10	µM	1.00	100
Taq polymerase	5	u/µl	0.2	20
DNA template	100-200	ng	2	200
Total volume			20	2000

The PCR reaction mixture included the following: DNA, 10 ng/µl; 10X buffer; 0.02 mM of each dNTPs; 2 mM $MgCl_2$; 10 µM each of forward and reverse primers; 0.5 unit of Taq polymerase. The PCR profile started with 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, appropriate annealing for 1 min, extension at 72°C for 2 min. A final extension 72°C for (7-10) min was included. Only the annealing temperature varied with primers. The various primers and their annealing temperature are listed out in Table 4.

The thermosensitive male sterile gene associated with the imported variety was not known. Hence we randomly selected different markers based on the published articles. Marker data for all accessions are available at http://www.gramene.org. and are represented in Table 3.

Table 3. List of SSR primers used for analyzing TGMS gene

Sl. No.	Primer	Sequence	Map position	Expected PCR product size (bp)
1	RM2	FP-ACGTGTCACCGCTTCCTC RP-ATGTCCGGGATCTCATCG	Chr 7	150
2	RM11	FP-TCTCCTCTTCCCCCGATC RP-ATAGCGGGCGAGGCTTAG	Chr 7	140
3	RM174	FP-AGCGACGCCAAGACAAGTCGGG RP-TCCACGTCGATCGACACGACGG	Chr 2	208
4	RM5862	FP-TTAGTACCTCATCATAGCTG RP-CTCTAATCTTCTCTCATTATCA	Chr 2	223
5	RM5897	FP-GGCATCTTCCCCTCTCTCTC RP-CCAACCCAAACCAGTCTACC	Chr 2	141
6	RM21	FP-ACAGTATTCCGTAGGCACGG RP-GCTCCATGAGGGTGGTAGAG	Chr 11	157
7	RM224	FP-ATCGATCGATCTTCACGAGG RP-TGCTATAAAAGGCATTCGGG	Chr 11	157
8	RM27	FP-TTTTCCTTCTCACCCACTTCA RP-TCTTTGACAAGAGGAAAGAGGC	Chr 2	158
9	RM3351	FP-ATGGAAGGAATGGAGGTGAG	Chr 5	174

		RP-TACCCCTACGTCGATCGATC		
10	RM29	FP-CAGGGACCCACCTGTCTAC RP-AACGTTGGTCATATCGGTGG	Chr 2	250
11	RM257	FP-CAGTTCCGAGCAAGAGTACTC RP-GGATCGGACGTGGCATATG	Chr 9	147
12	RM2020	FP-ACACGTCTTTTCTGCAAGAT RP-GACGAACGTTATCGTGAAAT	Chr 5	160
13	RM216	FP-GCATGGCCGATGGTAAAG RP-TGTATAAAACCACACGGCCA	Chr 10	143
14	RM7215	FP-GCCTCTGCGTTCACAAGTAG RP-ATCTACCTATCTTGGCGGTG	Chr 2	110
15	RM6378	RP-ATCTACCTATCTTGGCGGTG FP-TAGGGTGGGTGTGCTGAAC	Chr 2	167
16	RM6247	FP-CGCTCTTGTCTTTACTCCCG RP-GCTGCTGCTGCTTCTTTTTC	Chr 2	103
17	RM7575	FP-GGTTTGATCTCGCGTCTCTC RP-CCAGCAGCGAGAGAGATAG	Chr 2	118
18	RM492	FP-CAAAAATAGCGCGAGAGAG RP-AGACGTACATGGGTCAGGC	Chr 2	224
19	RM6275	FP-CACTGAGCCCTTTTGTCCTC RP-CCCAGATCAGAATCGAAGG	Chr 2	161
20	RM440	FP-GGTAGGCACCAAAGAGTTTGACG RP-GGCATCACCTTATCCAATCACC	Chr 5	200
21	RM480	FP-GCTCAAGCATTCTGCAGTTG RP-GCGCTTCTGCTTATTGGAAG	Chr 5	225

22	RM215	FP-CAAAATGGAGCAGCAAGAGC RP-TGAGCACCTCCTTCTCTGTAG	Chr 9	150
23	RM219	FP- CGTCGGATGATGTAAAGCCT RP-CATATCGGCATTCGCCTG	Chr 9	203
24	RM259	FP- TGGAGTTTGAGAGGAGGG RP-CTTGTTGCATGGTGCCATGT	Chr 1	172
25	RM571	FP-GGAGGTGAAAGCGAATCATG RP-CCTGCTGCTCTTTCATCAGC	Chr 3	187
26	RM241	FP- GAGCCAAATAAGATCGCTGA RP- TGCAAGCAGCAGATTTAGTG	Chr 4	132
27	RM412	FP-CACTTGAGAAAGTTAGTGCAGC RP-CCCAAACACACCCAAATAC	Chr 6	188
28	RM270	FP- GGCCGTTGGTTCTAAAATC RP- TGCGCAGTATCATCGGCGAG	Chr 12	217
29	RM25	FP-GGAAAGAATGATCTTTTCATGG RP-CTACCATCAAAACCAATGTTC	Chr 8	146

Table 4. SSR Markers and details of annealing temperatures

Markers	Annealing Temperature
RM2, RM11, RM27, RM29, RM174, RM3351, RM224	62°C
RM5897, RM5862,	58°C
RM257	57°C
RM25,RM440	56°C
RM2020, RM21, RM216, RM7215, RM6378, RM492, RM6247, RM7575, RM6275	55°C
RM480, RM215, RM219, RM259, RM571, RM241, RM270, RM412	52°C

3.3.5 Agarose Gel Electrophoresis

PCR products were resolved on 3.5% agarose gel, in 1XTAE buffer at 65 V for 3 hours and then stained with ethidium bromide (0.5 µg/ml) and visualized on a UV transilluminator. Photograph was taken using gel documentation system. The primers which showed polymorphism were selected and again PCR reaction was performed by using the isolated DNA samples of F_1 and F_2 plants. The PCR products (3-4µl) were run in a 6 per cent denaturing polyacrylamide gel electrophoresis (PAGE) at 100 watts for 1 hour and amplified fragments were resolved by silver staining (Panaud *et al.,* 1996).

3.3.6 Polyacrylamide Gel Electrophoresis and Silver Staining

3.3.6.1 PAGE gel casting: (Sequi-Gen® GT, BIO-RAD sequencing gel, USA)

The small glass plates were soaked in 2% sodium hydroxide solution overnight and then the plates were cleaned using scrubber in tap water and again in distilled water. Both the small and large plates were again wiped with absolute alcohol. 2 ml of repellent was applied on large plate and spread uniformly with kim wipes. 2 ml of bind saline was applied on small plate and spread uniformly with kim wipes. Vaseline was applied to both the sides of the spacers. The spacers were placed with rubber adapter on either sides of the large plate and the small plate was placed on top of the large plate in such a way that it was seated uniformly on the edges and sides. Then the plates were clamped using side lever clamp set.

3.3.6.2 Gel matrix preparation and gel casting

100 ml of a 6% polyacrylamide denaturation solution was taken in a conical flask and 800 µl of 10 % ammonium persulphate (APS) solution and 80 µl of TEMED (Tetra methyl ethylene diamine) were added and mixed well. The plates were kept in slanting position in such a way that the gel matrix could flow freely into the plates without air bubble. The solution was poured between the plates with the help of 100 ml

syringe. After the matrix spread uniformly throughout the plate, the comb was placed. The plates were left for 1 hour for polymerization.

3.3.6.3 Sample loading and gel running (electrophoresis)

After polymerization, the comb was removed and the gel setup was mounted on an electrophoresis apparatus. After flushing the wells with running buffer (1X TBE), the gel was pre-run for 15-30 minutes. To the 20 µl PCR product, 8 µl gel loading dye was added and from this 3 µl sample was loaded. 3 µl of DNA (each PCR reaction) and 2µL of loading dye were taken for sample preparation. Samples were denatured at 95^0C for 5 minutes and snap cooled on ice. After flushing the wells again, the denatured DNA samples were loaded onto the gel. The electrophoresis was started and allowed to proceed at 100 watts (constant) for about 1-2 hours based on SSR product size (bp). Finally, plates were dismantled from the electrophoresis apparatus for silver staining.

3.3.6.4 Silver staining for PAGE gel Staining of gel

After careful removal of the small plate with gel from the assembly, the gel with plate was stained with washing treatments of various solutions. Silver staining was done through fixing, staining and developing. Then the gel was dried and scanned using a computer scanner.

3.4 SCORING AND STATISTICAL ANALYSIS

The bands and phenotypic characters were scored visually by numerical numbers. Then statistical analysis, “ANOVA single factor” was performed.

Results

4. RESULTS

The main objective of the study was to identify a functional marker associated with TGMS gene present in the TGMS variety, EC720903 obtained from IRRI. The mapping populations for this study were the F_2 plants developed from the cross between TGMS line and two ruling red rice varieties such as Uma and Jyothi. The DNA of all progenies were isolated and subjected to marker analysis and the plants were retained for phenotypic study. The pollen grains were collected from F_2 progenies and stained with 1% I_2-KI solution. The results obtained in the present study is given below.

4.1 GENERATION OF MAPPING POPULATION

With proximal hybridization method, F_1 progenies of the crosses Jyothi X EC720903 (TGMS line) and Uma X EC720903 (TGMS line) were generated (plate 2). For hybridization programme, TGMS plants were grown under sterility inducing condition. The fertility/sterility status of the pollen grains were determined using 1% I_2-KI solution. The sterile plants were hybridized with fertile pollen grains of Uma and Jyothi. The F_1 progenies of Uma and Jyothi have undergone self-pollination and produced F_2 population. This F_2 population was used for marker analysis with microsatellite markers. The pictures of the parents, F_1 progenies and F_2 progenies are represented in plate 1, plate 3, plate 4 and plate 5 respectively.

4.2 PHENOTYPIC EVALUATION

The phenotypic study was done through anther staining with 1% iodo potassium iodide solution. After staining the fertile anthers were appeared as blue colour and sterile anthers were appeared as yellow colour. The pictures of sterile and fertile anthers are shown in Plate 6. The morphological characters of anthers were studied by high resolution microscope (Motic BA310). It was found that the TGMS plants and sterile F_2 plants showed 100% pollen and spikelet sterility. Whereas male parents and fertile progenies showed pollen sterility ranges from 0.8% to 3.5% and that of spikelet sterility ranges from 20% to 34%. All the sterile plants including TGMS plants produced white coloured anther while all the fertile plants including male parents were produced yellow

coloured anthers. The length of anthers shared similarities between sterile and fertile plants and it was ranges from 1.82mm to 2.15mm. The diameter measurements of anthers suggested that sterile plants including TGMS line had small diameter as compared with fertile lines. The diameters of sterile and fertile anthers ranged from 0.20mm to 0.26mm and 0.31mm to 0.49mm respectively. The phenotypic characters of parents and their progenies are represented in table 5.

UMA EC720903 (TGMS) JYOTHI

Plate 1. Parents used for hybridization

JYOTHI X EC720903 (TGMS) UMA X EC720903 (TGMS)

Plate 2. Proximal hybridization method


F_1 of UMA X EC720903 (U3) F_1 of JYOTHI X EC720903 (J3)

Plate 3. F_1 progenies of UMA and JYOYHI



Sterile Fertile (Homozygous) Fertile (Heterozygous)

Plate 4. F_2 progenies of UMA



Sterile Fertile (Homozygous) Fertile (Heterozygous)

Plate 5. F_2 progenies of JYOTHI

Table 5. Morphological characteristics of parents and progenies

Plant Name	Pollen Sterility (%)	Spikelet Sterility (%)	Anther Length (mm)	Anther Diameter(mm)	Anther Colour
Jyothi	0.80	20.0	2.15	0.39	Yellow
Uma	1.00	23.4	1.81	0.45	Yellow
EC7209O3	**100**	**100**	**2.04**	**0.23**	White
J3(F_1 of Jyothi)	2.40	28.0	2.19	0.38	Yellow
U3(F_1 of Uma)	2.74	26.3	2.16	0.46	Yellow
F_2(Jyothi)1	1.60	27.0	1.94	0.31	Yellow
2	1.00	21.0	2.01	0.35	Yellow
3	3.50	24.0	1.96	0.42	Yellow
4	2.20	27.2	1.95	0.29	Yellow
5	0.88	29.0	1.89	0.36	Yellow
6	1.10	28.0	2.03	0.39	Yellow
7	**100**	**100**	**2.03**	**0.20**	**White**
8	0.50	26.8	2.01	0.41	Yellow
9	2.50	28.2	2.02	0.38	Yellow
10	2.10	33.0	2.07	0.34	Yellow
11	1.40	27.3	2.01	0.36	Yellow
12	0.70	29.0	1.98	0.36	yellow
13	1.60	22.5	1.93	0.37	Yellow
14	1.90	21.6	1.90	0.39	Yellow
15	**100**	**100**	**1.99**	**0.21**	**White**
16	2.00	32.7	1.95	0.32	Yellow
17	1.90	25.9	1.91	0.38	Yellow
18	1.10	22.0	1.99	0.40	Yellow
19	2.40	26.0	2.01	0.39	Yellow
20	3.30	23.8	2.03	0.35	Yellow
21	3.00	2.60	1.96	0.36	Yellow
22	3.00	26.0	1.97	0.38	Yellow
23	2.10	28.6	1.87	0.37	Yellow
24	2.00	21.9	1.95	0.41	Yellow
25	2.30	27.1	1.96	0.34	yellow
26	1.40	34.0	1.82	0.36	yellow
27	1.60	24.8	1.85	0.39	yellow
28	3.50	25.7	1.96	0.41	yellow

Table 5. Continued

Plant Name	Pollen Sterility (%)	Spikelet Sterility (%)	Anther Length (mm)	Anther Diameter(mm)	Anther Colour
29	3.30	23.0	1.93	0.40	yellow
30	2.90	25.7	2.02	0.38	yellow
31	**100**	**100**	**2.03**	**0.24**	**white**
32	0.50	30.6	2.01	0.38	yellow
33	3.20	24.0	1.96	0.37	yellow
34	2.80	23.7	2.01	0.38	yellow
35	**100**	**100**	**2.02**	**0.25**	**white**
36	2.20	27.0	1.98	0.39	yellow
37	0.60	22.8	2.01	0.39	yellow
F_2(uma)1	1.00	25.6	1.82	0.45	yellow
2	1.30	29.7	1.85	0.43	yellow
3	1.60	26.1	1.90	0.45	yellow
4	3.30	29.4	1.96	0.49	yellow
5	2.70	23.3	1.95	0.39	yellow
6	**100**	**100**	**1.96**	**0.26**	**white**
7	2.10	27.2	1.93	0.46	yellow
8	**100**	**100**	**1.98**	**0.24**	**white**
9	2.50	25.3	1.88	0.38	yellow
10	**100**	**100**	**1.96**	**0.21**	**white**

4.3 DEVELOPMENT OF MARKERS

4.3.1 DNA Extraction

The genomic DNA isolated from Uma, Jyothi, 03 (TGMS line), F_1 progenies and F_2 progenies were confirmed by agarose gel electrophoresis. All the isolated DNAs appeared as discrete bands in 0.8% gel indicating good quality DNA (Plate 7).

4.3.2 DNA Quality Confirmation

The quality of isolated DNAs were confirmed by measuring the absorbance at 260nm and 280nm (Table 6). The DNA samples showed 1.8 as A_{260}/A_{280} ratio it indicates the 100% purity, while others were showed the ratio below 1.8 indicated the presence of proteins contamination and above 1.8 indicated the presence of RNA contamination in the sample.

4.3.3 Quantification of DNA

Quantity of DNAs was calculated based on the absorbance of sample at 260nm are given in Table 6. The Quantity of the isolated DNAs were ranges from 600 ng/µl to 3840 ng/µl.

4.3.4 Screening of primers by Polymerase Chain Reaction

PCR reactions were performed using selected primer by providing appropriate PCR conditions. Out of twenty nine primers, three of them showed polymorphism in 3.5% agarose gel electrophoresis. RM3351, RM257 and RM5869 were the polymorphic markers. RM3351 showed polymorphic bands with ~174bp length, RM257 showed polymorphic bands with ~147bp length and RM5897 showed polymorphic bands with ~141bp length. The polymorphic markers were subjected to PCR reaction for checking the banding pattern of F_1 and F_2 progenies in 3.5% agarose gel but it didnot produce proper heterozygous bands. So urea PAGE was performed. The banding patterns obtained from Urea page showed that, out of three primes only one is effective for selecting progenies. RM3351 and RM257 produced multiple bands and all bands were

monomorphic. While RM5897 produced polymorphic bands with ~141bp length. Banding pattern of amplified products with RM3351, RM257 and RM5897 in urea PAGE gel is shown in plate 11, plate 12 and plate 13 respectively.

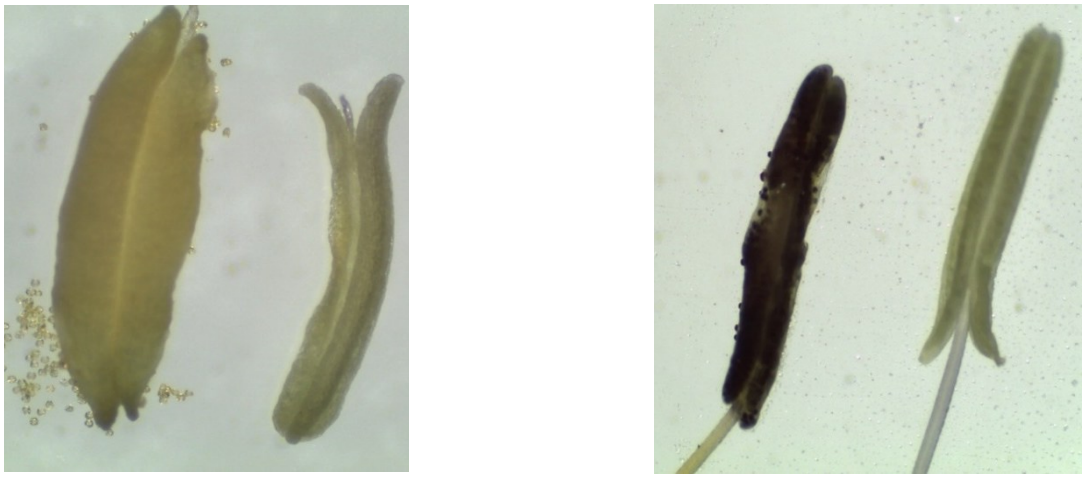
The phenotypic evaluation was performed in same progenies. The data obtained from phenotypic studies were correlated with the genotypic data obtained. Hence it is concluded that the marker RM5897 is a functional marker for distinguishing the fertile lines from sterile lines. The phenotype and genotype comparison data are shown in table 7.

Table 6. Concentration and purity of isolated DNA identified by spectrophotometric method.

Plant Name	A_{260} Value (nm)	A_{280} Value (nm)	A_{260}/A_{280} (ratio)	Quantity of DNA (ng/µl)
Jyothi	0.087	0.049	1.78	2610
Uma	0.102	0.059	1.73	3060
EC720O3	0.069	0.041	1.68	2070
J3(F_1 of Jyothi)	0.096	0.054	1.79	2880
U3(F_1 of Uma)	0.075	0.039	1.92	2250
F_2 (Jyothi)1	0.103	0.056	1.84	3090
2	0.109	0.06	1.82	3270
3	0.052	0.030	1.73	1560
4	0.087	0.047	1.85	2610
5	0.098	0.056	1.75	2940
6	0.066	0.038	1.74	1980
7	0.099	0.054	1.83	2970
8	0.067	0.035	1.91	2010
9	0.057	0.029	1.97	1710
10	0.069	0.038	1.82	2070
11	0.035	0.021	1.67	1050
12	0.098	0.059	1.66	2940
13	0.067	0.038	1.76	2010
14	0.102	0.055	1.85	3060
15	0.046	0.025	1.84	1380
16	0.123	0.069	1.78	3690
17	0.02	0.012	1.67	600
18	0.128	0.075	1.71	3840
19	0.058	0.031	1.87	1740
20	0.028	0.015	1.87	840
21	0.094	0.055	1.71	2820
22	0.079	0.046	1.72	2370
23	0.046	0.026	1.77	1380
24	0.095	0.054	1.76	2850
25	0.116	0.069	1.68	3480
26	0.099	0.054	1.83	2970
27	0.041	0.022	1.86	1230

Table 6. Continued

Plant Name	A_{260} Value (nm)	A_{280} Value (nm)	A_{260}/A_{280} (ratio)	Quantity of DNA(ng/µl)
28	0.099	0.052	1.90	2970
29	0.056	0.031	1.81	1680
30	0.034	0.019	1.79	1020
31	0.126	0.063	2.00	3780
32	0.079	0.044	1.80	2370
33	0.098	0.057	1.72	2940
34	0.084	0.041	2.04	2520
35	0.094	0.054	1.74	2820
36	0.087	0.041	2.12	2610
37	0.065	0.04	1.63	1950
F_2(Uma)1	0.088	0.049	1.80	2640
2	0.097	0.056	1.73	2910
3	0.076	0.041	1.85	2280
4	0.086	0.049	1.76	2580
5	0.051	0.028	1.82	1530
6	0.063	0.033	1.91	1890
7	0.099	0.057	1.74	2970
8	0.076	0.039	1.95	2280
9	0.047	0.026	1.81	1410
10	0.067	0.035	1.91	2010



Fertile Sterile Fertile Sterile

Plate 6. Fertile and sterile anthers (a) Before staining with 1% IKI (b) After staining with 1% IKI

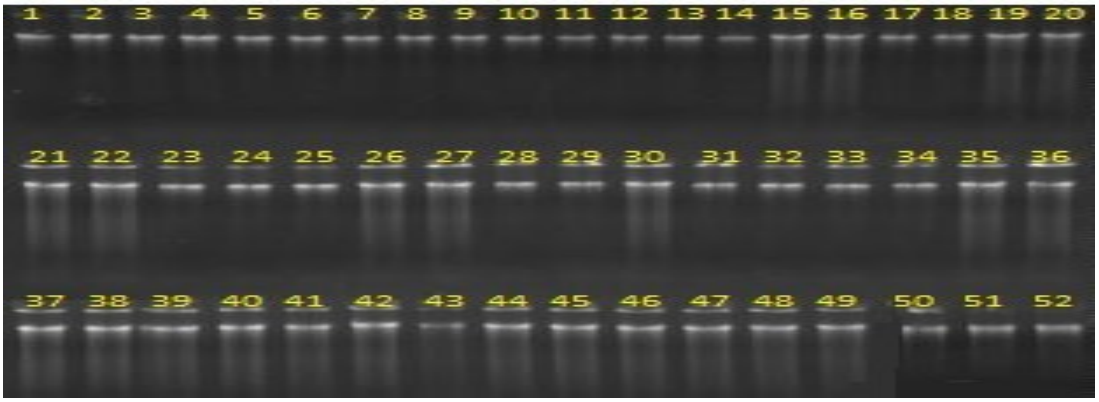


Plate 7. The quality of DNA isolated from parents and their progenies. (1-Jyothi, 2 - Uma, 3-EC720903, 4 - F_1 of Jyothi, 5 - F_1 of Uma, (6 to 42) - F_2 of Jyothi, (43 to 52) - F_2 of Uma).

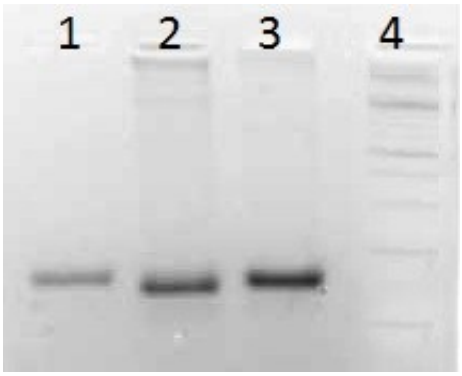


Plate 8. PCR amplification of genomic DNA of parents using the marker RM5897 (Line1-Jyothi, Line2-EC720903, Line3-Uma and Line4-100bp ladder).

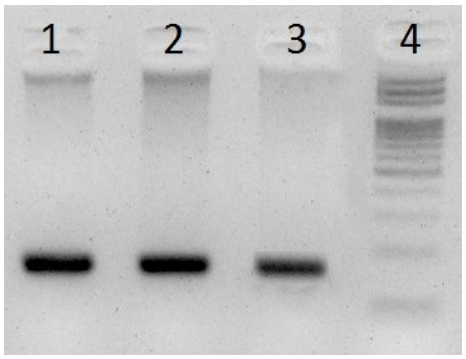


Plate 9. PCR amplification of genomic DNA of parents using the marker RM3351 (Line1-Jyothi, Line2-Uma, Line3- EC720903 and Line4-100bp ladder).

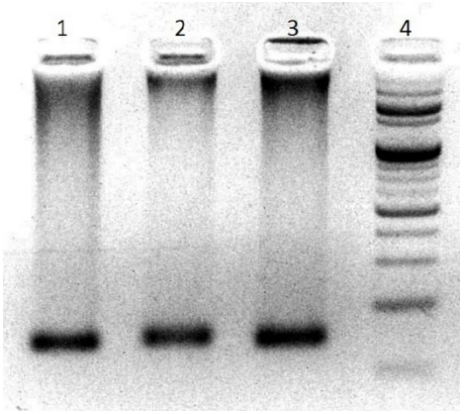


Plate 10. PCR amplification of genomic DNA of parents using the marker RM257 (Line1-EC720903, Line2- Jyothi, Line3-Uma and Line4-100bp ladder).

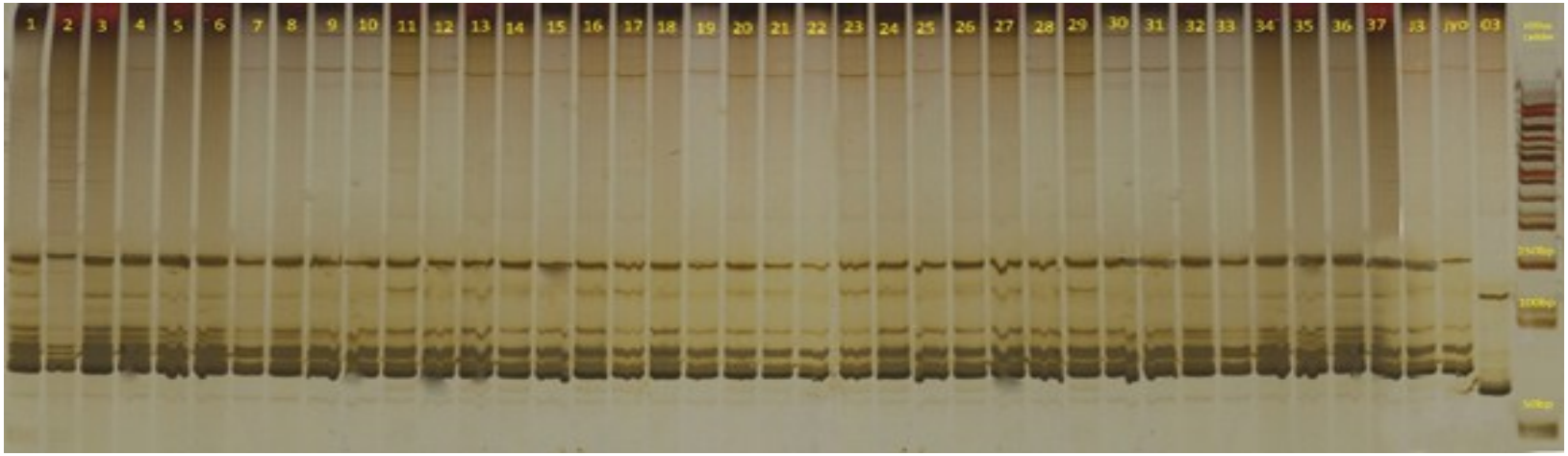


Plate 11(a). Amplified product on urea page (1-37(F_2 progenies of Jyothi X EC720903), J3 (F_1 progenies of Jyothi X EC720903), Jyo (Jyothi), EC720903) with the marker RM3351

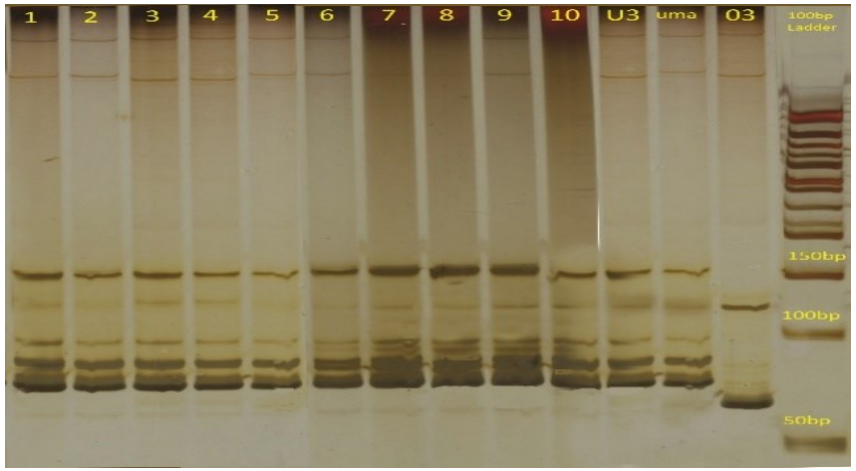


Plate 11(b). Amplified product on urea page (1-10(F_2 progenies of Uma X EC720903), U3 (F_1 progenies of UMA X EC720903), Uma, EC720903) with the marker RM3351.

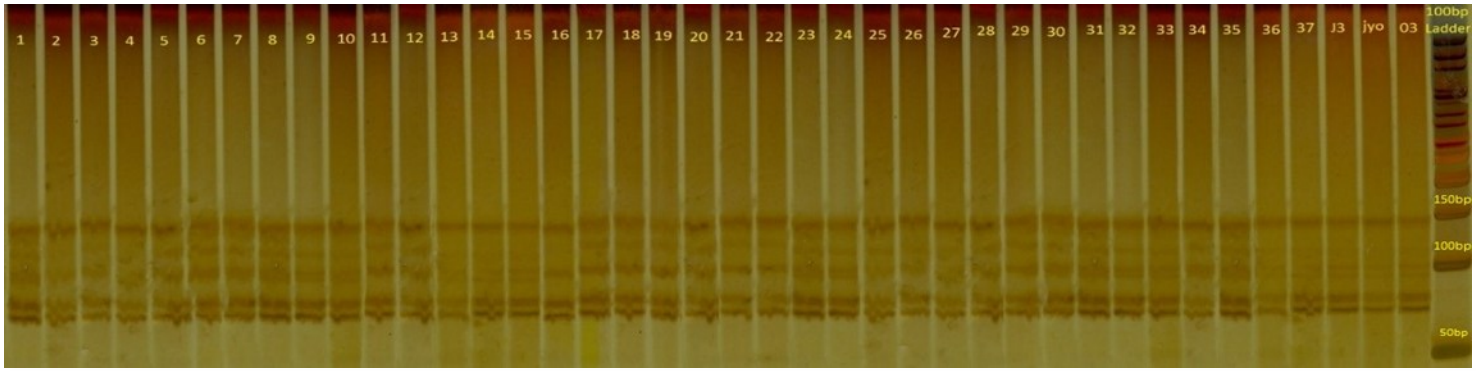


Plate 12(a). Amplified product on urea page (1-37(F_2 progenies of Jyothi X EC720903), J3 (F_1 progenies of Jyothi X EC720903), jyo (Jyothi), EC720903) with the marker RM257.

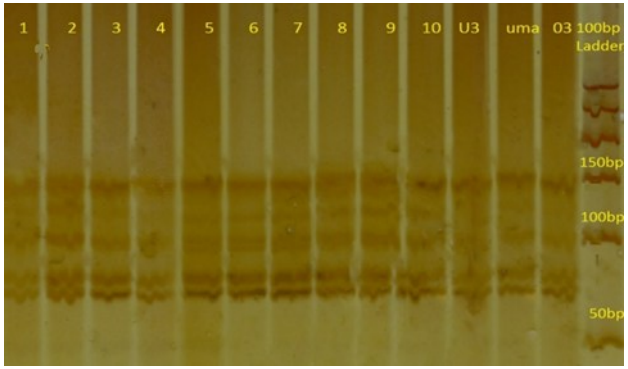


Plate 12(b). Amplified product on urea page (1-10(F_2 progenies of Uma X EC720903), U3 (F_1 progenies of Uma X EC720903), Uma, EC720903) with the marker RM257.

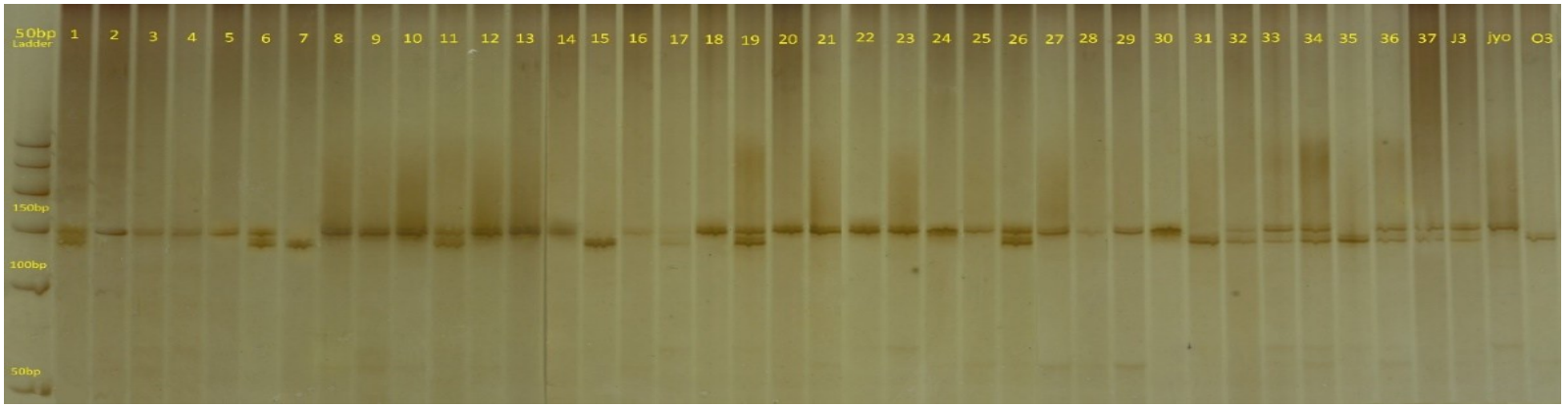


Plate 13(a). Amplified product on urea page (1-37(F_2 progenies of Jyothi X EC720903), J3 (F_1 progenies of Jyothi X EC720903), Jyo (Jyothi), EC720903) with the marker RM5897.

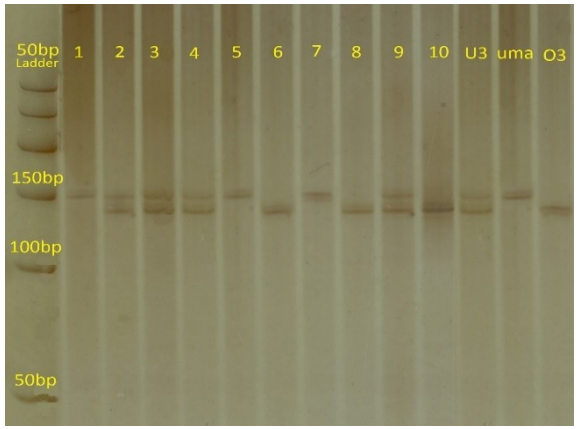


Plate 13(b). Amplified product on urea page (1-10(F_2 progenies of Uma X EC720903), U3 (F_1 progenies of Uma X EC720903), Uma, EC720903) with the marker RM5897.

Table 7. Phenotypic and genotypic characters (sterile/ fertile)

Plant Name	Genotype (Sterile(S)/Fertile(F)/Fertile Heterozygous(F(Hetero)))	Phenotype (Sterile(S)/Fertile(F))
Jyothi	F	F
Uma	F	F
EC7209O3	**S**	**S**
J3(F_1 0f jyothi)	F(Hetero)	F
U3(F_1 of uma)	F(Hetero)	F
F_2(Jyothi)1	F(Hetero)	F
2	F	F
3	F	F
4	F	F
5	F	F
6	F(Hetero)	F
7	**S**	**S**
8	F	F
9	F	F
10	F	F
11	F(Hetero)	F
12	F	F
13	F	F
14	F	F
15	**S**	**S**
16	F	F
17	F(Hetero)	F
18	F	F
19	F(Hetero)	F
20	F	F
21	F	F
22	F	F
23	F	F
24	F	F
25	F	F
26	F(Hetero)	F
27	F	F
28	F	F
29	F	F

Table 7. Continued

Plant Name	Genotype (Sterile(S)/Fertile(F)/Fertile Heterozygous(F(Hetero)))	Phenotype (Sterile(S)/Fertile(F))
30	F	F
31	**S**	**S**
32	F(Hetero)	F
33	F(Hetero)	F
34	F(Hetero)	F
35	**S**	**S**
36	F(Hetero)	F
37	F(Hetero)	F
F2(uma)1	F	F
2	F(Hetero)	F
3	F(Hetero)	F
4	F(Hetero)	F
5	F	F
6	**S**	**S**
7	F	F
8	**S**	**S**
9	F(Hetero)	F
10	**S**	**S**

4.4 STATISTICAL ANALYSIS

Based on the phenotypic data derived from anther staining and genotypic data obtained from banding pattern were scored and given in Table 8. Score 1 represents the upper bands similar to male parental line (Jyothi) and score 3 for lower band similar to female parental line EC720903 (TGMS line). When sterility was present, they were scored phenotypically as 2 and if absent, they were scored as 5. Table 9 represents the ANOVA table for RM5897.

Table 8. Statistical analysis-phenotypic and genotypic scores for ANOVA single factor with RM5897 marker.

F_2 plants obtained from the cross between Jyothi and EC720903(TGMS line)	Genotypic score		Phenotypic score
	RM5897a	RM5897b	
1	1	3	5
2	1	1	5
3	1	1	5
4	1	1	5
5	1	1	5
6	1	3	5
7	**3**	**3**	**2**
8	1	1	5
9	1	1	5
10	1	1	5
11	1	3	5
12	1	1	5
13	1	1	5
14	1	1	5
15	**3**	**3**	**2**
16	1	1	5
17	1	3	5
18	1	1	5
19	1	3	5
20	1	1	5
21	1	1	5
22	1	1	5
23	1	1	5
24	1	1	5
25	1	1	5
26	1	3	5
27	1	1	5
28	1	1	5
29	1	1	5
30	1	1	5
31	**3**	**3**	**2**
32	1	3	5
33	1	3	5
34	1	3	5
35	**3**	**3**	**2**
36	1	3	5
37	1	3	5

Note: a-the upper band similar to male parent (Jyothi), b-lower band similar to female parent (EC720903).

Table 9. Analysis of variance for RM5869b marker

ANOVA						
Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P-value	F critical value
Between Groups	5.71	1	5.71	7.57	0.009	4.12
Within Groups	26.4	35	0.75			
Total	32.11	36				

Here the F calculated value (7.57) is greater than F critical value (4.12), so this data is significant and the marker RM5897 can be used for further marker assisted selection programme.

Discussion

5. DISCUSSION

The study entitled "identification of functional markers associated with thermosensitive genic male sterile rice (*Oryza sativa* L.)" was conducted to identify a molecular marker associated with TGMS gene and to transfer TGMS character to red rice varieties such as Uma and Jyothi. The results of this study presented in chapter 4 are discussed here.

Two line system of hybrid rice production is a very good alternative to that of three line breeding systems. The commonly available male sterile lines for two line breeding system are thermosensitive and photosensitive genic male sterile lines (TGMS and PGMS). In the present study the female parent used was a TGMS line (EC720903). These lines can be easily multiplied in winter season because of the availability of low temperature (Virmani *et al.*, 2003). Evaluation of TGMS lines for flowering, morphological and agronomic traits is an important tool to find out commercially usable TGMS lines (Virmani *et al.*, 1997; Kalaiyarasi and Vaidyanathan, 2002 and Niya Celine, 2014).

For successful hybrid production as part of molecular marker development, it is necessary to ensure perfect synchronization with respect of flowering time and duration of anther production between parental lines (Ramakrishna *et al*., 2006). The morphological characteristics of parental lines (Jyothi, Uma and 03(TGMS)) and their progenies share similarities. Their anther length ranged from 1.81mm - 2.15mm and the diameter of anthers ranged from 0.20mm - 0.49mm and pollen sterility ranged from 0.50% - 3.5% and that of spikelet sterility ranged from 20% - 32.71% respectively. TGMS line IR73834-21-26-15-25-4 showed the potential for commercial utilization in two line rice hybrid seed production. It had an anther length of 2.14 mm and stigma length of 2.0 mm (Akhter *et al*., 2008). But the TGMS line (EC720903) and F_2 plants (which are homozygous to sterility) showed complete (100%) spikelet and pollen sterility. They also had white colour in anther wall, which might be due to the lack of pollen grains in the anther. TGMS lines with bigger pollen grains would have very less

fertility chances under appropriate conditions (Virmani *et al.,* 2003). The pollen fertility ranged from 0.5% to 82% and spikelet fertility from 0 to 87% in hybrids of some cytoplasmic male sterile lines. In parents, while the pollen fertility varied from 28% to 97% and that of spikelet fertility varied from 73% to 91%. In earlier studies, it was observer that spikelet fertility percentage varied widely among hybrids and many hybrids had lower spikelet fertility percentage than their parents (Joshi *et al.,* 2007). Some studies also indicated that the TGMS lines with maximum length of anther and stigma may be useful to produce higher outcrossing rate as they correlate positively (Ali, 1993). The adaptability of hybrid seed production directly depend on the outcrossing rate (Abeyesekera *et al.,* 2003).

The genotypic identifications of plants can be achieved through molecular marker technology. The advantage is that, it will give the genotypic information in the seedling stage itself. Selecting the progenies obtained from successive crosses can be done through Marker Assisted Selection (MAS) programme. The widely used and efficient marker for Marker Assisted Selection (MAS) programme is Simple Sequence Repeats (SSR) or microsatellites markers, because of their abundance, co-dominant nature and inherent potential for variation in next generation. Several studies in rice have contributed to the development of several hundred simple sequence repeats markers and a genetic map consisting of 320 SSRs (Wu and Tanksley, 1993; Akagi *et al.,* 1996; Temnykh *et al.,* 2000). These markers can be used for genetic diversity analysis (yang *et al.,* 1994; Olufowote *et al*., 1997; Cho *et al*., 2000; Harrington, 2000) and gene identification using both inter and intra specific crosses (Xiao *et al*., 1998; Bao *et al*., 2000; Zou *et al*., 2000; Bres-Party *et al*., 2001; Moncada *et al*., 2001). SSRs are increasingly been used for integrating the genetic, physical and sequence based maps of rice. They simultaneously provide breeders and geneticists with a useful tool to correlate phenotypic and genotypic variations.

It is a time consuming and difficult task to transfer the TGMS trait to different background. Marker assisted selection using tightly linked molecular markers will aid

the transfer of TGMS gene to different backgrounds in a better way. A well-defined marker once identified for the unknown TGMS gene residing in the particular male sterile line, could easily be used in marker facilitated transfer. These markers could also be used for distinguishing heterozygous fertile plants from homozygous sterile/fertile plants in the seedling stage itself and plants can be grouped into homozygous sterile and heterozygous fertile. These homozygous plants could be directly used for back cross breeding while heterozygous plants should be emasculated before back crossing under sterility inducing conditions, preferably at lower altitude, where the average temperature is above fertility inducing temperature.

Many *tms* genes controlling TGMS trait in rice have been reported, starting from *tms1* (Wang *et al*., 1995) to *tms9* (Sheng *et al.,* 2013). The TGMS gene present in the TGMS variety EC720903 is not known clearly. In general the TGMS trait was found to be controlled by a single recessive gene in nucleus (Jia *et al*., 2001). Codominant markers are needed to identify molecular markers of TGMS gene in F_2 population. Hence simple sequence repeats (SSR) markers (codominant marker) was used for screening the TGMS and non-TGMS lines. The PCR products separated on 3.5% agarose gel gave three positive primers which showed polymorphism in parents. But all the three markers (RM257, RM3351 and RM5897) identified were linked to different *tms* genes (*tgms, tms5* and *tms6*) and found situated in different chromosomes such as chromosome 9, chromosome 5 and chromosome 2 respectively.

TGMS gene, designated as *tms6*, was fine mapped between markers RM3351 (0.1 cM) and E60663 (1.9 cM) in chromosome 5 by Lee et al. (2005). It is a reported marker for *tms6* gene. Hence the possibility of presence of *tms6* gene cannot be ignored. Rongbai, *et al.* (2005) reported that a new TGMS source, UPRI 95-140TGMS line has shown digenic inheritance. On the basis of this report, it is inferred that the TGMS line (EC720903) have got multigene (*tgms, tms5* and *tms6*) for controlling male sterility.

The banding pattern of F_1 and F_2 progenies amplified using these primers on 3.5% agarose gel revealed that all the bands were homozygous bands even if the F_1

progenies are heterozygous. To further confirmation, urea PAGE was performed for getting more resolution in polymorphism. Urea PAGE analysis clearly distinguished the bands which denoted fertility and sterility. Analysis using urea PAGE, revealed that out of three markers only one showed exact polymorphism between parents and it also showed heterozygous banding pattern in F_1 progenies. That identified marker was RM5897 having ~141bp length and it is situated in chromosome 2. Yang *et al.* (2007) constructed a genetic map (Plate 14) covering the *tms5* gene from marker analysis data using MAPMAKER software. It was reported that RM 5897 is closely linked with the gene, *tms5* at a distance of 1.45 cM. Later Hoan, N. T. (2014) identified that RM5862 and RM5897 also the linked markers of *tms* gene in CL64S rice variety.

The *tms5* gene was first mapped in AnnongS-1 on chromosome 2. The studies conducted by Jia and co-wokers (2000) indicated that the TGMS gene *tms5* is very tightly linked to the SSR marker, RM 174 with a distance of 0.0 cM, but here, did not produce polymorphism when the PCR reaction was performed with the same marker, RM174. While Dagang *et al*. (2006) identified twelve different SSR markers which are linked to *tms5* gene. The identified SSR markers were, RM5780, RMAN13, RMAN7, RMAN54, RM4702, RMAN81, RMAN43, RMAN42, RMAN20, RMAN2, C365 and RMAN8, which showed polymorphism between the plants obtained from the cross between the lines (Annong S-1 X IR36) and (Xiang125S X BAR68).

Wang *et al*. (2003) reported that RM492, RM279, RM327, RM324, RM341, RM6, RM263, RM262, RM166, RM208 and OSR28 are the co-segregating SSR markers of *tms5* gene in RIL (recombinant inbred line) population from the cross between AnnongS-1 and Nanjing11. Further studies with more number of the RIL

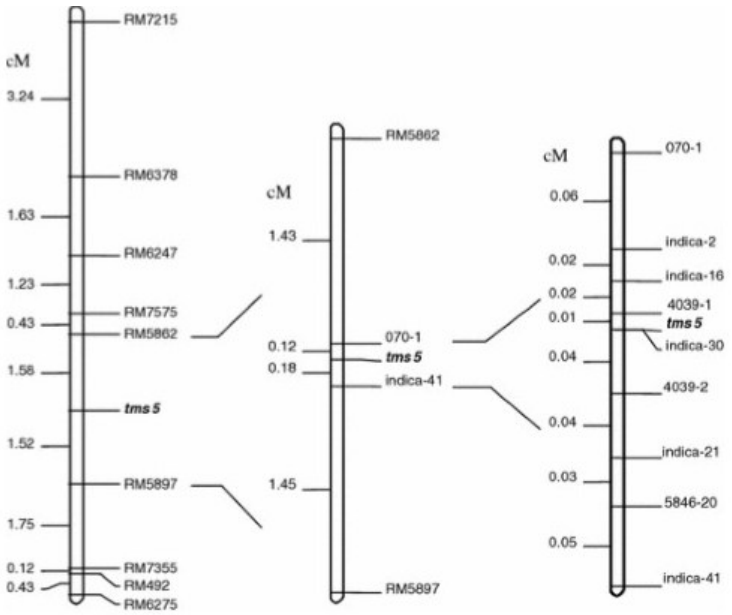


Plate14. The genetic map of *tms5* gene on the short arm of chromosome 2

plants indicated that the marker, RM492 on the small arm of chromosome 2 near the centromere region. The chromosome mapping study indicated that, it was located at a distance of 5.4 cM from the *tms5* gene and also found the map distance of another SSR marker, RM279 was located on the other side of *tms5* gene at a distance of 19 cM. In this study they also identified several STS, RAPD, SCAR and AFLP markers.

Hien *et al*. (2015) identified SSR markers linked to unknown TGMS gene located on chromosome 2. The SSR markers, RM154, RM211 and RM71, showed polymorphism between two bulks. Based on the constructed linkage map (Temnykh *et al.,* 2000; Mc Couch *et al*., 1997), these three markers were located on the chromosome 2 with the map positions of 4.8 cM for RM154, 10.0 cM for RM211 and 41.0 cM for RM71. The position of *tms-103S* gene in this study was found to coincide with *tms5* gene based on the SSR marker analysis. Thus the *tms-103S* may be allelic or closely linked to *tms5* gene (Hien and Yoshimura*,* 2015). In the same way the unknown gene present in TGMS line, EC720903 may be allelic or closely linked to *tms5* gene.

The statistical analysis was performed for checking whether the data obtained from this marker is significant or not. For that the bands in polyacrylamide gel were scored. The bands similar to fertile line (upper bands, denoted as RM5897a) was scored as 1 and the bands similar to sterile line (lower bands, denoted as RM 5897b) was scored as 3. Based on the phenotype, score 5 for fertility and score 2 for sterility were given. Then single factor ANOVA was performed. From the statistical ANOVA analysis the F calculated value was greater than F critical value for the lower band that is RM5897b and so the marker data is significant and RM5897b (lower band) is the band which produced real polymorphism. Thus the marker RM5897 can be used for identifying progenies having desired trait.

The phenotypic evaluation by pollen staining with 1% iodo potassium iodide solution was correlated with the data generated genotypically. Hence the marker RM5897 is found as a functional marker associated with TGMS gene, *tms5* in thermosensitive genic male sterile rice EC720903.

Summary

6. SUMMARY

The study entitled "Identification of functional markers for Thermosensitive genic male sterile rice (*Oryza sativa* L.)" was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram. The main aim of the study was to identify a functional SSR (Simple Sequence Repeats) marker for the selection of progenies obtained from the crosses between Uma and Jyothi with TGMS line (EC720903). Anther staining is a method of plant selection, but it is not accurate because change in temperature will changes the fertility, sterility characters and it will not give the information about heterozygosity. The sterility is a recessive character so that both the heterozygous and homozygous to fertility show same result when stained.

The thermosensitive genic male sterility (TGMS) system has great potential for revolutionizing hybrid rice production through simple, less expensive and more efficient seed production technology. For the successful utilization of male sterility, the genetic information of the particular gene must be known. In this study we aimed to identify a functional marker, which is linked to the unknown gene linked to male sterility present in the TGMS line, EC720903. Twenty nine different reported SSR (Simple sequence Repeats) located in different chromosomes for screening were selected. For marker evaluation, cross between female parent (TGMS-EC720903) and male parents (Uma and Jyothi) were made by proximal hybridization method. The F_1 obtained from the crosses were allowed to self-pollinate to produce F_2 population. That F_2 population was the mapping population for this experiment.

In the present study, the genomic DNA of parents and progenies were isolated for marker screening. The genomic DNA of parents were subjected to PCR reaction with 29 reported markers. PCR products were separated on 3.5% agarose gel. The documented data of these gels suggested that only three out of twenty nine primers showed polymorphism in the parents. The primers which showed polymorphism were RM257, RM3351 and RM5897. Then all these three primers were subjected to second set of PCR reaction in progenies. The product obtained from the PCR reaction were

resolved in Urea PAGE. After gel running, the gel was subjected to stain with silver nitrate for observing the bands. Based on the banding pattern from the stained gel, it is found that only one marker out of three showed exact polymorphism and segregation in F_2 population. The marker is identified as RM5897 and this marker is situated on chromosome 2 and it is found that this marker is very tightly linked to the thermosensitive male sterile gene *tms5*. So it is assumed that the unknown gene may be *tms5*.

Then scoring of the gel and subsequent statistical analysis by single factor ANOVA test was carried out. ANOVA test result suggested that the marker data is significant and it is efficient for further marker assisted programme.

Future line of work:

The identified SSR marker RM5897 is very efficient and it is co-segregating in successive generations. Thus it can be used for further marker assisted selection programmes, thereby we can identify the required male sterile plants in its seedling stage itself without phenotypic staining. The marker obtained in this study may be sequenced and utilized in further marker assisted selection programmes.

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appendices

8. APPENDICES

8.1 CHEMICALS FOR PLANT DNA ISOLATION

Dellaporta Extraction Buffer (50 ml):

1M Tris (pH 8.0)	2.5 ml
0.5M EDTA (pH 8.0)	2.5 ml
5M NaCl	2.5 ml
Distilled water	42.5 ml

5M Potassium Acetate (100 ml)

Potassium acetate	29.6 g
Acetic Acid	11.5 ml
Distilled water	28.5 ml

1X TE Buffer (100 ml)

1M Tris HCl (pH 8.0)	100 µl
0.5M EDTA (pH 8.0)	20 µl

This was dissolved and made up to 100ml using autoclaved distilled water.

8.2 CHEMICALS FOR AGAROSE GEL ELECTROPHORESIS

Gel loading dye

Formamide	50 ml
Xylene cyanol	50 mg
Bromophenol blue	50 mg
0.5 M EDTA	1 ml

10X TBE Buffer (Tris-Borate EDTA): 1000 ml

Tris base	107 g
Boric acid	55 g

Na_2EDTA	9.8 g

8.3 CHEMICALS FOR PAGE GEL ELECTROPHORESIS

40% Acrylamide stock (19:1)

Acrylamide	38 g
Bis- acrylamide	2 g

5% Polyacrylamide denaturing stock solution

Urea	210.0 g
40% Acrylamide	62.5 ml
Distilled water	200.0 ml
10X TBE	50 ml

10X TBE Buffer

Tris base	107.8 g
Boric acid	55.0 g
Na_2-EDTA	8.2 g

Bind saline (For 500 ml salinizing solution)

Ethanol	497.5 ml
Glacial acetic acid	2.5 ml
Bind saline	1.0 µl

8.4 CHEMICALS FOR SILVER STAINING

Fixer

10 % Acetic acid solution

Staining solution

Silver nitrate ($AgNO_3$)	2 g
Formaldehyde	3 ml

Distilled water	2000 ml

Developing solution

Sodium carbonate	60 g
Distilled water	2000 ml
1% sodium thiosulphate	400 µl
Formaldehyde	3 ml

Gel loading dye

Formamide	50 ml
Bromophenol blue	50 mg
Xylene cyanol	50 mg
0.5M EDTA	1000 µl

8.5ANTHER STAINING SOLUTION

1% I_2-KI (Iodo potassium iodide) solution (250ml)

Iodin	500 mg
Pottasium iodide	5 g

This was dissolved and made up to 250ml using autoclaved distilled water

"IDENTIFICATION OF FUNCTIONAL MARKERS FOR THERMOSENSITIVE GENIC MALE STERILE RICE (*Oryza sativa* L.)"

By

VIPIN MOHAN

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Abstract of Thesis

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9. ABSTRACT

The project entitled "Identification of functional markers for thermosensitive genic male sterile rice (*Oryza sativa* L.)" was conducted in Department of Plant Physiology, College of Agriculture, Vellayani, Thriruvananthapuram, during 2015-2016. The main objective of the study was the identification of a functional molecular marker (SSR-Simple Sequence Repeats) to identify the unknown TGMS gene. The pollen donors for this particular study were the two red rice varieties, Uma and Jyothi. The TGMS line used for this study was EC720903 imported from IRRI, Philippines.

The mapping population for this particular study was the F_1 and F_2 populations obtained from the crosses between Uma and Jyothi with TGMS line (EC720903). For obtaining the F_1 population we adopted proximal hybridization method. The F_1 plants were allowed to self-pollinate for getting F_2 population.

In this present study, twenty nine SSR primers have been used for initial screening with parents. Out of twenty nine SSR primers only three showed polymorphism viz; RM5897, RM257 and RM3351 in 3.5% agarose gel. One of these polymorphic markers showed polymorphism in urea PAGE. That marker was RM5897 with product size 141bp, which was associated with *tms5* gene with a distance of 1.45 cM and located on chromosome number 2. The data obtained from urea PAGE analysis was in correlation with the phenotypic data obtained from anther staining of F_2 plants at flowering stage with 1% Iodo potassium iodide. The present study revealed that the gene conferring male sterlity present in this TGMS line is *tms5* and which is located in chromosome number 2. The identified marker RM5897 associated with *tms5* gene is functional for selecting progenies for further backcross breeding programmes (so that identification of male sterile plants in seedling stage itself will be possible).