MARKER ASSISTED TRANSFER OF THERMOSENSITIVE GENIC MALE STERILITY TO HIGH YIELDING RED KERNELLED VARIETIES OF RICE (*Oryza sativa* L.)

by

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Thesis

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DECLARATION

I hereby declare that this thesis entitled "**Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis entitled **"Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)"** is a record of research work done independently by **Smt. Niya Celine V. J. (2009-21-107)** under my guidance and supervision and it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Thiruvananthapuram

(Niya Celine, V.J.)

Dedicated to

My Beloved Father

*Late. Sri.**A. R. Vijaya Raj***

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LIST OF ABBREVIATIONS

%	Percent
^{0}C	degree Celsius
d	Day
mg	milligram
cm	centimeter
ie.	that is
ddH_2O	double distilled water
bp	base pair
TGMS	Thermosensitive genic male sterility
DBH	Days before heading
SSR	Simple sequence repeat
et al.	and co-workers/ co- authors
CD (0.05)	Critical difference at 5% level
CV	Coefficient of variance
EU	Enzyme units

Introduction

1. INTRODUCTION

Rice is the staple food for more than half of the world's population, especially in Asian, African and Latin American countries. Hence it stands unique among the food grains consumed around the globe. With the current rate of increase in population, the demand for rice would be about 800 million tons by 2020, nearly 50% more than what is being produced today. Moreover, most of the crops are facing a phenomenon "plateuing of yield" during last few decades. In order to minister this food security issues with limited land resources, there should be a combined adoption of various technologies like diverse breeding methods and molecular techniques. Asian cultivated rice (*Oryza sativa* L.) belongs to the family Graminae, is a model system for developmental biology studies. As a model organism with a fully sequenced genome, it offers plenteous choices for various molecular tools. Being a self-pollinated crop, rice can very well be improved by hybrid rice technology. Hybrid rice seed production involves the use of male sterility systems. Male sterility can be defined as a condition in which the pollen grain is unviable or cannot germinate and fertilize normally to set seeds.

Among the various male sterility systems utilised for the development of hybrids, the three-line breeding (Cytoplasmic male sterility-CMS) is the widely used mechanism. CMS is a maternally inherited condition in which a plant is unable to produce functional pollen. Here male sterility is induced by a cytoplasmic gene. The negative effects of this cytoplasm is limiting the wide spread use of CMS system.

The environmental genic male sterility system (EGMS), or two-line system, involves a line in which male sterility is induced by an environmental factor (such as temperature or photoperiod) of a particular range or duration at a sensitive stage of plant development. In China, photoperiod- sensitive genic male sterility PGMS is extensively exploited. The differences in the photoperiod regulate the male sterility/ fertility conditions. In two-line breeding, the genes for male sterility

can be transferred to any genetic background and more diversity among the female parents help in reducing the genetic vulnerability among the hybrids. There is a wide scope of using any fertile pollen parents. Restorer gene in male parent is not a pre-requisite in EGMS.

In India, where the photoperiodic differences are marginal or negligibly small, the ideal EGMS male sterility system will be thermosensitive genic male sterility (TGMS). In TGMS system, hybrid seeds can be produced at high temperatures when the TGMS line is male-sterile, while at low temperatures, seed multiplication of the male-fertile TGMS line can be done by normal self-pollination. TGMS plants exposed to higher temperatures 1–2 weeks after panicle initiation exhibit male sterility. These hybrids are capable of giving higher yield due to hybrid vigour or heterosis. It is reported in rice to exhibit 15-20% increase in yield over the traditional varieties. There is also a higher probability of finding heterotic hybrids because the number of parental lines is not limited by the presence or absence of restorer genes. Male sterility expression in TGMS lines is influenced by temperature alteration of environmental condition with temperature shift that can restore fertility in TGMS (Liu *et al.,* 2001; Wang *et al.,* 2003). Thus, TGMS plants can be used not only as male-sterile lines but also as maintainer lines, providing the opportunity to produce hybrid seeds in rice through a simple, less expensive and more efficient two-line seed production system.

Several TGMS genes in rice have been reported from various countries, China (Sun *et al.,* 1989), Japan (Maruyama *et al.,* 1991), and the International Rice Research Institute (IRRI) in the Philippines (Virmani and Voc, 1991; Virmani *et al.,* 1998). The TGMS trait was identified to be controlled by a single recessive gene (Yang *et al.,* 1992; Borkakati and Virmani, 1996; Reddy *et al.,* 2000). The system has advantages in much simpler and economic hybrid seed production and broader choice of male parents for enhancing yield potential as the maintainer and restorer lines employed in the currently used three-line hybrid breeding system based on male sterility are not required (Rongbai *et al.,* 2005). In

TGMS lines, pollen abortion is controlled by nuclear genes and is usually monogenic recessive (Kaul, 1988). Some TGMS lines were spontaneous mutants, where some others were developed by irradiation breeding or were selected from filial generations following hybridisation (Zhou *et al.*, 1991).

TGMS system is the best method to develop hybrid red rice in tropical region like Kerala. Red pericarp is ubiquitous among the wild ancestors of cultivated rice and the red colour of rice pericarp is attributed by the flavonoid group of compounds called proanthocyanidin which is condensed tannin (Oki *et al.*, 2002). This compound plays a significant role in protection against UV radiation, attraction of insects for pollination, plant protection and plant defense responses etc. (Harborne and Williams, 2000). They contribute some beneficial effects in humans too. It serves as a powerful antioxidant to decrease the formation of antherosclerotic plaque formation associated with cardiovascular diseases (Ling *et al.*, 2001). People of Kerala prefer red rice which further pressurises the development of red rice hybrids using this technology. With the advent of molecular biology and biotechnology it is now easy to identify the DNA markers linked to TGMS genes. Marker assisted selection paves the way to transfer the gene efficiently from one genetic background to another (Collard *et al.*, 2005). Studies conducted by Lopez *et al.*, 2003 concluded the use of microsatellite markers as a powerful screening method in the case of large population and for identification of TGMS lines in the early stage of the crop, without the exposure of materials to required temperature. The male sterility gene was transferred from Norin PL 12, a TGMS line from Japan to a popular aromatic Thai rice cultivar, KDML-105.

It has been shown during the past two decades that hybrid rice technology can increase yields by 20–30% under irrigated conditions in China and some other countries (Yuan, 1998; Virmani, 2003). Hence the main objectives in the present study were

(1) To develop molecular markers associated with TGMS gene and

(2) To transfer TGMS character to red rice background.

Review of literature

2. REVIEW OF LITERATURE

Rice is the world's single most important food crop and a primary food source for more than a half of the world's population. More than 90% of the world's rice is grown and consumed in Asia. Asian cultivated rice *(Oryza sativa* L.) occupies a significant position among the domesticated crop species. Rice contributes 43 % of total food grain production and 46 % of total cereal production. FAO's latest projection indicates that total rice consumption would increase from the present 395.4 million metric tons to 472 million metric tons in 2015 and to 533 million metric tons in 2030. India annually plants rice on a total area of 43.9 million hectares, which produces an average of 130 million tons of rice. Under current consumption rate of rice, the production needs to reach 158 million tons in the 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).

The miracle of green revolution was outweighed by the burgeoning population and limited geographical arable land. Moreover, the yield plateauing in major crops is aggravating the condition. If the rate of population growth is continuing in a similar way, this rice production pace will not be sufficient for satiating the hungry mouths. FAO, in 2009 predicted that the food production should be accelerated by 2050, to about 70% to meet 9 million burgeoning population.

Being a self-pollinated crop, rice crop offers numerous alternatives for its improvement. Programs were initiated to incorporate several traits into cultivated rice varieties to develop better ones. At this juncture, the scope of hybrid rice came as a boon in rice research and breeding arena. The discovery of male sterility systems in rice has essentially resolved the constraint of controlling fertility/sterility and paved the way for the development of hybrids.

Hybrid vigour or heterosis expresses a better level of performance in growth, vigour, stress resistance, adaptability, reproductive capacity, grain quality, yield and so on. Development of hybrids is found to be a better alternative to ensure food security. Hybrids are found to yield 15-20% more than the inbred high yielding varieties (Sanchez and Virmani, 2005). Hybrid rice technology was pioneered by China by the discovery of wild rice population of a single male sterile line. In 1972, Chinese Professor Long Ping Yuan successfully developed the genetic material necessary for breeding hybrid rice and encouraged the growing of hybrid rice. From 1976 to 2001, the area under hybrid rice in China increased to over 270 M ha, and the total rice production increase to 380 M tons (Ma and Yuan, 2003). The technique was later spread to Japan (Maruyama *et al*., 1991) and to International Rice Research Institute (Virmani and Voc, 1991). The success in this technology aroused interest in other Asian countries, especially India.

Currently, the cytoplasmic male sterility (CMS) system is the most widely used male sterility system for hybrid rice breeding. In cytoplasmic genetic male sterility, male sterility is controlled by the interaction of a genetic factor S present in the cytoplasm and nuclear gene(s). It is now known that the male sterility factor S is located in the mitochondrial DNA. In CMS system there will be three lines, A, B and R. A line is the CMS line, B line is the maintainer and R line is the restorer. The CMS A line will be crossed with B line for maintaining sterility and the hybrid seeds are obtained through a cross between A line and R line to restore fertility. While effective and reliable, this system is cumbersome for hybrid seed production since CMS requires a third or maintainer line for seed multiplication of the male-sterile parent. The use of specific maintainer and restorer lines restricts exploitation of many elite lines. The complicated breeding procedures, longer breeding period of CMS lines and costly maintenance of the parental system are the other limitations. There is a potential risk of genetic vulnerability caused by the possible association of cytoplasm with susceptibility to a biological stress (Ullstrup, 1972). Yield of three-line hybrids has also reached a plateau

(Yuwei and Mingwei, 1992). Consequently, a search has begun to identify alternative genetic tools to develop rice hybrids. Thus the widely used system of male sterility, the cytoplasmic male sterility (CMS) system was alternated by another efficient, practicable and simple system in the tropics called environment-sensitive genic male sterility (EGMS) (Shukla and Pandey, 2008).

The EGMS system requires only 2 lines. In EGMS system, there is no need for a maintainer line for seed multiplication, thus making seed production simpler and more cost-effective. Environment-sensitive genic male sterility is controlled by nuclear gene expression, which is influenced by environmental factors such as temperature, daylength, or both. The EGMS in rice first became possible when Nongken 58S was discovered in China by Shi (1985).

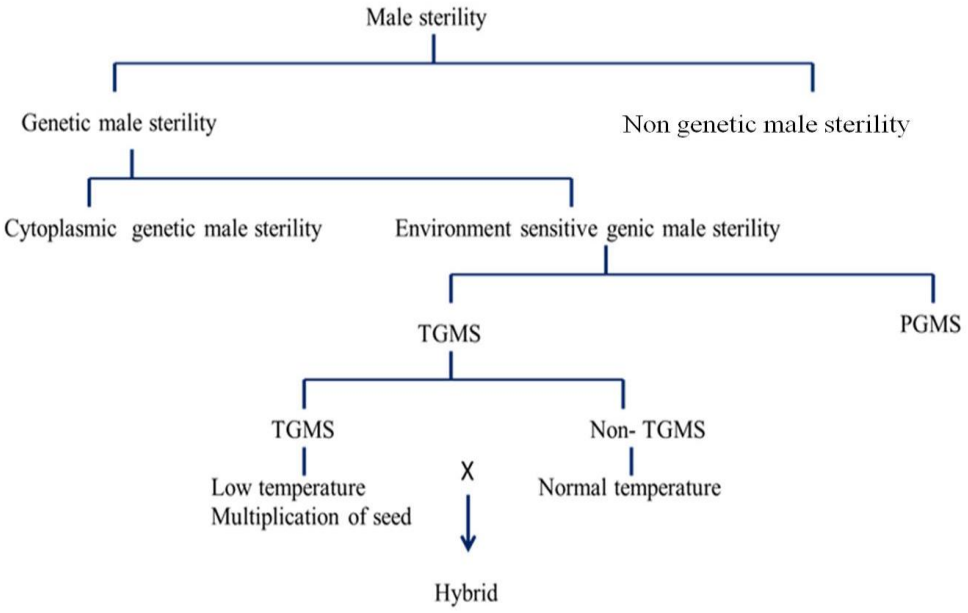


Fig.1 Brief outline of male sterility system

Brief outline of Male Sterility system is given as Fig.1. The following genetic and non-genetic male sterility systems are known for developing rice hybrids: cytoplasmic genetic male sterility, environment-sensitive genic male sterility, chemically induced male sterility etc. The genic male sterility can be

regulated by environmental factors like temperature, photoperiod etc. called environment-sensitive genic male sterility (EGMS). EGMS systems are classified according to the environmental factors affecting the sterility/fertility expression: thermosensitive male sterility (TGMS), photoperiod-sensitive genic male sterility (PGMS), photothermosensitive male sterility (PTGMS), rTGMS: reverse temperature-sensitive genic male sterility and rPGMS: reverse photoperiod-sensitive genic male sterility.

In the tropics, where photoperiod differences are minimal, but wide temperature differences exist among different altitudes or different seasons, it is more practical to use the TGMS system (Virmani and Ilyas-Ahmed, 2001). The EGMS makes use of photoperiod and/or temperature to control the sterility/fertility behaviour of the female parent. Under certain conditions (usually short photoperiod and/or low temperature), the EGMS line is male-fertile; therefore, it can be multiplied by selfing without the use of a maintainer line. The two-line system increases the frequency of heterotic hybrids, since any fertile line can be used as a male parent. The frequency of heterotic combination is more in the case of TGMS system (36-67%) (Lopez and Virmani, 2000), when compared to that of three line (16-31%). The multiplication of EGMS lines and hybrid seed production are restricted by space and season. This means that an EGMS line is used in a given region and season. The two line breeding technology could be used as an ideal system for the development of hybrids in a tropical country like India. The two line hybrids have a yield advantage at the rate of 5-10% when compared to that of the three line ones (Thiyagarajan *et al*., 2010). This system was found to be efficient with increased seed production and grain yield (Yuan *et al.,* 1998; Lu *et al*., 1998; Lopez and Virmani, 2000).

Deploying the TGMS system for developing two-line hybrids has several advantages over the conventional CMS system. TGMS utilizes specific temperature regimes in maintaining fertility and sterility forms in the rice plant without any ill effects. Male sterility expression in a TGMS line is heritable but

regulated by temperature (Virmani *et al*., 1997; Virmani, 2003). At certain high temperatures occurring after panicle initiation, the TGMS line becomes completely male sterile and is used as the female parent for hybrid seed production. Under low temperature, the same male sterile individual regains its fertility. Therefore, a hybrid production system based on TGMS can avoid using any maintainer line, which is required in the three-line system. Since the cytoplasm is not involved in sterility expression, plant breeders can develop hybrids with diverse cytoplasmic backgrounds to reduce the risk of potential genetic vulnerability.

Achieving complete male sterility in TGMS lines is essential to produce pure hybrid seed. Broad spectrum gametocides ensure higher seed yield and genetic purity. The potential use of male gametocides (chemicals and growth regulators) to convert partially sterile lines to completely male sterile lines in rice is well established. Various chemicals tried so far include ethylene- releasing compounds, highly carcinogenic arsenic compounds, and growth hormones etc.

2.1 DEVELOPMENT OF MOLECULAR MARKERS ASSOCIATED WITH TGMS GENE

DNA markers that are absolutely heritable and environmentally insensitive can be efficiently used to tag the TGMS gene for using the linked markers as indirect selection tools. Among the various types of molecular markers available, microsatellites have recently received greater attention, especially for breeding purpose. Microsatellites consist of tandemly arrayed di-, tri, and tetra-nucleotide repeats, which are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Microsatellite DNA markers, which can be directly amplified by PCR, have been developed using the unique sequences that flank microsatellites (Tautz, 1989; Weber and May, 1989). In several crop plants, including soybean (Akkaya *et al*., 1992), rice (Wu and Tanksley, 1993) and maize (Senior and Heum, 1993), specific amplification of microsatellite loci indicated

that microsatellite DNA markers are more variable than RFLPs. Thus, microsatellite DNA markers which are highly polymorphic and easily assayed by PCR with small samples of genomic DNA, should be of value for breeding programs. In addition to the above special feature of microsatellite DNA markers, information about these loci could be quite valuable, because they can be used as co-dominant landmarks of chromosomes.

Since their emergence as a PCR-based genetic marker over a decade ago (Weber and May, 1989), microsatellites or simple sequence repeats (SSRs) have become a major source of molecular markers for a diverse range of studies in plants and animals (Wang *et al*., 1994; Dib *et al*., 1996). One of the distinguishing features of SSR loci is their hypervariability. This feature, in combination with the ease of PCR amplification, the co-dominant profiles and the potential for automation, have contributed to the widespread use of these markers in a range of genetic studies. However, the precise nature of microsatellite variability is far from clear.

In the past, the advantages of microsatellite markers were partially offset by the difficulty inherent in marker development, as laborious use of rice genomic DNA library screening with SSR probes were required to isolate microsatellite containing sequences (Panaud *et al*., 1996; Chen *et al*., 1997). As random rice EST sequences became available, they provided a new source of SSR markers (Akagi *et al*., 1996; Temnykh *et al*., 2000), but the chromosomal positions of these markers had to be determined by genetic mapping. More recently, the growing pool of DNA sequence information being generated by the International Rice Genome Sequencing Project (IRGSP) and by other organizations (e.g., http:// www.rice-research.org) allows high-throughput in silico identification of SSR loci in sequenced regions, often with known map position, providing an excellent starting point for marker development. Conversely, mapped SSR markers that have been associated with phenotypes of interest provide a direct link to

sequenced regions that can be carefully annotated to identify candidate genes underlying the target trait.

Little is known about the TGMS gene at the molecular level. TGMS trait is controlled by *'tms'* genes located in different chromosomes in rice. To date there are 12 *tms* genes reported in rice. Some of the reported markers for TGMS are RM 239 for *tms1* (Wang *et al*., 1995) on chromosome 8, RM 11 for *tms*2 (Maruyama *et al*., 1990) on chromosome 7, RM 257 for *tms*4 (Dong *et al*., 2000, Reddy *et al*., 2000) on chromosome 2, and RM 3351, RM3476, and RM 440 for *tms*6 (Lee *et al*., 2005 and Wang *et al*., 2004) on chromosomes 5 and 3, respectively.

Genetic studies at IRRI (Borkakati and Virmani, 1996) indicated that the TGMS trait in Norin PL 12 and IR32364 TGMS was controlled by a single recessive gene. Allelic relationship studies indicated that the TGMS genes in the two mutants were different (Borkakati and Virmani, 1996). Because the TGMS gene in line 5460s from China was designated as *tms1*, and the one in Norin PL 12 from Japan as *tms2* (Kinoshita, 1992), the TGMS gene in the IR32364 TGMS mutant was tentatively designated as *tms3(t).* Its allelic relationship with the *tms1* gene present in TGMS mutant 5460S could not be studied due to non availability of the mutant, 5460S. Recently, the *tms3(t)* TGMS gene has been located on the short arm of chromosome 6 using molecular markers (Subudhi *et al*., 1997). Because the *tms1* gene of 5460S TGMS is now known to be located on chromosome 8 (Wang *et al*., 1995), it was concluded that *tms3(t)*of IR32364 TGMS is not allelic to *tms1*.

2.1.1 Phenological Characterisation of TGMS Lines

2.1.1.1 Determination of Critical Sterility Point (CSP)

The thermo-sensitive genic male-sterile (TGMS) gene in rice can alter fertility in response to temperature and is useful in the two-line system of hybrid rice production. A sudden decrease in temperature at a sensitive period in panicle development would cause the TGMS line to revert to fertility, thus decreasing the purity of hybrid seeds. If the TGMS line is partially sterile because of a reduction in temperature or one or more rains during the thermosensitive phase, it creates a problem by self seed setting. The selfed seed gets mixed up with hybrid seed and causes impurity and non-uniformity in the hybrid population.

One of the strategies being employed to overcome this problem is to breed for TGMS lines with low critical sterility point (CSP). The CSP is the critical temperature during the sensitive stage of a TGMS line that results in complete sterility. When used in hybrid seed production, TGMS lines possessing low CSP remain completely sterile despite sudden temperature changes during the sensitive growth stage. This eliminates selfing. Hybrid rice breeders use either of the two parameters in determining the CSP of a TGMS line: maximum temperature or mean temperature, depending on geographical location. Viraktamath and Virmani, (2001) have shown that it is the maximum temperature that influences the fertility/sterility expression of the TGMS line in the tropics. The ideal CSP for a TGMS line under tropical conditions is at maximum temperatures between 30 and 32°C (Ali *et al.*, 1995). In China, which is a temperate country, the daily mean temperature is used to identify the CSP of TGMS lines, since it is a function of both minimum and maximum temperatures (Mou *et al.*, 1998). The ideal CSP for a TGMS line is a daily mean temperature of 23-25°C (Ying, 1999).

The critical sterility period vary with TGMS lines. The sensitive stage usually exists between 15-24 days before heading (Ali *et al.,* 1995, Lohithaswa *et al.,* 2001, Reddy, 1997, Ramakrishna *et al.,* 2006) and it coincides with pollen mother cell development to secondary branch primordial formation. Yamaguchi and his co-workers in 1997 found that the sterility inducing temperature of a japonica line, Reimei is above 31^{o}C and three weeks (21days) before heading is the critical sterility inducing period.

2.1.1.2 Phenological, Floral, Morpho-agronomic Characterisation of TGMS lines

Many TGMS sources were identified from worldwide. The success of the two line breeding depends on extend of maintaining stability in changing the fertility and sterility status (Nair, 2006). Each TGMS sources have their own specific temperature regimes in exhibiting the sterility/ fertility forms. So, it is highly essential to know the specific temperature for inducing sterility and fertility in every line.

Detailed meteorological data (such as minimum and maximum temperature, daylength, sunshine hours, humidity, etc.) are essential to characterize EGMS lines under field conditions at a given location. To characterize given EGMS lines, the following procedure can be used: 1. Identify 3–4 distinct periods of high and low temperatures during the year. Likewise, determine the longer and shorter daylength durations during the year and over locations. 2. Seed/plant EGMS lines at 15–25-day intervals in such a way that their heading coincides with the high temperature or longer photoperiod. 3. Study pollen fertility of the EGMS lines from the top five spikelets of primary panicles under the microscope. 4. Relate the pollen sterility data to temperatures/ daylengths prevailing during the period of 15–25 days before heading (“tracking technique”). The temperature or daylength that is just sufficient to make the plant completely pollen sterile must be noted among the several temperature or daylength regimes to which plants were exposed during the period. Such a

temperature point or daylength at which complete pollen sterility is obtained is termed the critical sterility point and the number of days before heading during which this behaviour is expressed is designated as the sensitive stage. 5. Likewise, determine the critical fertility point (i.e., the lowest temperature at which maximum pollen fertility is achieved) by using the tracking technique. Verify the critical sterility period (CSP) and critical fertility period (CFP) information for each of the EGMS lines under growth chamber or phytotron conditions after the field characterization studies have been done.

The proportion of EGMS plants in a population of more than 1,000 plants during the critical sterility period should be 100%, the pollen sterility of each male sterile plant should be more than 99.5%, EGMS lines should have clearly defined sterility- fertility alteration regimes, the male sterile phase should last for more than 4 consecutive weeks, seed setting during the fertile phase should be more than 30%, the critical temperature or photoperiod for inducing sterility should be as low as possible for more stability of the EGMS lines. In addition, these lines should have a good plant type possessing high yield, acceptable grain quality, pest and disease resistance, and adaptability to the target environment (good phenotypic acceptance).

Good phenotypic acceptability and higher out crossing rate are highly essential for a stable TGMS line. Study of floral and morphological traits was found to be highly essential for the release of a commercially viable TGMS line (Virmani *et al*., 1997, Kalaiyarasi and Vaidyanathan, 2002). An availability of more TGMS lines with a favourable genetic background and desirable features of stable sterility and outcrossing potential will intensify two-line breeding programs. Low seed yield has been identified as the major limitation to hybrid rice technology. To enhance hybrid seed yield, the natural outcrossing of more sterile lines should be increased, which depends upon many floral traits such as panicle exsertion, stigma exsertion, duration of glume opening, etc. (Robin *et al*., 2010).

2.1.1.3 Biochemical and Physiological Analysis

The pollen fertility in thermosensitive genetic male sterility (TGMS) lines is regulated by temperature. Male reproductive development is considered as the most sensitive stage to environmental fluctuations than compared to vegetative and female reproductive phase (Sakata *et al*., 2008). The mechanism behind the thermosensitive genic male sterility in rice is not yet understood. The physiological and biochemical analyses of the sterile and fertile plants showed a significant influence of various enzymes during these conditions.

Free radical scavenging enzymes, Catalase (CAT) is mainly involved in the destruction of hydrogen peroxide and oxidation of hydrogen donors. The enhanced production of most reactive hydroxyl radicals, which cause peroxidation of unsaturated lipids of cell membrane and damaging the structural integrity of the membranes, change the structure and functions of proteins including enzymes and nucleic acids, is due to the impaired activity of catalase.

Liang and Chen (1993) and Zou *et al*., (1993) reported a reduction of CAT activity in sterile Photoperiod / Thermo Sensitive Genic Male Sterility (PTGMS) lines. They suggested the weaker oxygen scavenger system along with lipid peroxidation in anthers might be responsible for male sterility in CMS and TGMS lines. Peroxidase (POX) is the enzyme, which is very sensitive to environmental fluctuations and is considered as the measure of a plant's resistance to stress. The major function of POX is the catalytic conversion of H_2O_2 produced by SOD to water. In sterile TGMS lines, there was upto 37 % reduction of POX activity in leaves compared to fertile TGMS lines.

In PSGMS, sterility is caused by pollen abortion which results through the degradation of tapetal cells (Papini *et al*., 1999). Most sensitive stage of male fertility induction is during the period between secondary branch differentiation

and pollen mother cell (PMC) formation. Tapetal cell development and differentiation are found to be crucial for early male reproductive development.

In a detailed functional characterization of rice Ugp1, Chen *et al*., (2009) indicated that temperature-sensitive splicing is the molecular basis for fertility reversion of TGMS in Ugp1-cosuppressing plants. Ugp1 is essential for PMC meiosis and microspore development in rice. Silencing of Ugp1 by RNA interference or co-suppression affects callose deposition during pollen wall development and leads to the degeneration of the pollen mother cell (PMC) at the early meiosis stage, resulting in male sterility in rice.

Shi *et al*., (2009) postulated that pollen abortion in Photoperiod Sensitive Genic Male Sterility (PSGMS) rice first occurs before the pollen mother cell (PMC) stage, and continues during the entire process of pollen development until pollen degradation. The abortive process was closely associated with the abnormal behaviour of the tapetum. Although tapetum degeneration in Photoperiod Sensitive Genic Male Sterility in rice initiates already at the PMC stage, it proceeds slowly and does not complete until the breakdown of the pollen. Such cytological observations were supported by the results of the TUNEL (TdT-mediated dUTP Nick End Labeling) assay, which detects DNA fragmentation resulting from programmed cell death (PCD), indicating that the premature tapetum degeneration is in the process of PCD.

Genome-wide identification has uncovered large amount of lncRNAs in Arabidopsis, Rice, Maize and Wheat, and more information from other plant species will be expected with the aid of deep sequencing technologies. Similar to other species, lnc RNA mediated gene regulation also widely exists in plants (Zhang *et al*., 2013). In rice, an lncRNA LDMAR was cloned for controlling PSMS (Photo-sensitive male sterility) originated from an elite japonica rice variety Nongken 58N (NK 58N). Nongken 58S (NK58S) was a spontaneous mutant exhibiting PSMS, i.e. its pollen becomes completely sterile when grown

under long-day conditions, whereas the pollens are viable under short-day growth conditions. LDMAR is 1236 bases in length and non-protein-coding, essentially an lncRNA (Ding *et al*., 2012).

Zhou *et al*., (2012) postulated that photoperiod- and thermo-sensitive genic male sterility in rice are caused by a point mutation in a novel noncoding RNA that produces a small RNA. They mapped and cloned a major locus, p/*tms*12-1 (photo- or thermo-sensitive genic male sterility locus on chromosome 12), which confers PGMS in the japonica rice line Nongken 58S (NK58S) and TGMS in the indica rice line Peiai 64S (PA64S, derived from NK58S). A C-to-G mutation in this locus confers PGMS in the japonica line NK58S and TGMS in the indica line PA64S. Therefore, they renamed this locus p/*tms*12-1 (photo- or thermo-sensitive genic male sterility locus on chromosome 12.

2.2 TRANSFER OF TGMS TRAIT TO RED RICE BACKGROUND

Any line can be converted into a TGMS line as TGMS is controlled by a single recessive nuclear gene (Borkakati and Virmani, 1996; Ali and Khan, 1997). There were also some reports on thermo-sensitive male sterility controlled by dominant genes such as the Pingxiang male-sterile line (Yan, 1989) and the 8987 male sterile line (Li *et al*., 1999), though this kind of male sterile line was difficult to use in rice breeding. Any line also can be used as a pollen parent, thus paving the way for widening the genetic base of hybrids. Transfer of TGMS character to different genetic background is easy in TGMS system. There is a report by Lopez and his co-workers in 2003 for the transfer of TGMS trait to aromatic Thai rice background by molecular marker-aided breeding. They derived a F2 population from a cross between Norin PL12 (a TGMS line from Japan) and KDML 105 (a popular aromatic Thai rice cultivar). The monogenic recessiveness of the TGMS gene to be integrated in classical breeding involves a long gestation cycle, which demands substitution of environments for sterility observation and seed

production since the gene is under the influence of the environment (Borkakati and Virmani, 1996).

Transfer of the TGMS gene *tms*2 from Norin PL 12 into indica rice at IRRI has also resulted in several indica TGMS lines that are being shared with collaborating national programs interested in developing two-line rice hybrids. Their utility in breeding two line hybrids will depend on their stability of sterility expression, extent of fertility reversion, and outcrossing rate under field conditions. In order to identify molecular markers tightly linked with the TGMS gene and to map the gene onto a specific rice chromosome, Bulked segregant analysis of an F2 population from 5460s (a TGMS mutant line) x 'Hong Wan 52' was conducted. RAPD markers linked to the rice TGMS gene were identified (primer OPB-19). The TGMS gene was located on chromosome 8.

An F2 population, derived from the cross between fertile and TGMS indica lines, was used to construct a microsatellite-based genetic map of rice for understanding the genetic control of thermosensitive genic male sterility (TGMS) in rice. Alcochete *et al*., (2005) found that the TGMS phenotype showed a continuous variation in the segregant population. The phenotypic and genotypic data allowed for the identification of three new quantitative trait loci (QTL) for thermosensitive genic male sterility in indica rice. Two of the QTL were mapped on chromosomes that, so far, have not been associated with the genetic control of the TGMS trait (chromosomes 1 and 12). The third QTL was mapped on chromosome 7, where a TGMS locus (*tms*2) has recently been mapped.

Lee *et al*., (2005) conducted genetic analysis and molecular mapping based on SSR, STS and EST markers, revealed that a single recessive gene locus involved the control of genic male sterility in Sokcho-MS. By using an F2 mapping population derived from a cross between Sokcho-MS and a fertile indica variety Neda, the new TGMS gene, designated as *tms*6, 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). Subsequently, *tms*6 was fine mapped to the interval between markers RM3351 (0.1 cM) and E60663 (1.9 cM).

An indica rice TGMS mutant, 0A15-1, was crossed with a fertile indica line "Guisi-8" by Wang and his co-workers in2004 to map the gene responsible to the TGMS. A RAPD (Random Amplified Polymorphic DNA) marker, S187-770, linked to the TGMS gene at a distance of 1.3 cM was identified. The S187-770 was then cloned and sequenced to develop a dominant SCAR (sequence characterized amplified region) marker. This SCAR marker can be used in the marker-assisted transfer of this gene to different genetic background. As no other TGMS gene has been mapped on rice chromosome 3, the gene from 0A15-1 is a new TGMS gene and tentatively designated *tms* 6(t) (Wang *et al*., 2004).

Hussain *et al*., (2012) used the RAPD primers UBC 345830, UBC 313927 and microsatellites RM224 and RM21 produced putative markers for differentiating parents and bulks from sterile parent and sterile bulks. The RAPD analysis of individual F2 plants with the primer UBC345830 showed perfect marker–phenotype cosegregation with a distance of 0.8 cM. TGMS locus was mapped onto chromosome 11 using RM21 and RM224, flanking it at a distance of 4.3 and 3.0 cM, respectively. The DNA markers tightly linked to TGMS gene (*tms*8) in F 61 can be cost effectively used for marker-assisted selection of TGMS trait. Gene pyramiding is considered a viable approach to attaining stable phenotype to rice male sterility. Nas *et al*., in (2005) pyramided three thermosensitive genetic male sterility (TGMS) genes – *tms*2, tgms and *tms*5 – using linked microsatellite markers.

The introgression of TGMS gene through conventional breeding is quite cumbersome; it involves identification of TGMS plants in segregating generations and the subsequent induction of fertility by ratooning at the appropriate temperature. In order to accelerate the development of TGMS lines in different genetic background, marker-assisted selection (MAS) through use of DNA

markers offers an attractive alternative. But, to improve selection efficiency in MAS, identification of tightly linked markers and subsequent development of PCR markers from them constitutes two important steps. The theory and advantages of molecular marker based selection over selection based solely on the phenotype has been reviewed (Tanksley *et al*., 1989; Paterson *et al*., 1991). It provides a new solution for selecting and maintaining more durable important genes in rice. MAS is also helpful in transfer of useful genes from exotic germplasm into cultivated lines.

There are about 65 hybrids released from all over India both from private and public sectors. The three line system is the popular method used to develop hybrids. APHR1 and 2 were the first hybrids released from APRRI, Maruteru, Hyderabad. It was reported to have an yield of 7.14 t/ha. It was recommended for cultivation in Andhra Pradesh. Short duration hybrids like DRH-775, JRH-8 etc. with duration of 97 days and 105-110 days respectively were also released. Private agencies like Pioneer Overseas Corporation, Hyderabad, Bayer Bioscience, Hyderabad, Parry Monsanto Seeds Ltd. Bangalore, Mahyco Ltd. Aurangabad also shared the country's hybrid rice production. A maximum yield of 9.41 t/ha was reported from a hybrid developed by RARS, Karnal, Haryana. The last released hybrid was TNRH174 from Tamil Nadu Agricultural University, Coimbatore in the year 2013. DRR, Hyderabad has released DRRH1 and 2 during the year 1996 and 2005 respectively. CRRI, Cuttack also has developed a hybrid named CRHR-32 with a yield potential of 5.43 t/ha.

Hybrid rice technology is the best option for increasing the production of rice in Kerala by exploiting the hybrid vigour. Generation of hybrids using cytoplasmic male sterility (CMS) system have many technical difficulties. This system will yield slightly-scented rice hybrids which are not generally preferred in Kerala and also with limited choice of male parental lines as restorers. Being a tropical zone with significant variation of temperature between seasons, Kerala is best suited for hybrid production using thermosensitive genic male sterile

(TGMS) lines. TGMS lines are male sterile at higher temperature ie. above 31^{o}C (Yamaguchi *et al.,* 1997), 36/22°C (max/min) and can be used as female parent for hybridisation and behave fertile at low temperature 30/13°C (max/min) and can be used for seed multiplication (Latha *et al.,* 2005). In these lines, the male sterility is controlled by recessive nuclear gene(s), which can be transferred to any background.

As people of Kerala prefer red rice, the development of red rice hybrids can be done using this technology. Kerala has no hybrids. Molecular markers have enormous potential to tag the gene of interest and help easier selection of individual at the early stage of development without the requirement of higher temperature. At this juncture, it is high time to develop hybrid rice with red rice background using microsatellite marker assisted breeding.

2.2.1.1 Development of Background Selection Marker

The trait, "red colour pericarp" was denoted by the gene 'rc'. The red coloured and white coloured rice differs in 14bp in rc gene. Red coloured pericarp character was reported to be a trait that disappeared during domestication (Sweeney *et al*., 2006).

Materials and methods

3. MATERIALS AND METHODS

The present investigation entitled "Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)" was conducted in the field and rain out shelter maintained by the Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University during the years from January, 2011 to December, 2013. The main objectives were to develop molecular markers associated with TGMS gene and to transfer TGMS character to red rice background. The materials and methods adopted for the study are given below.

Two TGMS lines (EC720903 and EC720904) were imported from IRRI through Standard Material Transfer Agreement (SMTA) through National Bureau for Plant Genetic Resources (NBPGR), New Delhi. EC720903 and EC720904 will be denoted hereafter as TGMS1 and TGMS2 respectively. The lines TGMS1 and TGMS2 are from two different TGMS sources ID24 and IR 32364 respectively. The present investigation was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University. The latitude and longitude of the location is 8.50N and 76.90E respectively with a height of 29 m above MSL. The TGMS lines along with two popular red rice varieties of Kerala state, Uma and Jyothi were the source material for the study. The pedigrees of the two TGMS lines are shown in the fig. 2 and 3. The details regarding the TGMS genes present in both TGMS sources, ID24 and IR 32364 is furnished in the Table 1.

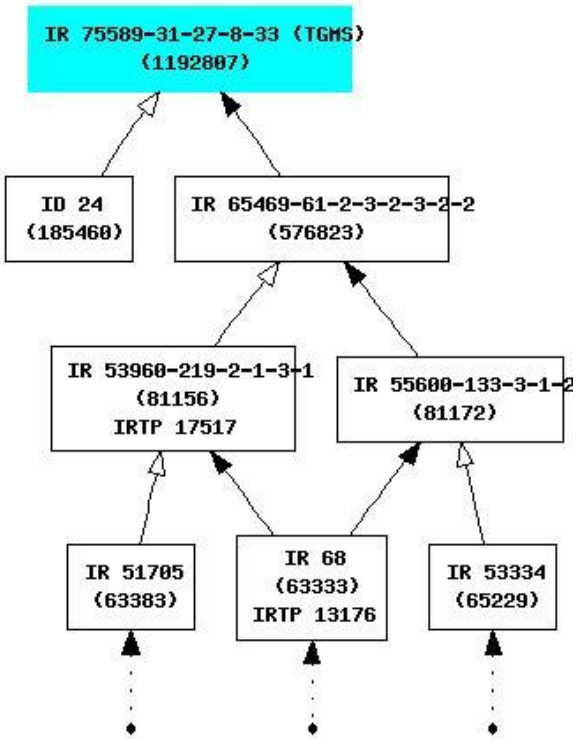


Fig. 2 Pedigree of the TGMS rice line, TGMS1

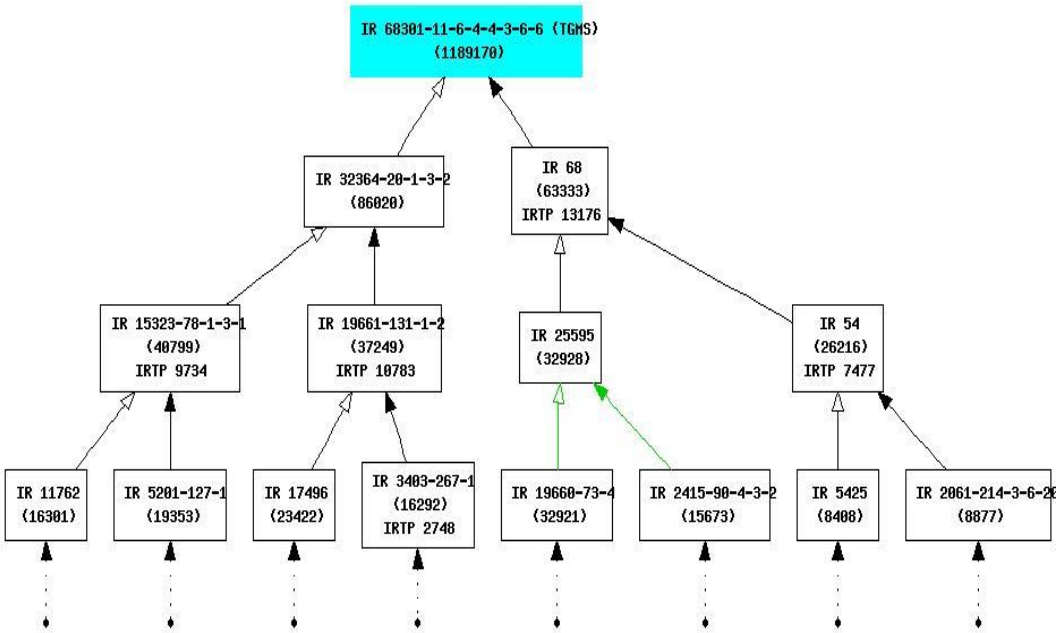


Fig. 3 Pedigree of the TGMS rice line, TGMS2

3.1 DEVELOPMENT OF MOLECULAR MARKERS ASSOCIATED WITH TGMS GENE

There are several *tms* genes reported so far. The list of *tms* genes reported is listed in the table (2) below. Before development of a molecular marker associated with TGMS gene in the present TGMS lines, a few evaluation and characterisation studies need to be done.

3. 1.1 Phenological Characterisation of Selected TGMS Lines and Red Rice Lines and Selection of Stable TGMS Lines

The crop life cycle can be divided into vegetative, reproductive and ripening stages. The vegetative period is divided into basic vegetative and photoperiod sensitive phases. Vegetative period comprises of germination, emergence and tillering stages. Panicle initiation stage marks the starting of the reproductive stage. Panicle initiation stage is the stage at which the panicle has grown about 1mm long. This stage denoted the top dressing window for nitrogenous fertilizer. Internode elongation starts during this time and continue till heading. Usually the top five internodes were elongated during heading. Floral structure emerges out of the swollen flag leaf succeeding the "booting stage". Heading occurs about 25-30 days after panicle initiation. Heading is followed by ripening stage.

3.1.1.1. Determination of Critical Sterility Period

The critical sterility temperature and critical sterility period of TGMS lines were characterised in the field. The crop was raised in pots and was exposed to field conditions round the years, 2011-'13. Each pot was labelled separately for making observation during different stages of plant growth. Five plants were selected at random for taking observation. During the time of anthesis, five spikelets from each plant were sampled and anthers were stained in 1% Iodine Potassium Iodide (I-KI) solution to analyse the fertility and sterility status of pollen grains. The anthers were crushed using a needle and debris was removed.

A cover slip was placed and observed under stereo microscope (Leica). Round and darkly stained pollen grains were taken as fertile and irregularly shaped lightly stained ones were taken as sterile pollen. Average of the pollen fertility value of five pots was expressed in percentage. Till maturity, each panicle was covered with paper bags to avoid cross pollination. Number of filled grains was counted and the percentage of spikelet fertility was also assessed.

The temperature sensitizing period was determined by physical cum morphological index method and through tracking method (Ali, *et al.*, 1995). Under opened condition, the plants were grown at sterility inducing condition ie. when the maximum/ minimum temperatures are 36°C /22°C respectively for a period of ten days ie. 15-24 days before heading as the most sensitive phase of panicle development, the stamen-pistil primordial stage, lies between 24 and 15 days (Ramakrishna *et al.*, 2006). The day on which first spikelet emerges out was taken as the date of panicle emergence. The date of panicle emergence and fertility status of the pollen was recorded on daily basis. The date on which complete sterility was occurred was taken as the tracking date. Five spikelets were taken for pollen fertility/ sterility studies. and environmental parameters like maximum and minimum temperatures, relative humidity and sunshine hours during the period was recorded. The primary tiller was also split opened to study the critical phase of panicle and correlated the panicle size with length of the flag leaf. Twenty plants in each line were taken to characterize these floral traits, morphological traits, agronomic traits and quality traits during the crop growth period.

3.1.1.2 Phenological, Floral, Morpho-Agronomic Characterisation of TGMS Lines

The sterile lines, TGMS1 and TGMS2 and pollen parents were staggeredly sown on monthly interval. They were grown in plastic pots and maintained in the field for the initial evaluation studies. The set of twenty plants were observed

from each line for performing the phenological, floral, morpho-agronomic characterisation of parental lines.

Table 1. TGMS genes present in popular indica TGMS lines under study

TGMS lines	Parental source	Genes	Chromosome
TGMS1	ID 24	*tms1*	No.8
TGMS2	IR32364	*tms3*	No. 6

Siddiq and Ali, 1999

3.1.1.2.1 Phenological Characterisation of Stable TGMS Lines

The critical stage in the lifecycle of rice plant in the present study is the reproductive stage, especially panicle initiation. The development stage of panicle is given in the Table 3.

3.1.1.2.1.1 Stages of panicle development can be determined by various methods

Physical method

The stages of panicle development from the day of its initiation were determined by periodically split-opening the main tillers of the TGMS line.

Morphological index method

The flag leaf length was used as a morphological index to determine the stage of panicle development (Yoshida, 1981) (Table 4). Yoshida in 1981 reported 7 stages of panicle development and the corresponding length of the

developing panicle. When the flag leaf is about 4-5 cm long, or 20% of the length of the preceeding leaf, the panicle developing inside is at stage V.

Table 2. List of *tms* genes identified from different sources

Sl. No.	TGMS gene	Source	Chromosomal location	Linked markers	Reference
1	*tms 1*	5460S (Spontaneous)	8	OPB-19(RAPD), RM 239	Wang *et al.*, 1995
2	*tms 2*	Reimei (X-ray)	7	RM 2,11	Yamaguchi *et al.*, 1997
3	*tms 3*	IR 32364 (X- ray)	6	OPF-18, OPAC-3, SCAR	Subudhi *et al.*, 1997
4	*tms 4*	VN1	2	RM27, AFLP, RFLP	Dong *et al.*, 2000
5	*tms 5*	Annong S-1 (spontaneous)	2	RM 5862, RM 5897, RM174	Wang *et al.*, 2003
6	*tms 6*	Sokcho- MS	2,5	RM3351, RM 2020	Lee *et al.*, 2005
7	*tms 7(t)*	-	7		Rongbai *et al.*, 2005
8	*tms 8*	-	11	RM21, RM224, RAPD, SCAR	Hussain *et al.*, 2012
9	*tms 9*	Zhu 1S	2	Indel 37, Indel 57	Sheng *et al.*, 2013
10	*Ms-h*	-	9	-	Koh *et al.*, 1999
11	*rtms-1*	J 207S	10	-	Jia *et al.*, 2001

Table 3. Developmental stages and morphological characteristics of panicle

Sl. No.	Developmental stages	Panicle length
1.	Necknode differentiation stage	-
2.	Branch differentiation stage	-
(a)	Primary branch differentiation	-
(b)	Secondary branch differentiation	0.5-0.9 mm
3.	Spikelet differentiation stage	1.0 -15.0 mm
4.	Pollen mother cell differentiation stage	1.5-5.0 cm
5.	Reduction division stage of pollen mother cell	5.0-20.0 cm
6.	Exine formation stage	Full length
7.	Ripe pollen stage	Full length

(Yoshida, 1981)

Table 4. The relative growth of flag leaf indicating the different stages of panicle development

Flag leaf		Panicle	
Length (cm)	Relative length	Size (cm)	Stage
4.0	1/5 of (n-1)th leaf	0.2	IV (pistil – stamen primordia formation)
6-8	3/5 of (n-1)th leaf	2.5-3.5	V (pollen mother cell formation)
	4/5 of (n-1)th leaf		VI (beginning of meiosis)
Collar- auricle of flag leaf and (n-1)th leaf at same level			VII (meiosis in the middle of panicle)

(Yoshida, 1981)

Tracking method

The date of panicle emergence and pollen fertility status of each test line raised in adequate population at one-day intervals in the field were recorded daily. The date when the first spikelet protruded out was taken as the day of panicle emergence. Five spikelets were examined on the same day for pollen fertility. The observations were continued till the pollen was found to be totally sterile in the newly emerging panicles and 3-4 such days were chosen as tracking dates. On the basis of earlier reports suggesting that the most sensitive phase of panicle development, i.e. the stamen-pistil primordia stage, lies between 24-10 days before heading, the day of critical stage was determined with the help of weather chart showing maximum-minimum temperatures. Days were counted backward from the tracking date between 10 and 24 days and the dates, when the maximum temperature in this period was above 30°C were noted. The temperature record for the 24-10 day pre heading period of TGMS rice line was examined for three tracking dates and the dates on which the maximum temperature was above 30°C were regarded as critical temperature days coinciding with the sensitive stamen-pistil primordia phase. In the event of more than one date qualifying as critical temperature days, the day with maximum mean temperature was taken as critical. If that too was not discriminative enough, high minimum temperature was used as the criterion for judgement. Among the prospective days, the n^{th} day having the highest mean temperature was taken as the day of critical temperature during the sensitive stage. If several days were identical in respect of all the foregoing parameters, then two consecutive critical days with the highest mean temperatures were taken together as the sensitive period.

3.1.1.2.2 Floral, Morpho-agronomic Characterisation of TGMS Lines

Morphological, agronomic and floral traits were studied during the specific crop growth stage and were recorded as per the standard evaluation systems (SES)

given by International Rice Research Institute (IRRI). Based on the SES, the TGMS lines were scored and the stable line with ideal characters was identified.

3. 1.1.3 *Biochemical and Physiological Analysis*

The plants were grown in the department field till stage III and at stage III they were transferred to the rain out shelter. The plants were maintained in the rain out shelter till stage VII of panicle development and physiological and biochemical analyses were done immediately after stage VII. The leaf samples were collected from fertile and sterile plants during this time and used as samples for the analyses. Index leaf was used for the purpose.

3. 1.1.3.1 *Estimation of Total Sugars*

Weighed 100 mg of the leaf sample (of sterile and fertile plants) into a boiling tube and hydrolysed by keeping it in a boiling water bath for three hours with 5 mL of 2.5 N HCl and cooled to room temperature. It was neutralised with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 mL and centrifuged. Collected the supernatant and took 0.5 and 1 mL aliquots for analysis. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard (Standard glucose: Stock—Dissolved 100 mg in 100 mL water. Working standard—10 mL of stock diluted to 100 mL with distilled water. Stored, refrigerated after adding a few drops of toluene) '0' served as blank. Made up the volume to 1 mL in all the tubes including the sample tubes by adding distilled water and then added 4 mL of anthrone reagent (Dissolved 200 mg anthrone in 100 mL of ice-cold 95% H_2SO_4.). Heated it for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm. A standard graph was plotted with concentration of the standard on the X-axis versus absorbance on the Y-axis. Calculated the amount of carbohydrate present in the sample tube from the graph.

3. 1.1.3.2 *Estimation of Reducing Sugars (DNS Acid Method)*

The estimation of reducing sugars in plants was done following Dinitro Salicylic acid (DNS) method (Sadasivam and Manickam, 2008). The leaf samples from sterile and fertile plants were weighed (100 mg) and the sugars were extracted with hot 80% ethanol, twice. The supernatant was collected and evaporated by keeping it on a boiling water bath at 80^0C. The sugars were dissolved by adding 10 ml water. Aliquots of 0.5 to 3 ml were pipetted out into test tubes and the volume was equalized to 3 ml with distilled water in all the test tubes. To this 3 ml of DNS reagent was added. The test tubes were heated in a boiling water bath for 5 minutes. Rochelle salt solution (40%, w/v) (1 ml) was added to the test tubes when the contents were hot. Then the test tubes were cooled and the intensity of dark red colour was read at 510 nm. A series of the standard, Glucose, (0 to 500 µg) was run and a standard curve was plotted. The amount of reducing sugars in the sample was calculated from the standard graph.

3. 1.1.3.3 *Estimation of Total Soluble Protein (Bradford method)*

The total soluble proteins were estimated following the Bradford method (1976). A series of protein samples were prepared in PBS. The experimental samples (leaf) were harvested from sterile and fertile plants and prepared in 10 microliter of PBS. A known volume (5 ml) of diluted dye binding solution was added to each tube. The solution was mixed well and allowed to develop a blue colour for at least 5 min but no longer than 30 min. The red dye turns blue when it binds protein and its absorbance was measured at 596 nm. A standard curve was plotted using the field plot absorbance verses concentration. The protein in the experimental sample was calculated using the standard curve.

3. 1.1.3.4 *Estimation of Proline*

The proline content in the plant leaf samples was estimated following the method described by Sadasivam and Manickam (2008). The leaf sample from sterile and fertile plants (0.5 g) was extracted by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. The homogenate was then filtered through Whatmann No. 2 filter paper. Aliquot of 2 ml was drawn from the filtrate in a test tube and 2 ml of glacial acetic acid and 2 ml of acid ninhydrin was added to it. The mixture was heated in a boiling water bath for one hour. The reaction was terminated by placing the tube in ice bath. Toluene (4 ml) was added to the reaction mixture and stirred well for 20- 30 seconds. The toluene layer was then separated and warmed to room temperature. The red colour intensity was read at 520 nm. A series of standard with pure proline was run in a similar method to prepare the standard curve. The amount of proline in the sample was calculated from the standard curve. The proline content is expressed as follows.

µ moles per g tissue ={[(µg proline / ml) x ml toluene]÷ 115.5} x (5/ g sample)

where 115.5 is the molecular weight of proline

The content of Proline can be calculated using the formula given below,

$$\text{Proline content} = \left\{ \frac{\text{Concentration (ug)} * \text{OD of the sample}}{\text{OD of the standard}} \right\} * \frac{V}{W} \quad (\text{ug/g})$$

3. 1.1.3.5 *Estimation of Catalase (E. C. 1.11.1.6):*

The CAT activity in sterile and fertile plants was quantified following the method described by Luck (1974). A 20% homogenate of the leaf sample was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay. The H_2O_2-phosphate buffer (3.0 ml) was taken in an experimental cuvette, followed by the rapid addition of 40 µl of

enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer. The enzyme solution containing H_2O_2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 24 0nm by 0.05 units.

3. 1.1.3.6 *Estimation of Peroxidase (POX)*

The peroxidase activity in sterile and fertile plants was estimated following the method described by Reddy *et al*., (1995). A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the sample, clarified by centrifugation and the supernatant was used for the assay. To 3.0 ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5 ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

3. 1.1.3.7 *Estimation of Chlorophyll and Carotenoids (DMSO method)*

A weighed quantity of leaf sample (0.5g) was taken from sterile and fertile plants and cut into small bits. These bits were put in test tubes and incubated overnight at room temperature, after pouring 10 ml DMSO: 80 % acetone mixture (1:1 v/v). The coloured solution was decanted into a measuring cylinder and made up to 25 ml with the DMSO- acetone mixture. The absorbance was measured at 663, 645, 480 and 510 nm using a spectrophotometer. The chlorophyll content was measured by substituting the absorbance values in the given formulae (Hiscox and Tsraelstam, 1979).

Total Chl (a + b) = (8.02 x A_{663} + 20.2 x A_{645}) x V/1000 x 1/ Fresh weight

Carotenoid = [(7.6 x A_{480}) – (1.49 x A_{510}) x V] / (w x 1000)

3.1.2 Production of F_1Plants from Selected TGMS Lines and Red Rice Lines

3.1.2.1 Production of F_1

The TGMS and non TGMS plants were crossed to obtain F_1. For hybridisation programme, TGMS plants were grown under sterility inducing condition. The fertility/ sterility status of the pollen grains was determined using the 1% IKI solution. The sterile plants were dusted with the pollen grains from male parents, Uma or Jyothi to facilitate and got F_1 plants. Different methods of hybridisation like proximal hybridisation, focussed dusting and excision method were adopted. In proximal hybridisation, the panicles of the two parents are placed close to each other and kept for pollination to happen naturally. In focussed dusting method, the pollen parent will be selected and the pollen is collected during the time of anthesis, then the pollen will be dusted on to flower of the female TGMS line. In excision method of hybridisation, the female flowers are cut open for the better receival of pollen grains from male flowers. The focussed dusting method yielded a better percentage of seed set when compared to other two methods.

3. 1.3 Development of Mapping Population and Standardisation of Markers

3.1.3.1 Production of F_2 and Total DNA Isolation

The stable TGMS parent, TGMS1 was crossed with a popular red rice variety, Uma and F_1s were produced. F_1 s were then selfed and the selfed seeds were harvested separately from each F_1 plants. This F_2 population was used for

marker analysis. Microsatellite analysis was done to find the SSR markers polymorphic to the *tms* gene. The DNA was isolated from the 50 F_2 plants.

Total genomic DNA was isolated from fresh-frozen leaf tissue of TGMS and red parental rice lines along with the F_1 using IRRI protocol (Modified CTAB Method). Ground tissue (50 µl) was taken in 2 ml microtubes. 500 µl of 2x CTAB buffer (containing 40 µl/ 20 ml *b*-mercapthoethanol (for older leaves) or optional or replaced it with SDS) was added, warmed to 65ºC. Mixed thoroughly and incubated at 65ºC for 30 minutes to 1 hour. Briefly cooled and added 500 µl chloroform-isoamyl alcohol (24:1). Centrifuged at 5000 rpm for 15 minutes after shaking at room temperature for 20 minutes. Decanted aqueous phase (top phase) into a new 2 ml tubes and added 500 µl isopropanol (0.5 ml, 1 volume) and kept for incubation at -20ºC for 30 minutes. Centrifuged at 5000 rpm for 10 minutes (12,000 rpm for 5 min: try 30 secs to avoid unwanted carbohydrates). Decanted isopropanol and washed the pellet with 500 µl 70% ethanol and then drain dry. Then dissolved the pellet in (100 µl) TE. Added (1.0 µl) RNAse (10 mg/ ml) and incubated at 37ºC for 30 minutes. Added 1/10 volume sodium acetate (10 µl) and 2 volumes of absolute ethanol (200 µl). Kept for incubation at -20ºC for 1 hour or overnight. Centrifuged at 5000 rpm for 15 min, drained and rinsed the pellet with 70% ethanol. Air dried and dissolved the pellet in 100-200 µl TE. Centrifuged for 5 minutes at 5000 rpm to pellet the debris. Stored the stock solution in –20ºC and the working solution at 4ºC. [2X CTAB (1l) – 20 g CTAB dissolved in 860 ml sterile ddH_2O. (Added 81.82g NaCl, 100 ml 1M Tris pH 8.0, 40 ml 0.5M EDTA pH 8.1, autoclaved and stored at room temperature. Added 40 µl *b*-mercapthoethanol per 20 ml solution just before use).

3.1.3.2 Checking of DNA Concentration in Gel

DNA was checked for its quality and quantity by 0.8% agarose gel electrophoresis. Isolated DNA was used for the PCR amplification.

3.1.3.3 SCAR Marker Analysis and Standardisation of Polymerase Chain Reaction (PCR)

Sequence Characterised Amplified Region (SCAR) is a codominant marker. The details of SCAR primers used in the investigation are presented in the Table 5. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The PCR components for respective primers were standardized to obtain the optimum condition for PCR amplification profile.

Table 5. List of SCAR markers used for the present study

Primer Name	Sequence
RM9-F	TTCCCGGGTTCCACTAGGAT
RM9-R	AGAGAGTGATCTATGCCCTG
RM10-F	TTCCCGGGTTGGGTTAGGGG
RM10-R	GCGGACCGTGGAAGCTGGGG

3.1.3.4 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

The PCR products were checked in 1.0% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 50 or 100 bp ladder. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.1.3.5 Standardisation of Polymerase Chain Reaction (PCR)

A set of 45 SSR primers were used for the present investigation. The list of primers used for the study is given below (Table 6). These were selected on the basis of genetic distance from the gene of interest (www.gramene.org). The PCR reaction was carried out using Taq polymerase in 20 ml reaction volume containing 1.5X PCR buffer, 2 mM $MgCl_2$, 0.02 mM of each dNTPs, 1 mM of forward and reverse primers, 0.5 µl (3 units) Taq polymerase and 50 ng genomic DNA. Amplification profile was standardized after giving varying temperature condition and concentrations of the components of PCR mix.

Table 6. List of SSR markers used for the study

Sl. No.	Name	Sequence	
		Forward	Reverse
1	RM2	ACGTGTCACCGCTTCCTC	ATGTCCGGGATCTCATCG
2	RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
3	RM214	TTTTCCTTCTCACCCACTTCA	TCTTTGACAAGAGGAAAGAGGC
4	RM27	CAGGGACCCACCTGTCATAC	AACGTTGGTCATATCGGTGG
5	RM29	AGCGACGCCAAGACAAGTCG CC	TCCACGTCGATCGACACGACGG
6	RM174	ATGGAAGGAATGGAGGTGAG	TACCCCTACGTCGATCGATC
7	RM3351	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
8	RM5897	GGCATCTTCCCCTCTCTCTC	CCAACCCAAACCAGTCTACC
9	RM3859	TTGCAGATCGGTTTCCACTG	GGTCCTGGATTCATGGTGTC
10	RM7121	GGAGATGGCACACGTCAAAC	AGGATCCCGTTTTGTAGCAG
11	RM6776	AGCCCGGACATGCAAAAC	GAAGCAGGCGAAATCTCCTC
12	RM21197	CGGTGAGAATGGTACTCTGTC CTACC	ATGGGCAAGGGCAATTAAAGG
13	RM239	TACAAAATGCTGGGTACCCC	ACATATGGGACCCACCTGTC
14	RM589	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG

Table 6. continued

Sl. No.	Name	Forward	Reverse
15	RM 224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
16	RM 212	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG
17	RM 227	ACCTTTCGTCATAAAGACGAG	GATTGGAGAGAAAAGAAGCC
18	RM 252	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG
19	RM 261	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC
20	RM 26	GAGTCGACGAGCGGCAGA	CTGCGAGCGACGGTAACA
21	RM 25	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC
22	RM 205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG
23	RM 219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
24	RM 244	CCGACTGTTCGTCCTTATCA	CTGCTCTCGGGTGAACGT
25	RM 258	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC
26	RM 228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
27	RM 229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT
28	RM 202	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA
29	RM 204	GTGACTGACTTGGTCATAGGG	GCTAGCCATGCTCTCGTACC
30	RM 225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
31	RM 23	CATTGGAGTGGAGGCTGG	GTCAGGCTTCTGCCATTCTC
32	RM 31	GATCACGATCCACTGGAGCT	AAGTCCATTACTCTCCTCCC
33	RM 38	ACGAGCTCTCGATCAGCCTA	TCGGTCTCCATGTCCCAC
34	RM 80	TTGAAGGCGCTGAAGGAG	CATCAACCTCGTCTTCACCG
35	RM 41	AAGTCTAGTTTGCCTCCC	AATTTCTACGTCGTCGGGC
36	RM 44	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC
37	RM 210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG
38	RM 223	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG
39	RM 201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA
40	RM 216	GCATGGCCGATGGTAAAG	TGTATAAAACCACACGGCCA
41	RM 206	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG
42	RM 83	ACTCGATGACAAGTTGAGG	CACCTAGACACGATCGAG
43	RM 247	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG
44	RM 207	CCATTCGTGAGAAGATCTGA	CACCTCATCCTCGTAACGCC
45	RM 253	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC

3.1.3.6 SSR Marker Analysis and Capillary Electrophoresis

Genomic DNA was used as template for PCR amplification. Standardisation of PCR condition was done. Allele sizing was determined using Capillary electrophoresis using four parental lines. One micro litre of the PCR product was added to 10 µl Hi-Di formamide (Applied Biosystems) and 0.5 µl GeneScan 500 Liz - Size Standard and run on the ABI 3500 Genetic Analyzer. Data was analysed using GeneMapper ID-X v1.4 software. Set of random SSR markers (http://archive.gramene.org/markers/microstat/) were used in the analysis of parental lines to find polymorphism.

3.1.3.7 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

The PCR products were checked as described in 3.1.3.4. The SSR-PCR product was analyzed on 3% agarose gel and was visualized by staining with ethidium bromide and transillumination under shortwave UV light. DNA ladder used in the electrophoresis was 100 bp.

3.1.3.8 Linkage Analysis and Calculation of Segregation Ratio

Fig. 4 shows the linkage map of rice with different SSR markers. In the present study, 50 F_2 plants were used for marker analysis. MAPMAKER/EXP version 3.0 (Lander *et al.*, 1987) were used to locate the SSR markers in the map and for construction of the map. The recombination frequencies were converted to the map distances, centimorgans using the option of Kosambi's function in the software. The value of the interval mapping was 0.1, the LOD score higher than 3was used as threshold for putative locus.

The 50 F_2 individuals scored as fertile and sterile were subjected to Chi-squared (χ^2) test for goodness of fit to test the deviation of the observed segregation data from the theoretically expected Mendelian segregation ratio.

$\chi^2 = \sum(O - E)^2 / E$ where, O = Observed number of individuals

E = Expected number of individuals

3. 2. TRANSFER OF GMS CHARACTER TO RED RICE BACKGROUND

In order to transfer TGMS character to red rice lines, hybridisation between stable TGMS and pollen parents was carried out initially, and backcrossing was done to fix those characters. F_2 mapping population generated was used for the marker analysis and phenotypic validation. The plants were split into two with one half of the tillers growing under fertile and the other half under sterility inducing conditions.

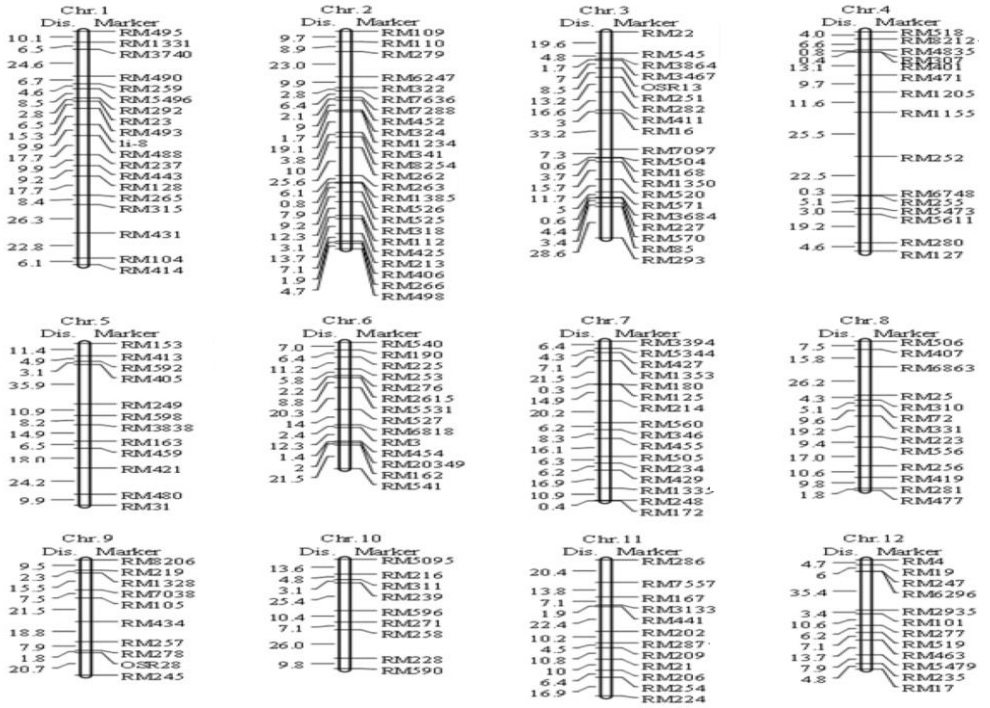


Fig. 4 Linkage map of rice showing various SSR markers

3.2.1 Validation of Markers by Phenotyping Male Sterile Rice Lines

The markers which were able to distinguish the TGMS and pollen parental lines were used for screening the mapping population. The genotypic evaluation was further confirmed using phenotypic observation. The F_2 plants which were found sterile as per the TGMS markers were checked for their sterility status when grown in sterility inducing condition. Thus the markers were validated. The association between the TGMS trait and the markers was confirmed after marker analysis.

3.2.1.1 Development of Background Selection Marker

The present investigation aims at the transfer of TGMS trait to high yielding red rice background.

3.2.1.1.1 Primer Designing and Multiple Sequence Alignment

A marker was designed to distinguish the red plants from the white ones using the 14bp deletion. The sequences of the red coloured pericarp and white coloured pericarp were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/nucleotide/) and aligned using Multiple sequence alignment tool from the website (http://www.ebi.ac.uk/Tools/msa/clustalw2/) Clustal W2. The sequences differing between them ie.14 bp) was used to design the primers. The primer was designed using Primer3 (http://simgene.com/Primer3), a primer designing tool.

3.2.1.1.2 Standardisation of Polymerase Chain Reaction (PCR)

The PCR conditions were standardized for the Rc primer designed. The annealing temperature of the respective primers was optimized doing gradient PCR.

3.2.1.1.3 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

After standardization of the PCR conditions, the PCR products were run in 3% agarose for separation of bands. A molecular ladder of 50 bp was used. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.2.1.2 Phenotyping Red Rice Lines

Sterility and red pericarp colour were the two traits under study. The $F_1$and F_2 progenies were checked for the fertility/ sterility status using 1% IKI stain and pericarp colour was assessed visually. The marker association of TGMS trait was confirmed using phenotyping. The sterile plants showing genotypical difference were grown under low temperature conditions and the fertility transformation was assessed.

Results

4. RESULTS

Two TGMS lines were imported from IRRI through Standard Material Transfer Agreement (SMTA) through National Bureau for Plant Genetic Resources (NBPGR), New Delhi. The lines TGMS1 and TGMS2 are from two different TGMS sources ID24 and IR 32364 respectively. The main objectives of the study were to develop molecular markers associated with TGMS gene and to transfer the TGMS trait to red rice background. The results obtained in the present study are given below.

4. 1 Development of Molecular Markers Associated with TGMS Gene

4.1.1 Phenological Characterisation of Selected TGMS Lines and Red Rice Lines and Selection of Stable TGMS Lines

4.1.1.1 Determination of Critical Sterility Period

The critical sterility temperature and critical sterility period of TGMS plants were determined by physical cum morphological index method and tracking method (Ali, *et al.*, 1995).

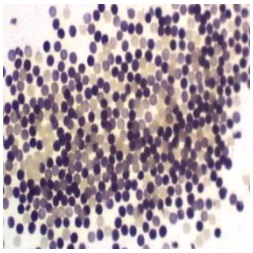
Fig. 5 shows the sterility/ fertility status of the pollen grains of TGMS1 when stained in 1% IKI dye during the time of anthesis. 20.09.12, 19.10.12 and 01.11.12 were considered as the three dates of total sterility (tracking dates). Environmental parameters like maximum and minimum temperatures, and average temperature during 15-24 days before heading (critical sterility period) were recorded and given in the Table 7.

According to Ali *et al.*, (1995), days with higher maximum temperature above 30^{o}C is considered as the critical day. In the present study, the highest maximum temperature is 30.6^{o}C ie. on 22nd day. Hence, that day was assumed as

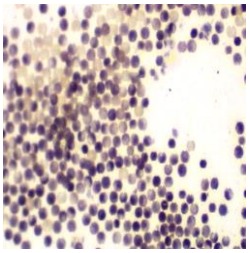
the critical day. The same day has 27.25ºC as the highest average temperature. So, the critical sterility period for the TGMS line, TGMS1 was assumed to be 15-22 days before heading with maximum temperature 30.6ºC and minimum temperature 23.9ºC (Table 8). (As the TGMS line, TGMS2 has shown differential sterility, most of the studies were concentrated to TGMS1). In my study it was observed that a daily average temperature of 27.25ºC and above can result in sterility.

In the present study, day with highest maximum temperature was considered as the critical day. The fertility percentage of the pollen grains of TGMS1 plants were also calculated by growing them at low temperature conditions. The maximum, minimum and average temperatures during the crop growth were assessed. The results have shown that the maximum temperature below 25.75ºC caused 100% fertility (Table 9).

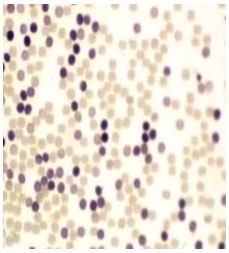
100% fertility



50% fertility



25% fertility



15% fertility

100% sterility

Fig. 5 Sterility/ fertility status of the pollen grains of TGMS1 stained in 1% IKI dye

Table 7. Weather data in the field for the determination of Critical Sterility Temperature (CST) of TGMS line (Tracking method)

TGMS line	Dates of total sterility in 2012	Maximum and minimum temperatures (°C) on different days prior to heading										Critical temperatures (°C)	
		15	16	17	18	19	20	21	22	23	24	Max	Min
TGMS1	20.09.12	30.7/ 23.8	30.4/ 24.7	30.5/ 24.3	30.7/ 24.5	30.7/ 24.2	30.4/ 24.2	30.7/ 24.0	30.7/ 24.4	30.8/ 24.7	31.1/ 24.5	30.7	24.4
	19.10.12	30.4/ 23.3	30.7/ 23.7	28.9/ 23.9	30.3/ 24.2	29.4/ 23.7	30.6/ 23.7	30/ 24.1	30.6/ 23.7	29/ 23.5	30.5/ 23.7	30.6	23.7
	01.11.12	29/ 23.5	30.3/ 21.5	30.2/ 23.4	27/ 23.4	30.2/ 23.4	30.6/ 23	30.9/ 22.4	30.5/ 23.7	30.9/ 21.3	29.9/ 23.5	30.5	23.7
Mean Maximum Temperature		30.03	30.47	29.87	29.3	30.1	30.5	30.5	30.6	30.23	29.9		
Mean Minimum Temperature		23.5	23.3	23.87	24.03	23.77	23.63	23.5	23.9	23.17	23.9		
Average		26.77	26.89	26.87	26.67	26.94	27.07	27	27.25	26.7	26.9		

Table 8. Critical Sterility Temperature (CST) in TGMS1

TGMS line	Dates of total sterility	Critical temperature (°C)		Days before heading
		Maximum	Minimum	
TGMS1	20/09/12	30.7	24.4	22
	19/10/12	30.6	23.7	
	01/11/12	30.5	23.7	
	Average	30.6	23.9	

Table 9. Maximum temperature causing fertility in TGMS1 rice line

TGMS line	Sowing date	Maximum temperature causing fertility (°C)	Fertility (%)
TGMS1	01/03/2012	25.50 (15/04/2012)	100
		25.75 (24/04/2012)	100
	05/05/2012	22.25 (11/06/2012)	100
		22.25 (12/06/2012)	100

4. 1.1.2 Phenological, Floral, Morpho-agronomic Characterisation of TGMS Lines

4. 1.1.2.1 Phenological Characterisation of Stable TGMS Lines

Staggered sowing of TGMS lines along with two popular red rice varieties of Kerala state, Uma and Jyothi was done on monthly intervals for assessing their phenological stages. The date of panicle emergence and pollen fertility status of TGMS lines were noted along with the climatic data like maximum, minimum and average temperature. The date when the first spikelet protruded out was taken as the day of panicle emergence. Five spikelets were examined on the same day for pollen fertility. The observations were continued till the pollen was found to be totally sterile in the newly emerging panicles and three such days were considered as tracking dates. Three such tracking dates were taken and maximum and minimum temperatures during the period (15-24 days) were taken and the average values of three dates were also recorded. Days were counted 15 and 24 days backward from the tracking dates and the days when the average of maximum temperatures was above 30°C, were noted. It was found that highest maximum temperature was on the 22ndday (30.6^{o}C). It was also found that the mean temperature (27.25^{o}C) was highest in this particular day. Hence a period of 15-22 days before heading was assumed as the critical sterility period in the case of TGMS line, TGMS1. Critical sterility temperature calculated was around a daily average of 27.25^{o}C. Above the daily average temperature of 27.25^{o}C TGMS1 plants will be sterile.

The characterisation of TGMS lines was done using different methods like Physical method, morphological index method, and tracking method. The stages of panicle development from the day of its initiation were determined by periodically split-opening the main tillers of the TGMS line. For the determination of panicle initiation, main shoots were selected from the centre of the plant and the roots were cut off just above the root ball. The shoot was sliced lengthwise

with a sharp knife. The panicle was located above the air space. At panicle initiation it appeared as a "furry tip" 1 to 2 mm long. A magnifying glass was used for better identification. Panicle initiation marks the beginning of the reproductive stage. The male reproductive part of rice plant is reported to be mostly affected by high temperature. The main tillers in the plant was pulled out and dissected transversally and the emerging panicle was measured. The panicle at different stages of development is shown in the Fig. 6. Table 10 shows the developmental stages of panicle on different dates before heading. The primary tiller was split open to study the critical phase of panicle and correlated panicle size with length of the flag leaf. The size of panicle was around 0.2 cm during the critical sterility period ie. 15-22 days before heading and the corresponding flag leaf size was 4.2 cm (Table10).

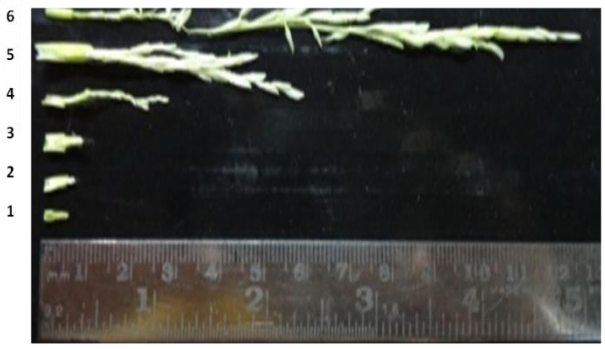


Fig. 6 Different stages of panicle development in male sterile line, TGMS1

(Tiller dissected through Physical method showing the emergence of panicle (1-6) at 0.2, 0.5, 1.0, 2.8, 6.0 and 12.6cm length)

Table 10. Panicle size and comparative flag leaf length on different dates before heading

TGMS line	Panicle size (cm)	Days before heading	Flag leaf length (cm)
TGMS1	0.2	22	4.2
	0.5	19	4.8
	1.0	17	5.5
	2.8	15	6.3
	6.0	13	21.1
	12.6	8	28.1

4.1.1.2.2 Floral, Morpho-agronomic Characterisation of TGMS Lines

Morphological, agronomic and floral traits were studied during the specific crop growth stage and were recorded as per the standard evaluation systems (SES) given by International Rice Research Institute (IRRI). Based on the SES, the TGMS lines were scored and the stable line with ideal characters was identified. As part of the phenological study, a crop calendar showing the days required for the four parental lines is presented in the Table 11. The calendar gives an idea about time of sowing for experiencing sterility condition in TGMS lines for hybridisation purpose. Sowing during March- April and August- September months can be utilised for hybridisation and November- December sowing can be done for seed multiplication.

Twenty plants were selected in the two TGMS lines and the morphological, agronomic and floral traits like ligule length, panicle length, panicle exertion, glume opening, stigma exertion, spikelet fertility and hundred grain weight were studied. Statistical analysis of T-test with twenty replications was performed and a T-stat value at 5% level of significance was used for comparison. It was found

that the characters like panicle length, panicle exertion, glume opening, stigma exertion, spikelet fertility, 100 grain weight was significantly varying between both the lines, TGMS1 and TGMS2.

Panicle exsertion in TGMS1 is 76.38 %, which means that about 76.38% of the spikelets are exserted in the panicle, favouring outcrossing. TGMS2 has lower rate of outcrossing due to lesser panicle exsertion. The stigma of TGMS1 plants is exserted more than the TGMS2 line. The length of the panicle and number of filled grains/ panicle is also more in the case of TGMS1. Glume opening in TGMS1 is about 25.05, which is higher than the other line, TGMS2. Hence, the percentage of self-seed setting is more in TGMS1.

TGMS1 was having desirable breeding traits when compared to the other line TGMS2 (Table 12). Hence the TGMS line, TGMS1 was selected as a better candidate over the other line TGMS2.

Table 11. A crop calendar of selected parental lines in Vellayani conditions

	Days to 50% flowering					
	March- April sown*	May- June sown	July- August sown	Sept- Oct sown	Nov- Dec sown**	Jan- Feb sown
TGMS1	113	100	97	95	83	110
TGMS2	127	123	102	110	84	83
Uma	121	115	115	113	113	115
Jyothi	105	102	95	95	91	95

*March- April and August- September months sowing can be for hybridisation purpose

**November- December sowing can be done for seed multiplication

Table 12. Important breeding traits of TGMS lines

	Ligule length (cm)	Panicle length (cm)	Panicle Exertion (%)	Glume opening (^{o})	Stigma exertion (^{o})	Spikelet Fertility (%)	Number of filled grains/ panicle	100 grain weight (g)
TGMS1 (Mean)	2.29	16.605	76.38	25.05	65.935	70.45	68.725	2.07
TGMS2 (Mean)	2.13	11.315	66.19	20.145	55.36	42.1	39.795	1.14
T value	6.96 *	10.402 *	4.402 *	64.181 *	6.956 *	15.552 *	16.784 *	15.603 *
CV of TGMS1	0.72	11.71	10.38	1.15	8.68	8.25	8.03	8.66
CV of TGMS2	4.91	9.40	9.42	0.84	6.03	12.88	12.80	16.5

CV- Coefficient of variation, *-Significance at 5% level

Table 13. Floral biology and morphological characterization of four parental rice lines

Traits	Pedigree	Anther colour	Stigma colour	Pollen sterility (%)	Basal leaf sheath colour	Collar colour	Ligule colour	Ligule shape	Auricle colour	Apiculus colour	Panicle type	Plant height (cm)	Number of productive tillers
TGMS1	ID 24 x IR 65469-61-2-3-2-3-2-2	White	White	100*	Green	Light green	White	Truncate	Light green	White	Intermediate	53	4
TGMS2	IR 32364-28-1-3-2 x IR 68	White	White	90-100*	Green	Light green	White	Truncate	Light green	Light green	Intermediate	58	3
Uma (MO16)	MO6 X Pokkali	Yellow	White	0	Green	Light green	Light green	Truncate	Light green	Light green	Compact	64	4
Jyothi (PTB39)	Ptb10 X IR8	Yellow	White	0-5	Green	Light green	Light green	Truncate	Light green	Light green	Intermediate	62	3

*- During sterile phase for TGMS

A few floral and morphological characters were also studied (Table 13). TGMS1 line was found short stature than the other TGMS line. Number of productive tillers in line TGMS1 was found to be 4 and TGMS2 was 3. Uma and Jyothi had yellow anther whereas the sterile lines had white anther. Stigma colour, pollen sterility, basal leaf sheath colour, collar colour, ligule length, ligule shape, auricle colour, apiculus colour and panicle type were assessed.

Characters like stigma exersion, number of filled grains per panicle, 100 grain weight were higher in the case of TGMS1. From these results, the choice of TGMS1 as a better TGMS source in further breeding programs is clear.

4.1.1.3 Biochemical and Physiological Analysis

To understand the mechanism behind the male sterility, biochemical parameters like catalase, peroxidase, soluble protein, reducing sugars and proline and physiological parameters like carotenoids and total chlorophyll were estimated for twenty plants from sterile and fertile plants of TGMS1 and TGMS2 lines.

Analysis of variance was conducted using CRD design with 20 replications. The result reveals that there is a significant difference between catalase and peroxidase content between fertile and sterile counterparts (Table 14). This explains the possible role of these antioxidant enzymes in causing male sterility.

Table 14. Differences in biochemical and physiological parameters between fertile and sterile plants

	Catalase enzyme activity (EU/g protein)	Peroxidase enzyme activity (EU /g protein)	Carotenoids (mg/g)	Total chlorophyll (mg/g)	Soluble Protein (mg/g)	Reducing sugar (mg/g)	Proline (mg/g)
TGMS1 Fertile	187 (100.0)	33 (100.0)	0.154 (100.0)	0.363 (100.0)	7.169 (100.0)	47.135 (100.0)	0.942 (100.0)
TGMS1 Sterile	59 (31.6)	25 (75.76)	0.137 (88.96)	0.259 (71.35)	5.338 (74.46)	45.475 (96.48)	2.006 (212.95)
TGMS2 Fertile	178 (100.0)	53 (100.0)	0.154 (100.0)	0.361 (100.0)	4.998 (100.0)	45.635 (100.0)	0.474 (100.0)
TGMS2 Sterile	39 (21.91)	43 (81.13)	0.139 (90.26)	0.276 (76.45)	3.962 (79.27)	44.45 (97.40)	1.472 (310.55)
CD (P=0.05)	11.7 *	1.1*	0.002*	0.016*	0.132*	0.619*	0.195*

Values in parentheses are per cent change in sterile plants as compared to its fertile counterparts,

EU- enzyme units, CD- Critical difference

4.1.2 Production of F_1 Plants from Selected TGMS Lines and Red Rice Lines

4.1.2.1 Production of F_1

The TGMS and non TGMS plants were crossed to obtain F_1. For hybridisation programme, TGMS plants were grown under sterility inducing condition. Among the three different methods of hybridisation (Fig. 7) mentioned previously, focussed dusting was found better. The fertility/ sterility status of the pollen grains was determined using the 1% IKI solution. The sterile plants were dusted with the pollen grains from male parents, Uma or Jyothi. Panicle of the hybrid between TGMS1 and Uma is shown below along with the hybrid seeds (Fig. 8 (a) and (b)). The morphology of the following plants (a) TGMS1, (b) Hybrid plant TGMS1xUma and (c) Uma are given below in the fig. 9

4.1.3 Development of Mapping Population and Standardisation of Markers

4.1.3.1 Production of F_2 and Total DNA Isolation

The stable TGMS parent, TGMS1 was crossed with a popular red rice variety, Uma and F_1s were produced. (For the purpose of developing the hybrids, only Uma was used as a pollen parent. Jyothi has shown differential sterility). F_1 s were then selfed to get F_2 population. This F_2 population was used for marker analysis. Microsatellite analysis was done to find the SSR markers polymorphic to the *tms* gene. The DNA was isolated from the 50 F_2 plants. Fig. 10 shows the F_2 seeds. The sterile and fertile F_2 plants are given Fig. 11. Fig.12 displays the sterile and ferile anthersof F_2 plants when stained in 1% IKI dye.

Total genomic DNA was isolated from fresh-frozen leaf tissue of TGMS and red parental rice lines along with the F_1 and F_2 progeny using IRRI protocol (Modified CTAB Method).

Fig. 7 Different hybridisation methods followed (1) Excision method, (2) Proximal hybridisation and (3) Focussed dusting

Panicle of hybrid (TGMS1xUma) Seeds of hybrid (TGMS1xUma)

Fig. 8 The (a) panicle of hybrid (TGMS1 xUma) and (b) seeds of hybrid (TGMS1xUma)

TGMS1 Hybrid plant (TGMS1xUma) Uma

Fig. 9 Morphology of the plants (a) TGMS1, (b) Hybrid plant (TGMS1xUma) and (c) Uma

Fig. 10 Seeds of F_2

Fig. 11 Sterile and fertile F2 plants

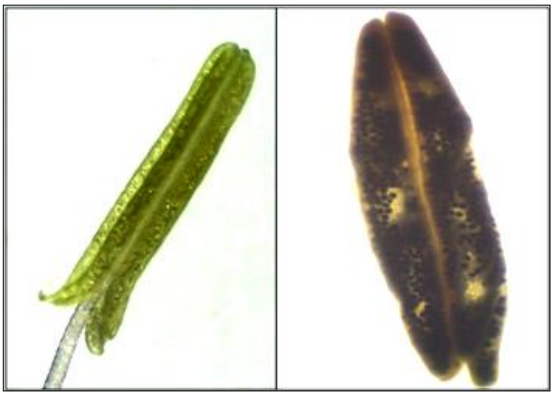


Fig. 12 Sterile and fertile anthers of F_2 rice plants when stained in 1% IKI dye

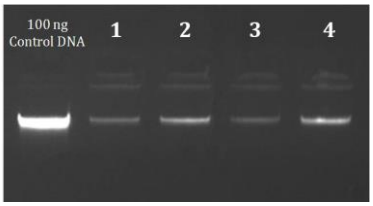


Plate 1. The quality of DNA of Uma (1), Jyothi (2), TGMS1 (3) and TGMS2 (4) in (0.8%) agarose gel electrophoresis

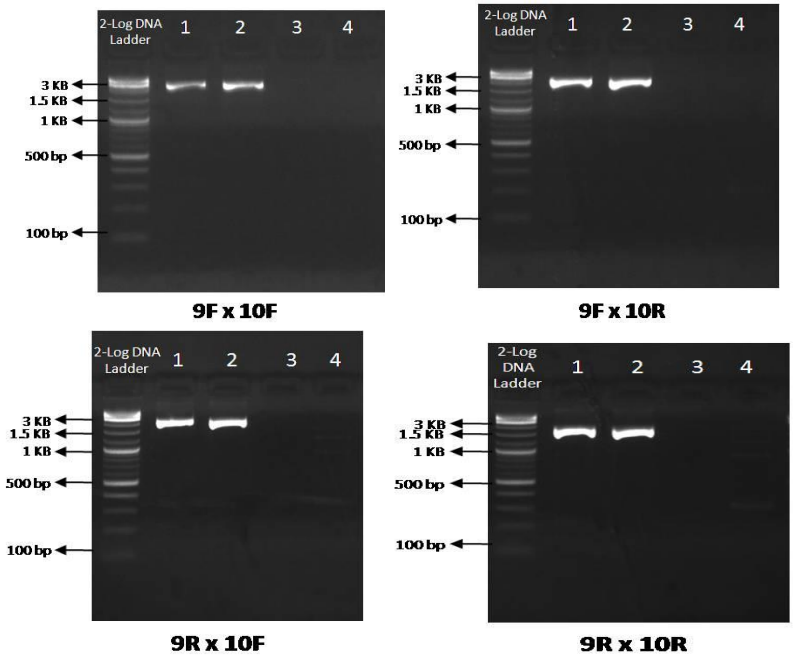


Plate 2. Amplification profile of four genotypes using SCAR markers

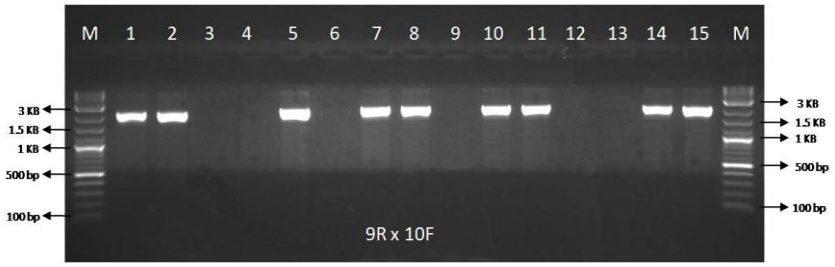


Plate 3. Amplification profile of four genotypes along with F_1 and F_2 using SCAR marker 9RX10F (1- Uma, 2- Jyothi, 3- TGMS1, 4- TGMS2, 5- Neeraja, 6- Mushoori, 7- TGMS1X Uma, 8-10- F_2 plants of (TGMS1X Uma) F_2 1, F_2 2 and F_2 3, 11- TGMS1X Jyothi, 12-15- F_2 plants of (TGMS1X Jyothi))

4.1.3.2 Checking of DNA Concentration in Gel

The quality of extracted good quality DNA is shown in the Plate 1.

4.1.3.3 SCAR Marker Analysis and Standardisation of Polymerase Chain Reaction (PCR)

The standardised PCR amplification profile is as follows: Initial denaturation was given at 98 ^{o}C for 30 sec, followed by denaturation at 98 ^{o}C for 5 sec, primer annealing at 60 ^{o}C for10 sec and primer extension at 72 ^{o}C for 15 sec. The cycle was repeated for 40 cycles. Final extension was given at 72 ^{o}C for 1.00 min.

4.1.3.4 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

The amplification profile of four genotypes using SCAR marker, 9Fx 10F, 9F X10R, 9R X 10F and 9R X 10R is given in the Plate 2.

When SCAR markers were used for PCR amplification, a product was obtained at a size around 3Kb for the two non TGMS parents, Uma and Jyothi. Hence it was used for validating a few F_2 plants along with F_1s and non TGMS plants (Neeraja and Mashoori). Neeraja and Mashoori were used as other non TGMS rice lines. But the SCAR markers were not able to differentiate the Mashoori genotype which was having a band near 3Kb. This SCAR marker was a dominant marker and hence cannot be used for differentiating the heterozygotes.

A representative plate with amplification picture of the SCAR marker 9RX10F is given above (Plate 3).

4.1.3.5 Standardisation of Polymerase Chain Reaction (PCR)

PCR amplification profile for RM primers (RM2, RM11, RM214, RM27, RM29, RM174, RM 3351), RM589 and RM224 is as follows. The template DNA was initially denatured at 98^{o}C for 30 sec, followed by 40 cycles of PCR amplification under the following conditions- denaturation at 98^{o}C for 5 sec, primer annealing at 62^{o}C for 10 sec and primer extension at 72^{o}C for 15 sec. A final incubation of 1.00 min was given for the completion of primer extension at 72^{o}C. For primers RM 5897, RM 3859, RM 7121, RM 6776 and RM 21197, a primer annealing temperature of 58^{o}C was given and all the primers except the above given, including RM 239, the same PCR condition was provided except with primer annealing temperature of 56^{o}C. The difference in the protocol is only the annealing temperature. The varying annealing temperatures given for the different markers were given in the Table 15.

Table 15.The different annealing temperatures of the given markers

Markers	Annealing temperature
RM2, RM11, RM214, RM27, RM29, RM174, RM 3351, RM589 and RM224	62 ^{o}C
RM 5897, RM 3859, RM 7121, RM 6776 and RM 21197	58 ^{o}C
Remaining SSR markers	56 ^{o}C

4.1.3.6 SSR Marker Analysis and Capillary Electrophoresis

Among the total number of 45 SSR markers used, 3 primers could differentiate Uma, Jyothi and TGMS1, TGMS2. 21 polymorphic markers could differentiate TGMS1 and Uma.

The capillary electrophoresis has shown that microsatellite markers RM 7121, RM 6776, RM 239, RM 44, RM 223, RM 201, RM174, and RM 29

produced monomorphic banding pattern irrespective of TGMS and non TGMS lines. RM2 could differentiate Uma and TGMS1 parents with a 2 base pair difference and RM21197 produced a difference of 4 bp. The primers RM224, RM3859, RM 212, RM 252, RM 261, RM 25, RM 219, RM 244, RM 258, RM 228, RM 229, RM 202, RM 204, RM 225 and RM5897 also could differentiate these parents distinctly. The SSR markers RM 11, RM 27, RM 5897, RM 3859, RM 212, RM 261, RM 26, RM 25, RM 219, RM 244, RM 228, RM229, RM 202, RM 204, RM 225 and RM 21197 showed polymorphism between the parents TGMS1 and Jyothi. The primers RM 3351, RM 23 and RM 31 gave polymorphic bands between the TGMS and non TGMS lines. RM 3351 produced an amplification of 173bp in both non TGMS lines, while 179bp in TGMS. These primers were further validated using a larger F_2 population. The electrophoregram showing the allele sizing in Uma, Jyothi, TGMS1 and TGMS2 using SSR marker, RM 3351 is given below as Fig. 13. The allele sizing in Uma, Jyothi, TGMS1 and TGMS2 using different SSR markers is given in the Table 16.

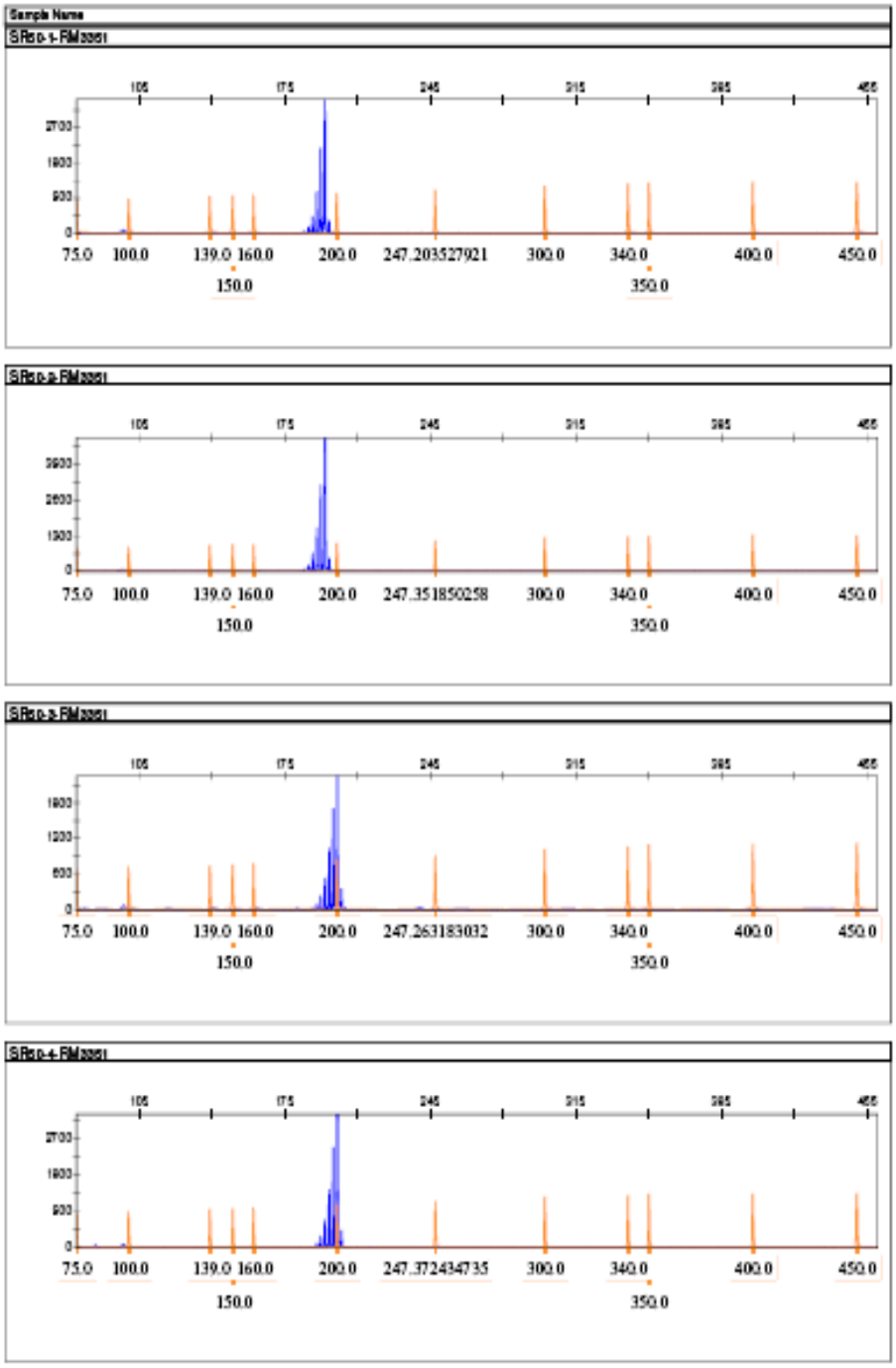


Fig. 13 Electrophoregram showing the allele sizing in Uma, Jyothi, TGMS1 and TGMS2 using SSR marker, RM 3351

Table 16. The allele sizing in Uma, Jyothi, TGMS1 and TGMS2 using different SSR markers

Sl. No.	Primers	Uma		Jyothi		TGMS1		TGMS2	
1	RM2	143	143	145	145	145	145	145	145
2	RM11	143	143	121	121	143	143	143	143
3	RM27	157	157	159	159	157	157	157	157
4	RM3351	173	173	173	173	179	179	179	179
5	RM224	131	131	152	152	152	152	154	154
6	RM5897	155	155	152	152	145	145	145	145
7	RM21197	86	86	88	88	82	82	82	82
8	RM212	130	130	130	130	112	112	108	108
9	RM227	92	92	98	98	98	98	98	98
10	RM252	260	260	215	215	215	215	220	220
11	RM261	124	124	121	121	120	120	119	119
12	RM26	109	109	97	97	109	109	109	109
13	RM25	147	147	141	141	145	145	127	127
14	RM205	111	111	119	119	111	111	119	119
15	RM219	213	213	215	215	219	219	219	219
16	RM244	158	158	158	158	154	154	162	162
17	RM228	105	105	125	125	103	103	147	147
18	RM229	158	158	158	158	154	154	162	162
19	RM202	185	185	160	160	163	163	185	185
20	RM224	130	130	151	151	151	151	153	153
21	RM204	103	103	103	103	169	169	105	105
22	RM225	143	143	141	141	133	133	141	141

Table 16. continued

Sl. No.	Primers	Uma		Jyothi		TGMS1		TGMS2	
23	RM23	142	142	142	142	145	145	145	145
24	RM31	142	142	142	142	139	139	139	139
25	RM207	126	126	126	126	126	126	124	124
26	RM253	137	137	137	137	137	137	139	139
27	RM3859	162	162	162	162	183	183	-	-
28	RM258	136	136	-	-	138	138	132	132
29	RM589	173	173	173	173	173	173	203	203
30	RM224	131	131	152	152	152	152	154	154
31	RM210	151	151	151	151	151	151	151	-
32	RM44	99	99	99	99	99	99	99	99
33	RM223	157	157	157	157	157	157	157	157
34	RM201	153	153	153	153	153	153	153	153
35	RM29	233	233	233	233	233	233	233	233
36	RM174	205	205	205	205	205	205	205	205
37	RM7121	128	128	128	128	128	128	128	128
38	RM6776	162	162	162	162	162	162	162	162
39	RM239	143	143	143	143	143	143	143	143

*-Not worked

4.1.3.7 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

The SSR-PCR product was analyzed on 3% agarose gel and was visualized by staining with ethidium bromide and transillumination under shortwave UV light. DNA ladder used in the electrophoresis was 50bp.

4.1.3.8 Linkage Analysis

The fifty F_2 plants were used for marker analysis. MAPMAKER/EXP version 3.0 was used to locate the SSR markers in the map and for construction of the map. The value of the interval mapping was 0.1, the LOD score higher than 3was used as threshold for putative locus. Seven groups were obtained as below: Group1 has RM23 and RM212 with a mapping distance of 55.9cM in chromosome number 1, Group 2 with RM261 and RM252 with a distance of 84cM, Group 3 with RM3351 and RM31 with a mapping distance of 54.2 cM in chromosome no. 5. Group 2 is residing in chromosome no. 4. Group 4 has RM204 and RM225 in chromosome 6 with a distance of 0.9cM, group 5 with RM3859, RM2 and RM21197 with 15.1 and 8.2 cm respectively in chromosome7 respectively. Chromosome 10 has the group 6 with RM244, RM258 and RM228 with mapping distance of 50.2 and 34cM respectively and chromosome 11 has the last group, group 7 with RM202, RM229 and RM224 with a mapping distance of 18.4 and 56.4cM respectively.

The linkage map obtained as the output from the MAPMAKER software is given in Fig. 14. The following result concludes that these markers may be associated to this *tms* gene.

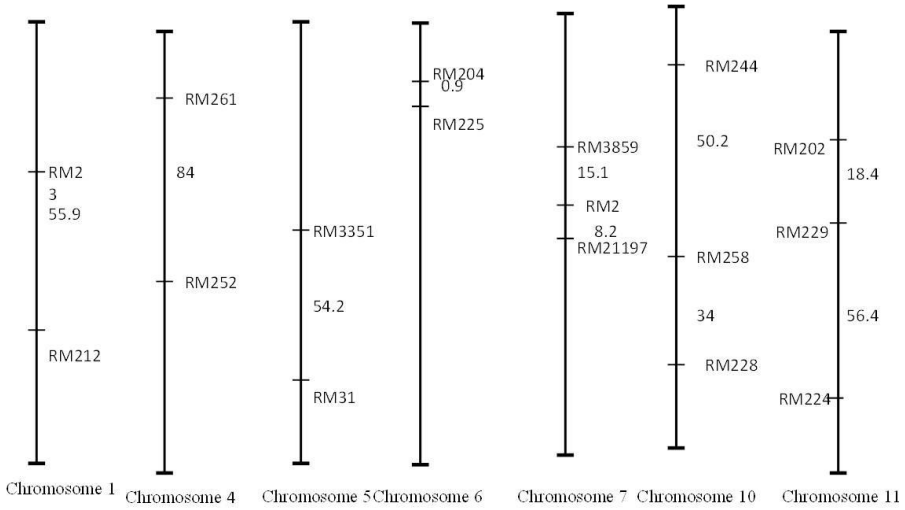


Fig. 14 The linkage map of polymorphic markers in TGMS1

4. 2 TO TRANSFER TGMS CHARACTER TO RED RICE BACKGROUND

Inorder to transfer TGMS character to red rice lines, hybridisation between stable TGMS and non TGMS parents was carried out initially. F_2 mapping population was developed for the marker analysis and phenotypic validation. 50 F_2s were used as mapping population. The DNA was isolated from these plants and is given in Plate 4.

4 2.1 Validation of Markers by Phenotyping Male Sterile Rice Lines

The markers which were able to distinguish the TGMS and non TGMS parental lines were used for screening the mapping population. From the 45 primers used for capillary electrophoresis, 21 primers were polymorphic and were able to distinguish Uma and TGMS1. Hence, these 21 markers were used for further validation of 50 F_2 plants. The plate 5 shows the amplification of 50F_2s using RM31and RM23 respectively. The genotypic evaluation was further confirmed using phenotypic observation. The F_2 plants which were found sterile as per the TGMS markers were checked for their sterility status when grown in sterility inducing condition. The plants were split into two with one half of the tillers growing under fertile and the other half under sterility inducing conditions. Sterile and fertile plants were checked for their genotype and phenotype. Thus the markers were validated. The association between the TGMS trait and the markers was confirmed after statistical analysis.

Among the 50 F_2 plants tested, 36 plants were fertile and remaining 14 were sterile phenotypically (Lanes 37-50 in both Plate 5 (a) and (b). Hence F_2 plants exhibited 2.57 for fertile to sterile ratio which fitted significantly with the Mendelian ratio of 3:1 by Chi-square test ($\chi^2_{(0.01,1)} = 6.635$) (Table 17).

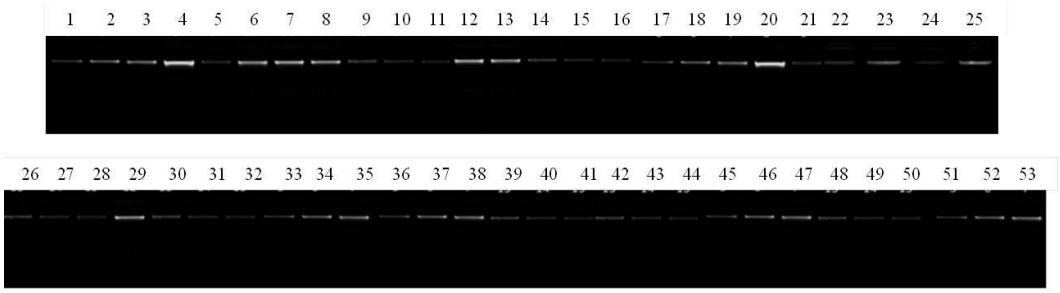
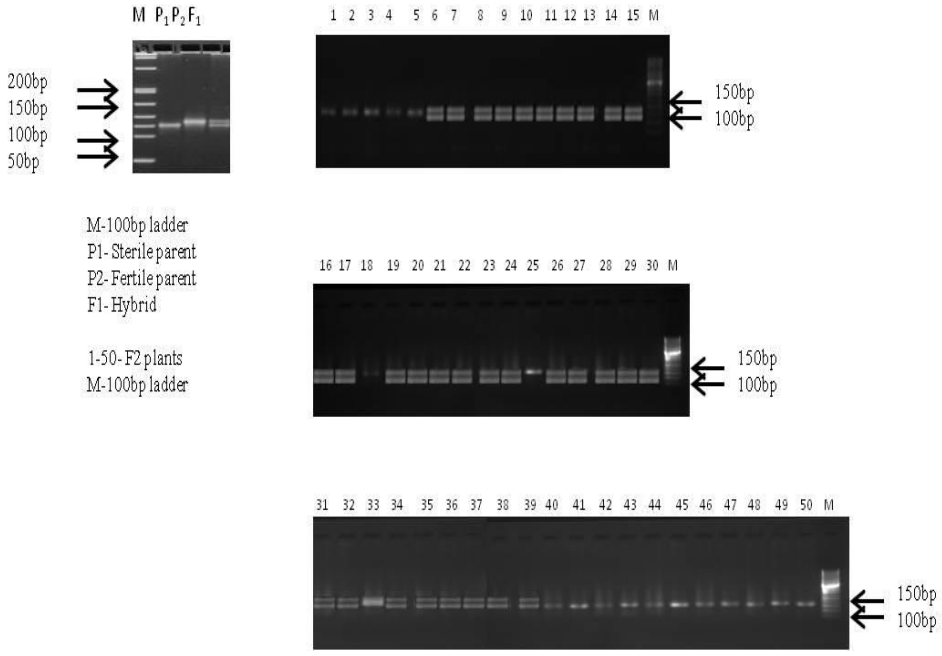
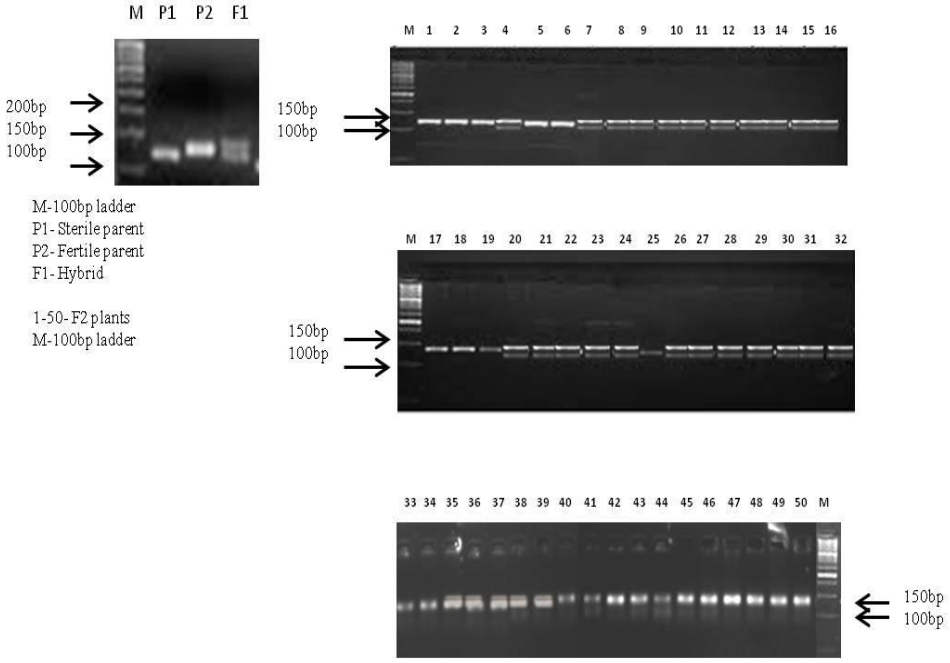


Plate 4. Picture showing the quality of DNA isolated from parental, hybrid and F_2 lines in 0.8% agarose gel



(a)



(b)

Plate 5. PCR amplification pattern of 50 F_2s along with parents, TGMS1 and Uma, F_1 using (a) RM31 and (b) RM23 respectively

Table 17. The segregation ratio of fertile to sterile plants

Generation	Fertile	Sterile	Total	$\chi 2$	Ratio of fertile to sterile plants
F_2	36	14	50	0.24	2.57

4.2.1.1 Development of background selection marker

4.2.1.1.1 Primer designing and multiple sequence alignment

The sequences of the red coloured pericarp and white coloured pericarp were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/nucleotide/) (Fig. 15) and aligned using Multiple sequence alignment tool from the website,

(http://www.ebi.ac.uk/Tools/msa/clustalw2/) Clustal W2 (Fig. 16). The sequences differing between them ie.14 bp was used to design the primers. The primer was designed using Primer3 (http://simgene.com/Primer3), a primer designing tool (Fig. 17).

ggactactcctgaaggaagtgatgacaacaagaccatgatcagtccaggcaccacacagagaatgctcaagagcatcctgatgattgttccca
gtagtcactgcagttacaggggagcagaaacacctgaatcaaggggcgggaaaggcgcaagtggaacgcgaaaagtcggtgccatccaa
ggtgatttcagtgccaaccatgtgctgaaagagaggagaagaagagagaagctcaatgagaagttcataattctgcgatctttggtacctttcat
gacaaaggtaattaagtactccctctatttctataaagccgtatttgactagttatcttatttagaaagtatgtgcaaatatgtaaaatataagtcatact
taaaagaacttttaatgttattaaataataagtcacaccaaaaataaaacatatatatttttaataagataaatgattaaatgtatatataaaaattaata
gcgtcacatattttaaaatagaggggtatttaagtacccacaggatcatcaaaattcagttatcttttcttaagcctctaacgaacattggaagatcc
tcactaatgggcagcatgaatctagggttcactatttcggaatgcaaaatatgttttgccgggcatccgatttttaaaaaatt

Fig. 15 Sequence information of Rc gene governing the red pericarp colour in rice

```
Surjamukhi      CATCAAGAACTACTTGCCAGTTTCAGAGAAATCATCATTCTCCAGATGGACTACTCCTGA 4994
Rc              CATCAAGAACTACTTGCCAGTTTCAGAGAAATCATCATTCTCCAGATGGACTACTCCTGA 4993
H               CATCAAGAACTACTTGCCAGTTTCAGAGAAATCATCATTCTCCAGATGGACTACTCCTGA 5037
Jefferson       CATCAAGAACTACTTGCCAGTTTCAGAGAAATCATCATTCTCCAGATGGACTACTCCTGA 5038
                ************************************************************

Surjamukhi      AGGAAGTGATGACAACAAGACCATGATCAGTCCAGGCACCACACAGAGAATGCTCAAGAG 5054
Rc              AGGAAGTGATGACAACAAGACCATGATCAGTCCAGGCACCACACAGAGAATGCTCAAGAG 5053
H               AGGAAGTGATGACAACAAGACCATGATCAGTCCAGGCACCACACAGAGAATGCTCAAGAG 5097
Jefferson       AGGAAGTGATGACAACAAGACCATGATCAGTCCAGGCACCACACAGAGAATGCTCAAGAG 5098
                ************************************************************

Surjamukhi      CATCCTGATGATTGTTCCCAGTAGTCACTGAAGTTACAGGGGAGCAGAAACACCTGAATC 5114
Rc              CATCCTGATGATTGTTCCCAGTAGTCACTGCAGTTACAGGGGAGCAGAAACACCTGAATC 5113
H               CATCCTGATGATTGTTCCCAGTAGTCACTGCAGTTACAGGGGAGCAGAAACACCTGAATC 5157
Jefferson       CATCCTGATGATTGTTCCCAGTAGTCACTGCAGTTACAGGGGAGCAGAAACACCTGAATC 5158
                ****************************** ******************************

Surjamukhi      AAGGGGCGGGAAAGGCGCAAGTGGAACGCGAAAAGTCGGTGCCATCCAAGGTGATTTCAG 5174
Rc              AAGGGGCGGGAAAGGCGCAAGTGGAACGCGAAAAGTCGGTGCCATCCAAGGTGATTTCAG 5173
H               AAGGGGCGGGAAAGGCGCAAGTGGAACGCGAAAAGTCGGTGCCATCCAAGGTGATTTCAG 5217
Jefferson       AAGGGGCGGGAAAGGCGCAAGTGGA--------------TGCCATCCAAGGTGATTTCAG 5204
                ************************              ********************

Surjamukhi      TGCCAACCATGTGCTGAAAGAGAGGAGAAGAAGAGAGAAGCTCAATGAGAAGTTCATAAT 5234
Rc              TGCCAACCATGTGCTGAAAGAGAGGAGAAGAAGAGAGAAGCTCAATGAGAAGTTCATAAT 5233
H               TGCCAACCATGTGCTGAAAGAGAGGAGAAGAAGAGAGAAGCTCAATGAGAAGTTCATAAT 5277
Jefferson       TGCCAACCATGTGCTGAAAGAGAGGAGAAGAAGAGAGAAGCTCAATGAGAAGTTCATAAT 5264
                ************************************************************

Surjamukhi      TCTGCGATCTTTGGTACCTTTCATGACAAAGGTAATTAAGTACTCCCTCTATTTCTATAA 5294
Rc              TCTGCGATCTTTGGTACCTTTCATGACAAAGGTAATTAAGTACTCCCTCTATTTCTATAA 5293
H               TCTGCGATCTTTGGTACCTTTCATGACAAAGGTAATTAAGTACTCCCTCTATTTCTATAA 5337
Jefferson       TCTGCGATCTTTGGTACCTTTCATGACAAAGGTAATTAAGTACTCCCTCTATTTCTATAA 5324
                ************************************************************

Surjamukhi      AGCCGTATTTGACTAGTTATCTTATTTAGAAAGTATGTGCAAATATGTAAAATATAAGTC 5354
Rc              AGCCGTATTTGACTAGTTATCTTATTTAGAAAGTATGTGCAAATATGTAAAATATAAGTC 5353
H               AGCCGTATTTGACTAGTTATCTTATTTAGAAAGTATGTGCAAATATGTAAAATATAAGTC 5397
Jefferson       AGCCGTATTTGACTAGTTATCTTATTTAGAAAGTATGTGCAAATATGTAAAATATAAGTC 5384
                ************************************************************
```

Fig. 16 Multiple sequence alignment of red and white rice lines using Clustal W2

Primer3 OUTPUT

WARNING: assuming you want to pick PCR primers

<< Back

PAIR 1

Left Primer1: Primer_1_F

Sequence: agaccatgatcagtccaggc

Start: 30 Length: 20 Tm: 60.080 GC%: 55.000 ANY: 6.00 SELF: 6.00

Right Primer1: Primer_1_R

Sequence: ggttggcactgaaatcacct

Start: 201 Length: 20 Tm: 59.973 GC%: 50.000 ANY: 4.00 SELF: 4.00

Product Size: 171 Pair Any: 6.00 Pair End: 3.00

DISPLAY

```
1     ggactactcc tgaaggaagt gatgacaaca agaccatgat cagtccaggc
51    accacacaga gaatgctcaa gagcatcctg atgattgttc ccagtagtca
101   ctgcagttac aggggagcag aaacacctga atcaaggggc gggaaaggcg
151   caagtggaac gcgaaaagtc ggtgccatcc aaggtgattt cagtgccaac
201   catgtgctga aagagaggag aagaagagag aagctcaatg agaagttcat
251   aattctgcga tctttggtac ctttcatgac aaaggtaatt aagtactccc
301   tctatttcta taaagccgta tttgactagt tatcttattt agaaagtatg
351   tgcaaatatg taaaatataa gtcatactta aaagaacttt taatgttatt
401   aaataataag tcacaccaaa aataaaacat atatattttt aataagataa
451   atgattaaat gtatatataa aaattaatag cgtcacatat tttaaaatag
501   aggggtattt aagtacccac aggatcatca aaattcagtt atcttttctt
551   aagcctctaa cgaacattgg aagatcctca ctaatgggca gcatgaatct
601   agggttcact atttcggaat gcaaaatatg ttttgccggg catccgattt
651   ttaaaaaatt
```

Fig. 17 Primers designed for the Rc gene using Primer3

4.2.1.1.2 Standardisation of Polymerase Chain Reaction (PCR)

Standardization of the PCR conditions was done with the following protocol. Template DNA was initially denatured at 98ºC for 30 sec, followed by 40 cycles of PCR amplification under the following conditions- denaturation at 98ºC for 5 sec, primer annealing at 56ºC for 10 sec and primer extension at 72ºC for 15 sec. A final incubation of 1.00 min was given for the completion of primer extension at 72ºC.

4.2.1.1.3 Agarose Gel Electrophoresis of PCR Products and Visualization of PCR Products

PCR products when run in 3% agarose gel, an amplification of ~150bp were obtained.

4.2.1.2 Phenotyping Red Rice Lines

The F_1 progenies and F_2 population were checked phenotypically for their pericarp colour. Among the fifty F_2 plants, amplification of 29 ones was found polymorphic (Plate 6, Lane No. 1, 2, 3, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 28, 29, 30, 31, 32, 33, 34, 35, 36, 44, 45, 46, 47, 48, 49, 50) and the rest were monomorphic. Lanes with polymorphic bands were actually red pericarped rice and the lanes with monomorphic lower (only) band were expected to be loaded with white pericarped rice DNA samples. Plate 6, Lane No. 4, 6, 10, 23, 26, 41& 42 produced monomorphic band (with upper band alone) were red pericarped. Plate 6, Lane No. 5, 7, 8, 9, 21, 22, 24, 25, 27, 37, 38, 39, 40, 43 (marked as yellow asterisk) were expected to be white pericarped rice, but only (Fig. 18) Grain No. 5, 7, 8, 9, 21, 22 were found white pericarped. Hence this marker was having an accuracy of 70% in determining the correct phenotype.

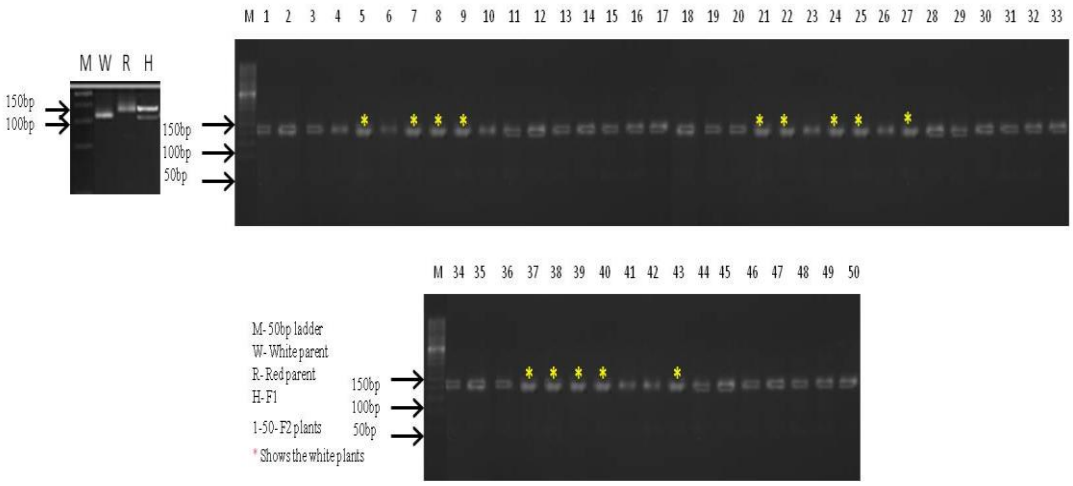


Plate 6. Gel picture showing the amplification of Rc primer in F_2 population

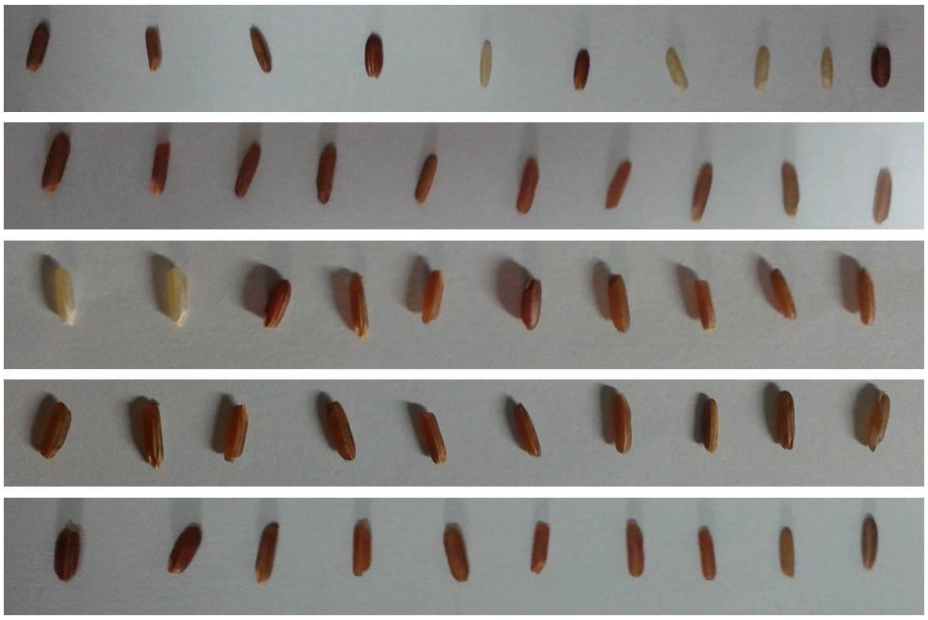


Fig. 18 The red and white phenotype of the rice grains of F_2 plants

(First row – Grain of 1-10 F_2 plants, Second row- Grain of 11-20 F_2 plants, Third row- Grain of 21-30 F_2 plants, Fourth row- Grain of 31-40 F_2 plants and Fifth row -Grain of 41-50 F_2 plants)

4.2.1.3. Statistical Analysis

The sterility and the marker association were checked statistically by performing single factor ANOVA in the Microsoft Excel. This was based on the method called single dose restriction fragmentation proposed by Wu *et al.,* 1992. The amplification profile in agarose gel was scored initially. The sterile plants were given a score of '1' and the fertile plants were given a score '3'. The heterozygotes were represented as '2'. Along with these genotypic data, phenotype was also scored. Phenotypic sterility/fertility scores of F_2 progenies of TGMS1 X Umais given in the table 18. The sterile plants were given a score '1' and fertile plants were given a score '5'. This score data was used for performing ANOVA (single factor) in the MS Excel software. The significant markers were further used for Chi-square test.

Twenty one polymorphic markers were scored and analysed in Excel. Among twenty, six (RM23, RM31, RM3351, RM 212, RM258, RM244) were found significant in stat analysis. Score tables and their ANOVA tables are given below (Table 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30). These were used for analysis using Chi-squared test (Table 31). Results have shown a higher level of significance in all these six markers at 5% significance level (χ^2 table value-3.841).

This reveals the possible association between the sterility phenotype and marker.

Table 18. Phenotypic sterility/fertility scores of F_2 progenies of TGMS1 X Uma

Progeny lines	Phenotype	Sterility/ fertility score	Progeny lines	Phenotype	Sterility/ fertility score
1	F	5	26	F	5
2	F	5	27	F	5
3	F	5	28	F	5
4	F	5	29	F	5
5	F	5	30	F	5
6	F	5	31	F	5
7	F	5	32	F	5
8	F	5	33	F	5
9	F	5	34	F	5
10	F	5	35	F	5
11	F	5	36	F	5
12	F	5	37	S	1
13	F	5	38	S	1
14	F	5	39	S	1
15	F	5	40	S	1
16	F	5	41	S	1
17	F	5	42	S	1
18	F	5	43	S	1
19	F	5	44	S	1
20	F	5	45	S	1
21	F	5	46	S	1
22	F	5	47	S	1
23	F	5	48	S	1
24	F	5	49	S	1
25	F	5	50	S	1

F-Fertile pollen, S-Sterile pollen

Table 19. One way ANOVA to test the association of SSR marker, RM23 with thermosensitive genic male sterility

	RM 23 Score 1		RM 23 Score 3
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	
1	5		
1			

Table 20. Analysis of variance for RM23 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P- value	F critical
Between Groups	17.69026	1	17.69026	5.9136	0.018806	4.042652
Within Groups	143.5897	48	2.991453			
Total	161.28	49				

Table 21. One way ANOVA to test the association of SSR marker, RM31 with thermosensitive genic male sterility

RM31 Score 1	RM31 Score 3		
1	5	5	5
1	5	5	5
1	5	5	1
1	5	5	1
1	5	5	1
1	5	5	
1	5	5	
1	5	5	
1	5	5	
1	5	5	
1	5	5	
	5	5	
	5	5	
	5	5	
	5	5	
	5	5	
	5	5	
	5	5	

Table 22. Analysis of variance for RM31 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P- value	F critical
Between Groups	116.9723	1	116.9723	126.72	4.56E-15	4.042652
Within Groups	44.30769	48	0.923077			
Total	161.28	49				

Table 23. One way ANOVA to test the association of SSR marker, RM3351 with thermosensitive genic male sterility

RM3351 Score 1		RM3351 Score 3
5	5	5
5	5	5
5	5	5
5	5	5
5	5	5
5	1	5
5	1	5
5	1	5
5	1	5
5	1	5
5	1	5
5	1	5
5	1	5
5	1	
5	1	
5	1	
5	1	
5	1	
5	1	
5	1	

Table 24. Analysis of variance for RM3351 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P- value	F critical
Between Groups	19.80632	1	19.80632	6.72	0.012597	4.042652
Within Groups	141.4737	48	2.947368			
Total	161.28	49				

Table 25. One way ANOVA to test the association of SSR marker, RM244 with thermosensitive genic male sterility

RM244 Score 1	RM244 Score 3		
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
	5	5	1
	5	5	1
	5	5	1
	5	5	1
	5		1

Table 26. Analysis of variance for RM244 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P- value	F critical
Between Groups	91.0361	1	91.0361	62.208	3.26E-10	4.042652
Within Groups	70.2439	48	1.463415			
Total	161.28	49				

Table 27. One way ANOVA to test the association of SSR marker, RM212 with thermosensitive genic male sterility

RM212 Score 1	RM212 Score 3		
5	5	5	5
5	5	5	5
5	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	5
1	5	5	1
	5	5	1
		5	1
		5	1
		1	1
		1	

Table 28. Analysis of variance for RM212 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P- value	F critical
Between Groups	35.28	1	35.28	13.44	0.000615	4.042652
Within Groups	126	48	2.625			
Total	161.28	49				

Table 29. One way ANOVA to test the association of SSR marker, RM258 with thermosensitive genic male sterility

RM258 Score 1	RM258 Score 3		
5	5	5	5
5	5	5	5
5	5	5	5
5	5	5	5
5	5	5	5
5	5	5	1
1	5	5	1
1	5	5	1
1	5	5	1
1	5	5	1
1	5	5	1
1	5	5	
1	5	1	

Table 30. Analysis of variance for RM258 marker

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P-value	F critical
Between Groups	18.77688	1	18.77688	6.324706	0.01531	4.042652
Within Groups	142.5031	48	2.968815			
Total	161.28	49				

Table 31. Chi square test of expected and observed segregation ratio (1:1) for six primers

SSR marker	Parents		F_2 genotypic class	Observed values			χ^2 value
	Sterile	Fertile		Score 1	Score 3	Total	
RM23 (145/142)	—	—	— — —	39	11	50	7.84*
RM31 (139/142)	—	—	— — —	11	39	50	7.84*
RM3351 (179/173)	—	—	— — —	38	12	50	6.76*
RM244 (154/158)	—	—	— — —	9	41	50	10.24*
RM212 (112/130)	—	—	— — —	10	40	50	9*
RM258 (138/136)	—	—	— — —	13	37	50	5.76*

*- Significant at 5% level

Discussion

5. DISCUSSION

The experiment entitled "Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)" was conducted to develop molecular markers associated with TGMS gene and to transfer TGMS character to red rice background. The results of the experiment, presented in the previous chapter are discussed here.

5. 1 DEVELOPMENT OF MOLECULAR MARKERS ASSOCIATED WITH TGMS GENE

Simple sequence repeats (SSR) or Microsatellites are random repeats of short DNA motifs (1–6 bp in length). They frequently exhibit variation in the number of repeats at a particular locus. Because of their abundance, codominant nature and inherent potential for variation, they have become a valuable source of genetic markers. Several studies in rice have contributed to the development of several hundred microsatellite markers and a genetic map consisting of 320 SSRs (Wu and Tanksley, 1993; Akagi *et al*., 1996; Temnykh *et al*., 2000). These markers have been used to analyze diversity (Yang *et al*., 1994; Olufowote *et al*., 1997; Cho *et al*., 2000; Harrington, 2000) and to locate genes and QTLs on rice chromosomes using both intra- and interspecific crosses (Xiao *et al*., 1998; Bao *et al*., 2000; Zou *et al*., 2000; Bres-Patry *et al*., 2001; Moncada *et al*., 2001). SSRs are increasingly useful for integrating the genetic, physical, and sequence-based maps of rice. They simultaneously provide breeders and geneticists with an efficient tool to link phenotypic and genotypic variation.

It is a cumbersome task to transfer the TGMS trait to different background. Marker assisted selection (MAS) using closely linked molecular markers will aid the transfer of TGMS gene to different backgrounds in a better way. A well defined marker once developed for the TGMS gene residing in the particular male sterile line, then there could be an easy marker facilitated transfer. Then these markers could be used for differentiating a heterozygous fertile from homozygous

sterile in early stage itself. Since heterozygous fertile plants segregate the trait, such plants can be discarded in early nursery stage itself. Homozygous sterile plants screened using this marker could be used for multiplication under fertile condition, preferably at higher altitudes, where the average temperature is below sterility inducing temperature.

For successful F_1hybrid production as part of marker development, it is essential to ensure perfect synchronization with respect of flowering and time and duration of anthesis between parental lines (Ramakrishna *et al.,* 2006). Hence, the TGMS lines and pollen parents were staggeredly sown on monthly interval. A crop calendar gives the duration of the crop when grown in different months of the year. This helps to choose the best time for hybridisation and seed multiplication procedures. Sowing of seeds during March- April and August-September months will make plants experience high day temperature (>30^{o}C), which can cause plants sterile and hence can be utilised for hybridisation purpose. November- December sowing can be done for seed multiplication as the plants experience a lower night temperature <23^{o}C.

The crucial factor under consideration behind any hybrid rice program is the knowledge about the sterility- fertility alteration behaviour of each TGMS source. There will be a different critical sterility and fertility inducing temperature and period in each line. The critical sterility temperature (CST) is the critical temperature during the sensitive stage of a TGMS line that results in complete sterility. The CSP of a TGMS line is identified as the temperature corresponding to ≤ 0.5% spikelet fertility. This is the acceptable spikelet fertility rate for TGMS lines in their sterile phase (Lu *et al.,* 1998).

The tracking method (Ali et al., 1995; Ramakrishna *et al.,* 2006) was followed for determining CST. The maximum and average temperature during 15-24 days before heading was used for the determination of CST. The TGMS1 used in the present study yielded a maximum and minimum temperature regime of

30.6ºC/ 23.9ºC with an average temperature of 27.25ºC in causing sterility. Similar findings were reported by Gong *et al.,* (2000) and Roystephen and Thangaraj (2000) under phytotron condition. The TGMS line, TGMS1 found to have a critical sterility period of 15-22 days before heading. The critical sterility inducing period of 22 days before heading coincided with the secondary branch primordial formation to stamen pistil primordial formation stage. The above results are in line with previous studies. According to Ali *et al.,* (1995), the critical stage in most TGMS lines ranged between 15-24 days before heading.

Yuan *et al.,* (1988) identified the stage from secondary rachis branching and spikelet primordia differentiation to pollen mother cell formation in the process of panicle development as the sensitive stage for fertility alteration in Nongken 58S. Zhang *et al.,* (1993) also reported the stages between the formation of secondary rachis branch and the formation of PMC as the critical stage of thermosensitivity in TGMS lines. Maruyama *et al.,* (1990) found that the critical stage for Norin PL 12, as 22 to 26 DBH and that even high temperature for an hour would adversely affect the seed setting.

Zeng (1991) reported that the daily mean temperature of >28ºC during 13 to 25 DBH and during 10 to 18 DBH were crucial for complete pollen sterility. Senthil *et al.* in 2004 identified 10-25 DBH as critical thermosensitive stage for fertility alteration, which corresponds to differentiation of secondary branch primordium, differentiation of pistil and stamen, formation of PMC and early meiosis stages (Stage 111 to IV). This supports the findings of Lakshmipraba and Thangaraj (2000) and Gong *et al.,* (2000). Yao *et al.,* (1995) reported that 5-20 DBH was critical stage in TGMS lines.

In the present study, minimum temperature was not used because Viraktamath and Virmani (2001) had shown that between the maximum and the minimum temperature, it is the maximum day temperature that influences the fertility/sterility expression of the TGMS lines. They have also shown that it is the

maximum day temperature that influences the fertility/sterility expression of the TGMS line in the tropics. Ali *et al.,* (1995) found that the ideal CSP for a TGMS line under tropical conditions is at maximum day temperatures between 30 and 32°C.

In China, the daily mean temperature is used to identify the CSP of TGMS lines, since it is a function of both minimum and maximum temperatures (Mou *et al.*, 1998). The ideal CSP for a TGMS line is a daily mean temperature of 23-25°C (Ying, 1999). Zhang and Lu (1992) reported that pollen sterility of some TGMS lines was positively correlated with the daily air temperatures at around 10 to 16 days before heading.

Maximum temperatures and daily mean temperatures from 1 to 30 days before heading corresponds to the panicle development of a rice plant from the differentiation of the first bract primordium to the time the spikelets are completely formed (Virmani and Sharma, 1993). The importance of maximum and average temperatures in inducing sterility is evident in the present study. It is assumed that the maximum temperature and average temperature above 30.6 ^{o}C and 27.25 ^{o}C can induce sterility in TGMS1. This result goes side by side with the above findings.

Exposure of the mapping population to fertility inducing temperature (ie. an average temperature >27.25^{o}C) at the critical stage (around 15-22 days before heading) during the months of December-February induced all plants to produce fertile panicles. The sterile plants had either unstained, withered or spherical sterile pollen grains, or had pollen free anthers. The reversion of the fertile plants to sterile plants under high day temperature (March- May) in the critical stage indicated the presence of TGMS gene. Through this strategy, the segregation of pollen-fertile and pollen- sterile plants in the population, and the conversion from fertility to sterility of TGMS plants was observed. This result is in agreement with

Lopez and Virmani (2000). The results indicate that this TGMS line is a good TGMS gene source.

For tropical conditions, low CSP-Iow CFP (20-24 ^{o}C) type appears to be desirable (Ali *et al.,* 1995). Low CSP group has a critical sterility maximum temperature between 30-32 ^{o}C. The line TGMS1 belongs to low CSP group. (The male sterile rice line, TGMS2 could not be used for this evaluation study due to its differential sterility condition). One of the constraints in adapting 2-line hybrid rice breeding in commercial scale is the contamination of selfed seeds within the TGMS line resulting from temperature fluctuations occurring during seed production. A sudden fall in day/ night temperature during the sensitive period in panicle development would cause the TGMS line to revert to fertility, thus decreasing the purity of hybrid seeds. Low CSP is one of the strategies being employed to overcome this problem is to breed for TGMS lines with low critical sterility point (CSP). When used in hybrid seed production, TGMS lines possessing low CSP remain completely sterile despite sudden temperature changes during the sensitive growth stage. This eliminates selfing.

ID24 is the TGMS parent of TGMS1. The CSP of ID24 was found to be around 10-14 days before heading with maximum and average temperatures of 29.5^{o}C and 25.9^{o}C respectively (Sanchez and Virmani, 2005). In the present study, the critical sterility temperature and period varied in the progeny of ID24, TGMS1. The change in CSP when a TGMS gene transferred to different backgrounds has also been observed by Mou *et al.,* (1998) and Viraktamath and Virmani, 2001). According to Zhang and Lu, (1992), conditions inducing fertility alteration in EGMS lines vary among different lines due to different source of male sterile genes and due to the effect of genetic backgrounds.

For the evaluation of CSP, the wet period of the year September-November was utilised in the present study. Sanchez and Virmani (2005) reported that wet season (when panicle initiation to flowering occurs between August and

September) is the best time for identifying the CSP (low CSP group). The CSP was confirmed using the physical method and morphological index method (Ramakrishna *et al.,* 2006). In this study also, the three methods like tracking method, physical method and morphological index method were used for characterisation of TGMS lines. It was found when the flag leaf has a length of 4.2 cm, the panicle is in the critical sterility inducing condition (0.2 cm), which coincides with the stamen- pistil primordia to pollen mother cell formation stage.

The TGMS line should also have characteristics that commercially useful CMS lines should have, ie, wide adaptability, high outcrossing ability, good combining ability, and acceptable grain quality (Virmani *et al.,* 1997). Evaluation of TGMS lines for floral, morphological and agronomic traits is a pre requisite to find out commercially useable TGMS lines (Virmani *et al.,* 1997 and Kalaiarasi and Vaidyanathan, 2002).

Floral and morpho-agronomic characterization of TGMS lines is highly essential for hybrid rice production through TGMS. TGMS lines with maximum anther length and stigma length may be useful to achieve higher out crossing rate as they correlate positively (Ali *et al.,* 1993 and Virmani and Atwal, 1973).

Results revealed that the vital floral traits like the panicle exsertion, stigma exsertion and glume opening played an important role in seed setting behaviour. In the TGMS line TGMS1, panicle exsertion, stigma exsertion and glume opening was found significantly higher than the other line TGMS2. This resulted in higher number of filled grains, which is unison with the study reported by Jayaramaiah (2007). Panicle exsertion of 76.38% long with wider glume opening (25.05^{o}) has resulted in higher number of filled grains in TGMS1. Short stature with more productive tillers, early maturation and better phenotypic acceptability made TGMS1 a better candidate for selection as a TGMS source for developing a male sterile red rice parent for hybrid seed production in Kerala. This trait is useful in hybrid seed production and self-seed multiplication of TGMS lines.

High frequency of exerted stigma facilitates stigma reception of naturally out crossed pollen grains and higher seed set. Oka (1988) stated that out-crossing in rice depended on the capacity of stigma to receive alien pollen. Out crossing rate in TGMS lines is largely influenced by glume angle, panicle exertion and stigma exertion. In DRR 1S (41%) maximum out-crossing rate was observed, which can be ascribed to wider glume angle (36°), panicle exertion (71.5%) and stigma exertion (45%). The observations are in agreement with earlier reports (Ganesan, 2000; Banumathy *et al.,* 2002). The lines with complete pollen sterility at high temperature and more than 30% self-seed set at low temperature are considered as promising TGMS lines for commercial exploitation (Lu *et al.,* 1994).

The physiological and biochemical analyses of the sterile and fertile plants showed a significant influence of various antioxidant enzymes during sterility/ fertility conditions. It is the catalase (CAT) enzyme, which is mainly involved in the destruction of hydrogen peroxide and oxidation of hydrogen donors. Catalase activity was reduced in sterile TGMS lines under high temperature. Due to lack of sufficient antioxidant enzymes, there will be higher production of hydroxyl radicals, which cause peroxidation of unsaturated lipids of cell membrane and damage the structural integrity of the membranes, change the structure and functions of proteins including other enzymes and nucleic acids.

In present investigation, the reduction in the catalase activity was upto 68.4 % in leaves compared to fertile TGMS1 plants during different stages of panicle development. The sterile TGMS2 plants were showing 78.09 % reduction in catalase activity when compared to fertile plants. Shen and Gao (1992), and Zhang *et al.,* (1994) also reported a reduction of CAT activity in sterile PTGMS lines. The findings of the present experiment confirmed the results of Roystephen and Thangaraj (2000) who reported the role of active oxygen species in causing pollen abortion in TGMS lines. They observed a reduction in photochemical efficiency and disturbance in protein and nucleic acids metabolism besides lower

enzyme activities due to lower CAT activity under sterile condition. Similar reasons were also suggested by Zou *et al.,* (1993) for pollen sterility in photo sensitive genie male sterile (PGMS) lines.

In this present experiment, the level of catalase enzyme activity was 31.6% and 21.91% respectively in the case of sterile TGMS1 and TGMS2 plants respectively (Zhang *et al.,* 1994). This result was in agreement with the previous reports. Liang and Chen (1993) suggested that the weaker oxygen scavenger system together with a high level of lipid peroxidation in anthers was responsible for male sterility in CMS and TGMS lines.

In sterile TGMS lines, there was upto 24.24% per cent reduction of POX activity in leaves compared to fertile TGMS lines. Roystephen and Thangaraj (2000) reported similar results in TGMS lines under high temperature and suggested that the abnormal activity of POX might be associated with male sterility through strengthening the role of active oxygen species, which causes pollen abortion in TGMS lines.

The reduction in POX activity was marked even at the beginning of secondary branch primordia differentiation. He and Xiao (1993) and Zhang *et al.,* (1994) and also confirmed the increased POX activity under fertile conditions during sensitive stages. Chen and Zhou (1997) found decreased activity of POX in PTGMS lines under long days with high temperature.

By effecting about 25.54 % reduction in soluble protein content, apparently the pollens might have been rendered sterile by CST condition in the case of TGMS1. Many authors (Peng and Wang, 1991; Qing-liu *et al.*, 1994; Roystephen and Thangaraj, 2000) attributed reduced availability of aminoacids and reduced nitrogen assimilation for low soluble protein content which could cause male sterility in TGMS lines.

By increasing the temperature above the critical sterile average temperature of 27.25^{o}C, proline content was almost doubled in TGMS1. It is apparent that, in a thermosensitive line like TGMS2, the production of proline is of high order (three times) culminating in induction of pollen sterility. The findings of Elsy (1997) and Roystephen (1998) showed that 38% proline increase appears to be insufficient to effect pollen sterility.

The carotenoid content also decreased 11.04% in TGMS1 sterile plants when compared to fertile plants. There was a reduction of 28.65% in total chlorophyll in sterile plants when compared to fertile plants. The reducing sugars reduced at the rate of 3.52% in sterile plants when compared to fertile ones.

The results of the present experiment revealed that the activities of free radical scavenging enzymes were reduced significantly in sterile plants compared to the fertile plants. The better status of biochemical molecules, greater activity of enzymes and the resultant better assimilation potential and effective partitioning of assimilates to the reproductive part enhanced good seed setting in the TGMS lines grown under fertile conditions. The major cause of sterility was found to be the accumulation of active oxygen species due to an impaired scavenging system in the sterile plants of TGMS lines.

There are many *tms* genes controlling the TGMS trait in rice beginning from *tms*1 (Wang *et al.,* 1995) to *tms*9 (Sheng *et al.,* 2013). The TGMS gene present in the TGMS lines, TGMS1 and 2 is not known clearly. But there are reports informing the presence of *tms1* and *tms 3* genes in ID24 and IR 32364, the TGMS source parents of TGMS1 and 2 respectively (Siddiq and Ali, 1999). Since TGMS trait was controlled by a recessive gene (Jia *et al.,* 2001), codominant markers are needed to identify molecular markers of TGMS gene in F2 population. Hence the codominant marker, SCAR was used for screening the present TGMS and non TGMS lines. The used SCAR markers were not found to be a codominant marker. The two SCAR markers which could perform polymorphism in parent population

were not able to be differentiating between sterile and fertile F_2 plants as it was a dominant marker with a single band. Also, the same SCAR marker was not able to show similar polymorphism with other non TGMS lines.

As a result of microsatellite analysis to find the SSR markers polymorphic to the *tms* gene, presence of 21 polymorphic markers for differentiating Uma and TGMS1 plants were obtained. Even these polymorphic markers were not able to distinguish TGMS1 and Jyothi, suspecting its similarity with TGMS lines. These TGMS lines were developed from IRRI and Jyothi has one of its parents, IR8, which was also from IRRI.

5.2 TO TRANSFER TGMS CHARACTER TO RED RICE BACKGROUND

Developing TGMS lines is one of the basic steps in obtaining superior two-line rice hybrids. Transferring the TGMS trait to different background is very tedious and cumbersome. Identification of closely linked molecular markers is a first step towards expediting the transfer of TGMS gene to different backgrounds through marker assisted selection (MAS). For a trait like TGMS, which is controlled by a single recessive gene and would be difficult to identify in field conditions using conventional approaches, MAS will facilitate early selection with greater accuracy.

Transfer of TGMS character to red rice background was another objective in the present study. A similar study was aimed to develop TGMS lines with aromatic Thai rice background by molecular marker-aided breeding by Lopez and his co-workers in 2003. They derived a F2 population from a cross between Norin PL12 (a TGMS line from Japan) and KDML 105 (a popular aromatic Thai rice cultivar).

Data on the segregation of fertile and sterile plants inF_2 were done on the basis of fertility/ sterility status of pollen grains. Results indicated that in F_2

mapping population, the segregation ratio of fertile to sterile plants obtained were 3:1 which fitted significantly to the Mendelian ratio with X^2 test of 2.57 ($X^2_{(0.01,1)}$ = 6.635). This result is similar to that of the findings by Rongbai *et al.,* (2005), Dong *et al.,* (2000), Jia *et al.,* (2001) and Lopez *et al.,* (2003), Reddy *et al.,* (2000), Yang *et al.,* (1992), Maruyama *et al.,* (1991), Borkakati and Virmani, (1996). This shows that the inheritance of fertility gene in TGMS plants is following a Mendelian ratio and hence substantiating that the TGMS trait is controlled by a monogenic recessive gene. Sastry (1978) and Sahu (1991) also reported similar gene action for the red pericarp in rice.

In the present study, linkage analysis of these 21 polymorphic markers has shown the presence of seven linkage groups. In this investigation, the polymorphic markers obtained to screen the parents, TGMS1 and Uma were loosely scattered in the chromosomes. As the number of polymorphic markers obtained was lesser in number and size of mapping population was 50, which is comparatively a low score, the resolution of these markers on the chromosome was very less.

Present study resulted in the identification of three polymorphic markers, RM3351, RM23 and RM31 which could distinguish TGMS1 and 2 from Uma and Jyothi. These can be used for further validating a bigger F_2 population. 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*. Hence the possibility of presence of *tms6* gene in the TGMS1 cannot be ignored. RM31 also resides in chromosome 5 at a mapping distance of 54.2 cM from RM3351, whereas RM23 is located in chromosome 1. RM31 and RM23 may also have a possible association with sterility.

The association between the trait and the marker was studied using a statistical method described by Wu and his co-workers. Wu *et al.,* (1992) proposed a method for mapping polyploids based on the segregation of single-

dose restriction fragment length polymorphisms (SDRFs). A SDRF is produced at a single locus among all the homologous loci and is identified by its presence in one parent, absence in the other, and a 1:1 (presence: absence) segregation in the progeny. Barnes and Bester, (2000) reported the use of SDRF in sugarcane genetic mapping purpose.

As a background selection marker, a gene specific marker for red colour pericarp was designed. “Rc” gene a domestication-related gene present in the chromosome7 is required for red pericarp in rice (*Oryza sativa*). The red grain colour is ubiquitous among the wild ancestors of *O. sativa*, in which it is closely associated with seed shattering and dormancy. “Rc” encodes a basic helix-loop-helix (bHLH) protein for the pigment, proanthocyanidin. The red pigment in rice grains is proanthocyanidin, also called condensed tannins (Oki *et al.,* 2002). Proanthocyanidins are a branch of the anthocyanin pathway and share many of the same biosynthetic genes (Winkel-Shirley, 2001). Proanthocyanidins have been shown to have important deterrent effects on pathogens and predators, so it is not surprising that spontaneous mutations that inhibit pigment production would be selected against in the wild (Shirley, 1998). On the other hand, white grain appears to be associated with the domestication syndrome and remains under strong selection in most rice breeding programs today.

The red pigment is of interest for nutritional reasons. It serves as a powerful antioxidant that has been demonstrated to reduce atherosclerotic plaque formation, a risk factor associated with cardiovascular disease (Ling *et al.*, 2001). On the negative side, proanthocyanidin pigments reduce the bioavailability of iron, protein, and carbohydrates (Carmona *et al.*, 1996 and Glahn *et al.*, 2002), which has important implications for people with low nutritional status.

The Rc primer was only 42.85% successful in differentiating the white and red rice lines. The red pericarp colour is having a complex inheritance. The red pericarp colour was reported to have a different sort of inheritance – a single

dominant gene, two genes, three genes involving two major genes either of them complementing with another dominant gene, to be responsible for the trait. Pavithran *et al.,* (1995) reported similar gene interaction for expression of red pericarp colour governed by three genes involving two dominant complementary genes and another independent recessive gene. Tomar *et al.,* (2000) reported that three dominant complementary genes controlled the red colour. Pigmentation of various parts of the rice plant involves a complicated system of the gene interaction, ranging from epistasis to pleiotropic effects (Chang, 1960).

Summary

6. SUMMARY

The experiment entitled “Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)” was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani. The main objectives of the study were to develop molecular markers associated with TGMS gene and to transfer TGMS character to red rice background.

As part of the investigation, two TGMS lines were imported from IRRI through Standard Material Transfer Agreement (SMTA) through National Bureau for Plant Genetic Resources (NBPGR), New Delhi. The lines TGMS1 and TGMS2 are from two different TGMS sources ID24 and IR 32364 respectively.

The TGMS sources along with two popular red rice varieties of Kerala state, Uma and Jyothi were sown on monthly intervals. Inorder to determine the critical sterility temperature and critical sterility period of TGMS lines were raised in pots and maintained in the field. The temperature sensitizing period was determined by physical cum morphological index method and through tracking method. The critical sterility period for the TGMS line was assumed as 15-22 days before heading with maximum temperature 30.6ºC and minimum temperature 23.9ºC. A daily average temperature of 27.25ºC or above can result in sterility. The results have shown that the maximum temperature below 25.75 caused 100% fertility. The size panicle was around 0.2 cm during the critical sterility period ie. 15-22 days before heading and the corresponding flag leaf size was 4.2 cm.

Based on the standard evaluation systems (SES) given by International Rice Research Institute (IRRI), the TGMS lines were scored and the stable line with ideal characters was identified. Results revealed that for the characters like plant height, days to 50% flowering, number of productive tillers, panicle length, panicle exertion, glume opening, stigma exertion, spikelet fertility and 100 grain weight, both the lines TGMS1 and TGMS2 were significantly varying. TGMS1

was having desirable traits when compared to the other line TGMS2. Hence the TGMS line TGMS1 was selected as a better candidate over the other line TGMS2.

Biochemical parameters like catalase, peroxidase, soluble protein, reducing sugars, proline etc. and physiological parameters like carotenoids, total chlorophyll etc. were estimated for twenty plants from sterile and fertile plants of TGMS1 and TGMS2 lines. The result revealed that there is a significant difference between catalase and peroxidase content between fertile and sterile counterparts. This explains the possible role of these antioxidant enzymes in causing male sterility.

The stable TGMS parent TGMS1 was crossed with a popular red rice variety, Uma and F_1s were produced. F_1s were then selfed to get F_2 population. This F_2 population was used for marker analysis. Microsatellite analysis was done to find the SSR markers polymorphic to the *tms* gene. As part of the work, SCAR markers were used for screening the TGMS1 and 2 lines from pollen parents. DNA was isolated from parental lines, F_1 hybrid and fifty F_2 plants. The PCR amplification profile was standardised. The F_2 population could not show the similar polymorphism, eventhough the SCAR marker could distinguish the parental lines TGMS1 and Uma. The other non TGMS lines were also not showing the similar band produced for the pollen parents, Uma and Jyothi. Hence the polymorphism exhibited was not linked to the TGMS trait.

SSR markers reported for the TGMS trait, along with some random markers were used for the screening. Capillary electrophoretic results concluded that three SSR markers namely, RM3351, RM23 and RM31 could differentiate the non TGMS lines under study, Uma and Jyothi from TGMS lines, TGMS1 and TGMS2. Among the 45 primers used for differentiating TGMS1 and Uma, 21 markers were found polymorphic. During validation of these markers in F_2 population of fifty plants, segregation ratio of 2.57 was obtained which fitted significantly with Mendelian ratio.

The 50 F2 plants were used for marker analysis. MAPMAKER/EXP version 3.0 was used to locate the SSR markers in the map and for construction of the map. The value of the interval mapping was 0.1, the LOD score higher than 3was used as threshold for putative locus. Seven groups were obtained in seven different chromosomes.

Inorder to transfer TGMS character to red rice lines, hybridisation between stable TGMS and non TGMS parents was carried out. Fifty F_2 plants were for the marker analysis and phenotypic validation.

For confirmation of true sterile TGMS plants, the sterile TGMS F_2 plants were grown in fertility inducing temperature condition. For that the plants were split into two with one half of the tillers and grown under fertile and the other half under sterility inducing conditions. The identified sterile TGMS lines were able to go fertile under low temperature and set seeds, confirming the presence of tgms gene.

As a background selection marker, a gene specific marker for red colour pericarp was designed. “Rc” gene is a domestication-related gene present in the chromosome7 is required for red pericarp in rice (*Oryza sativa*). “Rc” encodes a basic helix-loop-helix (bHLH) protein for the pigment, proanthocyanidin. Rc gene of the white rice lines lack 14 basepairs. This was expected to have lost during the process of domestication and evolution. The Rc primer was designed based on this deletion. The amplification using Rc primer produced six white lines and rest red lines with different hues. The result concluded the complex nature of inheritance of red pericarp colour in rice as it was not following the canonical Mendelian ratio of 3:1.

The outcome of the study is as follows:

- Identification of a stable TGMS line (TGMS1) suitable to Kerala
- Phenological, floral, morphological and agronomic characterisation of the TGMS1 line
- Biochemical and physiological analyses of fertile and sterile TGMS plants

- Development of F_1 hybrids and F_2 mapping population
- Development of twenty one polymorphic markers for distinguishing TGMS1and Uma
- Development of three polymorphic markers (RM3351, RM23 and RM31) for distinguishing TGMS1, 2 and Uma, Jyothi
- Standardisation of PCR conditions for these primers
- Validation of F_2 plants using these twenty one polymorphic markers
- RM3351, RM23, RM31, RM212, RM258 and RM244 markers might have an association with the sterility phenotype
- Primer (Rc) designed for red pericarp colour in rice and phenotyping

Future line of work:

The identified polymorphic markers can be used for screening mapping population 300-400 F_2 plants. Repeated backcrossing can be done for fixing the tgms character and red colour. Near isogenic lines may be also used as a non segregating mapping population. More number of polymorphic markers can be screened and utilized for understanding the *tms* gene in these lines.

The actual mechanism behind the phenomena of TGMS is quite unknown. Studies may be concentrated more on understanding the molecular mechanism behind it along with the identification of gene. Transcriptome analysis of fertile and sterile plants can be done for knowing the differential gene expression under fertile and sterile conditions. Identification of different and novel tgms sources can be done and used for pyramiding them for stable TGMS expression. Exploration of new generation markers is highly essential for the screening of the TGMS trait in large scale basis.

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MARKER ASSISTED TRANSFER OF THERMOSENSITIVE GENIC MALE STERILITY TO HIGH YIELDING RED KERNELLED VARIETIES OF RICE (*Oryza sativa* L.)

by

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Abstract of Thesis

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ABSTRACT

The project entitled “Marker assisted transfer of thermosensitive genic male sterility to high yielding red kernelled varieties of rice (*Oryza sativa* L.)” was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani during 2011 to 2014. The main objectives were to develop molecular markers associated with TGMS gene and to transfer TGMS character to red rice background. For the present investigation, two TGMS lines were imported from International Rice Research Institute (IRRI) namely, TGMS1 and TGMS2which are from two different TGMS sources ID24 and IR32364 respectively.

The TGMS lines along with two popular red rice varieties of Kerala, Uma and Jyothi were sown on monthly intervals for their initial phenological study. The critical sterility temperature and period of TGMS lines were characterised in the field using tracking method. The activity of antioxidant enzymes plays an important level in causing sterility in rice pollen grains, though the reason behind male sterility is unknown.

Morphological, agronomic and floral traits were studied during the specific crop growth stage and were recorded as per the standard evaluation systems given by IRRI. Phenological, floral and morpho-agronomic characterisation of TGMS lines revealed that the line TGMS1 performed better with short stature, early maturity, more productive tillers, wider glume opening, higher panicle and stigma exertion, more filled grains and longer panicles. Stages of panicle development were determined by various methods viz., physical method, morphological index method and tracking method. The results have shown that TGMS1 is a better candidate for Kerala condition with critical sterility period of 15-22 days before heading and the sterility inducing average temperature of 27.25 ^{o} C.

For hybridisation, TGMS1 plants were grown under sterility inducing condition and they were crossed with a popular red rice variety, Uma to produce F_1. The F_1 s were then selfed to get F_2 population. A gene specific primer was

designed for the red pericarp colour (Rc) which can be used as a background selection marker. Fifty F_2 plants were used for marker analysis. Microsatellite analysis was done to find out the SSR markers polymorphic to the *tms* gene. DNA was isolated from the 50 F_2 plants and performed PCR using 45 SSR markers. Capillary electrophoresis was done for the allele sizing of PCR products. Among 45 SSR markers used, three primers RM 3351, RM23 and RM31 could differentiate Uma, Jyothi andTGMS1, TGMS2. A set of twenty one primers were able to distinguish TGMS1 and Uma. The F_2 plants sterile as per the TGMS markers were found to be sterile under sterility inducing condition. Under low temperature their fertility was transformed indicating the presence of *tms* gene.

Linkage analysis using MAPMAKER version 3.0, seven linkage groups and a few unlinked primers were found. A segregation ratio of 2.57:1 was obtained between fertile and sterile lines. This ratio explains the monogenic nature of *tms* gene.Among 20 polymorphic markers, six (RM23, RM31, RM3351, RM 212, RM258 and RM244) were found significant. These on further analysis using χ^2-test revealed the possible association between the sterility phenotype and marker.

From the present study, the sterility *tms* gene got successfully transferred into 14 sterile F_2 plants and RM31, RM23, RM3351, RM212, RM244 and RM258 can be used for the evaluation of TGMS1 X Uma hybrids and their F_2s.