PHYSIOLOGICAL APPROACHES FOR MANIPULATING MALE STERILITY IN THERMOSENSITIVE GENIC MALE STERILE SYSTEM FOR HYBRID RICE SEED PRODUCTION

by

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(2015-21-010)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"PHYSIOLOGICAL APPROACHES FOR MANIPULATING MALE STERILITY IN THERMOSENSITIVE GENIC MALE STERILE SYSTEM FOR HYBRID RICE SEED PRODUCTION"** 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 **"PHYSIOLOGICAL APPROACHES FOR MANIPULATING MALE STERILITY IN THERMOSENSITIVE GENIC MALE STERILE SYSTEM FOR HYBRID RICE SEED PRODUCTION"** is a record of research work done independently by **Ms. Gayathri Rajasekharan** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ABBREVIATIONS

ABA:	Abscisic acid
BR:	Brassinosteroids
CAGR:	Compound annual growth rate
CEPA:	2-Chloroethyl phosphonic acid
CFP:	Critical fertility point
CGMS:	Cytoplasmic-genic male sterility
CHA:	Chemical hybridizing agents
CMS:	Cytoplasmic male sterile system
CST:	Critical sterility temperature
GA:	Gibberellic acid
GMS:	Genetic male sterility
IAA:	Indole-3-acetic acid
JA:	Jasmonic acid
MH:	Maleic hydrazide
MMC:	Microspore mother cells
PCD:	Programmed cell death
PGMS:	Photoperiod-sensitive genic male sterility
POP:	Package of practices
PPR:	Pentatricopeptide
ROS:	Rain Out Shelter
TGMS:	Thermo-sensitive genic male sterility

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INTRODUCTION

1. INTRODUCTION

Rice is the primary food grain of more than half of the world's population. India contributes 21% on world's rice production and is the second largest producer. It is reported that rice is cultivated in an area of 43.385 million ha with production of 104.317 million tonnes (NFSM, 2018). Global rice production in 2017-18 has decreased by 0.41% over previous years. However, it is not sufficient and approximately 116 million tonnes of additional rice production is required by 2035 to meet the increasing food demand (Jena and Nissila, 2017). Ensuring food security for the burgeoning population is a huge challenge and this necessitates the need for increased rice production. Development of hybrid rice is a major breakthrough in the history of rice breeding since hybrids are able to produce higher yield. Male sterility is an essential requirement for hybrid rice technology in which the plant fails to produce viable pollen grains to set selfed seeds normally.

Hybrids possess superior characteristics such as better performance and greater adaptability and they can produce 20 to 30% higher yield over conventional high yielding rice varieties. At present, the hybrid seed production utilizing cytoplasmic male sterile system (CMS: three line breeding system) is the widely adopted method. However, it is a tedious method in which a cytoplasmic male sterile source, a maintainer and a restorer line should be maintained for successful hybridization. Moreover, the male sterility gene in CMS system is controlled by mitochondrial genome which is maternally inherited and not transferable to any line of interest through hybridisation technique.

In the past few years, the area under hybrid rice cultivation in India ranged from 1.8 to 2.0 million hectares (USDA, 2018). More than 100 hybrids have been developed using CMS: three line breeding system by both public and private sectors in India. The rice hybrids released for commercial cultivation using CMS system are with white slender grain which is not suitable to the state of Kerala

since the people prefer to consume red bold grain. In this context, red rice hybrids with bold grains suitable to the state of Kerala need to be developed.

The two line breeding system aroused as an economically feasible, efficient and simple alternative to the three line breeding method which eliminates the requirement of a maintainer line for seed multiplication. Photoperiodic or thermo-sensitive genic male sterility (PGMS or TGMS) is the principal component of two line hybrid rice seed production as demonstrated in China. The P(T)GMS is a typical phenomenon of male sterility system which is controlled both by genes and environmental factors such as photoperiod or temperature. Thermo-sensitive genic male sterile plants become sterile when temperature is above critical sterility temperature (CST) and become fertile if the temperature is below CST during a specific thermosensitive stage. Furthermore, TGMS system is more useful than PGMS in tropical situations where the day length differences are only marginal but with significant variation in temperature between season and between altitudes.

The male sterility in TGMS is regulated by a single recessive nuclear gene that is sensitive to environmental conditions during a specific stage of panicle development. It can be transferred to any line of interest by hybridization thereby broadening the genetic background of rice hybrids (Borkakati and Virmani, 1996; Reddy *et al.,* 2000). The temperature during the panicle development is most critical in the expression of sterility or fertility in TGMS lines (Borkakati and Virmani, 1997). TGMS lines can be categorized as TGMS and reverse TGMS. Around 13 TGMS genes have been identified and mapped on different chromosomes in rice (Sheng *et al.*, 2015).

Successful implementation of TGMS system relies on the understanding of sterility/fertility behaviour as well as the ability of TGMS lines to maintain stable sterility under changing temperature conditions. Hence characterization of TGMS lines for their sterility and temperature requirement are mandatory. It also helps in finding out the ideal season for hybridization and for seed multiplication in a

specific location. The TGMS lines should be sown in such a way that the critical stages of panicle development would be exposed to appropriate temperature conditions for fertility alterations. In addition, identifying the season showing complete sterility, partial sterility and complete fertility would be helpful in generating a crop calendar for commercial hybrid rice program.

One of the major constrains with TGMS system is the frequent fluctuations or sudden decrease in temperature due to unexpected rainfall during their thermosensitive stage. This temperature reversion converts male sterile lines in to male fertile ones resulting in the failure of hybrid seed production. Assuring complete male sterility is a prerequisite for hybridization programmes involving TGMS lines. As an alternate strategy, use of plant growth regulators has opened new boundaries for sterility manipulation. Plant growth regulators known as gametocides or chemical hybridizing agents (CHA), have been reported to be an important tool for inducing male sterility for the production of hybrids. The gametocides selectively kill the male gametes or organs to maintain male sterility. Application of these chemicals at panicle initiation stage affects normal development of anthers and pollen mother cells resulting in the formation of non-functional pollen grains.

The TGMS hybrids have been reported to exhibit high heterosis and grain yield than three line hybrids (Lopez and Virmani, 2000). It is rather easier to develop TGMS hybrids with diverse genetic background, since the cytoplasm is not involved in the sterility gene expression. Thus TGMS lines can be exploited as a donor for male sterile gene in developing male sterile lines. Hence this system can be utilized for generating red rice hybrids with bold grains suitable for the state of Kerala. Therefore the potential of using TGMS line as female parent in Kerala condition should be evaluated.

The mechanism underlying sterility/fertility expression should be quite clear, since the TGMS gene is highly temperature regulated. However a comprehensive examination of the TGMS gene expression and protein profiling

under varying temperature is still lacking. Thus major focus has been given to analyse the gene expression pattern under fertility and sterility inducing conditions to analyse the mechanisms of male sterility in TGMS lines.

Hence the present study entitled 'Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was undertaken with the following objectives:

- Evaluating the environmental conditions required for complete male sterility of TGMS plants
- Manipulating the male sterility by using plant growth regulators
- Understanding the molecular mechanism associated with TGMS system

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Rice is the most important food grain and people around the world depend largely on rice as a primary staple food. To guarantee the food security for increasing population and due to the yield plateau of high yielding varieties, scientists have been in thought of developing rice hybrids since hybrids have 20-30% yield advantage over conventional varieties. Identification of male sterility in rice was a breakthrough in the development of rice hybrids. Alterations in stamen development lead to male sterile plants which can be utilized as female parent to generate hybrids.

There are different types of male sterility systems in plants namely genetic male sterility (GMS), cytoplasmic male sterility (CMS), cytoplasmic-genic male sterility (CGMS) and chemically induced male sterility (Virmani *et al.*, 1997). In genetic male sterility, the sterility is controlled by nuclear genes whereas in the case of cytoplasmic male sterility, cytoplasmic factor governs the sterility. Maternally inherited CMS is associated with unusual open reading frames (ORFs) found in mitochondrial genomes (Eckardt, 2006). Cytoplasmic dysfunction caused by mitochondrial gene (*orf79*) results in sterility and the fertility is restored by nuclear genes (*Rf*) which encodes pentatricopeptide (PPR) proteins that suppress cytoplasmic dysfunction. Sterility can also be introduced artificially by using chemical hybridizing agents.

The genetic male sterility includes environment sensitive male sterility and environment insensitive male sterility. In environment sensitive male sterility, the sterility expression is conditioned by environmental factors and is further classified into photoperiod sensitive and thermo sensitive male sterile systems (P/TGMS). The duration of day length decides the sterility/fertility expression in photoperiod sensitive male sterile system while temperature regulates the sterility expression in thermo sensitive male sterile system. During sterility, the plants produce nonfunctional pollen grains or pollen grains will be completely absent. At present, hybrid rice production system based on photoperiod and/or temperature

During stages 7 to 9, which is the most sensitive stages, microspore mother cells undergoes meiosis to form dyads and tetrads of haploid microspores. From stage 9, free microspores are released by the degradation of callose wall then the microspores are vacuolated and become round shape. The vacuolated microspores undergoes the first mitosis with asymmetric cell division generating a smaller generative cell and a vegetative cell with one vegetative nuclei. Afterwards, at stage 12 the generative cell in the microspore divides into two sperm cells and the mature pollen formed with three nuclei, *i.e.* two smaller sperm nuclei, and a larger vegetative nucleus. At stage 13, the lemma openes and the anther dehiscence occurres and at stage 14, the anther continues the release of mature pollen grains (Zhang and Wilson, 2009).

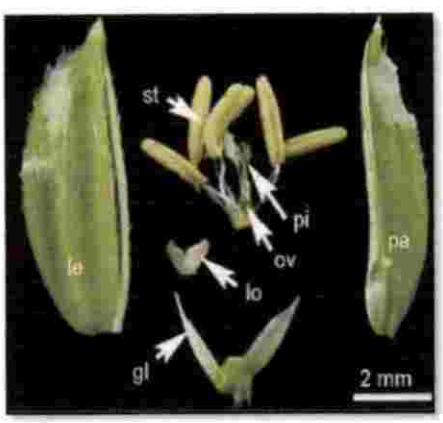


Plate 1. Mature rice flower (stage 12), glumes (gl), lemma (le), palea (pa), lodicules (lo), stamens (st), pistil (ps) and ovary (ov)

(Zhang and Wilson, 2009)

[P(T)GMS] known as two-line system is widely adopted in major rice producing countries like China, Indonesia, Vietnam and Philippines. The two line system of hybrid rice production was established in China. In the past decade, the percentage of area under two-line hybrid rice has increased significantly by 25% in China (Mi *et al.*, 2018).

The rice hybrid seed development exploiting P/TGMS overcomes the difficulties faced in the CMS system. A maintainer line is not required in two line system of hybrid rice production. The PGMS lines are sensitive to photoperiod whereas TGMS lines are responsive to temperature during a specific thermosensitive stage. Developing rice hybrids utilizing TGMS rather than PGMS is suitable in tropical countries since significant variations in temperature is available between seasons and altitudes. The temperature at or below which the TGMS plants produces high proportion of fertile pollen grains is the critical fertility point (CFP) and the temperature at or above which complete or maximum sterility can be induced in a TGMS line during its sensitive stage is the critical sterility point (CSP). Most of the TGMS lines maintain male sterility at a high temperature (maximum >30°C) and they can convert back to partial fertility at a lower temperature (maximum <30°C) and the critical sterility/fertility limit varies between genotypes. The critical thermosensitive stage for fertility variation in the TGMS line varies from 5-15 days after panicle initiation or 15 to 25 days before heading (Virmani *et al.*, 1997).

The two-line hybrid rice system was established by using a PGMS mutant discovered in 1973 by SHI Ming-song from a japonica cultivar 'Nongken 58' (Shi, 1985). Annong S1 was the first identified TGMS rice (*Oryza sativa* ssp. indica) found in 1987 as a spontaneous mutant isolated in the Huan Province, China (Siddiq and Ali, 1999). The TGMS trait in AnS1 and its derivatives were controlled by a single recessive locus, *tms5*. These lines remain sterile under high temperature of 28 to 33^{0}C and revert back to fertility under low temperature of 22 to 27^{0}C. Reverse TGMS lines showing fertility at high temperature and sterility at low temperature were also identified (Jia *et al.*, 2001). Reverse TGMS line J207S

exhibited complete sterility when the temperature was lower than 31°C. The reverse male sterility character in J207S is controlled by a single recessive gene which was first named as *rtms1*. Thus two-line hybrid rice seed prduction is an important innovation for the better exploitation of hybrid vigour with 10% additional yield (Zhou *et al*., 2012).

TGMS lines are sensitive to particular temperature when it is exposed to certain stage of floral development *i.e.* between panicle initiation and flowering. Panicle initiation is the stage at which rice begins its reproductive phase. Details of rice floral development with respect to anther and pollen development will be useful for understanding the sensitive stages of rice.

Spikelet is the basic unit of inflorescence in rice. One rice spikelet has only one floret which is surrounded by a pair of empty glumes. Rice floret has an asymmetric structure with five floral organs with one lemma and one palea in the first outer whorl, two lodicules in the second whorl, six stamens in the third whorl and one pistil in the fourth innermost whorl (Plate 1).

The rice anther development is divided into 14 stages, which is in consistence with the model plant *Arabidopsis*. Anther primordium is formed from cell divisions in the L1, L2 and L3 layers of the floral meristem at stage 1 (Table 1; Fig. 1). From stages 1 to 5, the anther primordia continues cell division and differentiation to form the characteristic structures of anther such as locule, wall, connective and vascular tissues. Archesporial cells in the four corners of the anther primordia generates distinct primary parietal cells and primary sporogenous cells. The primary parietal cells differentiate into two secondary parietal layers, the outer secondary parietal layer generates the endothecium layer and the middle layer and the inner secondary parietal layer developes the tapetum which provides nutrition to developing pollen grains. The primary sporogenous cells also divides to form secondary sporogenous cells and later generates microspore mother cells (MMC) within the locule.

During stages 7 to 9, which is the most sensitive stages, microspore mother cells undergoes meiosis to form dyads and tetrads of haploid microspores. From stage 9, free microspores are released by the degradation of callose wall then the microspores are vacuolated and become round shape. The vacuolated microspores undergoes the first mitosis with asymmetric cell division generating a smaller generative cell and a vegetative cell with one vegetative nuclei. Afterwards, at stage 12 the generative cell in the microspore divides into two sperm cells and the mature pollen formed with three nuclei, *i.e.* two smaller sperm nuclei, and a larger vegetative nucleus. At stage 13, the lemma openes and the anther dehiscence occurres and at stage 14, the anther continues the release of mature pollen grains (Zhang and Wilson, 2009).

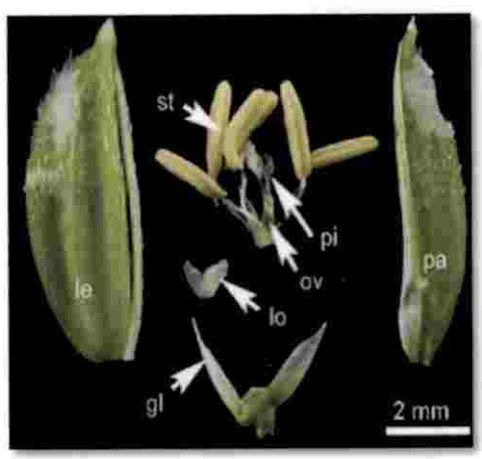


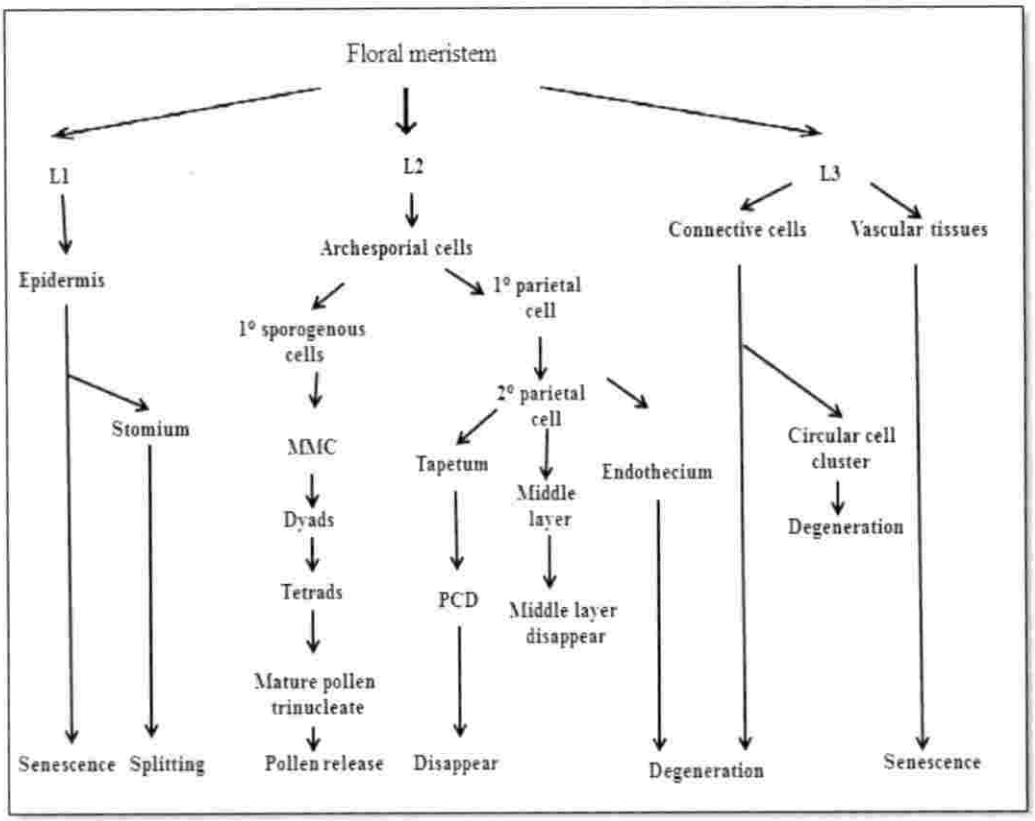
Plate 1. Mature rice flower (stage 12), glumes (gl), lemma (le), palea (pa), lodicules (lo), stamens (st), pistil (ps) and ovary (ov)

(Zhang and Wilson, 2009)

Table 1. The cytological events during anther development in rice

Stage	Events
1	Floral meristem differentiates into stamen primordia containing L1, L2 and L3 cellular layers.
2	Anther primordia with four corners is formed from stamen meristem. The L2 layer forms archesporial cells in each corner.
3	Archesporial cells divide and differentiate to form primary parietal cells.
4	Cell divisions continue to produce the secondary parietal layers and sporogenous cells.
5	Primary sporogenous cells divide and form the secondary sporogenous cells. The outer secondary parietal layer forms the endothecium layer. Middle layer and the inner secondary parietal layer develops into the tapetum, the nutritive layer.
6	Microspore mother cells (MMCs) surrounded by four-layered anther walls are developed.
7	Meiocytes which are associated with the tapetal layer start meiotic division and the middle layer becomes less visible.
8a	Dyads are formed; one meiocyte forms two nuclei separated by a cell plate at the end of meiosis I. Tapetal cells cytoplasm become condensed and Programmed Cell Death (PCD) is initiated.
8b	Tetrad containing microspores enclosed by the callose wall is formed after meiosis II. The tapetum becomes more condensed and vacuolated. Outside their surface, primexines are formed by microspores.
9	Spherical shaped free haploid microspores containing thin exines are released from the tetrads. Tapetal cells become condensed and form characteristic Ubisch bodies on the inner surface facing the microspores.
10	Tapetal cells become more degenerated and produce more Ubisch bodies. The microspore becomes more vacuolated and attains a round shape.
11	The microspore undergoes first mitotic division, generating a generative cell and a vegetative cell. The tapetum cells become completely degenerate into cellular debris and Ubisch bodies on the internal surface.
12	The generative cell undergoes the second mitosis and generates the mature pollen grain containing three invisible nuclei. The tapetum completely disappears.
13	The flower opens, pollen sacs become connected and anther dehiscence occurs.
14	The anther continues releasing mature pollen grains.

(Zhang *et al.*, 2011)



* L1, L2 and L3: three germ layers of the floral meristem which differentiates to form the anther primordium

Fig. 1 Major events during anther development in rice (Wang *et al.*, 2013)

2.1 WEATHER CONDITIONS SUITABLE FOR INDUCING STERILITY IN TGMS SYSTEM

The fertility behaviour of TGMS lines are influenced by the environmental conditions during floral development. The informations on climatic requirement for the expression of thermo-sensitive male sterile gene, critical fertility and sterility temperatures are necessary for the successful utilization of thermosensitive genic male sterility system. Each TGMS line should be characterized for determining its CST and critical thermosensitive stage. Any fluctuation in temperature during specific thermosensitive stage can impart a high risk in exploiting heterosis using TGMS system. The TGMS lines should be sown

in such a way that the critical stages of panicle development would be exposed to appropriate temperature for hybridization/seed multiplication. In the TGMS line 5460S, the critical temperature for fertility change from male sterile to partially fertile is about 28.5°C with the most sensitive stages from formation of pollen mother cell to late uninucleate stage of pollen grains (Zongxiu *et al.*, 1993).

The success of two line breeding technology depends on the stability of sterility and fertility of TGMS lines. The TGMS lines with complete pollen sterility under high temperature condition and more than 30 per cent self-seed set under low temperature condition are considered as promising lines for commercial exploitation (Lu *et al.*, 1994). The panicle developmental stages from stamen and pistil primordial differentiation to meiotic division of pollen mother cell have been reported as critical stages for different TGMS lines (Borkakati and Virmani, 1997). The TGMS rice lines showed complete pollen and spikelet sterility when exposed to maximum temperature higher than 30^0C one to two week after panicle initiation. Around 85.5% spikelet fertility was observed when these lines were exposed to 26^0C to 29^0C during the critical stage (Lopez and Virmani, 2000).

The fertility or sterility alterations in TGMS lines were predominantly influenced by the maximum temperature than minimum temperature (Viraktamath and Virmani, 2001). They also reported that period of four to eight days after panicle initiation was the stage most responsive to temperature fluctuations. Pollen and spikelet fertility were maximum at $27/21^0$C in the TGMS line IR68945-4-33-4-14.

The TGMS line TS 16 had pollen-free anthers indicating that the sensitive stage of TS 16 was around stage IV (stamen and pistil primordia) of panicle development (Kalaiyarasi and Vaidyanathan, 2003). The critical temperature for inducing sterility should be as low as possible for greater stability of the TGMS lines for successful hybrid seed production (Virmani *et al.*, 2003).

The thermosensitive stage in TGMS lines, DRR14S and DR39S was found to be 15 days prior to heading while DRR1S and DRR29S it was about 24 days

prior to heading whereas it was 16 and 19 days prior to heading respectively for DRR25S and DRR31S (Santosh, 2003). These findings indicate the need of characterizing individual TGMS lines for finding out line specific thermosensitive stage. The maximum temperature required for complete sterility ranged from 35°C to 37.2°C and the TGMS lines restored fertility between 24°C to 26°C.

The TGMS line T29S was sterile when they are exposed to daily mean temperatures of 24.1°C or above during the critical stage from 11 days before heading to 15 days before heading (Pham *et al.*, 2004). In addition to temperature (maximum, mean and minimum), relative humidity and sunshine hours also influence fertility alteration (Latha *et al.*, 2004). Lee *et al.* (2005) reported in the TGMS rice Sokcho-MS that it was completely sterile at a temperature higher than 27°C and fertile at the temperature ranging from 25 to 27°C regardless of the levels of day-length.

The purity of two line hybrid rice seeds is enhanced when the TGMS line possesses a low critical sterility point thereby minimizing the self-fertility induced by temperature fluctuations during hybrid rice seed production. The critical stage for sterility expression is approximately 24 days before heading to 5 days before heading (Sanchez and Virmani, 2005).

Genetic variability exist in TGMS lines for the CST. The critical sterility point ranged from 30°C (DRR IS) to 35.9°C (IR 73827-23S) and fertility was restored at a temperature range of 24 to 28°C in the TGMS lines DRR IS, DRR 5S, IR 3827-23S, IR 73834-21S, UPRI 95-104S and UPRI 95-167S (RamaKrishna *et al.*, 2006). The pattern of flowering and the number of spikelets that reach anthesis is greatly influenced by high temperature.

The TGMS lines *viz.*, GD 98014, GD 99017, GD 98029 and GD 98049 had stable sterile phase with 100 per cent pollen sterility during high temperature condition of 30/20°C maximum/minimum temperature and the same lines reverted back to 60% pollen fertility during low temperature condition of less than 30/20°C

(Chandirakala *et al.*, 2008). The daily mean temperature, relative humidity and photoperiod are found to influence fertility alteration in TGMS lines.

The correlation analysis of pollen sterility and weather parameters revealed that the daily maximum and mean temperature were the primary factors influencing fertility change, whereas sunshine hours, relative humidity and photo period were the secondary factors (Latha and Thiyagarajan, 2010). The critical temperature inducing fertility alteration was found to be between 24°C and 26°C in the TGMS lines TS 6, TS 16, TS 18, TS 29, TS 46 and TS 47. The TGMS lines COTGMS 02, COTGMS 07, COTGMS 10, COTGMS 11, COTGMS 12, COTGMS 13 and COTGMS 15 had complete pollen sterility under high temperature condition of 36°C (Thiyagarajan *et al.*, 2010).

Eight TGMS lines, DDR 1S, DDR 18S, DDR 19S, DDR 20S, DDR 23S, DDR 27S, DRR 28S and DDR 29 were characterized for analysing the sterility-sensitive stage at low altitude and high altitude regions (Salgotra *et al.*, 2012). The TGMS lines DRR 19S, DRR 20S and DRR 29S exhibited a sterility-sensitive stage at 21 days prior to normal heading at low altitude region. In the case of DRR1S a temperature of 36.6°C for complete sterility at 17 days prior to normal heading where as for the remaining seven lines, temperature for complete sterility induction ranged from 33.9°C to 35.8°C at low altitude region. All the eight lines showed acceptable seed set at high altitude region.

The TGMS line EC720903 was found to be a better choice for Kerala condition with critical sterility period of 15 to 22 days before heading and the sterility inducing average temperature of 26.9°C (Celine *et al.*, 2014). The critical temperatures of TGMS rice were reported to be around 23°C to 29°C with variation from line to line (Wongpatsa *et al.*, 2014). The night temperature in the range of 18 to 22°C has a greater effect on pollen viability compared to day temperature. The change in the temperature affects the ability to produce viable pollen.

The TGMS lines *viz*., TNAU 45S, TNAU 60S, TNAU 95 S, TNAU 19S and TNAU 39S were evaluated for their stability of pollen sterility under different temperature regimes (Manonmani *et al*., 2016). The daily mean temperature of 24 to 26°C was the critical temperature for fertility alteration with more than 60 per cent pollen and spikelet fertility. TGMS lines UPRI-99-71-1, UPRI-99-71-2, UPRI-99-73-1, UPRI-99-73-2, UPRI-99-73-4, UPRI-99-74-3, UPRI-99-60-1, UPRI-99-72-1, UPRI-99-74-1 and UPRI-99-74-4 showed complete sterility at an average temperature of 28.53 to 28.92°C (Kumar *et al*., 2016). The TGMS lines TNAU45S, TNAU60S, TNAU95S, GDR 61S and GDR 70S showed stable pollen sterility at a mean temperature of above 29°C and the critical stage for the TGMS lines as 26 days before heading to 5 days before heading (Rekha *et al*., 2017). TGMS line, EC720903 was completely pollen sterile with pollen free anthers above the CST of 26.9°C and fully fertile with 82.08% seed setting rate at high altitude region, where the mean temperature was below the CST (Gayathri and Roy, 2018).

2.2 PLANT GROWTH REGULATORS FOR REGULATING MALE STERILITY

Phytohormones play a significant role in male reproductive development through its differential distribution in plants. It regulates the anther development resulting in the formation of functional pollen grains. Therefore any instability in the hormone concentration during pollen development can result in the development of non-functinal pollen grains. Male reproductive development in flowering plants is complex which involves different stages that leads to the production of fertile or viable pollen for fertilization.

2.2.1 Role of Auxin in Pollen Development

The phytohormone auxin predominantly indole-3-acetic acid (IAA) plays a greater role in anther and pollen development. It has also been reported that the tapetum layer supply IAA to the developing pollen grains (Aloni *et al*., 2006). Decreased auxin levels in the tapetum results in reduced pollen development.

Auxin was principally accumulated in the anther during 10^{th} to 12^{th} stages of floral development (Feng *et al.*, 2006). The inhibition of auxin flow in anther filaments resulted in shortened filaments and significantly defected pollen grains and suppressed pollen mitosis processes.

Key enzymes in auxin biosynthetic pathway belong to *YUC* family of flavin mono oxygenases-which consists of 11 *YUC* genes in *Arabidopsis* (Cheng *et al.*, 2006). The *YUC* genes are chiefly expressed in meristems, young primordia, vascular tissues and reproductive organs. The expression of auxin biosynthesis genes *YUC2* and *YUC6* were confirmed in thecas, tapetum, endothecium and procambium (Cecchetti *et al.*, 2008). The auxin effects begin in anthers between the end of meiosis and the bilocular stage in the somatic tissues that are involved in the first step of dehiscence in the microspores. Higher accumulation of IAA was observed in the mature rice pollen (Hirano *et al.*, 2008).

Furthermore, down-regulation of auxin level during pollen development is essential for anther dehiscence. Dioxygenase for auxin oxidation (DAO) gene, encoding a putative 2-oxoglutarate- dependent-Fe (II) dioxygenase, is responsible for oxidizing free IAA into inactive OxIAA. It is required for pollen fertility and anther dehiscence in rice (Zhao *et al.*, 2013). Expression of *YUC* genes, *OsYUC1*, closely related to *Arabidopsis AtYUC1* and *AtYUC4* were observed in developing flowers of rice (Cardarelli and Cecchetti, 2014). Among the three PIN orthologs of rice, *OsPIN1*, *OsPIN1b* and *OsPIN1c*, *OsPIN1* were highly expressed in stamen filaments and at the junction between anther and filament during late anther development and *OsPIN3t* was expressed in mature anthers.

DII-VENUS is an auxin biosensor construct derived from the Aux/IAA-based sensor which is constitutively driven by maize ubiquitin-1 promoter. DR5-VENUS, a synthetic auxin-responsive promoter (DR5rev) was used to find out auxin distribution and signaling in rice tissues (Yang *et al.*, 2017). Auxin accumulation and active re-localization were observed at the initiation sites of inflorescence, spikelet primordia including branch meristems, female and male

organs. DR5-specified auxin and its transporters PIN1, *OsAUX1* signals were also found to participate in flower primodium initiation in rice.

Mutation in the *FT-INTERACTING PROTEIN7* in rice (*OsFTIP7*) produced completely sterile plants due to indehiscent anthers (Cardarelli and Costantino, 2018). At stage 9 of anther development, before pollen mitotic division, *OsFTIP7* was highly expressed in anthers. This facilitated nuclear translocation of homeodomain transcription factor, *Oryza sativa homeobox 1* resulted in the direct suppression of auxin biosynthetic gene, *OsYUCCA4* (Song *et al.*, 2018). The auxin level started to decline during the late development of anthers thereby regulating the auxin levels between meiosis and pollen mitosis, thus controls the timing of anther dehiscence during rice anthesis.

2.2.2 Role of Gibberellic Acid in Pollen Development

The plant hormone gibberellic acid has essential functions in male organ development. Sterile and semi-fertile phenotypes were shown by loss-of-function *slr1-1* (rice slender mutant) and gain-of-function *Slr1-d3* (GA-insensitive semidominant mutant) mutants respectively (Chhun *et al.*, 2007). Viable pollen production is mainly defective in GA-insensitive semidominant mutant *Slr1-d3*. Anther development arrested prematurely in mutants that were unable to synthesize or perceive bioactive GA which confirms the requirement of GA in the development of anthers.

Remarkable expression of GA signaling genes *viz.*, *GID1*, *SLR1*, *GID2*, *OsGAMYB* and *OsSPY* were noticed during the early stages of microsporogenesis in the rice anther (Hirano *et al.*, 2008). GA synthesis related genes *GA20ox3* and *GA3ox1* were largely accumulated in the rice pollen during bicellular and tricellular stages. On contrary, GA biosynthesis genes *CPS*, *KS*, *KO2* and *KAO*, encode the enzymes which catalyse the initial steps of biosynthesis were expressed at very low levels in the microspore during meiosis.

PCD is the selective elimination of cells during plant growth and development. The initial signal for tapetal PCD has been proposed to be produced first during the tetrad stage of pollen development (Kawanabe *et al.*, 2006). The physiological function of GA in anther development was analysed in GA-deficient, GA-insensitive and *gamyb* mutants and again confirmed the importance of GA in development of stamens and anthers (Aya *et al.*, 2009). All the three mutants exhibited similar defects in PCD of tapetal cell and exine and Ubisch bodies formation. Rice GA-insensitive *gid1-4* and *gid2-5* mutants showed deformation of pollen mother cell tetrads during anther development resulting in male sterility. Thus GA modulate the exine formation and the PCD of tapetal cells and directly activated *CYP703A3* (cytochrome P450 hydroxylase) by *GAMYB* is required for exine formation.

Gibberelic acid signalling regulates male sterility. It is required for filament elongation and pollen development in *Arabidopsis* and rice (Plackett *et al.*, 2011). Studies on role of GA in anther development show that most of the GA insensitive or deficient mutants exhibit defective pollen structure and development. GA-insensitive mutant showed severe reduction in the number of sporogenous cells and the abnormal enlargement of tapetal cells (Sakata *et al.*, 2014).

A central DELLA protein inhibits GA-associated development and GA induces the protease-mediated degradation of DELLA protein in GA signalling. In rice, downstream genes in the GA signalling pathway were negatively regulated by the DELLA protein *SLENDER RICE1* (*SLR1*) and it represses the expression of *GAMYB* (Kwon and Paek, 2016). The degradation of *SLR1*, caused by GA perception, de-represses the transcription of downstream genes in the GA signalling pathway. *GAMYB* activates the expression of downstream genes that regulate exine and Ubisch body formation in pollen by directly binding to their promoters. Ubisch bodies are small, granular structure, found in the extracellular matrix of tapetal layer. Thus, GA signaling plays a central role in floral organ formation.

2.2.3 Role of Cytokinins in Pollen Development

Cytokinins are involved in wide range of plant developmental processes including male reproductive development. The *CKX1* gene involved in oxidative cytokinin degradation was found to be accumulated in reproductive tissues of transgenic maize. It resulted in male-sterile plants due to abnormality in anther development (Huang *et al.*, 2003). Pollen sacs of anthers were completely absent at stage 5 of pollen development in such plants. Prominent accumulation of cytokinin accumulation was detected in the early stage of microspore/pollen formation in rice and it decreased in the later stages of microspore/pollen development (Hirano *et al.*, 2008).

The mutants of three cytokinin receptor genes: *CRE1*, *AHK2* and *AHK3* have lower levels of endogenous cytokinin and only two or three-lobed structures were formed in mutant anthers and number of pollen grains was less than wild type (Kinoshita-Tsujimura and Kakimoto, 2011). Cytokinin receptor gene *OsCKT1* was found to be expressed in young spikelet, glume and flower of rice (Ding, 2017). Autophagy is the selective degradation of intracellular components through vacuoles/lysosomes mediated by autophagosomes. Recently it has been shown to be required for postmeiotic anther development including anther dehiscence, programmed cell death of the tapetum and pollen maturation in rice. The bioactive cytokinin tZ (transzeatin) levels was lower in the autophagy-defective mutant of rice *Osatg7–1* at the flowering stage (Kurusu *et al.*, 2017).

2.2.4 Role of Ethylene in Pollen Development

Ethylene has a role in anther dehiscence for the release of pollen. It is involved in PCD in tapetal cells (Takada *et al.*, 2006). Male sterility is caused by the selective premature destruction of tapetum during anther development. The high amount of ethylene was also associated with femaleness through the suppression of staminal buds.

Notably, ethylene signalling related genes (ethylene receptor genes, *EIN2*, *EIN3*, *EIL3*, *ETO1*, *ERF*, etc.) expression were observed in microspore/pollen and tapetum tissues (Hobo *et al.*, 2008). This specifies the involvement of ethylene in regulating tapetum degeneration from the meiosis to uninucleate stages of pollen development. Ethylene signaling genes involved were recognized in the tapetal cells of rice, especially at the tetrad and uninucleate stages (Hirano *et al.*, 2008). In *Arabidopsis* over production of ethylene resulted in reduced fertility (Lin *et al.*, 2009).

2.2.5 Role of Abscisic Acid in Pollen Development

The phytohormone abscisic acid (ABA) is also involved in the pollen development in plants. Abscisic acid is known to suppress PCD (Fath *et al.*, 2000). Induction of its ABA deactivation enzymes at the later stages of tapetal development may have a significant role in promoting PCD in tapetum cells. ABA induced expression of gene *OsABI5* which belongs to a subfamily of bZIP transcription factor was observed in the rice panicle (Zou *et al.*, 2008).

The transcription factor *OsVP1* is required for the activation of ABA-regulated genes during embryo maturation. Significant increase in the level of *OsVP1* was noticed at the tricellular stage of the pollen and at the uninucleate stage of rice anther development (Hirano *et al.*, 2008).

2.2.6 Role of Jasmonic Acid in Pollen Development

Jasmonic acid plays a major part in final stages of anther dehiscence and pollen maturation. It induces and coordinates the elongation of anther filament, opening of stomium at the time of anthesis and the production of functional pollen. *Arabidopsis coi1* mutant, which is insensitive to jasmonate and related compounds was male sterile (Stintzi and Browse, 2000). The gene *DELAYED DEHISCENCE 1* encodes 12-oxophytodienoate reductase, an enzyme in the jasmonic acid biosynthesis pathway and the mutant in *Arabidopsis* were late to release pollen grains for pollination to take place (Sanders *et al.*, 2000). JA

synthesis and signalling were found to be active in tapetum at all stages in rice (Hirano *et al.*, 2008). The *delayed dehiscence1* defect was caused by a delay in the stomium degeneration program. JA-deficient *Arabidopsis* mutants were male sterile because of defects in filament elongation, completion of anthesis and anther dehiscence (Thines *et al.*, 2013).

EG1, a plastid-targeted lipase participates in JA biosynthesis and EG2/OsJAZ1 is a JA signalling repressor that interacts with a putative JA receptor, *OsCOI1b*, to trigger *OsJAZ1*'s degradation during spikelet development in rice (Cai *et al.*, 2014). *OsJAZ1* also interacts with *OsMYC2*, a transcription factor in the JA signalling pathway and represses *OsMYC2*'s role in activating *OsMADS1*, an E-class gene essential to the spikelet development. The *extra glume 1 (eg1)* and *eg2* mutants exhibited altered spikelet morphology. The developed spikelet was with abnormal floral organ identity and defective floral meristem determinacy. *OsAOS1* and *OsAOS2* are two genes involved in JA biosynthesis which encodes allene oxide synthase (AOS) (Liu *et al.*, 2015). *OsAOS1* and *OsAOS2* RNAi-silenced transgenic rice plants showed severe or complete sterility during the reproductive stage.

2.2.7 Role of Brassinosteroids in Pollen Development

Brassinosteroids are initially isolated from *Brassica napus* pollen (Grove *et al.*, 1979). BR biosynthesis and signalling mutants showed reduced pollen number, viability and release efficiency and it was related with abnormal tapetum and microspore development. BR synthesis and signaling were greatly expressed in tapetal cells in rice (Hirano *et al.*, 2008). In addition, BR is also important for exine formation through the regulation of sporopollenin synthesis or transport.

The transcription factor BES1 of brassinosteroid signalling directly binds to promoter regions of genes encoding transcription factors *SPL/NZZ*, *TDF1*, *MS1*, *MS2* and *AtMYB103* required for anther and pollen development in *Arabidopsis* (Ye *et al.*, 2010). Brassinosteroids promotes pollen development in rice by directly promoting expression of *Carbon Starved Anther* gene (*CSA*) (Zhu *et al.*,

2015). *CSA* encodes a MYB domain protein. Higher sugar accumulation in developing anthers caused by the over-expression of the BR-synthesis gene *D11* or BR-signaling factor *OsBZR1* results in functional pollen development.

2.2.9 Role of Salicylic Acid in Pollen Development

Salicylic acid is one of the phenolic compounds exhibiting thermogenic properties. In arum lily, salicylic acid triggers an increase in the alternate oxidase pathway which increases inflorescence temperature up to 14^0C (Raskin *et al.*, 1989). Therefore the salicylic acid induces metabolic heat, which would cause pollen sterility in specific plants. It has been suggested that PCD induced by oxidative stress was also associated with high levels of salicylic acid (Mazel and Levine, 2001). Salicylic acid induced PCD with increased proteolytic activity mediated by the up-regulation of specific cysteine proteases and down-regulation of protease inhibitors was also reported (Kovacs *et al.*, 2016).

2.2.9 Regulation of Male Sterility

Ensuring complete male sterility is necessary for the development of hybrids using TGMS lines. A sudden depression in temperature due to an unexpected rainfall during the critical thermosentive stage of TGMS lines can convert them to fertile ones. Similarly, attainment of complete fertility for the purpose of seed multiplication of TGMS lines is also required. The role of plant growth regulators in plant growth and development can be effectively utilized for inducing sterility and fertility.

The chemicals such as ethrel, salicylic acid and maleic hydrazide are reported to cause male sterility in rice and other crops. Spraying ethrel one week earlier to boot leaf stage or two sprays with, one week earlier to boot leaf stage and the other at boot leaf stage was very effective in inducing almost 90 per cent pollen sterility in rice (Parmar *et al.*, 1979).

Insufficient amount of active IAA and GAs would cause pollen abortion, which is a common reason for male sterility in crops (Tang *et al.*, 1996). The compounds ethrel (8000 to 10000 ppm) application during pre-boot and boot stage, monosodium methyl arsenate (2000 ppm) and sodium methyl arsenate (2000 ppm) were found to be useful in inducing male sterility in rice (Virmani *et al.*, 1997). Halogen substituted oxanilates like ethyl 4'fluoroxanilate and ethyl 4'bromooxanilate were reported to be highly efficient in inducing the pollen sterility (Ali *et al.*, 1999).

Exogeneous application of cytokinin overcomes GA-insensitivity effects in transgenic tobacco and the flowers restored the fertility by the application of 15 mg of kinetin (Huang *et al.*, 2003). Exogeneous application of IAA twice restored anther development in *Hordeum vulgare* under high temperature conditions (Sakata *et al.*, 2010).

The plant growth regulators like ethrel, salicylic acid and maleic hydrazide induced a significantly higher percentage of male sterility in the TGMS rice (Praba and Thangaraj, 2005). Maleic hydrazide (MH) is considered as an antiauxin and metabolic inhibitor which induced greater pollen sterility in the TGMS lines. Ideal concentration for inducing pollen sterility was 800 ppm ethrel.

Foliar treatment at panicle initiation, bolting and flowering stage was ideal for male gametocide (Sharma and Sharma, 2005). Application at panicle initiation stage (pre meiotic) affects normal development of anthers and pollen mother cells where as application at bolting stage (meiotic phase) and flowering stage (post-meiotic) disturbs normal development and f unctioning of the microspores.

Chemical hybridizing agents (CHA) were evaluated for inducing male sterility following the application at the pre-meiotic stage of wheat (Chakraborty and Devakumar, 2006). Among ethyl oxanilates, 4-fluoro, 4-bromo, 4-trifluoromethyl and 4-cyano derivatives and among pyridones, 4-chloro, 4-fluoro, 4-bromo and 4-trifluoromethyl derivatives were found to be the most promising treatments. The chemical compound (ethyl 4-fluoro oxanilate: 1500 ppm)

identified as a potent CHA and it induced significant male sterility (99.76 ± 0.37%) at 1500 ppm over 29 wheat cultivars.

The changes in the levels of phytohormones were closely associated with fertility alteration in TGMS wheat (Zhang *et al.*, 2006). The treatments with exogenous IAA, ABA, GA_3 and aminoethoxy vinylglycine (ethylene biosynthesis inhibitor) at the critical period for fertility alternation increased the frequency of fertile florets whereas the treatment with 2-chloroethyl phosphonic acid (CEPA), an ethylene releaser, increased the frequency of sterile pollen grains. IAA affects the differentiation and development of vascular bundles of the anther and the deficiency may result in poor differentiation of the vascular bundles resulting in nutrient deficiency leading to pollen abortion of TGMS wheat. Gibberellic acid helps to increase the content of endogenous IAA by decreasing the activity of IAA oxidase, which is responsible for the oxidation and decomposition of IAA. Gibberelic acid also stimulates the activity of proteinase and promotes the release of free IAA from bound IAA. Zhang *et al.* (2006) also mentioned that deficiency of ABA in the anthers may decrease their ability to resist adversities ultimately leading to anther abortion.

The chemical compounds like salicylic acid (800 ppm and 1000 ppm) and ethrel (ethephon; 800 ppm) were sprayed at the third and fifth stages of panicle development in TGMS rice (Vijayalakshmi and Bangarusamy, 2006). The salicylic acid spray at 800 ppm concentration has the ability to cause almost complete pollen sterility in TS 29. In another study it was reported that the application of maleic hydrazide 200 ppm sprayed three times at 20, 30 and 40 days after sowing resulted in 84.33% pollen sterility in okra (Deepak, 2007). It might have been due to abnormalities in the post meiotic stages and premature disintegration of tapetum which lead to male sterility.

The exogenous application of GA to the fertile inflorescence apex in early reproductive development induced male sterility due to arrest in microsporogenesis (Duca *et al.*, 2008). Moreover lack of sufficient IAA and GAs

and excessive ABA may induce pollen abortion (Tang *et al.*, 2008). The amount of ABA should be relatively low for normal anther and pollen development. The average contents of IAA and GAs decreased by 48.9% and 55.9% in Teyou559 (high temperature tolerant) and 20.4% and 30.2% in Shanyou63 (high temperature susceptible) hybrid rice cultivars, respectively compared to control plants.

The efficacy of ethrel (100, 200 and 300 ppm) and GA (400, 800 and 1200 ppm) in inducing pollen sterility in rice cultivars Ratnagiri-24 and Palghar-1 was evaluated (Khan *et al.*, 2011). The greatest pollen sterility was recorded for 300 ppm ethrel 51.28% and 47.19% for Palghar-1 and Ratnagiri-24 respectively.

Male sterility was induced in maize cultivars by the application of gametocide SQ-1, a pyridazinone derivative (Wei *et al.*, 2012). A dosage of chemical SQ-1 at 5.0 kg ha^{-1} was sprayed at 8 to 10 leaf stage exhibited more than 90% of male sterility by selectively hindering post-meiotic development of functional pollens. The phytohormone GA_3 was used in four different concentration 0, 100, 200, 300 ppm in CMS rice HIPA 8, HIPA 6, HIPA jatim 3 and HIPA 14 SBU. It increased plant height, stigma exertion, panicle exertion, duration of floret opening, angle of floret opening and panicle length in rice (Susilawati *et al.*, 2014). GA_3 concentration of 200 ppm gave the best results with highest seed set in all the CMS lines tested.

Maleic hydrazide 500 µM was effective in inducing pollen sterility in *Seteria viridis* (Green foxtail) and suppressed pollen viability (Rizal *et al.*, 2015). The efficiency of four chemical hybridizing agent ethrel, E4FO, 2,4-D and promalin were tested in cereal food crop Tef (*Eragrostis tef*). The results have shown that all the compounds induced pollen sterility and significantly reduced seed yield. The compound E4FO at 1500 to 3000 ppm and ethrel at 5000 ppm produced 99.50% pollen sterility (Ghebrehiwot *et al.*, 2015).

The sulfonylurea family herbicide monosulfuron ester (MES), an acetolactate synthase (ALS) inhibitor induced male sterility in rapeseed (Li *et al.*, 2015). The carbohydrate and lipid metabolism were altered by MES treatment

during anther development resulting in male sterility. It also affected the plastid ultrastructure and metabolite accumulation of the developing anthers.

Ethyl 4-fluorooxanilate (E4FO) and ethrel were evaluated on three genotypes of sorghum and the gametocides were sprayed at five concentrations at the booting stage (E4FO at 1, 1.5, 2, 2.5 and 3 ml L^{-1} and ethrel at 1, 2, 3, 4 and 5 ml L^{-1}). Ethyl 4-fluorooxanilate (2 mg L^{-1}) and higher concentrations of ethrel (3 ml L^{-1}) were efficient in inducing complete male sterility in sorghum (Amelework *et al.*, 2016). Male sterility was induced in okra by the application of maleic hydrazide at the rate 450 ppm at 45 mm bud size stage (Naveen, 2016).

Acetolactate synthase (ALS)-inhibiting herbicide amidosulfuron (1 µg per plant) was an effective gametocide that can be used to induce male sterility in rapeseed (*Brassica napus* L.). The pollen abortion was associated with abnormal behaviors both in the tapetal cells and pollen mother cells along with additional defects found in both cells at the later microspore stages (Liu *et al.*, 2017). Disruptions in carbohydrate metabolism and oxidative stress, along with premature tapetum degradation caused male sterility in SQ-1 treated wheat plants (Liu *et al.*, 2018).

2.2.10 Biochemical Changes during Thermosensitive Period of TGMS Lines

Plants respond to stress condition immediatly by changing metabolic content. Proteins which are the direct product of gene expression and plays an inevitable role in mitigating different stress conditions. Therefore, it provides an overall idea of the changes occurring under sterile and fertile conditions of TGMS lines. Other compounds like phenols and pigments also get altered under temperature stress. Leaves are the main source of photosynthates and other metabolites for the developing grain in rice (Yoshida, 1981). The translocation of carbohydrates synthesized during the grain formation and grain filling period has been found to be highest from the ear followed by flag leaf, first and second top leaves. 70% of the carbon fixed by these leaves after the flowering stage were recovered in the grain.

The accumulation of greater phenols in the leaves during fertility transformation was reported in different studies. A greater accumulation of total phenol content (9.6 mg g^{-1}, 10.82 mg g^{-1}, 9.6 mg g^{-1}) was observed in ethrel, salicylic acid and maleic hydrazide treated TGMS rice plants respectively compared to control (5.93 mg g^{-1}) (Praba and Thangaraj, 2005). Phenolic compounds have the ability to oxidize auxin by increasing IAA oxidase activity and suppress the auxin biosynthesis and transport resulting in male sterility.

Biochemical mechanisms of male sterility in rice was studied in TS 29 at Stage VII of panicle development (Vijayalakshmi and Bangarusamy, 2007). The male sterility in TGMS line was associated with accumulation of total phenolics and reduction in total soluble protein content.

The disruption of normal accumulations of proline and soluble proteins due to stresses may lead to drastic loss of pollen activity and even to sterility (Tang *et al*., 2008). That is under high temperature stress the amounts of soluble protein of high temperature tolerant Shanyou 63 and high temperature susceptible Teyou 559, two hybrid rice cultivars decreased dramatically. A change of soluble protein, soluble sugar and free proline contents in the young panicles of TGMS rice lines Zhun S and Zhu 1S were observed under fertility inducing condition (Song *et al*., 2015). The sugar, proline and soluble protein concentration in the young panicles increased in the Zhun S and Zhu 1S.

2.3 POTENTIAL OF USING TGMS LINE AS FEMALE PARENT

The TGMS lines which were completely sterile under high temperature condition and exhibits acceptable level of pollen and spikelet fertility under low temperature condition can be better exploited as TGMS donor for developing new TGMS lines. The correct choice of parents for hybridization decides the success of plant breeding programme. The outcrossing potential of the parental lines determines the seed yield in hybrid rice seed production.

The crosses involving TGMS lines IR68945, SA2 and ID24 with a set of normal pollen parents were observed for segregation of fertility in the F_2 and F_3 generation (Reddy *et al.*, 2000). They segregated in accordance with the expected ratio of 3 fertile: 1 sterile plant.

A study was carried out to evaluate six TGMS lines *viz.*, GD 98049, GD 98029, GD 99017, GD 98013, GD 98014 and TS 29 and their hybrids for yield and yield components (Sundar, 2003). The hybrids developed by crossing recorded significant positive heterosis. All the F_1 hybrids from TGMS lines DRR 14S, DRR 16S, DRR 25S, DRR 29S, DRR 31S and DRR 39S showed significant positive heterobeltiosis for morphological as well as yield and yield attributes (Santosh, 2003).

The heterosis of the F_1 hybrids between the TGMS line $T29^S$ and indica cultivars were examined (Pham *et al.*, 2004a). Most of the F_1 hybrids showed positive heterosis for grain yield per plant, number of spikelets per panicle and dry matter accumulation per plant.

The F_1 hybrids produced by crossing the TGMS $T29^S$ line with indica and japonica cultivars were evaluated (Pham *et al.*, 2004b). The results have shown that the F_1 hybrids showed a significantly positive heterosis over both the male parents and the mid-parents for the number of tillers, photosynthetic activity and dry matter accumulation per plant at all the growth stages.

Lower spikelet fertility at elevated temperature resulted in fewer filled grains, lower grain weight per panicle and decreased harvest index. The synchronization of the flowering time of TGMS lines and the pollen parents determines the crossing rate (Ramakrishna *et al.*, 2006).

The genetics of TGMS lines were studied in different crosses and found that F_1 of all the crosses were fertile with high pollen and spikelet fertility (>70%) (Shankar *et al.*, 2007). The F_1 rice hybrids produced from TGMS line with any fertile line would be useful not only for getting high yield but also for their

increased productivity on the account of their early maturity (Shukla and Pandey, 2008). Peiai64S (PA64S) TGMS rice has become the most widely used female parent for two line hybrid rice breeding in China with wide compatibility and good agronomic traits (Wang *et al*., 2013).

Seven cultivars of upland inbred rice were crossed with a TGMS line 103 (Cuong *et al*., 2014). The F_1 hybrids obtained were evaluated for characters of photosynthesis and dry matter production under drought stress at flowering stage and at ripening stage. The study revealed that under drought, all the F_1 hybrids exhibited very low negative heterosis for CO_2 exchange rate, stomatal conductance, mesophyll conductance and transpiration rate, but high positive heterosis for intercellular CO_2 concentration. The dry matter accumulation was maintained in all F_1 hybrids after drought recovery, which suggests the potential for using upland rice as male parent to produce F_1 hybrids from TGMS lines for drought tolerance.

Two line hybrids were evaluated for heterosis and combining ability using four TGMS lines *viz*., TS09 12, TS09 22, TS09 28 and TS09 410 and thirteen testers by line x tester method along with the checks improved white ponni and CORH 3 (Dhivyapriya and Kalaiyarasi, 2014). The potential hybrids identified were TS09 22 x T1408.10, TS09 28 x CO 43, TS09 12 x CO 43, TS09 22 x WGL 14 and TS09 22 x CO(R) 50 and they suggests that the TGMS system could be utilized for developing two line rice hybrids. Among the lines, TS09 22 and TS09 410 were found to be good combiner for number of productive tillers, panicle length, spikelet fertility, grain yield per plant. The best performing hybrid TS09 22 x T1408.10 showed high performance for yield contributing traits and produced 61.00g of grain yield per plant in 135 days which was 121.54 and 130.62 per cent high over standard checks CORH3 and Improved white ponni respectively.

A sufficient number of pollen grains must be deposited on stigma lobes of the spikelet of male sterile parents for successful hybrid seed production. The

TGMS lines TNAU60S, TNAU18S and TS-29-150GY had considerable outcrossing potential of 41.2%, 24.6% and 25.0% respectively (Arasakesary *et al.*, 2015). These lines exhibited an average of 60.7% pollen fertility rate during the fertility reversion period.

Two line hybrid combinations exhibited good performance and high heterosis for yield in a cross between TGMS lines and other pollen parents (Sasikala *et al.*, 2015). It is possible to transfer thermosensitive genic male sterile character to any line of interest. Suitable hybrid rice by transferring the TGMS gene to red rice background through molecular marker assisted selection were also developed (Roy *et al.*, 2016) .

Two photoperiod- and thermo-sensitive genic male sterile lines of polyploid rice, PS006 and PS012 were analysed for its combining ability (Zhang *et al.*, 2017). Both lines had high outcrossing rate, fertility alterations and good combining abilities. The hybrids obtained exhibited positive heterosis. PS006 hybrids showed heterosis for panicle number per plant, filled grain number per panicle, total grain number per panicle, seed-setting rate and grain weight per plant. PS012 hybrids had significantly positive plant height, panicle length and grain weight.

2.4 MOLECULAR MECHANISM OF MALE STERILITY IN TGMS LINES

The male reproductive development in plant is highly organized and sensitive to various environmental stresses, including high temperature. Therefore the molecular characterization and identification of TGMS rice is crucial for understanding the mechanism behind the male sterility. The male sterility in TGMS is controlled by a single recessive nuclear gene or a pair of recessive genes that are sensitive to environmental conditions and it is heritable (Borkakati and Virmani, 1996; Virmani *et al.*, 2003). Several TGMS/PGMS genes have been identified and mapped on different chromosomes in rice. To date around 15 TGMS genes were reported (Table 2). Reverse TGMS genes exhibiting sterility at

low temperature and fertility at high temperature were also identified (Dora *et al.*, 2017).

Table 2. TGMS genes reported and its location on chromosome

TGMS genes	Chromosome no.	References
tms1 (5460S)	8	Wang *et al.*, 1995
tms2 (Norin PL12)	7	Yamaguchi *et al.*, 1997
tms3 (IR32364)	6	Subudhi *et al.*, 1997
tms4 (TGMS-VN1)	2	Dong *et al.*, 2000
tms5 (Annong S1; DQ200047-12)	2	Wang *et al.*, 2003
tms6 (Sokcho-MS)	5	Lee *et al.*, 2005
tms6(t)	3	Rongbai *et al.*, 2005
tms7(t)	7	Rongbai *et al.*, 2005
tms8	11	Hussain *et al.*, 2012
tms9 (Zhu 1S)	2	Sheng *et al.*, 2013
tms9-1	9	Qi *et al.*, 2014
Ms-h (ms-h(t))	9	Koh *et al.*, 1999
rtms-1 (J207S)	10	Jia *et al.*, 2001
tmsX (XianS)	2	Yu *et al.*, 2017
Ptgms2-1	2	Xu *et al.*, 2011
PTMS 12-1(t)	12	Zhou *et al.*, 2012

Advances in molecular biology has tried to uncover the mechanism which results in male sterility in TGMS rice. Characterization of genes and proteins linked to male sterility expression helps to understand how male sterility occurs as well as gene expression pattern during fertility and sterility inducing conditions in TGMS rice.

Studies on fertile and sterile anthers revealed some possible relations between calcium content as well as distribution in the pollen grains with male sterility. Abundant calcium accumulation in the anther walls and on the surface of pollen grains during fertile anther development and Ubish bodies at the late developmental stage of the microspore (Tian *et al.*, 1998). But it was not observed in the cytoplasm of pollen grains. Followed by the accumulation of starch grains in pollen, calcium precipitates on pollen walls decreased and increased in parenchymatous cells of the connective tissue. Whereas in the case of sterile anther, calcium precipitates were found in the middle layer and endothecium but not in the tapetal cells like in fertile anther.

PCD can also be suggested as a cause of male sterility in TGMS rice. Analysis of TGMS rice line, 95850 ms revealed that the male-sterility by failure in pollen development was associated with premature PCD of the tapetum cells (Ku *et al.*, 2003). PCD initiated at an early stage of pollen development. It continued until the tapetal cells were completely degraded and resulted in pollen collapse. Abnormality in anther development at the tetrad stages and it became severe when it reached uni-nucleate stage at which the tapetum cells showed distinct cytoplasmic degradation and membrane blebbing.

The SDS-PAGE protein profile of TGMS lines, TGMS 6, TGMS 16 and TGMS 29 along with a normal variety ADT 39 were analysed under sterile and fertile condition (Senthil *et al.*, 2003). Under fertile conditions, TGMS lines leaf tissue exhibited an increased expression of 64, 54 and 24 kDa proteins while anther tissue showed an enhanced expression of 48, 38 and 22 kDa proteins. Under sterile condition these proteins were expressed in lower intensity except 14 kDa protein. They reported a reduction in the expression of larger subunit of Rubisco (54 kDa) and expression of smaller molecular weight protein (24 kDa) under sterility condition.

Microarray analysis of 4,304 cDNA clones of rice anthers showed that the hybridization signal of 396 cDNA clones increased more than six-fold (Endo *et al.*, 2004). RNA *in situ* analysis revealed that several genes showed temporal and spatial expression patterns during anther development. *Os-22* and *Os-25* exhibited sequence similarity with two key enzymes in fatty acid synthesis β-ketoacyl-ACP reductase and acyl carrier protein (ACP) respectively. Fatty acids are said to be a precursor of exine and pollen coat and these were specifically expressed during the floral meristem differentiation into stamen primordia *i.e.*, stage 1 of anther development.

Microarray study representing 3328 unique rice genes of Peiai 64S a P/TGMS rice was done in the young panicles under sterile and fertile conditions for studying gene expression patterns (Xiaoqin *et al.*, 2006). The results showed

that around 14.60% of genes displayed up-or down-regulated expressions at long day/high temperature compared with plants at fertile condition. Only four genes were up-regulated while 482 genes were down-regulated. Real-time PCR confirmed the differential expressions with all the up-regulated and 9 randomly selected down-regulated genes.

Recent developments on molecular characterisation of TGMS rice suggest that temperature-sensitive splicing is an important posttranscriptional regulatory mechanism in modulating gene expression for sterility/fertility expression. UDP-glucose pyrophosphorylase (*UGPase*) is expressed throughout the plant especially in pollen during anther development. UGPase is a key enzyme involved in carbohydrate metabolism, cell wall biosynthesis and callose deposition. It is required in pollen formation and maturation since PMCs and tetrads are surrounded by callose wall before meiosis. In addition, the pollen grain wall is also formed by the callose deposition in which *UGPase* is necessary.

Ugp1 silencing by RNA interference or cosuppression resulted in male sterility (Chen *et al.*, 2007). *Ugp1*-cosuppressing plants contained unprocessed intron containing primary transcripts. It undergoes temperature-sensitive splicing in florets, leading to thermosensitive genic male sterility. In *Ugp1*-silenced plants, pollen mother cells (PMCs) the normal callose deposition was disrupted during early meiosis resulting in complete pollen collapse. The degeneration of the tapetum and middle layer was also inhibited. This indicates the significance of sugar partitioning in maintaining rice pollen fertility in response to environmental signals.

Two-dimensional electrophoresis of around 2300 proteins revealed that 186 were differentially expressed in mature and germinated pollen (Dai *et al.*, 2007). These proteins were found to be involved in pollen wall metabolism, protein synthesis and degradation, cytoskeleton dynamics and carbohydrate metabolism. During high temperature treatment in rice approximately thirteen anther specific genes in secondary sporogenous cells, tapetum and middle layer cells were down

regulated. In contrast, the expression levels of *Osc6*, *OsRAFTIN* and *TDR*, tapetum-specific genes, were unaffected by high temperatures. Therefore tapetal genes are inhibited by increased temperatures and the tapetum itself is not degraded in high temperature conditions (Sakata and Higashitani, 2008).

Proteome analysis in the young panicle proteins of TGMS rice, Zhu 1S during different developmental stages under sterile and fertile conditions revealed that proteins expressed was closely associated with energy metabolism, protein biosynthesis, cell wall formation and stress responses in the pollen development (Xiao *et al.*, 2009). Down-regulation of fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase of young panicles under sterile condition indicates that the lack of energy resulted in pollen sterility.

Arabidopsis gene, *THERMOSENSITIVE MALESTERILE 1* (*TMS1*) plays an important role in thermotolerance of pollen tubes (Yang *et al.*, 2009). *TMS1* was expressed in pollen grains, pollen tubes and other vegetative tissues. Therefore *TMS1* is required for heat tolerance of pollen tubes in *Arabidopsis* by functioning as a co-molecular chaperone.

The high temperature may disrupt some of the tapetum functions which is required for pollen adhesion and germination (Endo *et al.*, 2009). High temperature-repressed genes were namely *YY1* and *YY2* as immature anther-specific genes. These were mainly expressed in the rice tapetum, *AK106843* (encoding *CYP703*, a plant-specific cytochrome P450) and *AK106946* (encoding a GDSL type lipase). *AK106823*, *AK105510*, *AK107501*, *AK112111*, *AK106747*, *AK105952*, *AK105540*, *AK102387*, *AK106946* and *AK106769* were also down-regulated in the anther of a gibberellin-insensitive mutant.

CYP703A3 (Cytochrome P450 family) regulate fatty acid hydroxylation pathway involved in the anther cuticle and pollen exine development (Li and Zhang, 2010). A key regulator of tapetal cell development and degradation in rice *PERSISTANT TAPETAL CELL1* (*PTC1*) was identified (Li *et al.*, 2011a). *PTC1*

encodes a PHD-finger protein, which is expressed specifically at stage 8 and 9 of anther development in the tapetum and microspore cells. The enzyme β-1,3-glucanase is essential for the timely degradation of callose deposition for releasing the microspores from tetrad assembly. β-1,3-glucanase encoding gene, *Osg1* was reported in rice which regulate the callose degradation (Wan *et al.*, 2011).

P/TMS12-1 (photo- or thermo-sensitive genic male sterility locus on chromosome 12) encodes a unique noncoding RNA. It produces a 21-nucleotide small RNA called as osa-smR5864w and is an important regulator of male sterility (Zhou *et al.*, 2012). A C-to-G substitution within the DNA sequence from NK58S-derived cultivars and mutation produces the loss of function of this RNA causing PGMS and TGMS in japonica and indica respectively.

Photoperiod-controlled male sterile line, *carbon starved anther* (csa), contains a mutation in R2R3 MYB transcription regulator of pollen development (Zhang *et al.*, 2013). This mutation rendered male sterility under short-day conditions and male fertility under long-day conditions. Photoperiod regulated *CSA* encodes a key regulator of sugar partitioning during anther development. *CSA* plays a significant role in improving sugar partitioning from leaf to anther by directly controlling the expression of *OsMST8*. *OsMST8* encodes a monosaccharide transporter and mutation in *CSA* resulted in severe reduction of *OsMST8* expression in anthers and decreased the amount of sugar accumulation in the anther under short day conditions resulting in male sterility.

The orosomucoids (ORM) are ER-resisdent polypeptides encoded by *ORM* and *ORMDL* (ORM-like) genes (Chueasiri *et al.*, 2014). The TGMS rice lines controlled by *tms2* contain a deletion of about 70 kb in chromosome 7 and four genes were expressed in panicles. *ORMDL* gene (*LOC_Os07g26940*) only showed differential expression under fertile and sterile conditions. The RNAi transgenic TGMS plants with low expression of *ORMDL* genes present in rice were sterile. The ORMDL proteins influence sphingolipid homeostasis and deletion of this gene results in abnormal pollen development.

The TGMS line Annong S-1 when grown under high temperature conditions produced empty (pollen free) anthers (Huang *et al.*, 2014). The premature tapetal PCD at the microspore mother cell stage (stage 6) resulted in early degradation of the tapetum. This caused a decline in the supply of nutrition and other components to the developing microspores, which were ruptured around stage 9. As a consequence, no pollen grains were produced in the TGMS line.

In the TGMS line TGMS-Co27, the male sterility was based on co-suppression of a *UDP-glucose pyrophosphorylase* (*Ugp1*) gene (Pan *et al.*, 2014). The expression level of total Ugp1 mRNA was higher and the UGPase protein was accumulated in TGMS-Co27 florets at low temperature. Temperature-sensitive splicing of *Ugp1* occurred during high temperature conditions resulting in male sterility. These findings suggest that both temperature-sensitive splicing and translational regulation may be required for fertility/sterility conversion in TGMS-Co27 plants.

In addition mutation of *tms5* in TGMS rice causes the male sterility through a loss of RNase Z^{S1} function (Zhou *et al.*, 2014). *TMS5* gene encodes an evolutionarily conserved ribonuclease Z (RNase Z) and it cleaves mRNAs that are preferentially expressed in MMCs. *TMS5* mRNA accumulates to comparatively high levels in pollen mother cells with in the anther and the *TMS5* protein is localized in the cytoplasm. A C-to-A transition at position 71 of *TMS5* in *tms5* plants produced low levels of UbL40 mRNAs without affecting normal pollen production at the low temperature condition. Three common mRNAs namely Ub 1 (*Os09g0452700*), Ub 2 (*Os03g0259500*) and Ub 4 (*Os09g0483400*) from genes of the conserved ubiquitin L40 60S ribosomal protein L40 family (Ub) accumulates (especially Ub 1 and Ub 4) at higher levels in *tms5* plants at the restrictive temperature. Thus high temperature caused an over accumulation of UbL40 mRNAs and resulted in defective pollen formation. Therefore regulation of Ub mRNA levels by *RNase* Z^{S1} is critical for MMC development at high temperature in TGMS rice.

Male sterility encoded by *tms9* in the TGMS rice Zhu1S was due to the failure of the tapetum to degenerate normally. It resulted in the starving of microspores and finally leading to pollen abortion (Sheng *et al.*, 2015). Gene *tms9* is located within a 30.2 Kb segment of chromosome 2 consisted of seven open reading frames namely *LOC_Os02g12240*, *12,250*, *12,260*, *12,270*, *12,280*, *12,290* and *12,300*. Comparitive study of transcripts of Zhu1S under sterile temperature condition and fertile temperature condition revealed that among seven open reading frames, *LOC_Os02g12290* (which encodes nuclear ribonuclease Z) was significantly transcribed. The level of transcription in the Zhu1S under sterile temperature condition was noticeably lower than that in the fertile ones.

Analysis of proteins from young panicles of TGMS rice lines Zhu 1S and Zhun S at the fertility alternation thermosensitive stage revealed that 83 protein spots were found to be significantly changed in abundance (Song *et al.*, 2015). They were involved in 16 metabolic and cellular processes with highest percentage associated with redox homeostasis (11%), transcription and translation regulation (11%) and carbohydrate metabolism (10%).

Functional analysis of differentially expressed genes related to anther development revealed that carbohydrate, cell wall and lipid metabolism pathways were significantly affected in the chemical hybridizing agent monosulfuron ester sodium treated plants (Li *et al.*, 2015). The starch and sucrose metabolism-related genes (*AGP*, *PGM*, *DPE1* and *PHS2*), sucrose phloem transport genes *sweet protein 11*, *AT3G48740* and lipid biosynthesis related genes were down-regulated in the MES-treated plants. Several substrate transport-related genes such as sugar, lipid, peptide, amino acid and nitrate transports and lipid degradation related genes were up-regulated in the early development stage anthers of the treated plants.

Transcriptomic studies in wheat and *Arabidopsis* showed that 5-10% of all transcripts were differentially expressed under short heat stress, including genes

that putatively encode proteins and transcription factors involved in phosphorylation, hormone biosynthesis and signalling, calcium, sugar and lipid signalling pathways, regulation of transcription and translation, primary and secondary metabolism and responses to different biotic and abiotic stresses (Muiller and Rieu, 2016). In rice, *Defective in Exine Formation 1* (*OsDEX1*), reported to be a Ca^{2+} binding protein, is required in the regulation of tapetal cell degradation as well as pollen formation (Yu *et al.*, 2016).

TMS10 and its close homolog *TMS10-Like* (*TMS10L*), two rice leucine-rich repeat receptor-like kinases, maintains tapetal cell layer and microspore/pollen viability under normal temperature conditions (Yu *et al.*, 2017). *tms10* exhibits male sterility under high temperature and fertility under low temperature condition. The kinase activity conferred by the intracellular domain of *TMS10* is essential for tapetal degeneration and male fertility under high temperatures. Therefore *TMS10* and *TMS10L* act as a key regulator in postmeiotic tapetal development and pollen development.

Gene *NO POLLEN 1* (*NP1*) in rice is involved in the biosynthesis and translocation of lipids for sporopollenin and cutin formation (Liu *et al.*, 2017). It encodes a putative glucose-methanol-choline oxidoreductase and mainly expressed in the tapetal layer from stage 8 to stage 10 of anther development. *np1* mutants were found to be male sterile due to abnormalities in cuticle, Ubisch bodies and the exine synthesis.

The outer pollen wall, exine, is composed of sporopollenin and it is secreted by the tapetal cells. ABC transporter proteins, which utilize energy from ATP, functions as a translocator of tapetum-derived materials to developing pollen grains for wall formation (Chang *et al.*, 2018). TGMS line HengnongS-1 when grown at high temperature condition produced pollen free anthers and normal pollen grains were developed at low temperature. The male sterility in HengnongS-1 was caused by *tms9-1*, a recessive SNP within *PERSISTANT TAPETAL CELL 1* (*PTC1* or rice *MALE STERILITY 1*, *OsMS1*) (Kim and Zhang,

2018). *PTC1* was expressed in tapetal cells, developing microspores and controls tapetal PCD and functional pollen production.

Role of proline in pollen development has recently been highlighted by observing the expression of proline biosynthetic genes *PYRROLINE-5-CARBOXYLATE SYNTHETASE 1* and *PYRROLINE-5-CARBOXYLATE SYNTHETASE 2* in *Arabidopsis* anthers (Mattioli *et al.*, 2018). It is required in maintaining proper pollen development and fertility.

Tandem mass tag (TMT) based proteomic analysis was done in TGMS rice AnnongS-1 anthers grown under fertile-low temperature and sterile-high temperature conditions (Wang *et al.*, 2019). High temperature condition resulted in differential accumulation of 89 proteins in the anther, in which 46 were increased in abundance and 43 were decreased in abundance. The differentially accumulated proteins were accumulated in cytoplasm and chloroplast where chloroplast had the greater accumulation. More than 40% of the differentially abundant proteins were enzymes involved in photosynthesis, energy metabolism, biosynthesis and catabolism of cellular components, metabolic regulation, defense and stress responses. The proteins related to protein metabolism accumulated 21.35%, defense and stress 12.36%, metabolic regulation 10.11% and carbohydrate metabolism 8.99%.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled 'Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani and Regional Agricultural Research Station (RARS), Ambalavayal, Wayanad during 2015 to 2018. The major objective of this study was evaluating the environmental conditions required for complete male sterility of TGMS plants and to manipulate the male sterility by using plant growth regulators and also to understand the molecular mechanism associated with TGMS system. The details of materials used and methods adopted are presented in this chapter.

3.1 GENERAL DETAILS

3.1.1 Location

The geographical co-ordinates of the location of Vellayani are 8° 5'N latitude and 76° 9'E longitude with an altitude of 29 m above Mean Sea Level (MSL) and of Regional Agricultural Research Station, Ambalavayal, Wayanad are 11°6' N latitude and 76°2' E longitude and 974 m altitude above MSL.

3.2 EXPERIMENTAL DETAILS

The stable Thermosensitive Genic Male Sterile (TGMS) line IR75589-31-27-8-33 (EC720903) was imported from International Rice Research Institute (IRRI), Philippines through Standard Material Transfer Agreement (SMTA) through National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The phenological characterisation of EC720903 was done through sequential monthly sowing for one year. The critical sterility temperature and the critical stage of thermo sensitivity of the TGMS line was identified at Department of Plant Physiology, College of Agriculture, Vellayani. The critical sterility temperature of EC720903 was recognized as 26.9°C and the critical thermosensitive phase was 15-22 days before flowering (Celine *et al.*, 2014).

This TGMS line was used as the donor parent for transferring the male sterile gene to popular red rice variety of Kerala, Jyothi. The F_1 plants were selfed to obtain F_2 generation. The sterile F_2 plants of Jyothi were backcrossed to yield BC_1F_2 for recovering maximum characters of red rice variety. This seeds of BC_1F_2 plants were sown in the experimental plot of the Department of Plant Physiology, College of Agriculture, Vellayani. The tillers of sterile plants identified using SSR markers were separated and transferred to RARS, Ambalavayal for seed multiplication. Three SSR markers RM3351, RM257 and RM5897 were identified which are associated with TGMS gene. Based on marker analysis the gene in the present TGMS line was identified as *tms5* located on chromosome no. 2.

3.2.1 Evaluation of Weather Conditions for TGMS System

The seeds multiplied at RARS, Ambalavayal were collected and used for evaluating the weather conditions required for the new TGMS red rice line developed. The TGMS red rice was raised as pot culture experiment in the open condition and Rain Out Shelter (ROS) maintained at the Department of Plant Physiology. The seeds were sown in pots at monthly interval for twelve months starting from June, 2017 to May, 2018 in order to identify the ideal season for hybridization and seed multiplication by observing pollen sterility and spikelet sterility. The plants were maintained healthy with proper nutrition, irrigation, pest and disease management throughout the crop period as per adhoc Package of Practice (POP) of Kerala Agricultural University (KAU, 2016). Each monthly sowing was considered as a treatment and the weather parameters were monitored throughout the crop period.

3.2.1.1 Observations Recorded

3.2.1.1.1 Morpho-physiological Observations

3.2.1.1.1.1 Plant Height (cm)

Five plants were selected from each treatment and height of the plant was measured. The plant height was recorded at the time of panicle initiation and flowering from soil surface to tip of the longest leaf of plant and average height was expressed in centimetre.

3.2.1.1.1.2 Number of Productive Tillers

Number of tillers which produces panicle were counted from five plants and expressed as the number of productive tillers per plant.

3.2.1.1.1.3 Days to First Flowering

Number of days taken for first flowering in five selected plants were counted from date of sowing and recorded as days.

3.2.1.1.1.4 Days to 50 Per cent Flowering

Number of days taken for 50 per cent flowering in the selected plants were counted from date of sowing and recorded as days.

3.2.1.1.1.5 Pollen Sterility (%)

Pollen sterility was observed using 1% Iodine-Potassium iodide (IKI) solution which was prepared by dissolving 1.3 g Potassium iodide and 0.636 g Iodine in alcohol and made upto 100 ml by adding water. Five florets each from the selected five plants were collected on or just before anthesis and anthers were separated, crushed and stained by keeping in the IKI solution on glass slide for few minutes. The fully stained grains represent fertile pollen and unstained, shrivelled and empty grains denote sterile pollen (Table 3). The number of pollen grains unstained to the total number of pollen grains was visually counted under compound microscope Leica DC 7.5 V (10 X) from three different microscopic

fields. The pollen sterility was calculated using the following formula and expressed as percentage.

Pollen sterility = [No. of pollen grains unstained/ Total no. of pollen grains] × 100

Table 3. Classification of pollen based on sterility/fertility

Sl. No.	Category	Classification
1.	Unstained withered	Sterile
2.	Unstained spherical	Sterile
3.	Stained round (light)	Sterile
4.	Stained round	Fertile

(Virmani *et al.*, 1997)

3.2.1.1.1.6 Spikelet Sterility (%)

The panicles were collected at maturity from five plants and number of grains unfilled to the total number of spikelets was counted. The spikelet sterility was expressed as percentage.

Spikelet sterility = [(No. of grains unfilled/Total no. of spikelets)] × 100

3.2.1.1.1.7 Scanning electron microscopic analysis of sterile TGMS anther

Anthers of TGMS plants maintained under open field and Rain Out Shelter were collected and scanning electron microscopic image was taken at STICS, CUSAT, Ernakulam.

3.2.1.1.2 Weather Parameters

All the weather parameters *viz.*, temperature, sunshine hours, relative humidity and rainfall were observed throughout the growing period from both open field and rain out shelter. The weather data of open field was collected from Department of Agricultural Meteorology, College of Agriculture, Vellayani.

The maximum and minimum temperature was observed using maximum-minimum thermometer at 7.30 am and 2.30 pm respectively from rain out shelter. The average temperature was also calculated by taking the mean of maximum and minimum temperature and represented as ^{0}C. Relative humidity inside the rain out shelter was observed at 7.30 am and 2.30 pm using hygrometer and expressed as percentage. Sunshine hour is the duration of sunshine in a given period for a given location and were recorded using sunshine recorder and was represented in hours. Rainfall with respect to Vellayani condition was recorded and expressed as mm.

3.2.2 Evaluation of Plant Growth Regulators for Inducing Male Sterility

The season showing fertility was identified and the TGMS line EC720903 was sown at RARS, Ambalavayal, high altitude zone in which the TGMS line shows fertility behaviour since the average temperature is below CST during June, 2017 to November, 2017. The weather data of RARS, Ambalavayal during the experimental period was also collected. The three plant growth regulators namely ethrel, salicylic acid and maleic hydrazide were applied as foliar spray at two stages *viz*., panicle initiation and two weeks after panicle initiation. The crop was given proper fertilization, irrigation, pest and disease management throughout the crop period as per adhoc Package of Practice (POP) (KAU, 2016) to maintain the plants healthy.

3.2.2.1 Treatments

Ethrel: 400 mg L^{-1}, 800 mg L^{-1} and 1200 mg L^{-1}

Salicylic acid: 400 mg L^{-1}, 600 mg L^{-1} and 800 mg L^{-1}

Maleic hydrazide: 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}

Control: Water spray

3.2.2.2 Observations Recorded

3.2.2.2.1 Pollen Sterility (%)

The pollen grains of five florets from three selected plants from each treatment were collected and pollen sterility was observed using 1% IKI solution and represented as percentage.

3.2.2.2.2 Spikelet Sterility (%)

The panicles were collected at maturity from selected three plants and number of grains unfilled to the total number of spikelets was counted. The spikelet sterility was expressed as percentage.

3.2.2.2.3 Seed Setting Percentage (%)

The mature panicles were collected from the selected experimental plants and counted the number of grains filled to the total number of spikelets and represented as percentage.

3.2.2.2.4 Pigment Composition (mg g^{-1})

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were estimated by the method suggested by Hiscox and Israelstam (1979). Leaf samples were collected at fifteen days after panicle initiation and at the time of flowering. For estimation, 100 mg leaf sample was added to 5 ml DMSO (Dimethyl Sulphoxide): 80% acetone mixture (1:1) and kept in darkness for overnight. The final volume was made up to 5 ml. Then the chlorophyll content was estimated using UV-VIS spectrophotometer (ELICO-SL 218 Double Beam) at two different wavelengths 645 nm and 663 nm and expressed as mg g^{-1} fresh weight of plant tissue. The carotenoid content was estimated at 480 nm and 510 nm and expressed as mg g^{-1} fresh weight of plant tissue. The calculation was done by the following formulae.

Chlorophyll 'a' = $[(12.7 \times A_{663}) - (2.69 \times A_{645})] \times V/1000 \times W$

Chlorophyll 'b'= $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times V/1000 \times W$

Total chlorophyll = $[(20.2 \times A_{645}) + (8.02 \times A_{663})] \times V/1000 \times W$

Carotenoid = $[(7.6 \times A_{480}) - (1.49 \times A_{510})] \times V/1000 \times W$

Where,

A = Absorption at given wavelength

V = Total volume of sample in extraction medium

W = Weight of sample in gram

3.2.2.2.5 Total Soluble Protein (mg g^{-1})

Total soluble proteins were estimated using Bradford method (1976). The assay is based on the ability of proteins to bind coomassie brilliant blue G 250 and a complex is formed whose extinction coefficient is much greater than that of free dye. Leaf samples were collected at fifteen days after panicle initiation and at the time of flowering. The total soluble protein from 500 mg of plant samples were extracted using 10 ml of Phosphate buffered saline (PBS) solution. The extracts were collected after centrifugation and 0.1 ml of extract was taken and made up to 3 ml by adding PBS. Dye (5 ml) solution was added to each sample. The solution was mixed well and allowed to develop blue colour. The red dye turned blue when it binds with protein and its absorbance was read at 595 nm. Bovine serum albumin was used as the protein standard and the protein concentration was calculated and expressed as mg g^{-1} fresh weight of plant tissue.

3.2.2.2.6 Phenol Content (µg g^{-1})

The phenol content was estimated by the Folin-Ciocalteau method suggested by Malick and Singh (1980). Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in the alkaline medium and produce blue coloured complex. Leaf samples were collected at fifteen days after panicle initiation and at the time of flowering. Five hundred milligram of plant sample was homogenised

in a mortar and pestle using 10 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The extract was collected and evaporated to dryness. Then the residue was dissolved in 5 ml distilled water. From the extract 0.1 ml was pipetted out and volume made up to 3 ml with distilled water and 0.5 ml Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% Na_2CO_3 was added. The catechol was used as the phenol standard and the absorbance was read at 650 nm and phenol content was expressed as $\mu g\ g^{-1}$ fresh weight of plant tissue.

3.2.3 Evaluating the Potential of Using TGMS Line as Female Parent

The season suitable for hybridization was selected and the TGMS line EC720903 was evaluated for its potential to be used as a female parent. The TGMS line was crossed with two rice varieties of Kerala Aiswarya and Swetha. The male parents were selected based on its grain colour. The red rice variety Aiswarya (PTB 52) is medium duration, with long bold grain and the white rice variety Swetha (PTB 57) is long duration, with short bold grain. The hybridization was done using proximal hybridization method. In proximal hybridization, the panicles of two parents are placed together and covered with butter paper cover on the previous day of anthesis for pollination to occur. On the next day during anthesis the crossing was assisted by tapping on the butter paper cover. The female parent TGMS line EC720903, male parents Aiswarya and Swetha were sown in the third crop season (December-January to March-April) for hybridization. The long duration rice variety Swetha was sown one week prior to Aiswarya and EC720903 inorder to synchronize the flowering period.

The mean temperature during the third crop season was above the CST and complete male sterility was obtained in the TGMS line which favoured the hybrid seed production.

3.2.3.1 Observations Recorded

The seed setting percentage and spikelet sterility percentage of hybridization of TGMS line EC720903 with Aiswarya and Swetha were calculated to find out its crossability when used as a female parent.

3.2.3.1.1 Spikelet Sterility Percentage (%)

The panicles were collected at maturity from the female parent and the number of grains unfilled to the total number of spikelets was counted and expressed as percentage.

3.2.3.1.2 Seed Setting Percentage (%)

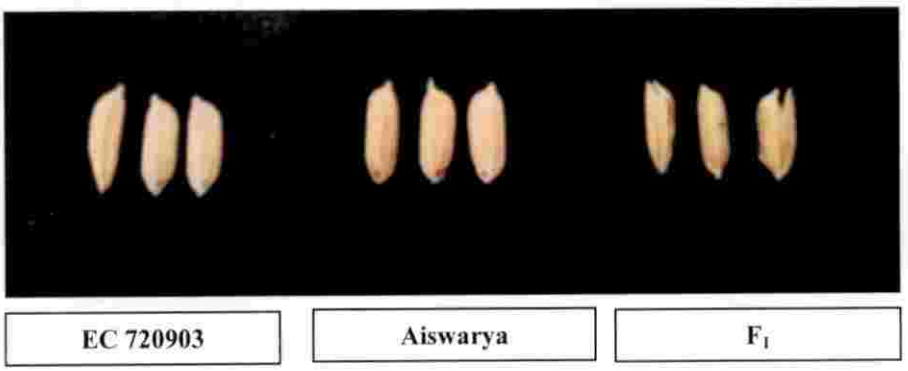
The mature panicles were collected from the female parent and counted the number of grains filled to the total number of spikelets and represented the seed setting as percentage.

3.2.3.2 Evaluation of F_1 Progenies in Comparison with their Parents

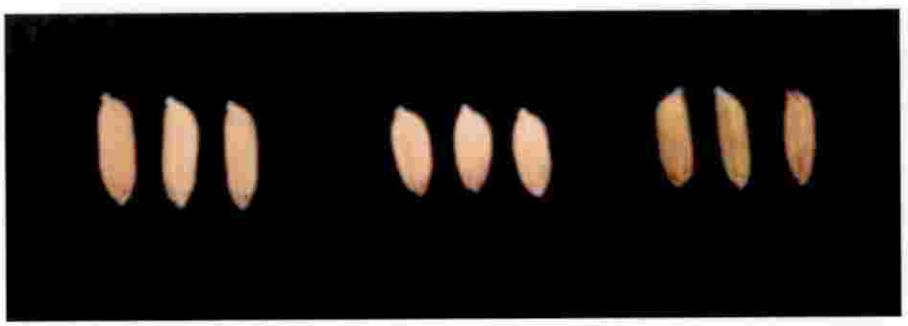
The F_1 progenies obtained from two crosses EC720903 x Aiswarya and EC720903 x Swetha (Plate 2) along with male parents Aiswarya, Swetha and the female parent EC720903 were sown in pots during the first crop season (April-May to September-October). The plants were maintained healthy throughout the crop period as per adhoc Package of Practice (POP) (KAU, 2016). The F_1 progenies were evaluated for its phenological, physiological and yield attributes in comparison with their parents.

3.2.3.2.1 Phenological Observations

Phenological observations *viz.*, days to first flowering, days to 50% flowering and days to physiological maturity were taken from F_1 progenies of EC720903 x Aiswarya and EC720903 x Swetha along with parents Aiswarya, Swetha and TGMS line EC720903 from selected plants. Number of days taken for first flowering in eight selected plants were counted from date of sowing and recorded as days. Number of days taken for 50 per cent flowering was counted from date of sowing from eight plants and recorded as days. Number of days taken for physiological maturity in the selected plants was counted from date of sowing and recorded number of days. At physiological maturity, rice panicles completely matures at top with golden yellow colour, few grains at bottom in the



EC 720903	Aiswarya	F_1



EC 720903	Swetha	F_1

Plate 2. F_1 progenies obtained from two crosses EC720903 x Aiswarya and EC720903 x Swetha

immature stage, senescence of the lower leaves and moisture content decreases from 40% to 20% in grains.

3.2.3.2.2 Physiological Observations

All the physiological observations were recorded two times *viz.*, at the time of panicle initiation and flowering from selected plants of F_1 progenies of EC720903 x Aiswarya and EC720903 x Swetha, Aiswarya, Swetha and TGMS line EC720903.

3.2.3.2.2.1 Plant Height (cm)

The plant height was recorded at panicle initiation stage and at the time of flowering from ground level to tip of the longest leaf of plant and expressed in centimetre.

3.2.3.2.2.2 Total Dry Matter (g)

The total dry weight of the selected plants were recorded by oven drying the whole plant at 80^0C for two days and expressed in gram.

3.2.3.2.2.3 Pigment Composition (mg g^{-1})

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were estimated by the method suggested by Hiscox and Israelstam (1979). The pigment content was expressed as mg g^{-1} fresh weight of plant tissue.

3.2.3.2.2.4 Total Soluble Protein (mg g^{-1})

The leaf samples from three plants were collected and total soluble protein content was estimated using Bradford method (1976) and expressed as mg g^{-1} fresh weight of plant tissue.

3.2.3.2.2.5 Photosynthetic characteristics

Photosynthetic characteristics such as photosynthetic rate (µmol CO_2 m^{-2} s^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}), stomatal conductance (mmol m^{-2} s^{-1}) and

leaf temperature (^{0}C) were measured using portable photosynthesis system (CIRAS-3 Ver. 1.06, Amesbury, USA) at the time of panicle initiation and flowering. Readings were taken between 9.00 AM and 11.30 AM using this instrument and a total of three measurements were taken in the same leaf from selected plants.

3.2.3.2.3 Yield Attributes

All the yield parameters *viz.*, number of productive tillers, number of spikelet per panicle, number of filled grains per panicle, 1000 grain weight, grain yield and straw yield were recorded from eight selected plants in each treatment.

3.2.3.2.3.1 Number of Productive Tillers

Number of tillers which produces panicle were counted and expressed as the number of productive tillers per plant.

3.2.3.2.3.2 Number of Spikelets per Panicle

Mature panicles were collected from selected plants and total number spikelets were counted from each panicle and expressed as number of spikelets per panicle.

3.2.3.2.3.3 Number of Filled Grains per Panicle

Rice grains were collected at maturity from selected plants and the number of filled grains were counted to the total number of spikelets per panicle from each panicle.

3.2.3.2.3.4 Spikelet Sterility Percentage (%)

The panicles were collected at maturity from selected plants and number of grains unfilled to the total number of spikelets was counted. The spikelet sterility was expressed as percentage.

3.2.3.2.3.5 Seed Setting Percentage (%)

The mature panicles were collected and counted the number of seeds set to the total number of spikelets and represented the seed setting as percentage.

3.2.3.2.3.6 Thousand Grain Weight (g)

One thousand grains were collected from selected panicles and their weight at 14% moisture level was recorded in grams using electronic balance (Sartorius CP 124).

3.2.3.2.3.7 Grain Yield (g plant^{-1})

Crop was harvested and weight of total grain per plant at 14% moisture level was recorded using Sartorius (CP 124) weighing balance and expressed as gram per plant.

3.2.3.2.3.8 Straw Yield (g plant^{-1})

Crop was harvested at maturity and the weight of straw from each plant was recorded using Sartorius (CP 124) weighing balance and expressed as gram per plant.

3.2.4 Analysing the Molecular Mechanism of Male Sterility in TGMS Lines

The TGMS line EC720903 was maintained simultaneously in sterility inducing (Rain Out Shelter) and fertility inducing conditions (Growth chamber) (Plate 3). Protein samples were collected from leaf and young panicle from both conditions at ten days after panicle initiation and analysed using SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Young panicles and leaf were collected from both conditions at ten days after panicle initiation and analysed using microarray technique.

3.2.4.1 Proteomic analysis of TGMS Lines using Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is the commonly used technique to separate macromolecules such as DNA, RNA and proteins by applying electric current based on their size. Electrophoresis using polyacrylamide gel (Polyacrylamide Gel Electrophoresis-PAGE) containing sodium dodecyl sulphate (SDS) is the most sensitive method to separate proteins under denaturing conditions. SDS PAGE analysis was done by the method suggested by Sadasivam and Manickam (2016).

3.2.4.1.2 Gel Preparation and Casting of Gel

The gel unit was cleaned thoroughly using distilled water and ethanol and dried and the glass plates were assembled properly. Resolving gel (10%) were prepared using 30% acrylamide stock solution, 1.5 M tris HCl (pH 8.8), 10% SDS, distilled water, 10% ammonium per sulphate solution and TEMED (N,N,N',N'-tetramethylethylene-1-diamine). It was mixed gently and carefully poured the gel solution between the glass plates to the three-forth portion. Overlaid with distilled water to prevent contact with air for proper polymerization and allowed to polymerize. Stacking gel (5%) was prepared using 30% acrylamide stock solution, 0.5 M tris HCl (pH 6.8), 10% SDS, distilled water, 10% ammonium per sulphate (APS) solution and TEMED. Overlaid water was removed and stacking gel was poured between glass plates. Comb was inserted properly and allowed to set. Placed the gel plate in the Biorad electrophoresis apparatus and poured electrode buffer containing tris base, glycine and SDS. Carefully removed the comb from the gel assembly.

3.2.4.1.3 Sample Preparation and Gel Loading

The wells were flushed with buffer using a syringe to remove residual gel reagents before loading samples. Protein concentration in each sample were adjusted to 40 µg and it was mixed well with sample buffer containing tris HCl (pH 6.8), bromophenol blue, β-mercaptoethanol, glycerol and SDS. Samples were

Rain Out Shelter

Growth chamber

Plate 3. TGMS line maintained simultaneously in sterility inducing (Rain Out Shelter) and fertility inducing conditions (Growth chamber)

heated at 93°C for 3 minutes before loading to ensure complete interaction between proteins and SDS. The protein samples were allowed to cool and samples along with pre-stained protein ladder (5 µl) were loaded in the wells. The position of wells on the glass plate were marked using a marker pen and the presence of bromophenol blue in the sample buffer facilitated the easy loading of samples.

3.2.4.1.4 Electrophoresis

Electrode buffer were poured into the electrophoresis apparatus and ran at 75 V for 1 to 1.5 hrs until dye front reaches the bottom.

3.2.4.1.5 Gel Staining and Destaining

After the run was complete, gel was removed carefully from the glass plates and immersed the gel in the staining solution for two to three hours with occational shaking. Staining solution was prepared by mixing coomassie brilliant blue R 250, glacial acetic acid, methanol and distilled water. The proteins absorb the coomassie brilliant blue present in the staining solution. After staining, gel was transferred to destaining solution containing all components except dye and allowed to destain till the bands are visible. Dye that was not bound to proteins were removed. Destaining should be stopped at correct stage to visualize the protein bands. The gel was photographed after proper destaining.

3.2.4.2 Microarray Gene Expression Study of TGMS Line under Sterility Inducing and Fertility Inducing Conditions

Microarray analysis techniques are used to study the expression state of a large number of genes. The TGMS line EC720903 were maintained simultaneously in sterility inducing and fertility inducing conditions. Young panicles and leaves were collected from both conditions at ten days after panicle initiation and analysed using microarray technique. The panicle initiation stage was confirmed by split opening the primary tiller longitudinally from base (Plate 4) and identified through microscope and the samples were collected after ten days.

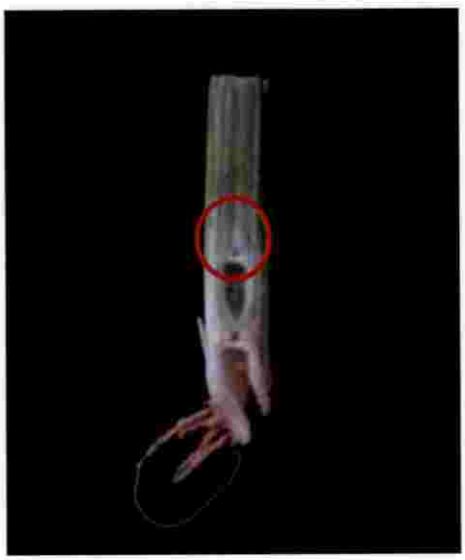


Plate 4. Panicle initiation stage confirmation by split opening the primary tiller longitudinally from base

Tissue sample preparation

The working area was cleaned with 1% hypochlorite to remove nucleic acid and RNaseZAP reagent to make RNase free. Flag leaf and ten days old panicle was dissected out from the primary tiller with a sterile scalpel and transferred immediately to clean petri-plate. Samples were dissected with new nitrile gloves, wiped with RNaseZAP. Tissue samples were washed twice in sterile 1X PBS taken in petri plate only for 10-15 seconds. Immediately transfered the tissue to a new sterile petri-plate containing RNALater and sliced tissue into fine pieces in RNALater in about 90 seconds since longer time might degrade RNA.

Tissue fragments with 3mm or less in size was placed in the sample vial and the samples were prepared in duplicates containing 15-20 small pieces. Time from dissection to transfer the specimen to tube should not be more than 120 seconds. RNA is very labile and can immediately start degrading. Immediately transfered the tissue to screw cap tube pre-chilled in liquid nitrogen using sterile forceps in liquid nitrogen. Snap freezed the tissue in liquid nitrogen and transfered immediately to tube on dry ice for shipping. Tissues were not allowed to thaw at any given point of time after snap freezing. Ensured tubes placed at the center of the ice box and not shifted to the periphery because 5-10 kg dry ice was advised for every 24hrs in transit.

Microarray analysis

The microarray analysis was done at Genotypic Technology Pvt Ltd., Bengaluru. Total RNA was isolated from TGMS samples and converted in to cDNA using Qiagen's RNeasy minikit Cat#74106. Quality Check (QC) of total RNA was done to check profile and integrity of RNA using Bioanalyzer. Samples were labeled using the Agilent's Quick-Amp labeling Kit (p/n5190-0442) and the QC was performed using NanoDrop. Labeling was done, Oligo dT primer T7 promoter based-linear amplification to generate labeled complementary RNA (One-Color Microarray-Based Gene Expression Analysis). Dye Cy3 was used to label the RNA for QC.

Plate 4. Panicle initiation stage confirmation by split opening the primary tiller longitudinally from base

Table 4. NanoDrop analysis of labeled cRNA

Sample ID	Dye	pmol/µl	Concentration ng/µl	260/280	Specific Activity (pmol dye/µg cRNA)
SO_8258_LS1	Cy3	4.5	181.1	2.23	24.85
SO_8258_LF1	Cy3	8.6	543.8	2.2	15.81
SO_8258_PS2	Cy3	1.3	56.5	2.22	23.01
SO_8258_PF1	Cy3	0.7	38.3	2.2	18.28

NanoDrop analysis of labeled cRNA revealed that specific activity (pmol dye/µg cRNA) of the four samples were greater than 8.0. Therefore the amplified and labeled cRNA is suitable for hybridization. The samples were labelled as LS1, LF1, PS2 and PF1 and hybridized. Agilent's In situ Hybridzation kit 5188-5242 was used for hybridization.

Image Quality Control

1. The images were manually verified and found to be devoid of uneven hybridization, streaks, blobs and other artifacts. Hybridization across the slide was good based on number of feature that were "g(r) is PosAndSignif" which indicates feature is positive and significantly above background.

2. The Microarray image was overall clean showing uniform intensity with very low background.

The normalization has been done using GeneSpring GX Software. Intra-array normalization deals with variability within a single array. In intra array normalization, gProcessed signal (dye normalized background subtracted signal intensity) is log transformed and then for each of the array the 75th percentile value is calculated separately. In each sample the log transformed intensity values for each probe is subtracted by the calculated 75th percentile value of the respective array and expression values are obtained.

Percentile shift normalization was done, which normalization is a global normalization, where the locations of all the spot intensities in an array are

adjusted. This normalization takes each column in an experiment independently, and computes the percentile of the expression values for this array, across all spots (where n has a range from 0-100 and n=75 is the median). It subtracts this value from the expression value of each entity.

Microarray analysis was done using Rice_8X60K Array AMADID: 064722. The leaves and panicles collected from fertility inducing condition were kept as control and the samples collected from sterility inducing condition were considered as test. Differentially regulated genes from each set *i.e*, Test_LS1 Vs Control_LF1 and Test_PS2 Vs Control_PF1 were identified.

Cutoff taken for up and down regulation

Differential expression was verified using fold change.

Fold Value > = 1 in the treated samples was taken for up regulation

Fold Value < = -1 in the treated samples was taken for down regulation

The functions and pathway analysis was done for the differentially regulated genes using DAVID database.

3.4 STATISTICAL ANALYSIS

The data were analysed using statistical software SPSS and the treatments were compared using the design CRD.

RESULTS

4. RESULTS

The study entitled 'Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was conducted to evaluate the environmental conditions required for complete male sterility of TGMS plants and to manipulate the male sterility by using plant growth regulators and to understand the molecular mechanism associated with TGMS system. The experimental data collected were tabulated, statistically analysed and are presented in this chapter.

The present study focussed on evaluating the environmental conditions required for complete male sterility of TGMS plants. The TGMS line in the red rice background developed in the Department of Plant Physiology was sown in pots under open field and Rain out Shelter (ROS) sequentially for twelve months in order to identify the ideal season suitable for hybridization and seed multiplication by analysing pollen and spikelet sterility. Prevailing weather conditions during the crop period from both open field and rain out shelter was also observed. The possibility of using plant growth regulators in inducing the male sterility in TGMS plants was studied. With the objective of assessing the potential of TGMS lines as a donor for developing new TGMS lines, the TGMS line EC720903 was hybridized with two rice varieties Aiswarya and Swetha. The experiment also aimed to understand the molecular mechanism associated with TGMS system. The TGMS plants were maintained simultaneously in sterility inducing and fertility inducing conditions during the thermosensitive stage for protein profiling and microarray gene expression analysis.

4.1 EVALUATION OF WEATHER CONDITIONS FOR TGMS SYSTEM

The stable TGMS line EC720903 imported from International Rice Research Institute (IRRI), Philippines was characterised through sequential monthly sowing for one year at Department of Plant Physiology, College of Agriculture, Vellayani. The CST was recognized as 26.9°C and the critical thermosensitive phase was 15-22 days before flowering (Celine *et al.*, 2014). The

male sterile gene of TGMS line was transferred to the popular red rice variety of Kerala, Jyothi. The sterile F_2 plants of Jyothi were backcrossed to yield BC_1F_2 for recovering maximum red character. This seeds of BC_1F_2 plants were sown at Department of Plant Physiology, College of Agriculture, Vellayani. The tillers of sterile plants were identified, separated and transferred to RARS, Ambalavayal for seed multiplication. The seeds multiplied were raised under open field and Rain Out Shelter for evaluating the weather conditions required for the new TGMS red rice line developed.

4.1.2 Weather Parameters

Weather parameters like temperature, relative humidity, sunshine hours and rainfall were recorded throughout the crop period from open field and rain out shelter (Appendix I, II). Analysis of weather data revealed that average temperature during the critical thermosensitive stage of all the monthly sown TGMS red rice under both conditions were above the critical sterility temperature of 26.9^{0}C. The observed CST is the specific critical sterility inducing average temperature of the parental line EC720903. The minimum temperature during the thermosensitive stage was in the range of 21.5^{0}C to 26.3^{0}C whereas the maximum temperature was in the range of 29.1^{0}C to 34.7^{0}C in the open field condition. In case of rain out shelter, minimum temperature was around 20.0^{0}C to 25.0^{0}C and the maximum temperature recorded 30.0^{0}C to 44.0^{0}C. The average temperature ranged between 26^{0}C to 30.4^{0}C in the open field and it was 26.35^{0}C to 34^{0}C under rain out shelter.

Relative humidity, sunshine hours and rainfall during critical thermosensitive stage were also recorded from both growing conditions (Appendix I, II). The relative humidity recorded in the open field condition was 77.9% to 92.4% on the other hand it was 46% to 80% inside the rain out shelter. Average sunshine hours ranged between 1.1 h to 9.7 h. The weekly rainfall in the open field has ranged from 0 mm to 56.30 mm.

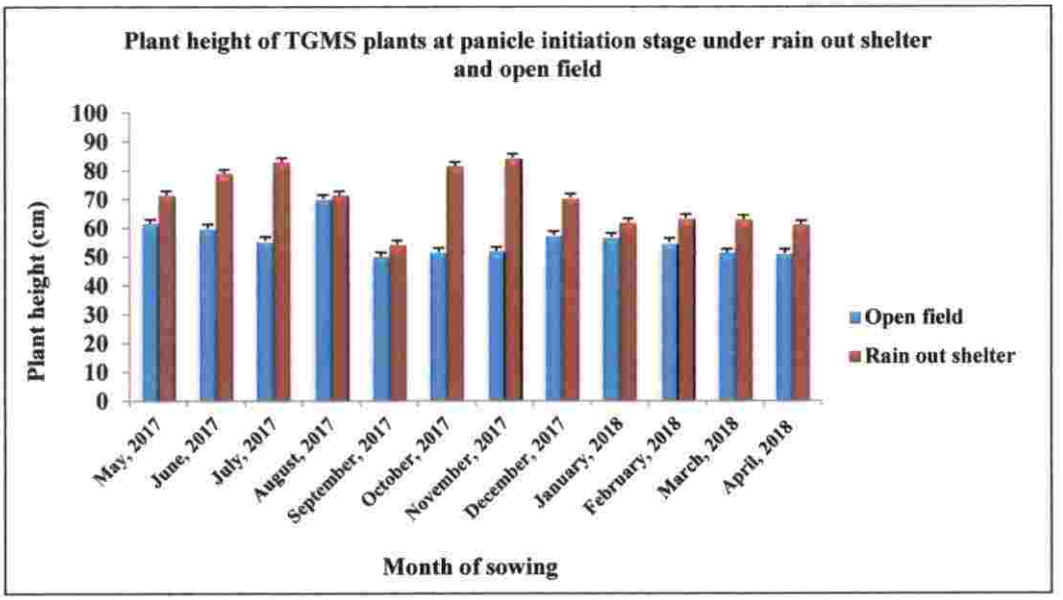


Fig. 2 Seasonal variation on plant height of TGMS red rice line at panicle initiation stage under two environmental conditions

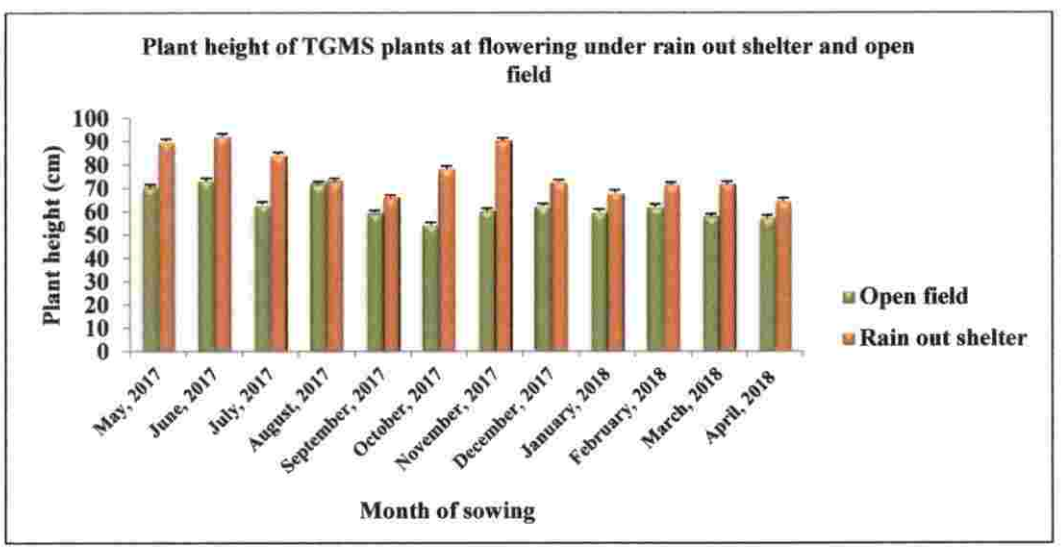


Fig. 3 Seasonal variation on plant height of TGMS red rice line under two environmental conditions at flowering

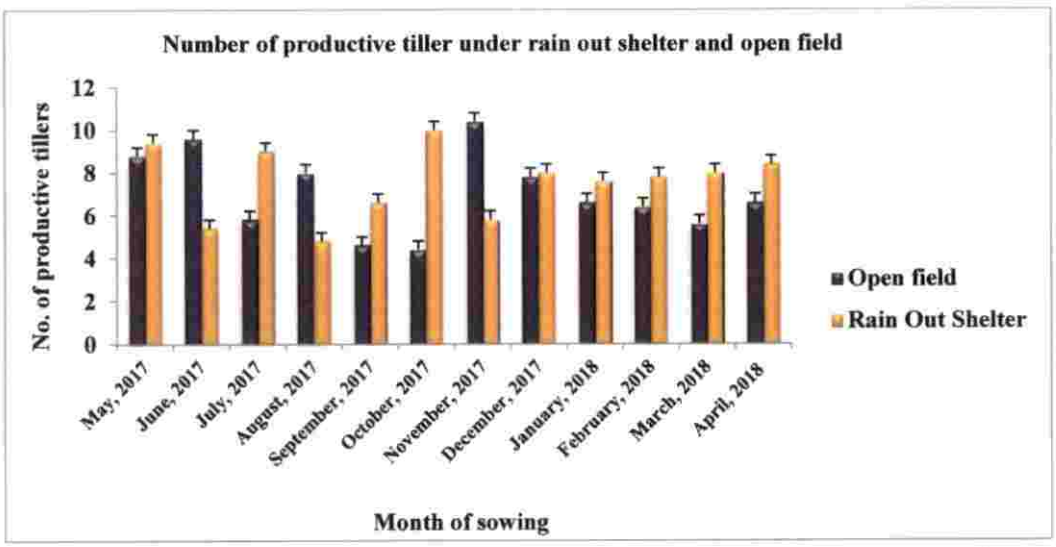


Fig. 4 Productive tiller number of TGMS red rice line under two growing conditions as influenced by time of sowing

4.1.2 Morpho-physiological Observations

4.1.2.1 Plant Height (cm)

The TGMS red rice developed in the Department of Plant Physiology was sown in open field and rain out shelter for twelve months starting from May, 2017 to April, 2018. The plant height of the TGMS red rice grown inside the rain out shelter was significantly higher in all the twelve months compared to open field crop at the time of panicle initiation and flowering (Table 5; Fig. 2, 3). During panicle initiation, August sown crop in the open field condition recorded maximum plant height of 70.22 cm whereas inside the rain out shelter maximum plant height of 84.48 cm was recorded by November sown crop. Lowest plant height was observed in September in the open field and rain out shelter with mean values of 50.18 cm and 54.34 cm respectively. On the other hand maximum plant height was observed in the month of June at the time of flowering in both conditions. The plant height observed was 73.22 cm and 92.42 cm in the open field and rain out shelter respectively. Minimum plant height was obtained in October (54.36 cm) in the open field and in the month of April inside the rain out shelter (64.74 cm).

4.1.2.2 Number of Productive Tillers

There was significant difference in number of productive tillers between two conditions throughout the experimental period (Table 6; Fig. 4). The productive tiller number was found to be higher inside the rain out shelter in all the months except June, August and November. Maximum number of productive tillers was observed in the month of November in the open field. In the rain out shelter, October sown crop recorded higher number of productive tillers. The mean values were 10.40 and 10.00 respectively. Lowest number of productive tillers was found in October (4.40) in the open field and it was in August (4.80) in the case of rain out shelter.

4.1.2.3 Days to First Flowering

Number of days taken to first flowering was counted from the date of sowing. It varied significantly between open field and rain out shelter (Table 7; Fig. 5). Early flowering was observed in plants under rain out shelter than open field. August sown TGMS red rice flowered early in both condition compared to all other months. It took only 77 days and 78 days respectively in rain out shelter and open field to flower. June sown crops had taken more time to flower in open field and rain out shelter. The mean values of days to first flowering in the month of June for rain out shelter and open field are 97 days and 99 days.

4.1.2.4 Days to 50% Flowering

Number of days to 50% flowering was counted from the date of sowing. Significant variation was found in the number of days to 50% flowering (Table 7; Fig. 6). Similar pattern followed in days to first flowering was observed in days to 50% flowering also. August sown crop attained 50% flowering faster under rain out shelter (82) and open field (83 days). TGMS line sown in the month of June reached 50% flowering late compared to all the other months in both conditions. The mean values were 100 days and 102 days inside rain out shelter and open field respectively.

4.1.2.5 Pollen Sterility and Spikelet Sterility (%)

The pollen sterility and spikelet sterility were observed throughout the experimental period (Table 8). There was no difference between open field and rain out shelter for the expression of pollen sterility. The new TGMS red rice line exhibited complete sterility throughout the study period at both the experimental locations.

Spikelet sterility did not show any difference between two growing conditions. Spikelet sterility at the time of maturity was observed and all the spikelets were sterile.

Table 5. Seasonal variation on plant height of TGMS red rice line under two environmental conditions

Month of sowing	Plant height at panicle initiation (cm)		Plant height at flowering (cm)	
	Open field	Rain out shelter	Open field	Rain out shelter
May, 2017	61.62	71.54	70.54	90.12
June, 2017	60.00	78.98	73.22	92.42
July, 2017	55.70	83.04	63.18	84.44
August, 2017	70.22	71.44	71.90	73.08
September, 2017	50.18	54.34	59.42	66.10
October, 2017	51.76	81.60	54.36	78.52
November, 2017	51.98	84.48	60.42	90.48
December, 2017	57.56	70.54	62.42	72.64
January, 2018	56.80	61.94	59.90	68.14
February, 2018	55.14	63.48	62.06	71.42
March, 2018	51.36	63.00	57.94	71.84
April, 2018	51.24	61.16	57.46	64.74
SEm (±)	C-0.39; T-0.96; C×T-1.36		C-0.31; T-0.76; C×T-1.08	
CD (0.05)	C-1.107; T-2.712; C×T-3.835		C-0.873; T-2.139; C×T-3.025	

Table 6. Productive tiller number of TGMS red rice line under two growing conditions as influenced by time of sowing

Month of sowing	Number of productive tillers	
	Open field	Rain Out Shelter
May, 2017	8.80	9.40
June, 2017	9.60	5.40
July, 2017	5.80	9.00
August, 2017	8.00	4.80
September, 2017	4.60	6.60
October, 2017	4.40	10.00
November, 2017	10.40	5.80
December, 2017	7.80	8.00
January, 2018	6.60	7.60
February, 2018	6.40	7.80
March, 2018	5.60	8.00
April, 2018	6.60	8.40
SEm (±)	C-0.12; T-0.28; C×T-0.40	
CD (0.05)	C-0.324; T-0.793; C×T-1.122	

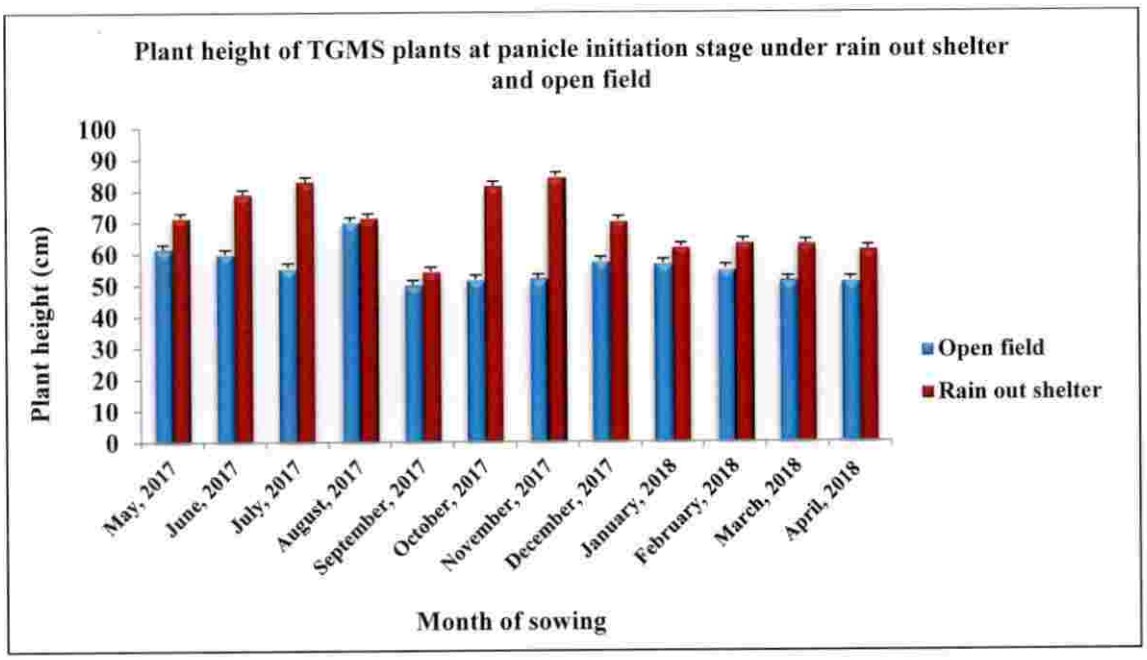


Fig. 2 Seasonal variation on plant height of TGMS red rice line at panicle initiation stage under two environmental conditions

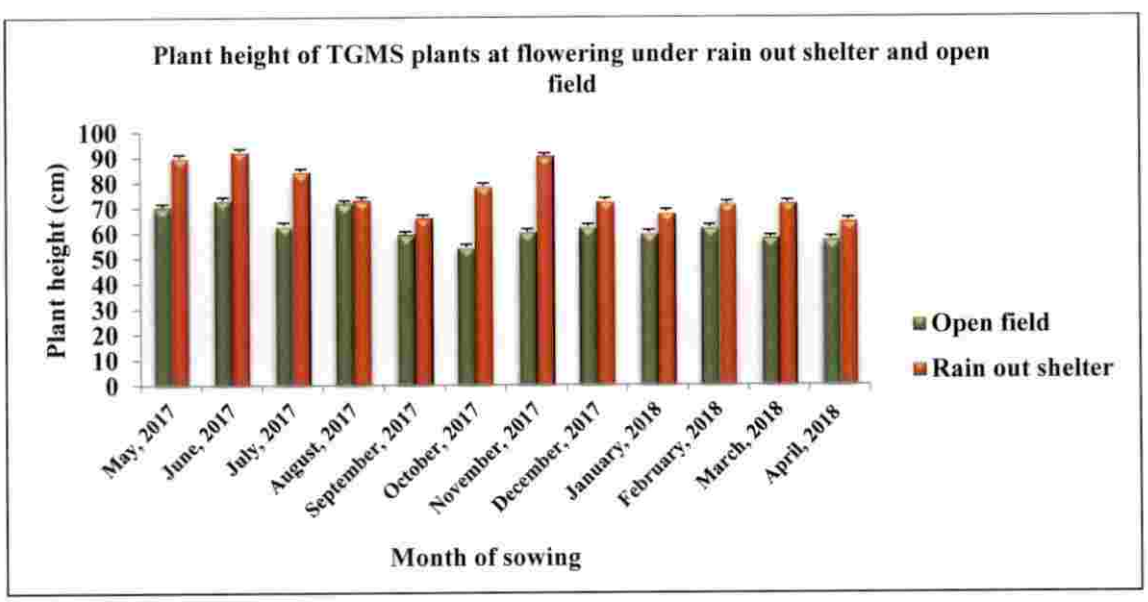


Fig. 3 Seasonal variation on plant height of TGMS red rice line under two environmental conditions at flowering

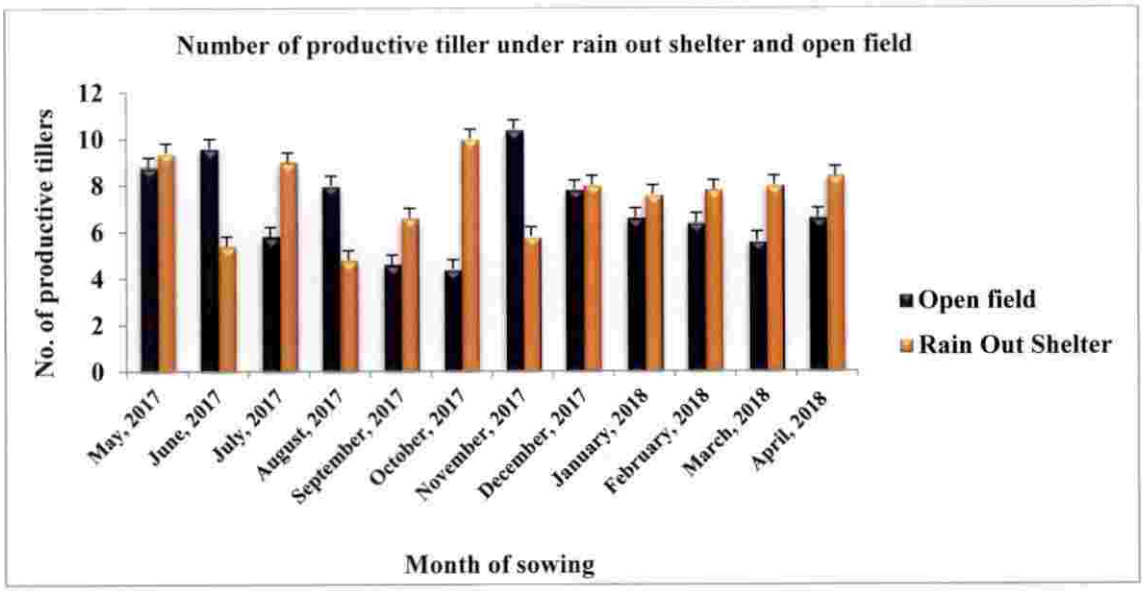


Fig. 4 Productive tiller number of TGMS red rice line under two growing conditions as influenced by time of sowing

4.1.2.6 Anatomical Analysis of TGMS Line under Sterility Inducing Condition

The anther of TGMS red rice line maintained under open field and Rain Out Shelter was observed under stereo microscope Leica, EZ4HD, longitudinal sections revealed it was pollen free (Plate 5). Scanning electron microscopic images of TGMS anther further confirmed that it was pollen free when the temperature was above CST during their critical thermosensitive stage (Plate 6).

Table 7. Seasonal influence on days to first flowering and 50% flowering of TGMS red rice line exposed to two growing situations

Month of sowing	Days to first flowering		Days to 50% flowering	
	Open field	Rain out shelter	Open field	Rain out shelter
May, 2017	91	88	94	91
June, 2017	99	97	102	100
July, 2017	90	87	94	91
August, 2017	78	77	83	82
September, 2017	89	83	92	87
October, 2017	93	83	97	87
November, 2017	90	87	93	90
December, 2017	83	80	87	85
January, 2018	91	88	95	92
February, 2018	92	90	96	93
March, 2018	91	87	95	91
April, 2018	91	89	94	94
SEm (±)	C-0.10; T-0.25; C×T-0.35		C-0.13; T-0.32; C×T-0.45	
CD (0.05)	C-0.281; T-0.689; C×T-0.974		C-0.364; T-0.893; C×T-1.263	

Table 8. Pollen and spikelet sterility of TGMS red rice as influenced by time of sowing

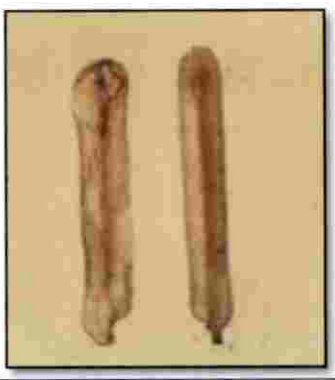
Month of sowing	Open field		Rain out shelter	
	Pollen sterility (%)	Spikelet sterility (%)	Pollen sterility (%)	Spikelet sterility (%)
May, 2017	100	100	100	100
June, 2017	100	100	100	100
July, 2017	100	100	100	100
August, 2017	100	100	100	100
September, 2017	100	100	100	100
October, 2017	100	100	100	100
November, 2017	100	100	100	100
December, 2017	100	100	100	100
January, 2018	100	100	100	100
February, 2018	100	100	100	100
March, 2018	100	100	100	100
April, 2018	100	100	100	100

Panicle showing sterile anthers

Floret showing sterile anthers

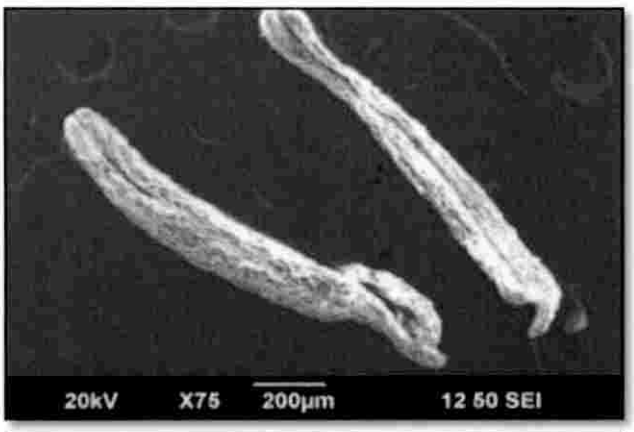
Microscopic image of floret showing sterile anthers

Stereo microscopic image of a single sterile anther

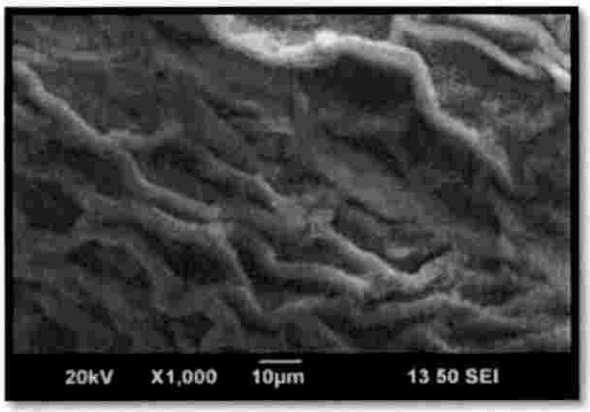


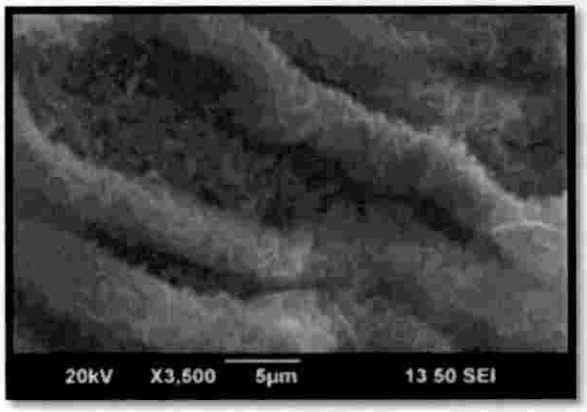
Longitudinal section of a single sterile anther under stereo microscope

Plate 5. Floret showing sterile anthers of TGMS line



Longitudinal section of sterile anther





Pollen free anthers

Plate 6. Scanning Electron Microscopic images of sterile anthers of TGMS line

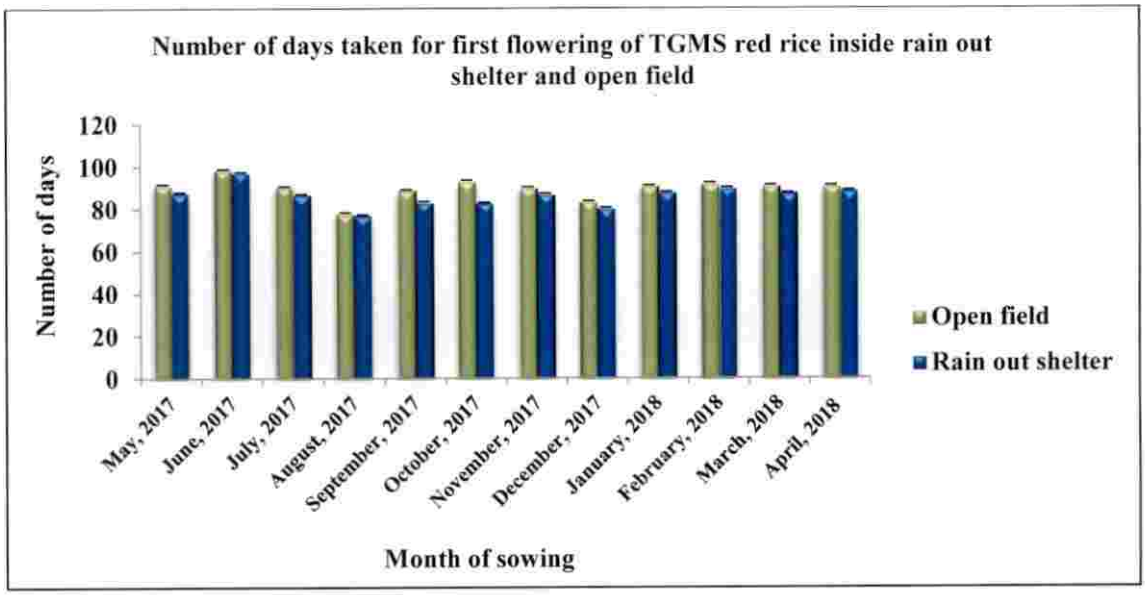


Fig. 5 Seasonal influence on days to first flowering of TGMS red rice line exposed to two growing situations

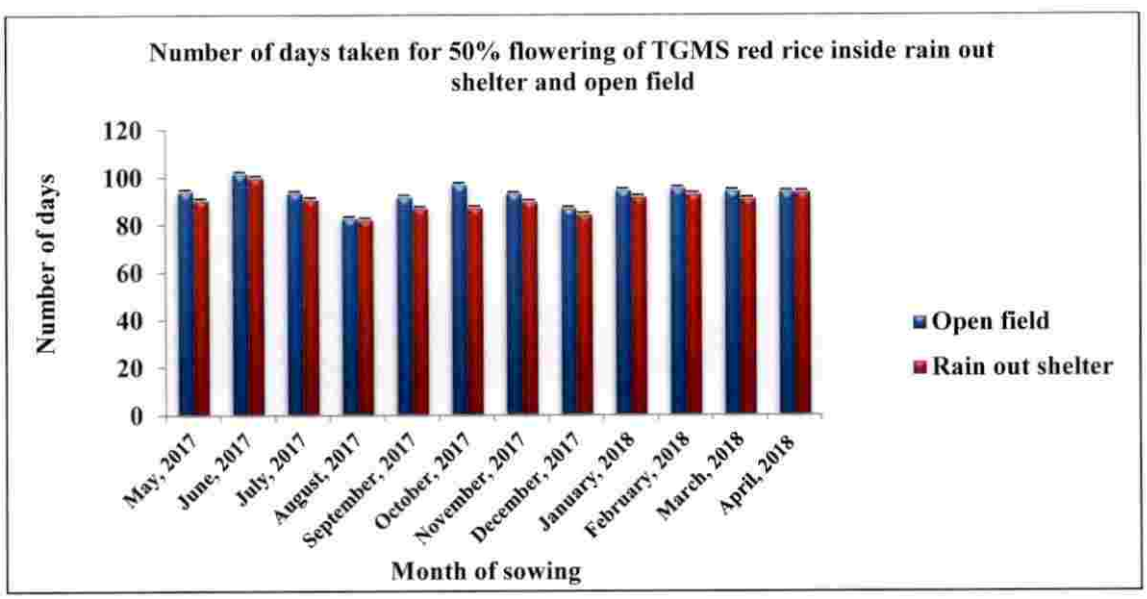


Fig. 6 Seasonal influence on days to 50% flowering of TGMS red rice line exposed to two growing situations

4.2 EVALUATION OF PLANT GROWTH REGULATORS FOR INDUCING MALE STERILITY

4.2.1 Effect of Plant Growth Regulators on Pollen Sterility, Spikelet Sterility and Seed Setting Percentage

The pollen sterility, spikelet sterility and seed setting percentage of TGMS lines treated with plant growth regulators are given in Table 9. The three growth regulators *viz*., ethrel, maleic hydrazide and salicylic acid were capable of enhancing pollen sterility in all the treated TGMS plants (EC720903) (Plate 7; Fig. 7). The pollen sterility percentage was significantly high in maleic hydrazide (1000 mg L^{-1}) treated plants which was on par with salicylic acid (600 mg L^{-1}), ethrel 400 mg L^{-1} and 800 mg L^{-1} and maleic hydrazide 800 mg L^{-1}. The mean values were 83.49%, 82.50%, 78.74%, 78.45% and 77.59% respectively. On other hand control plants recorded 19.92% pollen sterility. The pollen sterility percentage was minimum in the maleic hydrazide 600 mg L^{-1} treated plants (57.97%).

Similar to pollen sterility, the spikelet sterility was also found to be remarkably high in the maleic hydrazide (1000 mg L^{-1}) treated plants (70.46%), whereas the control plants showed the lowest spikelet sterility percentage of 17.92%. The minimum spikelet sterility was observed in the maleic hydrazide 600 mg L^{-1} treated plants (33.38%).

A significant reduction in seed setting percentage was observed in the maleic hydrazide 1000 mg L^{-1} treated plants. The mean value recorded was 29.54%. The control plants exhibited highest seed setting percentage of 82.08%. Among the treatments, maleic hydrazide 1000 mg L^{-1} was found to be more effective in inducing male sterility followed by salicylic acid 600 mg L^{-1}.

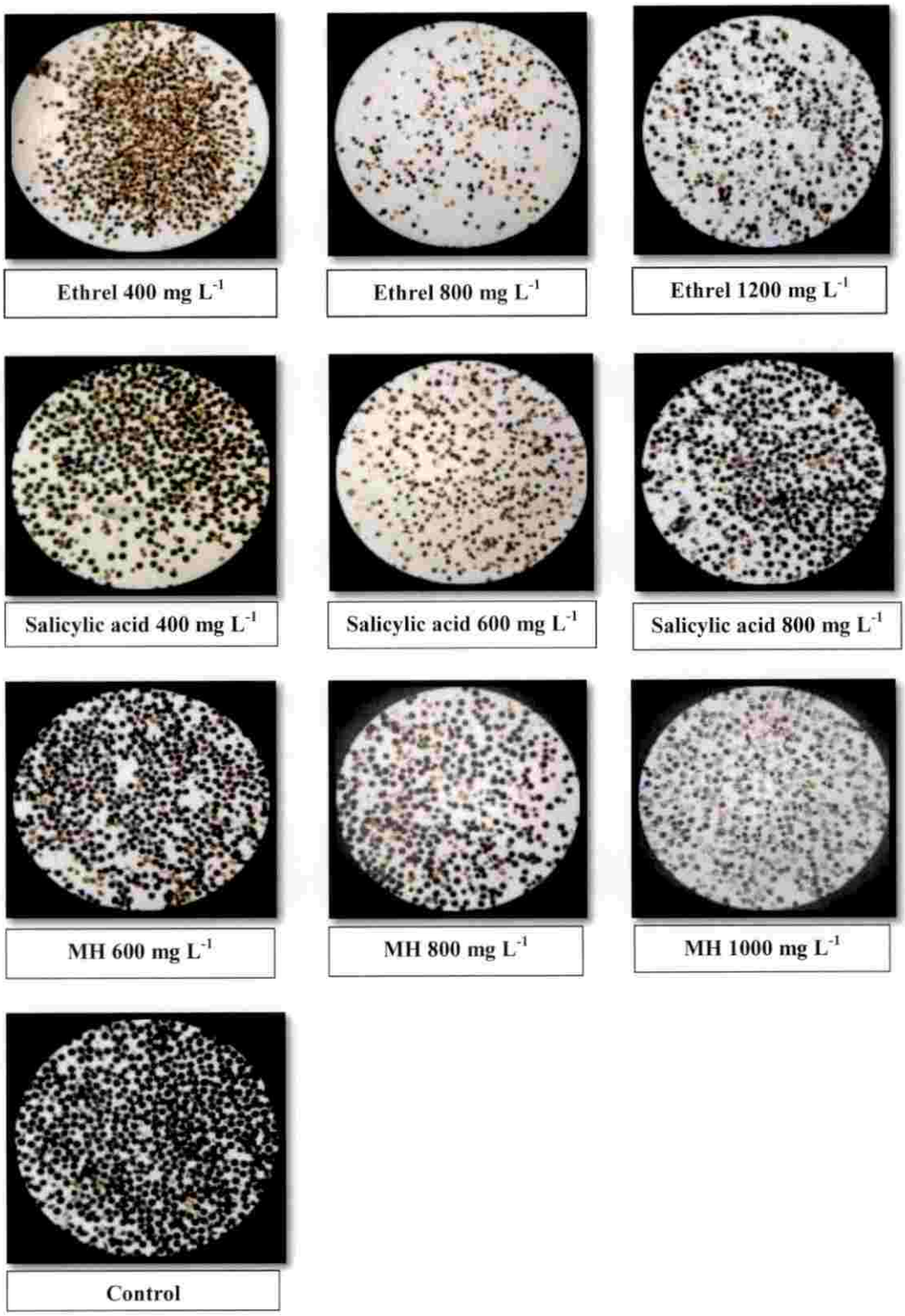


Plate 7. Effect of growth regulators on pollen sterility of TGMS rice

4.2.2 Effect of Plant Growth Regulators on Pigment Composition of TGMS Rice

The content of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid at fifteen days after panicle initiation and at the time of flowering are shown in Table 10. There was a reduction in pigment composition in all the treatments from panicle initiation stage to the time of flowering. The higher chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoid contents were observed in ethrel (1200 mg L^{-1}) treated plants whereas salicylic acid (600 mg L^{-1}) treatment recorded lower chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents at fifteen days after panicle initiation. The mean values for chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents in ethrel 1200 mg L^{-1} treated plants were 1.55 mg g^{-1}, 1.09 mg g^{-1}, 2.64 mg g^{-1} and 1.39 mg g^{-1}. The higher chlorophyll 'a', chlorophyll 'b' and total chlorophyll were observed in ethrel (800 mg L^{-1}) treated plants whereas the carotenoid content was higher in maleic hydrazide (800 mg L^{-1}) at the time of flowering. The chlorophyll 'a' and total chlorophyll content was lower for maleic hydrazide 800 mg L^{-1} whereas chlorophyll 'b' and carotenoid content was lower for salicylic acid 400 mg L^{-1} treated plants. The mean values of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents at the time flowering was ranged between 0.21 mg g^{-1} to 1.61 mg g^{-1}.

4.2.3 Effect of Plant Growth Regulators on Total Soluble Protein and Phenol Content of TGMS Rice

The total soluble protein content at fifteen days after panicle initiation and at the time of flowering is given in Table 11 (Fig. 8, 9). The protein content in all the treatments were reduced at the time of flowering except maleic hydrazide 600 mg L^{-1} and 800 mg L^{-1} compared to fifteen days after panicle initiation. Significantly higher protein content at fifteen days after panicle initiation was observed in ethrel 800 mg L^{-1} and the minimum value was obtained for ethrel 1200 mg L^{-1}. The mean values were 4.77 mg g^{-1} and 4.21 mg g^{-1} respectively. The protein content at

the time of flowering was higher for maleic hydrazide (800 mg L^{-1}) treated plants (5.68 mg g^{-1}) and lower for ethrel (1200 mg L^{-1}) treated plants (1.61mg g^{-1}).

Phenol content fifteen days after panicle initiation and at the time of flowering is given in Table 11 (Fig. 8, 9). The phenol content was also declined at the time of flowering compared to fifteen days after panicle initiation. The significantly high value of phenol content fifteen days after panicle initiation was recorded in the salicylic acid 600 mg L^{-1} treated plants and minimum phenol content was recorded in the ethrel 800 mg L^{-1} treated plants. The mean values obtained were 2.85 µg $g^{-1}$and 2.43 µg g^{-1} respectively. The ethrel 400 mg L^{-1} treated plants recorded higher phenol content at the time of flowering (2.50 µg g^{-1}) and the maleic hydrazide 1000 mg L^{-1} treated plants shown lowest content of 1.58 µg g^{-1}.

Table 9. Effect of plant growth regulators on pollen sterility, spikelet sterility and seed setting percentage

Treatments	Pollen sterility (%)	Spikelet sterility (%)	Seed setting (%)
Ethrel 400 mg L^{-1}	78.74^{ab}	52.42^{cd}	47.58^{b}
Ethrel 800 mg L^{-1}	78.45^{ab}	59.14^{d}	40.86^{b}
Ethrel 1200 mg L^{-1}	76.17^{b}	41.60^{b}	58.40^{cd}
Salicylic acid 400 mg L^{-1}	62.34^{cd}	43.21^{bc}	56.79^{c}
Salicylic acid 600 mg L^{-1}	82.50^{ab}	57.59^{d}	42.41^{b}
Salicylic acid 800 mg L^{-1}	64.59^{c}	38.58^{b}	61.42^{cd}
Maleic hydrazide 600 mg L^{-1}	57.97^{d}	33.59^{b}	66.41^{d}
Maleic hydrazide 800 mg L^{-1}	77.59^{ab}	52.92^{cd}	47.08^{b}
Maleic hydrazide 1000 mg L^{-1}	83.49^{a}	70.46^{e}	29.54^{a}
Control	19.92^{e}	17.92^{a}	82.08^{e}
SEm (±)	4.21	2.96	3.39
CD (0.05)	6.437	8.788	10.071

Table 10. Effect of plant growth regulators on pigment composition fifteen days after panicle initiation and at the time of flowering

Treatments	Chlorophyll 'a' (mg g^{-1})		Chlorophyll 'b' (mg g^{-1})		Total Chlorophyll (mg g^{-1})		Carotenoid (mg g^{-1})	
	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II
Ethrel 400 mg L^{-1}	1.41	0.79	0.65	0.23	2.05	1.02	1.02	0.48
Ethrel 800 mg L^{-1}	1.42	1.26	0.73	0.36	2.14	1.61	1.01	0.63
Ethrel 1200 mg L^{-1}	1.55	1.04	1.09	0.34	2.64	1.37	1.39	0.67
Salicylic acid 400 mg L^{-1}	1.46	0.74	0.75	0.21	2.21	0.95	1.09	0.43
Salicylic acid 600 mg L^{-1}	1.27	0.75	0.51	0.24	1.78	0.99	0.75	0.45
Salicylic acid 800 mg L^{-1}	1.42	0.84	0.70	0.28	2.13	1.12	1.00	0.47
Maleic hydrazide 600 mg L^{-1}	1.44	0.73	0.78	0.25	2.22	0.98	1.09	0.45
Maleic hydrazide 800 mg L^{-1}	1.48	0.62	0.96	0.30	2.44	0.92	1.22	0.69
Maleic hydrazide 1000 mg L^{-1}	1.37	0.78	0.54	0.26	1.91	1.04	0.76	0.47
Control	1.46	0.88	0.88	0.30	2.34	1.18	1.05	0.52
								NS

Stage I: Fifteen days after panicle initiation; Stage II: At the time of flowering

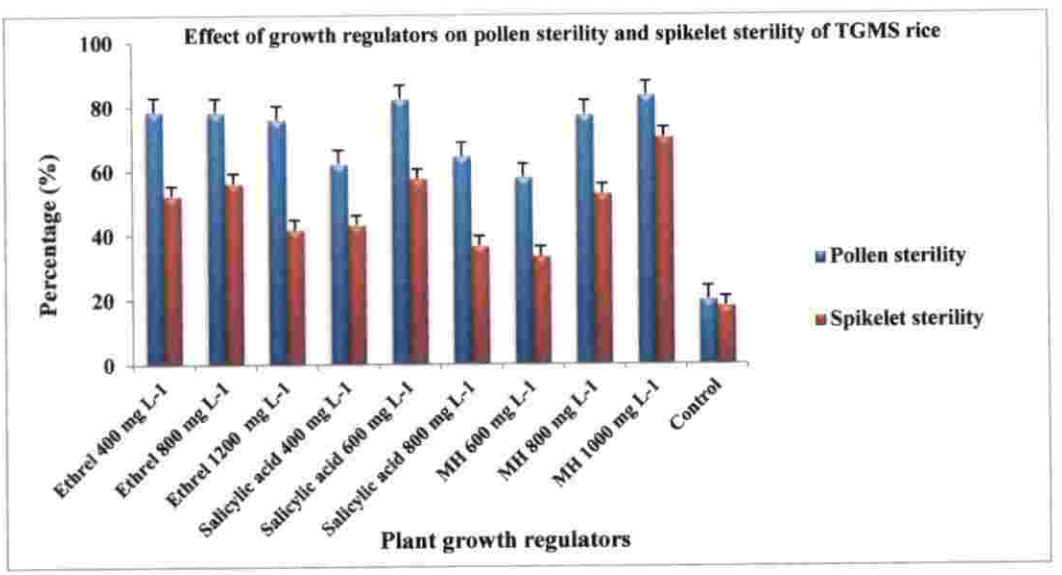


Fig. 7 Effect of plant growth regulators on pollen sterility and spikelet sterility of TGMS rice

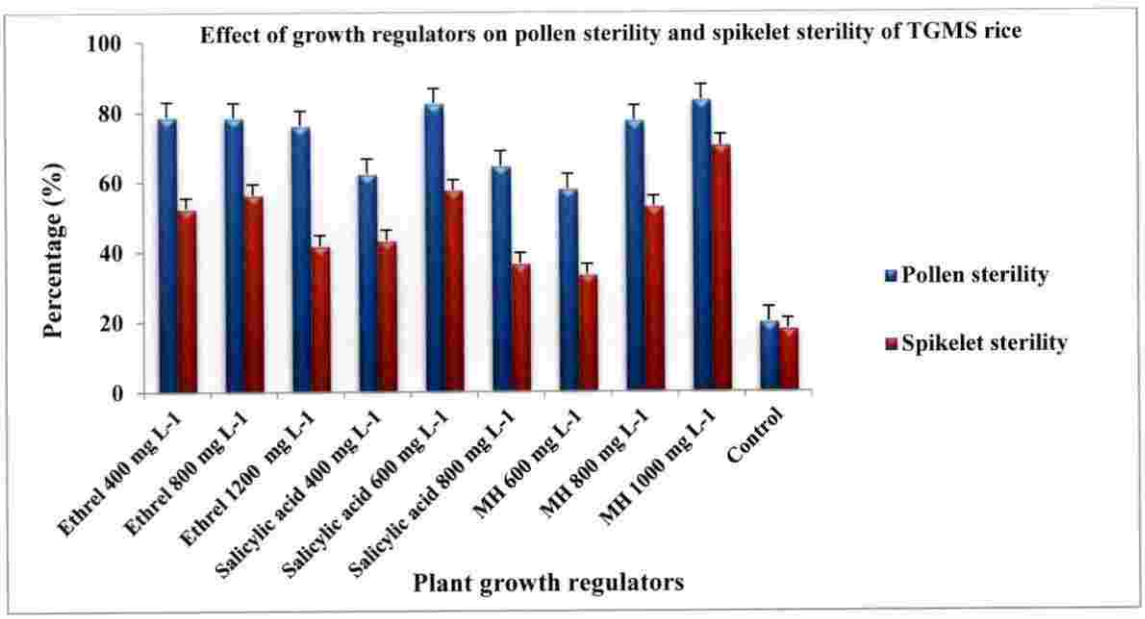


Fig. 7 Effect of plant growth regulators on pollen sterility and spikelet sterility of TGMS rice

Table 11. Effect of growth regulators on total soluble protein and phenol content in TGMS rice

Treatments	Total soluble protein content (mg g^{-1})		Phenol content (µg g^{-1})	
	15 Days after Panicle Initiation	At the time of Flowering	15 Days after Panicle Initiation	At the Time of Flowering
Ethrel 400 mg L^{-1}	4.32	3.56	2.65	2.50
Ethrel 800 mg L^{-1}	4.77	1.87	2.43	1.78
Ethrel 1200 mg L^{-1}	4.21	1.61	3.12	1.95
Salicylic acid 400 mg L^{-1}	4.56	1.74	2.52	1.82
Salicylic acid 600 mg L^{-1}	4.38	2.99	2.85	2.33
Salicylic acid 800 mg L^{-1}	4.48	3.64	2.76	2.03
Maleic hydrazide 600 mg L^{-1}	4.42	5.39	2.70	2.19
Maleic hydrazide 800 mg L^{-1}	4.54	5.68	2.55	1.79
Maleic hydrazide 1000 mg L^{-1}	4.49	2.75	2.49	1.58
Control	4.62	2.95	2.80	2.08
SEm (±)	0.06	0.32	0.05	0.11
CD (0.05)	0.188	0.950	0.147	0.312

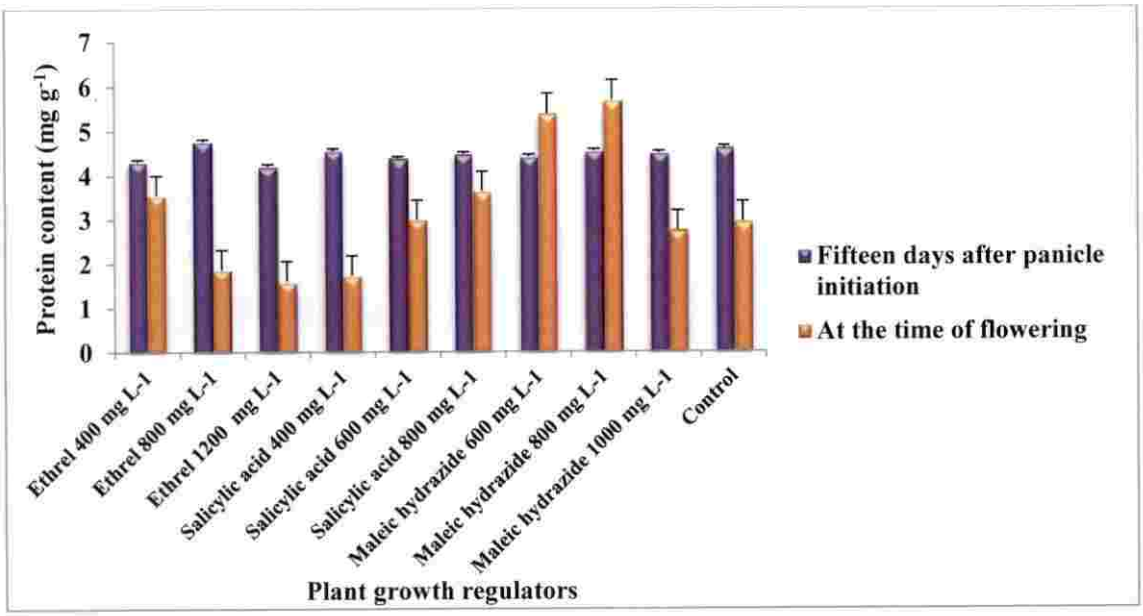


Fig. 8 Effect of plant growth regulators on total soluble protein content at fifteen days after panicle initiation and at the time of flowering

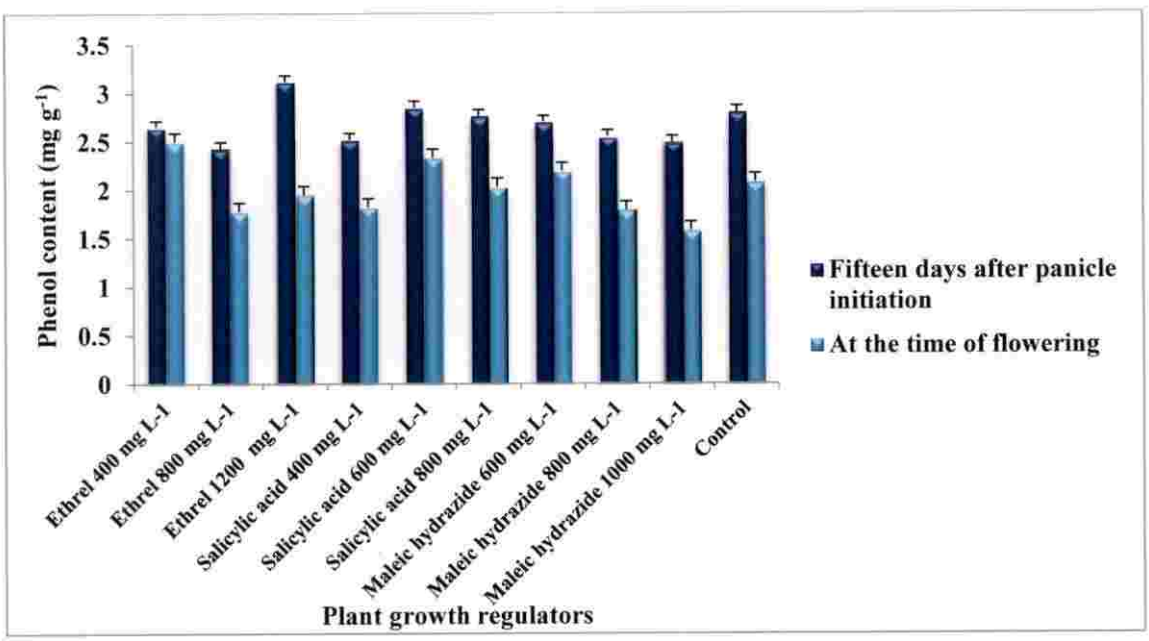


Fig. 9 Effect of plant growth regulators phenol content at fifteen days after panicle initiation and at the time of flowering

4.3 EVALUATING THE POTENTIAL OF USING TGMS LINE AS FEMALE PARENT

The season suitable for hybridization was selected and the TGMS line EC720903 was evaluated for its potential to be used as a female parent. The TGMS line was crossed with two rice varieties of Kerala Aiswarya and Swetha using proximal hybridization method. The hybridization was done during third crop season (December-January to March-April). The long duration rice variety Swetha was sown one week prior to Aiswarya and EC720903 inorder to synchronize the flowering period. The spikelet sterility percentage and seed setting percentage of hybridization was observed.

4.3.1 Spikelet Sterility (%)

The TGMS line EC720903 was hybridized with two rice varieties of Kerala Aiswarya and Swetha using proximal hybridization method (Table 12; Fig. 10). The percentage of spikelet sterility was significantly high for the hybrid EC720903 x Aiswarya (69.76%) and it was significantly lower for the hybrid EC720903 x Swetha (59.93%).

4.3.2 Seed Setting (%)

The seed setting percentage was found to be significantly different between EC720903 x Aiswarya and EC720903 x Swetha (Table 12; Fig. 10). Percentage of seed setting was significantly high for the hybrid EC720903 x Swetha with mean value of 40.07%. While in case of the EC720903 x Aiswarya hybrid, the mean value observed was 30.18%.

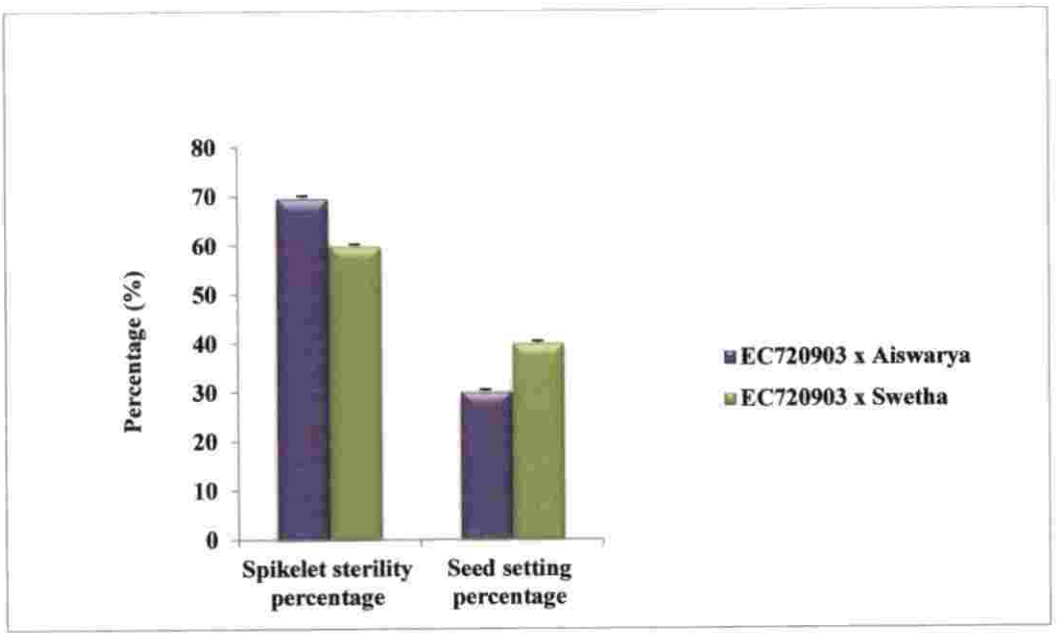


Fig. 10 Spikelet sterility percentage and seed setting percentage of hybrids of EC720903 with Aiswarya and Swetha

4.3.3 Evaluation of F_1 Progenies in Comparison with their Parents

4.3.3.1 Phenological Observations

4.3.3.1.1 Days to First Flowering

Number of days taken for first flowering varied significantly (Table 13). Both the F_1 progenies flowered early compared to their respective male parents, Aiswarya and Swetha. Among the hybrids, F_1 of EC720903 x Swetha flowered early compared to F_1 hybrids of EC720903 x Aiswarya. The mean values were 93 days and 95 days respectively. The male parents Aiswarya and Swetha flowered 97 days and 99 days from the date of sowing, while the female parent TGMS line EC720903 had taken only 91 days for flowering.

4.3.3.1.2 Days to 50% Flowering

Number of days taken for 50% flowering was found to be significantly different and similar to the number of days taken for first flowering, F_1 progenies attained 50% flowering early compared to their male parents (Table 13; Fig. 11). F_1 progenies of EC720903 x Aiswarya achieved 50% flowering at 98 days after sowing and at 95.88 days by F_1 progenies of EC720903 x Swetha from the date of sowing. The parents Aiswarya, Swetha and EC720903 reached 50% flowering stage about 99 days, 101 days and 94 days after sowing respectively.

4.3.3.1.3 Days to Physiological Maturity

The number of days to physiological maturity varied significantly and both F_1 progenies matured early compared to their respective male and female parents (Table 13; Fig. 11). The mean values were 109.75 days for F_1 progenies of EC720903 x Aiswarya and 114.75 days for F_1 progenies of EC720903 x Swetha. Significantly more number of days was taken by the variety Swetha (135.50 days). Whereas it was 121.25 days for Aiswarya and 120.13 days for TGMS line EC720903.

4.3.3.2 Physiological Observations

4.3.3.2.1 Plant Height (cm)

Significant variation was found in plant height recorded at the time of panicle initiation and flowering (Table 14; Fig 12). The plant height of EC720903 x Swetha (90.98 cm) at the time of panicle initiation was significantly high compared to the F_1 progenies EC720903 x Aiswarya (76.30 cm). The mean values were 76.23 cm, 84.66 cm and 66.53 cm respectively for parents Aiswarya, Swetha and EC720903. The plant height of F_1 progenies of Aiswarya were also significantly more when compared to the parents Aiswarya and EC720903 and the mean value obtained was 76.30 cm.

Similarly, the plant height at flowering stage was also significantly higher for F_1 progenies of Swetha than F_1 progenies of Aiswarya, male parent Swetha and the female parent EC720903. The plant height of F_1 hybrids of Swetha was 85.40 cm whereas it was 92.93 cm and 75.58 cm for Swetha and EC720903 respectively. The plant height of F_1 progenies of Aiswarya was also high compared to its male parent Aiswarya (84.89 cm) and EC720903 (75.58 cm).

4.3.3.2.2 Total Soluble Protein (mg g^{-1})

The protein content estimated at panicle initiation stage varied significantly whereas it did not show any significant difference during flowering stage (Table 15). Total soluble protein content in male parents was higher than their respective F_1 progenies. Total soluble protein content was significantly different at panicle initiation among F_1 progenies and the mean values were 1.55 mg g^{-1} and 1.44 mg g^{-1} respectively for F_1 progenies of Aiswarya and Swetha. Lower protein content was observed in EC720903 (1.18 mg g^{-1}). The protein content increased from panicle initiation to flowering in F_1 progenies and parents.

4.3.3.2.3 Pigment Composition (mg g^{-1})

Pigment composition, chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents were found to be non-significant difference during panicle initiation and flowering (Table 16). Chlorophyll a, chlorophyll b and total chlorophyll contents were more for F_1 progenies of EC720903 and Swetha whereas more carotenoid was observed in EC720903 during panicle initiation. The mean values were 0.90 mg g^{-1}, 0.48 mg g^{-1}, 1.39 mg g^{-1} and 0.76 mg g^{-1} respectively. Thereafter the pigment content decreased towards flowering. F_1 progenies of EC720903 and Swetha recorded more amounts of chlorophyll b and carotenoid during flowering. Total chlorophyll content was more in F_1 progenies of EC720903 and Aiswarya whereas more chlorophyll b content was recorded in EC720903 and Aiswarya. Male parent Swetha recorded lower chlorophyll a and total chlorophyll content. The mean values were 0.48 mg g^{-1} and 0.76 mg g^{-1} respectively. Chlorophyll b content was lower for F_1 progenies of EC720903 and Swetha and lower carotenoid content was observed in Aiswarya.

4.3.3.2.4 Photosynthetic Rate (µmol CO_2 m^{-2} s^{-1})

The photosynthetic rate recorded was found to be significantly different at panicle initiation stage and flowering (Table 17; Fig 13). F_1 hybrids of EC720903 x Swetha recorded higher photosynthetic rate of 14.78 µmol CO_2 m^{-2} s^{-1} at panicle initiation stage and later decreased to 10.59 µmol CO_2 m^{-2} s^{-1} during flowering. Whereas it was 13.21 µmol CO_2 m^{-2} s^{-1} for F_1 progenies of EC720903and Aiswarya at the time of panicle initiation and it decreased during flowering to 9.38 µmol CO_2 m^{-2} s^{-1}.

Table 12. Seed setting percentage and spikelet sterility percentage of F_1 progenies of EC720903 x Aiswarya and EC720903 x Swetha

	Spikelet sterility (%)	Seed setting (%)
EC720903 x Aiswarya	69.82[a]	30.18[b]
EC720903 x Swetha	59.93[b]	40.07[a]
SEm (±)	0.40	0.39
CD (0.05)	1.21	1.175

Table 13. Phenological characters of F_1 progenies of TGMS with Aiswarya and Swetha

Variety/Hybrid	Days to first flowering	Days to 50% flowering	Days to physiological maturity
F_1 (EC720903 x Aiswarya)	95[c]	98[c]	110[c]
F_1 (EC720903 x Swetha)	93[d]	96[d]	115[d]
Aiswarya	97[b]	99[b]	121[b]
Swetha	98[a]	101[a]	136[a]
TGMS line	91[e]	94[e]	120[e]
SEm (±)	0.27	0.27	0.26
CD (0.05)	0.775	0.842	0.758

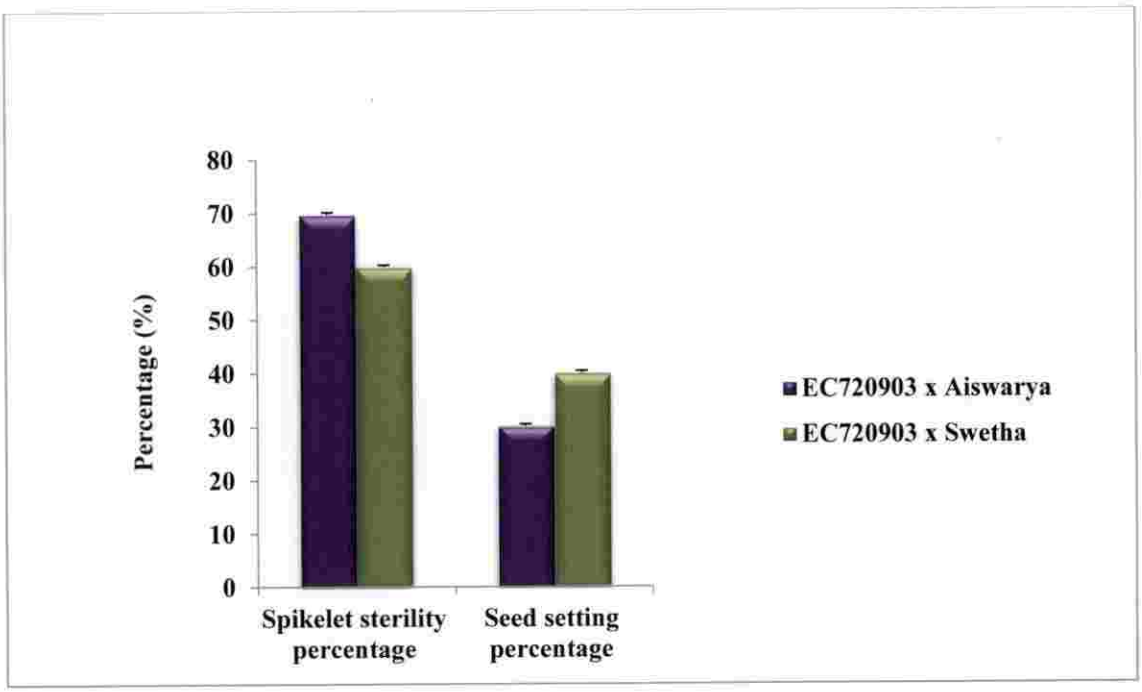


Fig. 10 Spikelet sterility percentage and seed setting percentage of hybrids of EC720903 with Aiswarya and Swetha

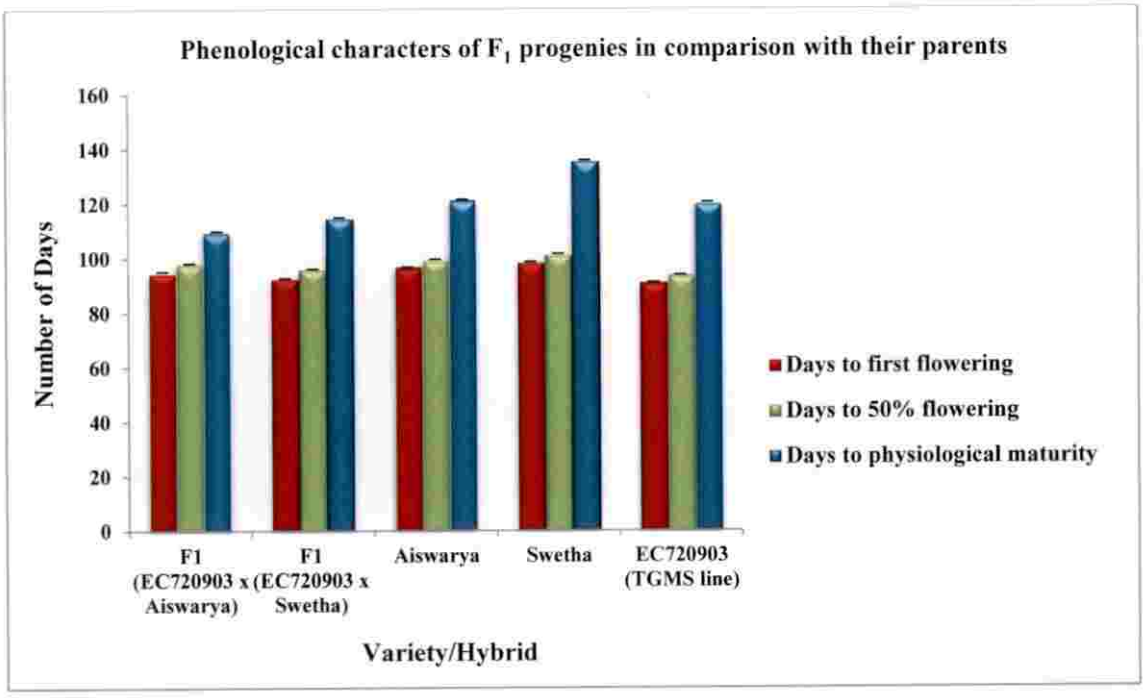


Fig. 11 Phenological characters of F_1 progenies in comparison with their parents

Table 14. Plant height of F_1 progenies along with TGMS line developed through crossing

Variety/Hybrid	Plant height (cm)	
	Panicle initiation	Flowering
F_1 (EC720903 x Aiswarya)	76.30[c]	85.40[c]
F_1 (EC720903 x Swetha)	90.98[a]	99.25[a]
Aiswarya	76.23[c]	84.89[c]
Swetha	84.66[b]	92.93[b]
TGMS line	66.53[d]	75.58[d]
SEm (±)	1.36	1.34
CD (0.05)	3.919	3.850

Table 15. Leaf total soluble protein of F_1 progenies with TGMS line along with their parents

Variety/Hybrid	Total soluble protein (mg g^{-1})	
	Panicle initiation	Flowering
F_1 (EC720903 x Aiswarya)	1.55[b]	5.56
F_1 (EC720903 x Swetha)	1.44[c]	6.06
Aiswarya	1.70[a]	5.05
Swetha	1.63[ab]	5.72
TGMS line	1.18[d]	5.28
SEm (±)	0.03	NS
CD (0.05)	0.100	NS

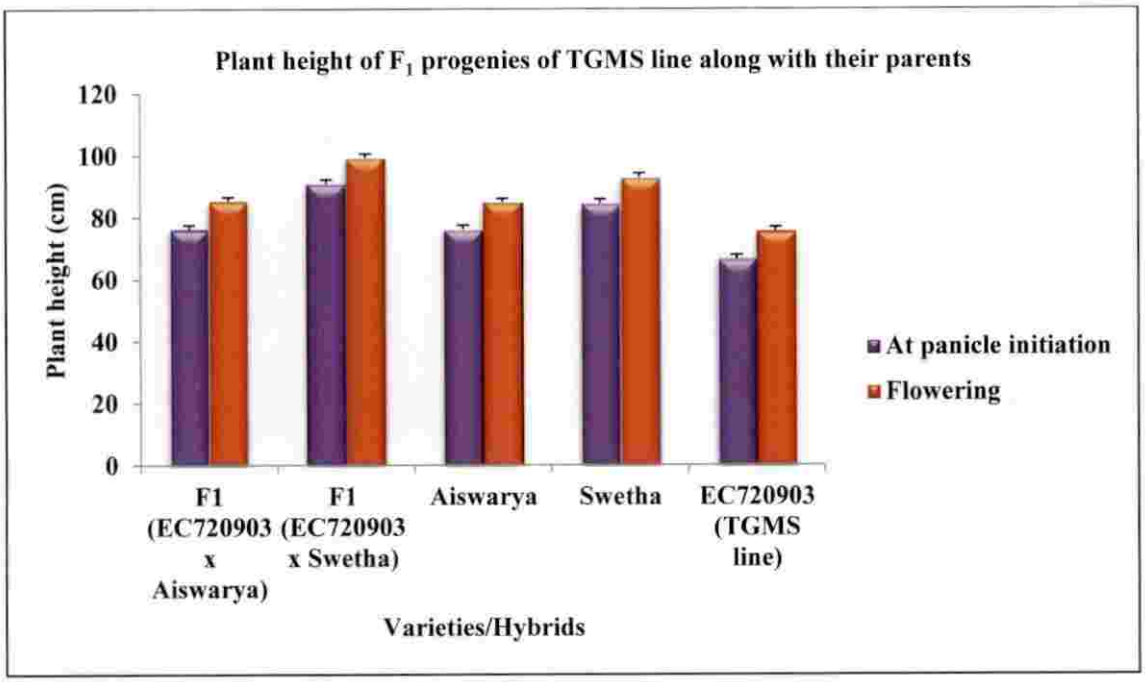


Fig. 12 Plant height of F_1 progenies of TGMS line along with their parents

Lower photosynthetic rate was shown by EC720903 and the mean value was 10.88 µmol CO_2 m^{-2} s^{-1}. In contrast it increased to 12.18 µmol CO_2 m^{-2} s^{-1} during flowering which was high compared to F_1 progenies and their male parents. The photosynthetic rate of male parents Aiswarya and Swetha were lower than their hybrids at panicle initiation and at flowering. The mean values were 11.10 µmol CO_2 m^{-2} s^{-1} and 12.19 µmol CO_2 m^{-2} s^{-1} during panicle initiation and 8.30 µmol CO_2 m^{-2} s^{-1} and 10.22 µmol CO_2 m^{-2} s^{-1} during flowering respectively for Aiswarya and Swetha.

4.3.3.2.5 Transpiration Rate (mmol H_2O m^{-2} s^{-1})

Significant variation was found in transpiration rate at panicle initiation and flowering (Table 17). F_1 progenies recorded high transpiration rate compared to their male parents and female parent EC720903. In contrast to photosynthetic rate higher transpiration rate at panicle initiation was recorded by F_1 progenies of Aiswarya and EC720903. The mean value was 2.80 mmol H_2O m^{-2} s^{-1} and it was 2.00 mmol H_2O m^{-2} s^{-1} for F_1 progenies of EC720903 and Swetha. The transpiration rate for Aiswarya was 1.79 mmol H_2O m^{-2} s^{-1} followed by EC720903 (1.35 mmol H_2O m^{-2} s^{-1}) and Swetha (1.06 mmol H_2O m^{-2} s^{-1}). Transpiration rate lowered during flowering in F_1 progenies and Aiswarya whereas it increased in EC720903 and Swetha. EC720903 and Swetha exhibited higher transpiration rate during flowering and F_1 progenies of EC720903 and Swetha showed lower transpiration rate. The mean transpiration rates were 1.93 mmol H_2O m^{-2} s^{-1}, 1.66 mmol H_2O m^{-2} s^{-1} and 1.50 mmol H_2O m^{-2} s^{-1} respectively.

4.3.3.2.6 Stomatal Conductance (mmol m^{-2} s^{-1})

The stomatal conductance showed significant difference at panicle initiation stage and flowering (Table 17). Similar to transpiration rate, stomatal conductance was more for F_1 progenies during panicle initiation with respect to parents and the male parent Swetha recorded lower stomatal conductance. Stomatal conductance decreased during flowering in F_1 progenies whereas it was enhanced in parents,

Aiswarya, EC720903 and Swetha in comparison with values obtained during panicle initiation. Lower conductance was recorded by the male parent Swetha and the mean value was 214.22 mmol m^{-2} s^{-1}.

4.3.3.2.7 Leaf Temperature (°C)

Leaf temperature was also found to be significantly different at panicle initiation and flowering (Table 17). Ambient temperature recorded at the time of panicle initiation was 34.33°C and it was 35.52°C at the time of flowering. F_1 progenies of EC720903 and Aiswarya recorded lower leaf temperature of 27.58°C at panicle initiation. F_1 progenies of EC720903 and Swetha recorded high leaf temperature which was on par with Aiswarya, Swetha and EC720903. The F_1 progenies of Aiswarya recorded 27.56 °C during flowering which was higher than their parents EC720903 (27.38 °C) and Aiswarya (27.32 °C). Whereas F_1 progenies of EC720903 and Swetha recorded lesser leaf temperature compared to its male parent Swetha.

Table 16. Pigment composition of F_1 progenies of TGMS crosses EC720903 x Aiswarya, EC720903 x Swetha and their parents

Variety/Hybrid	Chlorophyll a (mg g^{-1})		Chlorophyll b (mg g^{-1})		Total Chlorophyll (mg g^{-1})		Carotenoid (mg g^{-1})	
	PI	Flowering	PI	Flowering	PI	Flowering	PI	Flowering
F_1 (EC720903 x Aiswarya)	0.79	0.71	0.45	0.37	1.24	1.08	0.72	0.66
F_1 (EC720903 x Swetha)	0.90	0.92	0.48	0.12	1.39	1.04	0.75	0.73
Aiswarya	0.84	0.55	0.45	0.39	1.29	0.93	0.72	0.48
Swetha	0.79	0.48	0.40	0.28	1.18	0.76	0.72	0.65
TGMS line	0.85	0.59	0.43	0.39	1.28	0.98	0.76	0.70
	NS							

* PI: Panicle initiation

Table 17. Physiological parameters of F_1 progenies in comparison with their parents

Variety/Hybrid	Photosynthetic rate (µmol CO_2 m^{-2} s^{-1})		Transpiration rate (mmol H_2O m^{-2} s^{-1})		Stomatal conductance (mmol m^{-2} s^{-1})		Leaf temperature (^{0}C)	
	PI	Flowering	PI	Flowering	PI	Flowering	PI	Flowering
F_1 (EC720903 x Aiswarya)	13.21^{b}	9.38^{c}	2.80^{a}	1.66^{b}	377.33^{a}	316.56^{a}	27.58^{b}	27.56^{a}
F_1 (EC720903 x Swetha)	14.78^{a}	10.59^{b}	2.00^{b}	1.50^{c}	345.56^{a}	264.00^{c}	27.79^{a}	27.67^{a}
Aiswarya	11.10^{cd}	8.30^{d}	1.79^{bc}	1.67^{b}	267.78^{b}	315.33^{a}	27.83^{a}	27.32^{b}
Swetha	12.19^{bc}	10.22^{b}	1.06^{d}	1.66^{b}	156.00^{c}	214.22^{d}	27.84^{a}	26.88^{c}
TGMS line	10.88^{d}	12.18^{a}	1.35^{cd}	1.93^{a}	213.00^{b}	284.22^{b}	27.82^{a}	27.38^{b}
SEm (±)	0.40	0.15	0.17	0.03	19.22	1.73	0.05	0.05
CD (0.05)	1.150	0.443	0.482	0.071	55.149	4.967	0.156	0.136

* PI: Panicle initiation

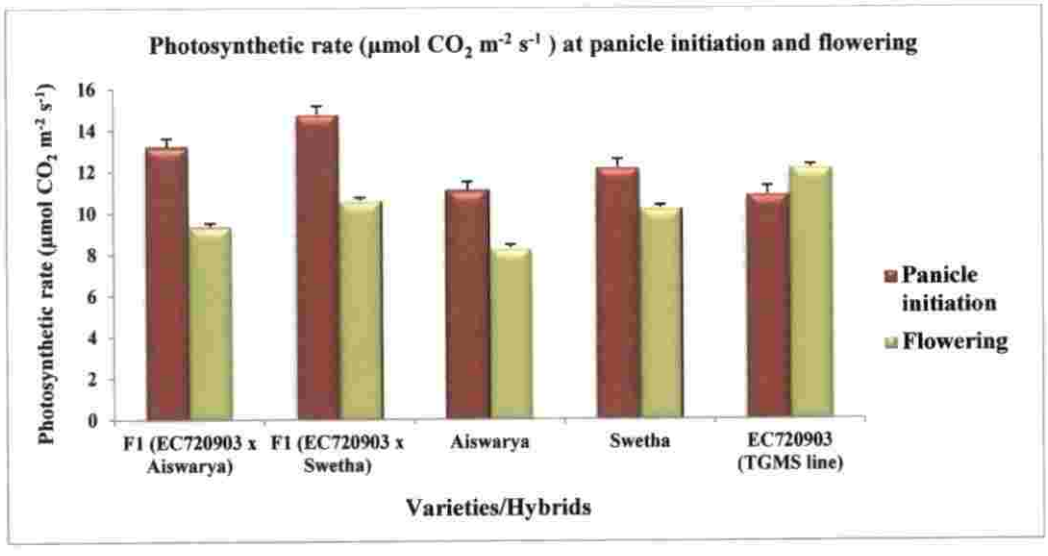


Fig. 13 Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) of F_1 progenies of TGMS line in comparison with their parents at panicle initiation and flowering

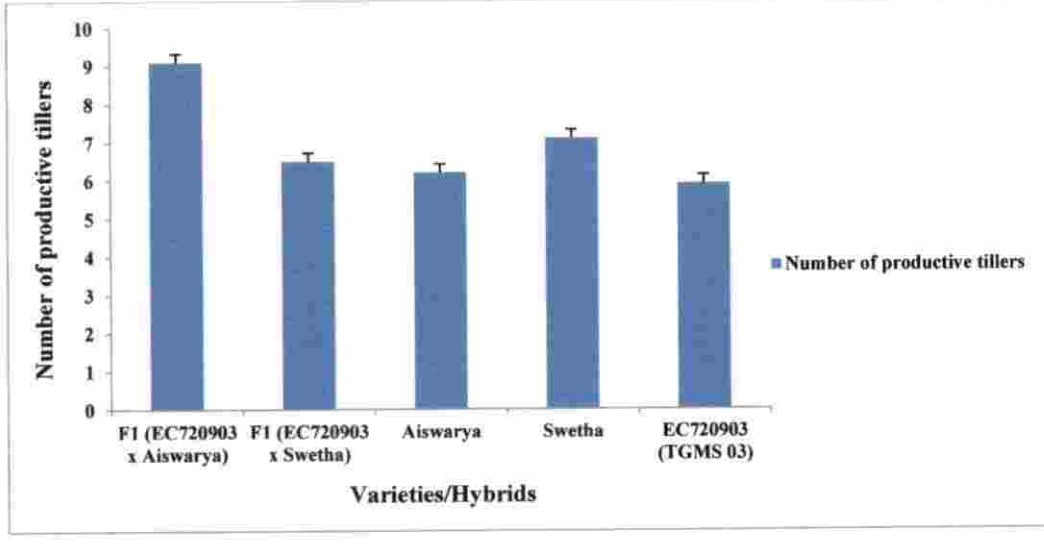


Fig. 14 Number of productive tillers of F_1 progenies in comparison with their parents

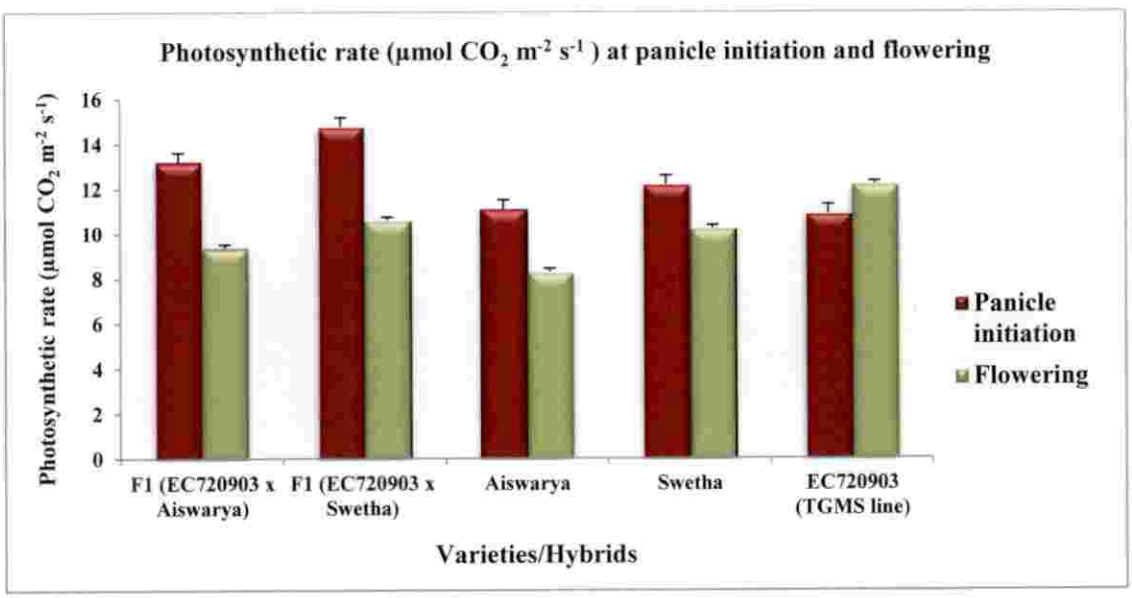


Fig. 13 Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) of F_1 progenies of TGMS line in comparison with their parents at panicle initiation and flowering

4.3.3.3 Yield attributes

4.3.3.3.1 Number of Productive Tillers

The number of productive tillers was found to be significantly different and it was high for F_1 progenies of EC720903 and Aiswarya followed by F_1 progenies of EC720903 and Swetha (Table 18; Fig. 14). The mean value observed for F_1 progenies of Aiswarya and TGMS line were 9.10 which were higher than both the parents EC720903 and Aiswarya. Minimum number of productive tiller was exhibited by TGMS line EC720903 with a mean value of 5.90.

4.3.3.3.2. Number of Spikelets per Panicle

Significant variation was observed in the number of spikelet per panicle also (Table 18; Fig. 15). It was significantly more for the male parent Swetha followed by F_1 progenies of EC720903 and Swetha and TGMS line EC720903. The mean values were 161.80, 130.50 and 118.50, respectively. The mean value obtained for F_1 progenies of Aiswarya was 95.50.

4.3.3.3.3 Number of Filled Grains per Panicle

The mean values observed for number of filled grains per panicle are shown in Table 18. The number of filled grains per panicle was significantly higher for Swetha followed by F_1 progenies of EC720903 and Swetha. The mean values were 116.30 and 108.20, respectively. The mean values recorded for F_1 progenies of EC720903 x Aiswarya and the male parent Aiswarya were 69.50 and 79.90 respectively. The seed set was absent in the female parent EC720903.

4.3.3.3.4 Spikelet Sterility (%)

Table 18 represents the mean values derived for spikelet sterility percentage. It varied significantly and complete spikelet sterility was exhibited by the TGMS line EC720903 (Fig. 15). The mean value obtained for Aiswarya was 28.88 %. The spikelet sterility was lower for F_1 progenies of EC720903 and

Swetha and F_1 progenies of EC720903 x Aiswarya. The mean values were 15.73 % and 27.23 %, respectively.

4.3.3.3.5 Seed Setting (%)

Significant variation was observed in the seed setting percentage also (Table 18). Significantly higher seed setting percentage was recorded by F_1 progenies of EC720903 and Swetha followed by F_1 progenies of EC720903 x Aiswarya. The mean values were 72.77 % and 84.27 % respectively. No seeds were set in the TGMS line EC720903.

4.3.3.3.6 Thousand Grain Weight (g)

The thousand grain weight obtained was significantly high for F_1 progenies (23.00 g) compared to corresponding male and female parents (Table 18). The male parents Aiswarya and Swetha showed 22.15 g and 21.15 g, respectively.

4.3.3.3.7 Grain Yield (g plant^{-1})

The F_1 progenies along with their parents were sown in pots. The grain yield recorded was significantly high for the male parent Swetha (Table 18). The mean value was 17.09 g plant^{-1} and the F_1 progenies of EC720903 x Swetha showed 12.27 g plant^{-1}. The F_1 progenies of EC720903 x Aiswarya recorded more grain yield of 11.11 g plant^{-1} compared to the male parent Aiswarya (10.30 g plant^{-1}). The TGMS female parent EC720903 exhibited complete spikelet sterility and no yield.

4.3.3.3.8 Straw Yield (g plant^{-1})

The straw yield was significantly more for the male parent Swetha and it was significantly lower for Aiswarya (Table 18). The mean values were 18.25 g plant^{-1} and 11.08 g plant^{-1}, respectively. The straw yield of F_1 progenies of EC720903 x Aiswarya was 12.86 g plant^{-1} and F_1 progenies of EC720903 x

Swetha was 13.11 g plant^{-1} whereas it was 14.60 g plant^{-1} for the TGMS line EC720903.

4.3.3.3.9 Total Dry Matter (g)

The total dry matter production was significantly high for Swetha (35.34 g) and significantly lower dry matter was produced by the TGMS line EC720903 (14.60 g) (Table 18). The dry matter production of F_1 progenies of EC720903 x Aiswarya were more compared to male parent Aiswarya and female parent EC720903. The mean value was 23.97 gram per plant. While the F_1 progenies of EC720903 x Swetha accumulated 25.37 g dry matter per plant.

Table 18. Yield attributes of F_1 progenies in comparison with parents

Variety/Hybrid	Number of productive tillers	Number of spikelet per panicle	Number of filled grains per panicle	Spikelet sterility percentage (%)	Seed setting percentage (%)	1000 grain weight (g)	Grain yield (g plant^{-1})	Straw yield (g plant^{-1})	Total Dry Matter (g plant^{-1})
F_1 (EC720903 x Aiswarya)	9.10^{a}	95.50^{d}	69.50^{c}	27.23^{b}	72.77^{b}	23.00^{a}	11.11^{c}	12.86^{c}	23.97^{c}
F_1 (EC720903 x Swetha)	6.50^{bc}	130.50^{b}	108.20^{a}	15.73^{c}	84.27^{a}	23.00^{a}	12.27^{b}	13.11^{c}	25.37^{b}
Aiswarya	6.20^{c}	112.80^{c}	79.90^{b}	28.88^{b}	71.12^{b}	22.15^{b}	10.30^{c}	11.08^{d}	21.37^{d}
Swetha	7.10^{b}	161.80^{a}	116.30^{a}	27.37^{b}	72.63^{b}	21.15^{c}	17.09^{a}	18.25^{a}	35.34^{a}
TGMS line	5.90^{c}	118.50^{bc}	0.00	100.00^{a}	0.00	0.00	0.00	14.60^{b}	14.60^{e}
SEm (±)	0.23	4.23	2.90	2.83	3.13	0.25	0.31	0.37	0.48
CD (0.05)	0.643	12.189	8.356	8.089	9.114	0.719	0.898	1.059	1.361

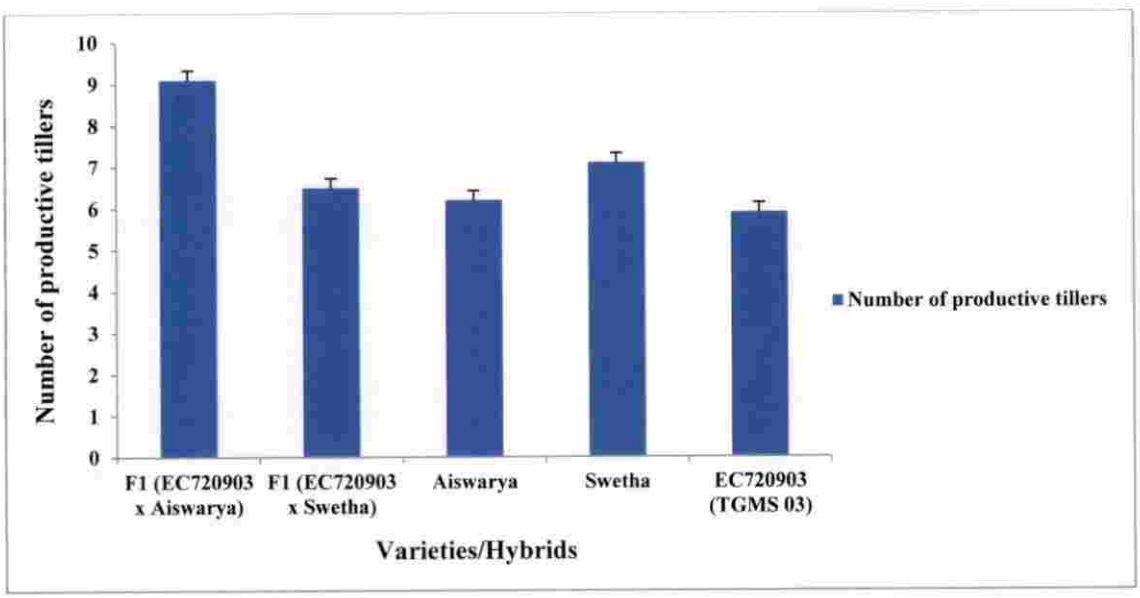


Fig. 14 Number of productive tillers of F_1 progenies in comparison with their parents

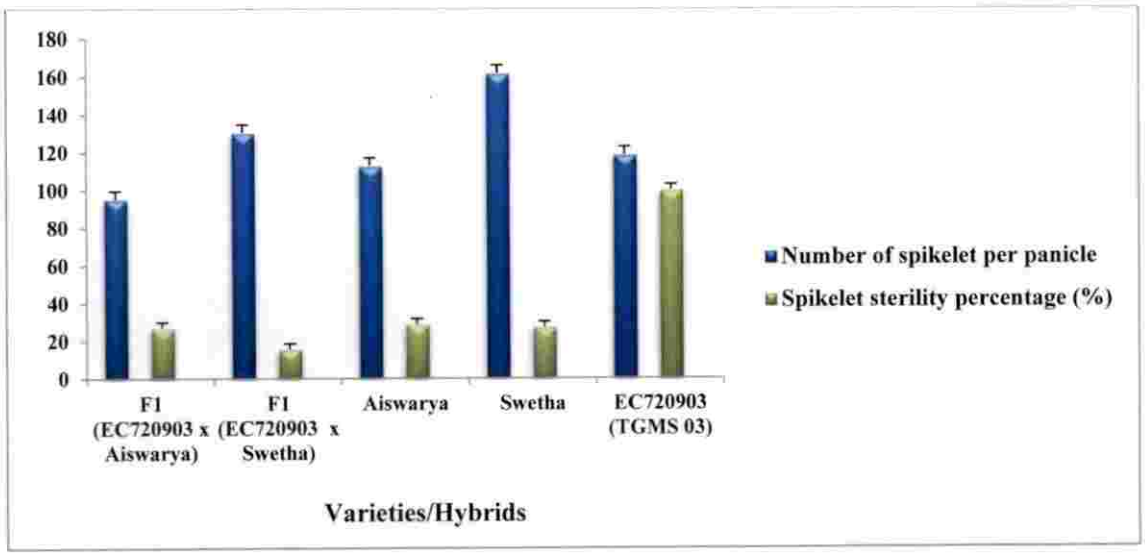


Fig. 15 Number of spikelets per panicle and spikelet sterility percentage of F_1 progenies in comparison with their parents

4.4 ANALYSING THE MOLECULAR MECHANISM OF MALE STERILITY IN TGMS LINES

4.4.1 Protein Profiling (SDS PAGE) of TGMS Lines under Sterile and Fertile Conditions

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis mediated protein profiling was done in the TGMS line EC720903 maintained at fertile and sterile conditions (Plate 8). Differential expressions of proteins were observed in leaf and panicle tissues collected at ten days after panicle initiation from fertile and sterile conditions. In TGMS leaf maintained in the fertility inducing condition, increased expression of proteins were observed at 25-35 kDa and 35-48 kDa whereas increased expression was observed at 245 kDa under sterility inducing condition. In case of panicle under fertility inducing condition, increased expression at 17 kDa was observed and it was between 48-63 kDa under sterility inducing condition. Presence of protein band at 245 kDa was observed in the panicle maintained at fertility inducing condition but it was absent under sterility inducing condition.

4.4.2 Microarray Analysis of TGMS Lines under Sterile and Fertile Conditions

TGMS line EC720903 was grown under sterile and fertile conditions simultaneously and microarray analysis was done with samples collected from leaf and young panicles ten days after panicle initiation. Differential regulation of gene expression was analysed by keeping fertile leaf and panicle of TGMS line as control and sterile leaf and panicle as test. Up-regulated and down-regulated genes were identified using fold change. The fold value >=1 in the sterile sample was considered for up-regulation and the fold value <=-1 in the sterile samples was used for finding down-regulated genes.

The results revealed that 4205 genes were up-regulated and 3559 genes were down-regulated in the leaves of TGMS plants under sterile condition

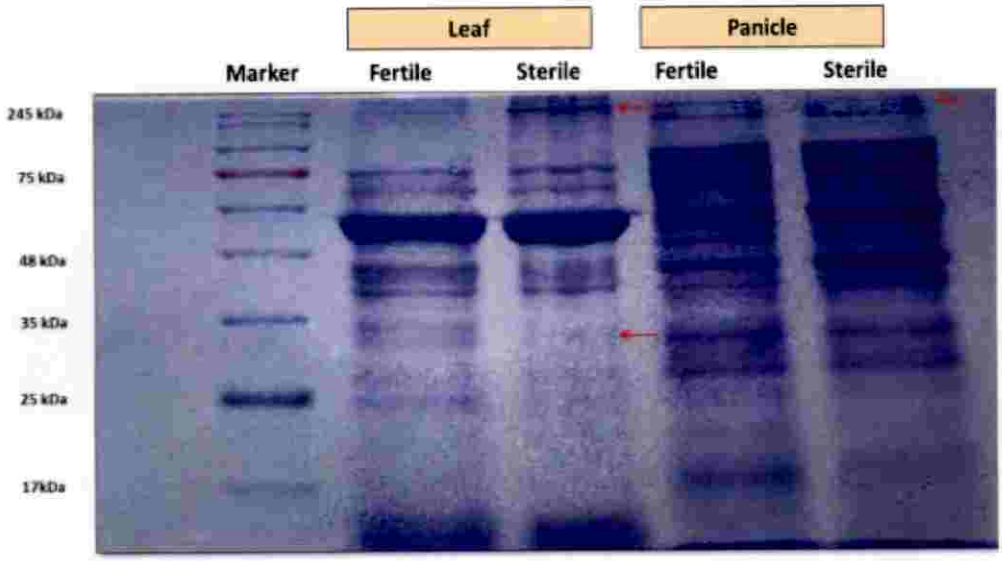


Plate 8. Protein profiling (SDS-PAGE) of TGMS lines under fertile and sterile conditions in leaf and panicle

compared to fertility inducing condition. Whereas 4867 genes were up-regulated and 2979 genes were down-regulated in sterile panicles compared to fertile panicles.

4.4.2.1 Genes Up-regulated in the Leaf of TGMS Line under Sterility Inducing Condition Compared to Fertility Inducing Condition

4.4.2.1.1 Genes Encoding Abiotic Stress Proteins

In the present study, heat shock protein/chaperone and other abiotic stress related genes were up-regulated in the TGMS leaf maintained under sterility inducing condition (Table 19). Genes *Os04t0107900-03* (Hsp 80), *Os04t0107900-01* and *Os04t0107900-04* (Hsp 82), *Os03t0245800-02* (Hsp 26), *Os01t0136200-01* and *Os01t0136100-01* (16.9 kDa class I Hsp 1), *Os11t0244200-01* Hsp 17.9, *Os02t0537400-01* and *Os02t0782300-01* (Hsp), *Os04t0675400-01* and *Os05t0210600-01* Hsp 40, *Os01t0184100-01* (Class II small Hsp 18) and *Os01t0157800-01*, *Os07t0620200-01*, *Os06t0195800-01* and *Os05t0579900-02* Hsp DnaJ, N-terminal domain containing protein providing heat tolerance were up-regulated in the TGMS leaf maintained under sterility inducing condition.

Os09t0491772-01, *Os01t0180800-01*, *Os11t0703900-01* and *Os02t0710900-01* (Hsp70), *Os01t0180800-02*, *Os05t0500500-01*, *Os01t0151100-01*, *Os10t0159600-01*, *Os10t0437700-01* and *Os12t0165200-01* (Hsp 20), *Os05t0530400-01* (Heat stress transcription factor Spl7), *Os09t0526600-02* (Heat shock factor protein 3), *Os01t0733200-01* (Heat shock transcription factor 29), *Os09t0109600-01* (small peptide involved in stress tolerance), *Os06t0716700-01* (Hsp 90) and *Os04t0445100-01* (22.7 kDa class IV heat shock protein precursor), *Os02t0217900-00* (Cytosolic class II small heat shock protein 4), *Os03t0669000-01* (DEAD-box RNA helicase involved in regulation of thermotolerant growth and rRNA homeostasis at high temperature), *Os10t0507800-01* (chaperone protein dnaJ 13) and *Os03t0267000-00* (low molecular mass heat shock protein Oshsp18.0) were also up-regulated.

Os10t0154700-01 and *Os09t0571400-01* (Cyclophilin Dicyp-2), *Os06t0567900-01* (L-ascorbate oxidase), *Os10t0550900-01* (proline oxidase), *Os06t0567200-01* (Ascorbate oxidase), *Os03t0285700-01* (L-ascorbate peroxidase), *Os07t0677500-00* (peroxidase precursor), *Os06t0486900-01* (formate dehydrogenase), *Os03t0320600-01* (VQ domain containing protein involved in abiotic cell stress responses), *Os05t0417100-01* (Chloroplast-targeted Deg protease protein required in chloroplast development and maintenance of PSII function under high temperatures) and *Os04t0473150-00* (photosystem II protein D1) were up-regulated under sterility inducing condition.

4.4.2.1.2 Genes Related to Calcium Signalling

Calcium associated genes such as *Os01t0824600-02* producing protein similar to CBL-interacting protein kinase 2 (calcinurein), *Os03t0397400-01* encoding protein similar to low affinity calcium transporter CAX2, *Os12t0132300-02* coding protein similar to Calmodulin (CaM) and *Os03t0167200-01*, *Os05t0541100-01* and *Os05t0197300-01* encoding IQ calmodulin-binding region domain containing protein, *Os02t0608400-02* producing protein similar to Ca^{2+} binding protein cbp1, *Os05t0370600-01* coding C2 calcium-dependent membrane targeting domain containing protein and *Os05t0495600-00* producing protein similar to autoinhibited calcium ATPase were up-regulated (Table 20). *Os02t0832000-01* and *Os12t0547600-01* (Calmodulin-like-domain protein kinase CPK2), *Os10t0539600-01* (Calcium-dependent protein kinase 3), *Os06t0695700-01* and *Os09t0309200-01* (Calmodulin-binding, plant family protein), *Os08t0360300-00* (Calmodulin binding protein-like domain containing protein), *Os01t0782800-00* coding similar to Cyclic nucleotide-gated ion channel 4 and *Os03t0832200-01* encoding protein similar to Calcium-binding protein precursor (Calreticulin) were up-regulated under sterility inducing condition.

Table 19. Genes encoding abiotic stress proteins up-regulated under sterility in the TGMS leaves

Gene Name	Fold change	Description
Os07t0677500-00	6.960	Similar to peroxidase precursor
Os04t0107900-03	5.986	Similar heat shock protein 80
Os04t0107900-01 *Os04t0107900-04*	5.112 3.073	Heat shock protein 82
Os03t0245800-02	5.513	Heat shock protein 26
Os01t0136100-01 *Os01t0136200-01*	4.710 2.248	16.9 kDa class I heat shock protein 1
Os11t0244200-01	3.165	Similar hsp 17.9
Os02t0537400-01 *Os02t0782300-01*	3.383 1.182	Heat shock protein
Os04t0675400-01 *Os05t0210600-01*	3.462 1.246	Hsp 40 (Similar to Chaperone protein dnaJ)
Os06t0486900-01	3.969	Formate dehydrogenase
Os01t0184100-01	2.989	Class II small heat shock protein (18)
Os01t0157800-01 *Os05t0579900-02* *Os06t0195800-01*	1.327 1.166 1.219	Heat shock protein DnaJ, N-terminal domain containing protein providing heat tolerance
Os09t0491772-01 *Os01t0180800-01* Os11t0703900-01 *Os02t0710900-01*	2.521 1.105 1.181 1.102	Hsp70
Os01t0180800-02 *Os05t0500500-01* *Os01t0151100-01* *Os12t0165200-01*	2.390 1.883 1.638 1.831	Hsp 20
Os05t0530400-01	2.531	Similar Heat stress transcription factor Spl7 (RHSF10)
Os09t0526600-02	1.023	Similar Heat shock factor protein 3 (HSF 3)
Os01t0733200-01	1.416	Similar Heat shock transcription factor 29
Os09t0109600-01	2.294	Small peptide involved in stress tolerance
Os06t0716700-01	1.165	Similar Heat shock protein 90
Os04t0445100-01	2.611	Similar to 22.7 kDa class IV heat shock protein precursor
Os02t0217900-00	1.001	Similar to Cytosolic class II small heat shock protein 4
Os03t0669000-01	1.020	DEAD-box RNA helicase regulating thermotolerant growth and rRNA homeostasis at high temperature
Os10t0507800-01	1.960	Similar to chaperone protein dnaJ 13
Os10t0154700-01 *Os09t0571400-01*	4.190 1.053	Similar to Cyclophilin Dicyp-2
Os03t0267000-00	1.974	Low molecular mass heat shock protein Oshsp18.0
Os06t0567900-01	2.305	L-ascorbate oxidase involved in regulating drought response
Os10t0550900-01	2.219	Similar to proline oxidase which degrades proline
Os06t0567200-01	1.113	Ascorbate oxidase involved in abiotic stress response
Os03t0285700-01	1.222	Similar to L-ascorbate peroxidase
Os03t0320600-01	2.808	VQ domain containing protein in abiotic stress responses
Os05t0417100-01	1.318	Chloroplast-targeted Deg protease protein, development and maintenance of PSII function under high temperatures
Os04t0473150-00	1.396	Similar to photosystem II protein D1

Table 20. Genes encoding calcium signalling proteins up-regulated under sterility in leaves of TGMS plants

Gene Name	Fold change	Description
Os01t0824600-02	3.478	Similar to CBL-interacting protein kinase 2 (calcinurein)
Os03t0832200-01	3.473	Similar to Calcium-binding protein precursor (Calreticulin)
Os03t0397400-01	1.734	Similar to Low affinity calcium transporter CAX2
Os12t0132300-02	1.338	Similar to Calmodulin (CaM)
Os05t0197300-01 *Os03t0167200-01* *Os05t0541100-01*	2.671 1.097 1.628	IQ calmodulin-binding region domain containing protein
Os02t0608400-02	1.163	Similar to Ca^{2+} binding protein cbp1
Os08t0360300-00	2.339	Calmodulin binding protein-like domain containing protein
Os01t0782800-00	2.483	Similar to Cyclic nucleotide-gated ion channel 4 (AtCNGC4) (Cyclic nucleotide-and calmodulin-regulated ion channel 4) (AtHLM1)
Os05t0370600-01	1.186	C2 calcium-dependent membrane targeting domain containing protein
Os05t0495600-00	1.250	Similar to autoinhibited calcium ATPase
Os02t0832000-01 *Os12t0547600-01*	1.459 1.904	Similar to Calmodulin-like-domain protein kinase CPK2
Os10t0539600-01	1.610	Similar to Calcium-dependent protein kinase 3
Os09t0309200-01 *Os06t0695700-01*	2.304 1.652	Calmodulin-binding, plant family protein

4.4.2.1.3 Genes Related to Biosynthesis and Signalling of Phytohormones

Fourteen genes related to phytohormone auxin were up-regulated in the TGMS leaf grown under sterility inducing condition (Table 21). *Os01t0764800-01* (fold change: 4.925) and *Os07t0576100-00* (1.977) genes encoding enzyme indole-3-acetic acid (IAA)-amido synthetase was up-regulated under sterile condition. *Os08t0520550-01* (2.849) gene coding protein similar to Auxin response factor 21, *Os12t0133800-01* (1.897) producing protein similar to Auxin efflux carrier, *Os04t0519700-01* (1.374) gene encoding protein similar to Auxin response factor 10, *Os01t0231000-02* (1.218) and *Os03t0742900-01* (1.251) genes coding protein similar to Auxin-responsive protein (Aux/IAA) were up-regulated. *Os01t0643300-01* (1.464: Auxin efflux carrier protein involved in auxin transport, PIN proteins), *Os09t0491740-01* (1.518: Auxin efflux carrier domain containing protein), *Os01t0805500-00* (2.734: similar to indole-3-acetate beta-glucosyltransferase), *Os08t0550700-00* (2.030) and *Os09t0527700-01* (1.246) genes producing protein similar to auxin induced protein, *Os12t0613700-02* (1.741: similar to Auxin response factor 25) and *Os05t0515400-01* (1.271) coding protein similar to Auxin response factor 14 were also up-regulated.

Gibberellic acid genes such as *Os01t0332200-01* (GA 2-oxidase2 involved in GA metabolism), *Os02t0630300-02* (GIBBERELLIN 2-OXIDASE 9 enzyme), *Os04t0522500-01*, *Os08t0480200-01*, *Os10t0559500-02*, *Os01t0830500-01* and *Os08t0392100-00* (2OG-Fe(II) oxygenase domain containing protein 6), *Os07t0580900-02* (similar to GGDP synthase: Geranylgeranyl diphosphate synthase 1), *Os01t0812000-02* and *Os01t0812000-01* (Myb-like transcription factor: GIBBERELLIN MYB GENE), *Os06t0605600-01* (GAMYB-like protein responsible for flower development and stem elongation at the reproductive stage and *Os01t0883800-01* (similar to GA C20oxidase2) were up-regulated (Table 21).

Table 21. Genes related to biosynthesis and signalling of phytohormones up-regulated under sterility in the TGMS leaves

Gene Name	Fold change	Description
Auxin		
Os01t0764800-01 *Os07t0576100-00*	4.925 1.977	Indole-3-acetic acid (IAA)-amido synthetase
Os08t0520550-01	2.849	Similar to Auxin response factor 21
Os01t0805500-00	2.734	Indole-3-acetate beta-glucosyltransferase in auxin conjugation
Os08t0550700-00 *Os09t0527700-01*	2.030 1.246	Similar to auxin induced protein
Os12t0133800-01	1.897	Similar to Auxin efflux carrier
Os04t0519700-01	1.374	Similar to Auxin response factor 10
Os01t0231000-02 *Os03t0742900-01*	1.218 1.251	Similar to Auxin-responsive protein (Aux/IAA)
Os01t0643300-01	1.464	Auxin efflux carrier PIN protein involved in auxin transport
Os09t0491740-01	1.518	Auxin efflux carrier domain containing protein
Os12t0613700-02	1.741	Similar to Auxin response factor 25
Os05t0515400-01	1.271	Similar to Auxin response factor 14
Gibberellic acid		
Os04t0522500-01 *Os01t0830500-01* *Os10t0559500-02*	3.383 3.341 2.116	2OG-Fe(II) oxygenase domain containing protein 6
Os01t0332200-01	2.777	Similar to GA 2-oxidase2, GA metabolism
Os02t0630300-02	1.479	GIBBERELLIN 2-OXIDASE 9
Os07t0580900-02	1.805	Similar to geranylgeranyl diphosphate synthase 1 synthase
Os01t0812000-02 *Os01t0812000-01*	1.212 1.375	Myb-like transcription factor which regulates of pollen development (GIBBERELLIN MYB GENE)
Os06t0605600-01	1.076	GAMYB-like protein for flower development and stem elongation
Os01t0883800-01	1.205	Similar to GA C20oxidase2
Ethylene		
Os12t0603300-01	2.771	AP2 domain containing protein (ERF 112), ethylene biosynthesis
Os06t0592500-01	2.397	Similar to Ethylene-responsive transcriptional coactivator
Os05t0149400-01	2.109	ACC oxidase enzyme in the ethylene biosynthesis
Os12t0623900-01	1.022	Similar to Ethylene-responsive methionine synthase
Abscissic acid		
Os07t0686100-01	2.628	Similar to Abscisic acid responsive elements-binding factor
Os01t0959100-01	1.884	Similar to Abscissic stress ripening protein 1
Brassinosteroid		
Os03t0266800-02	2.212	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
Os05t0458600-01	1.982	Laccase-like protein providing sensitivity of plants to brassinosteroids
Cytokinin		
Os08t0460600-01	1.322	Similar to Cytokinin dehydrogenase 11
Os01t0940000-01	1.069	Cytokinin oxidase/dehydrogenase
Os11t0143300-01 *Os12t0139400-01*	1.569 1.082	A-type response regulator, Cytokinin signalling
Jasmonic acid		
Os02t0732400-01 *Os04t0395800-01*	5.007 2.059	Tify domain containing protein
Os03t0225900-01	1.954	Allene oxide synthase (CYP74A2), biosynthesis of Jasmonic acid
Os12t0198700-01	1.109	Similar to Jasmonate-induced protein

Os08t0460600-01 gene coding enzyme similar to Cytokinin dehydrogenase 11, *Os01t0940000-01* gene encoding cytokinin oxidase/dehydrogenase, *Os11t0143300-02*, *Os11t0143300-01* and *Os12t0139400-01* genes coding A-type response regulator involved in cytokinin signalling were up-regulated in the TGMS leaf under sterility inducing condition (Table 21).

Os12t0603300-01 (2.771) gene encoding protein similar to AP2 domain containing protein (Ethylene response factor 112) required in ethylene biosynthesis, *Os06t0592500-01* (2.397) gene coding protein similar to Ethylene-responsive transcriptional coactivator, *Os05t0149400-01* (2.109) gene encoding ACC oxidase enzyme and *Os12t0623900-01* (1.022) gene coding protein similar to ethylene-responsive methionine synthase were also up-regulated (Table 21).

Genes *Os07t0686100-01* (similar to Abscisic acid responsive elements-binding factor) and *Os01t0959100-01* (similar to Abscissic stress ripening protein 1) were up-regulated (Table 21).

Os03t0266800-02 (2.212) gene encoding protein which is similar to BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 and *Os05t0458600-01* (1.982) gene coding Laccase-like protein providing sensitivity of plants to brassinosteroids were also up-regulated (Table 21).

Os02t0732400-01 and *Os04t0395800-01* genes encoding Tify domain containing protein, *Os03t0225900-01* coding enzyme Allene oxide synthase (CYP74A2) involved in the biosynthesis of jasmonic acid and *Os12t0198700-01* producing protein similar to Jasmonate-induced protein were up-regulated (Table 21).

4.4.2.1.4 Genes Regulating Flower Development

Os02t0214300-00 gene encoding enzyme similar to Nuclear ribonuclease Z was up-regulated (Table 22). *Os12t0232300-00* (1.891: similar to ZCN20 FLOWERING TIME LIKE GENE 7), *Os12t0209200-01* (1.599: similar to

CONSTANS-LIKE-a) and *Os01t0917500-01* (1.036) gene results in the production of Leucine-rich repeat receptor-like kinase which is required in the specification of anther cell identity were up-regulated in the TGMS leaf maintained under sterility inducing condition. *Os06t0614000-01* (1.099: multiple C2 domain and transmembrane region proteins (MCTPs) which mediate flowering time and regulation of florigen transport), *Os03t0268200-03* (1.298: similar to Protein kinase domain containing protein AUTOPHAGY ASSOCIATED GENE 1A), *Os03t0753100-01* (1.905: MADS-box transcription factor involved in inflorescence and spikelet development), *Os02t0682200-01* (1.817: MADS-box transcription factor involved in the regulation of floral organ identity and meristem fate) and *Os08t0562200-02* (1.348: Membrane-bound NAC-like transcription factor acts as transcriptional repressor involved in the suppression of flowering) were up-regulated.

Os01t0626400-01 (1.007) gene encoding WRKY transcription factor involved in the control of flowering time and plant height, *Os01t0126600-01* (1.118) gene encoding paralog of FACTOR OF DNA METHYLATION LIKE 1 (*OsFDML1*) required in regulation of flower development, *Os07t0222300-01* (1.127) gene producing Eukaryotic translation initiation factor 3 subunit E involved in organ growth and pollen development and *Os02t0662700-01* (1.542) gene encoding GRAS (GAI-RGA-SCR) plant-specific transcription factor required in the maintenance of shoot apical meristem indeterminacy and regulation of vegetative to reproductive phase change were up-regulated.

Os03t0719700-01 (1.148: similar to Phytochrome A), *Os02t0120500-01* (1.193: Basic helix-loop-helix (*bHLH*) transcription factor required in tapetum development and degeneration), *Os08t0482300-01* (1.209: Pistil-specific extension-like protein domain containing protein), *Os07t0182000-01* (1.875: Basic leucine zipper transcriptional activator), *Os12t0472500-01* (2.354: Homolog of the Arabidopsis *TAPETUM DETERMINANT1* (*TPD1*) protein), *Os03t0725400-02* (3.268: WD40 protein required in the promotion of flowering and panicle development) and *Os01t0578200-00* (3.061: similar to programmed

Table 22. Genes regulating flower development up-regulated under sterility in the TGMS leaves

Gene Name	Fold change	Description
Os01t0578200-00	3.061	Similar to programmed cell death protein 2
Os02t0214300-00	1.755	Similar to Nuclear ribonuclease Z
Os12t0232300-00	1.891	Similar to ZCN20 FLOWERING TIME LIKE GENE 7
Os12t0209200-01	1.599	Similar to CONSTANS-LIKE-a
Os01t0917500-01	1.036	Leucine-rich repeat receptor-like kinase which is required in the specification of anther cell identity, control of early sporogenic development and initiation of anther wall formation
Os06t0614000-01	1.099	Multiple C2 domain and transmembrane region proteins (MCTPs), mediate flowering time and regulation of florigen transport
Os03t0268200-03	1.298	Similar to Protein kinase domain containing protein AUTOPHAGY ASSOCIATED GENE 1A
Os03t0753100-01	1.905	MADS-box transcription factor involved in inflorescence and spikelet development
Os02t0682200-01	1.817	MADS-box transcription factor involved in the regulation of floral organ identity and meristem fate
Os08t0562200-02	1.348	Membrane-bound NAC-like transcription factor acts as transcriptional repressor involved in the suppression of flowering
Os01t0626400-01	1.007	WRKY transcription factor involved in the control of flowering time and plant height
Os01t0126600-01	1.118	Paralog of FACTOR OF DNA METHYLATION LIKE 1 (OsFDML1) required in regulation of flower development
Os07t0222300-01	1.127	Eukaryotic translation initiation factor 3 subunit E involved in organ growth and pollen development
Os02t0662700-01	1.542	GRAS (GAI-RGA-SCR) plant-specific transcription factor required in the maintenance of shoot apical meristem indeterminacy and regulation of vegetative to reproductive phase change
Os03t0719700-01	1.148	Similar to Phytochrome A
Os02t0120500-01	1.193	Basic helix-loop-helix (bHLH) transcription factor required in tapetum development and degeneration
Os08t0482300-01	1.209	Pistil-specific extensin-like protein domain containing protein
Os07t0182000-01	1.875	Basic leucine zipper transcriptional activator involved in grain filling
Os12t0472500-01	2.354	Homolog of the Arabidopsis TAPETUM DETERMINANT1 (TPD1) protein involved in the regulation of early anther cell differentiation
Os03t0725400-02	3.268	WD40 protein required in the promotion of flowering and panicle development

cell death protein 2) were up-regulated in the TGMS leaf under sterility inducing condition.

4.4.2.2 Genes Down-regulated in the Leaf of TGMS Line under Sterility Inducing Condition Compared to Fertility Inducing Condition

4.4.2.2.1 Genes Encoding Abiotic Stress Proteins

Genes encoding Hsp DnaJ family protein, Hsp70, dnaJ 11, Hsp 101, DnaJ domain containing protein, Hsp binding protein, Hsp needed for long-term acquired thermotolerance, Hsp40 and heat shock transcription factor 31 were down-regulated in the TGMS leaf maintained in the sterility inducing condition (Table 23). *Os10t0205700*-01 (-8.080) and *Os01t0899700-01* (-1.103) genes encoding Pollen Ole e 1 allergen/extensin domain containing protein, *Os07t0657100-01* (-2.951) gene coding protein Glyoxalase I, *Os01t0862800-01* (-2.520) gene encoding protein NAC (NAM ATAF1/2 CUC2) transcription factor responsible for early and transient regulator of abiotic stress responses, *Os03t0161900-01* (-2.440) gene producing protein similar to Isoform 2 of Heat stress transcription factor A-2d, *Os03t0782500-01* (-2.411) gene encoding phytochrome-interacting factor-like bHLH protein involved in stress-responsive transcription factor, *Os07t0673400-02* (universal stress protein) and *Os04t0568700-00* (heat stress transcription factor Spl7), Beta-glucosidase and beta-glucanase precursor were down-regulated.

Os01t0137250-00 (stress-induced receptor-like kinase 2), *Os06t0203800-01* (receptor-like kinase required in heat tolerance), *Os01t0835500-03* (respiratory burst oxidase-like protein J), *Os01t0835500-01* (respiratory burst oxidase protein) and *Os05t0413200-01* (beta-tubulin which regulates response to gibberellin and brassinolide) were down-regulated. Gene encoding plant peroxidase domain containing protein, Class III peroxidase GvPx2b, Peroxidase, Peroxidase BP 1 precursor, peroxidase 1, peroxidase 56, class III peroxidase 33, Peroxidase 47 precursor and Peroxidase2 precursor, proline transporter, sugar transporter-like

protein, sugar transporter family protein, sucrose synthase enzyme and Proline-rich protein APG-like were also down-regulated.

4.4.2.2.2 Genes Related to Calcium Signalling

C2 calcium-dependent membrane targeting domain containing protein encoding genes *Os07t0501700-01* (-3.356), *Os01t0853800-04* (-1.045) and *Os01t0242600-01* (-1.122) were down-regulated in the sterility inducing condition (Table 24). Other down-regulated genes include *Os12t0603800-00* (Calmodulin NtCaM13), *Os05t0380900-01* (Polcalcin Jun o 2: Calcium-binding pollen allergen Jun o 2), *Os07t0681400-01* (calcium-binding protein CAST), *Os03t0820300-01* (TFIIIA-type zinc finger protein, transcription activator needed in the abiotic stress tolerance), *Os12t0578300-01*, *Os12t0556500-01*, *Os01t0716200-02*, *Os06t0155300-02* and *Os12t0556300-01* (Calmodulin-binding, plant family protein), *Os10t0180800-02* (Calcium binding EGF domain containing protein), *Os02t0729400-01* (extracellular calcium sensing receptor), *Os01t0955100-01* and *Os01t0810300-01* (Calmodulin-like protein), *Os03t0425300-01* (DGK1: DIACYLGLYCEROL KINASE1; calcium ion binding/diacylglycerol kinase) and *Os05t0467000-02* (Calcium-dependent protein kinase) were down-regulated in the TGMS leaf in the sterility inducing condition.

Table 23. Genes encoding abiotic stress proteins down-regulated under sterility in the TGMS leaves

Gene Name	Fold change	Description
Os10t0205700-01 *Os01t0899700-01*	-8.080 -1.103	Pollen Ole e 1 allergen/extensin domain containing protein
Os01t0378100-01	-4.389	Plant peroxidase domain containing protein
Os02t0240300-01 *Os01t0205900-01*	-3.071 -1.199	Similar to Class III peroxidase GvPx2b (Fragment)
Os04t0513100-01 *Os03t0212800-01*	-2.772 -1.742	Similar to Beta-glucosidase
Os05t0375400-01	-1.330	beta-glucanase precursor
Os07t0657100-01	-2.951	Glyoxalase I involved in abiotic stress tolerance
Os01t0862800-01	-2.520	NAC responsible for early and transient abiotic stress responses
Os03t0161900-01	-2.440	Similar to Isoform 2 of Heat stress transcription factor A-2d
Os03t0782500-01	-2.411	Phytochrome-interacting factor-like bHLH protein for stress-response and regulator of reduced internode elongation under stress
Os07t0673400-02	-2.236	Similar to Universal stress protein
Os04t0568700-00	-1.965	Similar to Heat stress transcription factor Spl7 (RHSF10)
Os03t0683800-01	-1.478	Similar to Proline-rich protein APG-like
Os06t0212900-01	-3.640	Heat shock protein Hsp70 family protein
Os08t0548400-00	-1.581	Similar to chaperone protein dnaJ 11
Os05t0519700-02 *Os05t0519700-03*	-1.484 -1.327	Heat shock protein 101
Os03t0323600-01	-1.440	Similar to DnaJ domain containing protein
Os11t0216100-01	-1.358	Similar to Heat shock protein binding protein
Os05t0519700-01 *Os06t0682900-01*	-1.292 -1.160	Heat shock protein needed for long-term acquired thermotolerance
Os05t0587300-01	-1.237	Molecular chaperone, Hsp40, DnaJ domain containing protein
Os01t0625300-01	-1.101	Similar to Heat shock transcription factor 31 (Fragment)
Os01t0137250-00	-2.997	Similar to Stress-induced receptor-like kinase 2
Os06t0203800-01	-2.984	Receptor-like kinase required in heat tolerance
Os01t0835500-03	-2.431	Similar to Respiratory burst oxidase-like protein J
Os01t0835500-01	-2.412	Similar to Respiratory burst oxidase protein
Os05t0413200-01	-1.470	Beta-tubulin, Response to gibberellin and brassinolide (BL)
Os04t0688200-01 *Os03t0234900-01* *Os07t0677100-01* *Os01t0327400-01*	-1.972 -1.477 -1.335 -1.526	Similar to Peroxidase
Os01t0963000-01 *Os01t0963000-04*	-1.681 -1.434	Similar to Peroxidase BP 1 precursor
Os05t0134400-02	-1.455	Similar to peroxidase 1
Os01t0787000-02	-1.394	Similar to peroxidase 56
Os03t0121200-03	-1.090	Similar to Class III peroxidase 33
Os08t0113000-01	-1.089	Similar to Peroxidase 47 precursor (EC 1.11.1.7) (Atperox P47)
Os03t0762400-01	-1.058	Similar to Peroxidase2 precursor
Os01t0621200-00	-1.110	Similar to proline transporter
Os03t0363500-02	-1.105	Similar to Sugar transporter-like protein
Os11t0643800-01	-1.067	Similar to Sugar transporter family protein
Os02t0831500-01	-1.128	Similar to Sucrose synthase

Table 24. Genes related to calcium signalling down-regulated under sterility inducing condition in the TGMS leaves

Gene Name	Fold change	Description
Os07t0501700-01 *Os01t0853800-04* *Os01t0242600-01*	-3.356 -1.045 -1.122	C2 calcium-dependent membrane targeting domain containing protein
Os12t0603800-00	-3.350	Similar to Calmodulin NtCaM13
Os05t0380900-01	-2.863	Similar to Polcalcin Jun o 2 (Calcium-binding pollen allergen Jun o 2)
Os07t0681400-01	-2.338	Similar to Calcium-binding protein CAST
Os03t0820300-01	-2.074	TFIIIA-type zinc finger protein which is a transcription activator, abiotic stress tolerance
Os12t0578300-01 *Os12t0556500-01* *Os01t0716200-02* *Os06t0155300-02* *Os12t0556300-01*	-2.021 -1.078 -1.055 -1.033 -1.381	Calmodulin-binding, plant family protein
Os10t0180800-02	-1.973	Similar to Calcium binding EGF domain containing protein, expressed
Os02t0729400-01	-1.715	Similar to Extracellular calcium sensing receptor
Os01t0955100-01 *Os01t0810300-01*	-1.187 -1.017	Similar to Calmodulin-like protein (Fragment)
Os03t0425300-01	-1.146	Similar to DGK1 (DIACYLGLYCEROL KINASE1); calcium ion binding / diacylglycerol kinase
Os05t0467000-02	-1.139	Similar to Calcium-dependent protein kinase

4.4.2.2.3 Genes Related to Biosynthesis and Signalling of Phytohormones

Auxin biosynthesis gene *Os01t0645400-01* (-1.963) coding Flavin monooxygenase-like enzyme (YUCCA-like gene) was down-regulated in the leaf under sterility inducing condition (Table 25). AUX/IAA family protein genes *Os02t0723400-02*, *Os01t0231000-03*, *Os01t0231000-01*, *Os07t0182400-01* and *Os12t0609600-01*, *Os06t0702000-01*, *Os01t0768333-00*, *Os07t0475700-01*, *Os12t0626200-01*, *Os02t0445100-01* (-1.532) genes producing Auxin responsive/induced SAUR protein and *Os09t0457900-01* (-3.314) gene producing protein AP2/ERF transcription factor was down-regulated in the TGMS leaf under sterility inducing condition. Genes encoding GH3 auxin-responsive promoter family protein, Isoform 2 of Auxin-responsive protein IAA8, auxin efflux carrier domain containing protein, auxin induced protein, auxin response factor 5, auxin transporter-like protein 1, Bric-a-Brac/Tramtrack/Broad (BTB) complex and an NPH3domain (BTBN) involved in the regulation of auxin transport and Auxin-induced basic helix-loop-helix transcription factor were also down-regulated in the sterility inducing condition.

Gibberellic acid genes like *Os05t0560900-01* (gibberellin 2-beta-dioxygenase), *Os03t0439500-01*, *Os03t0439500-03* and *Os03t0856000-01* (2OG-Fe(II) oxygenase domain containing protein), *Os08t0560000-01* (gibberellin 20 oxidase 2), *Os03t0607200-01* (gibberellin regulated protein family protein) and *Os03t0856700-02* (GA 20-oxidase1), *Os03t0760800-01* (a member of the GAST (gibberellin (GA)-Stimulated Transcript) family required in the control of cell proliferation in meristems and panicles development) were down-regulated (Table 25).

Cytokinin signalling A-type response regulator, serine/threonine kinase in the cytokinin signalling, cytokinin dehydrogenase 2, cytokinin synthesis enzyme, cytokinin-activating enzyme and cytokinin oxidase/dehydrogenase were down regulated in the sterility inducing condition (Table 25).

Ethylene associated genes encoding ACC synthase enzyme involved in ethylene biosynthesis, Ethylene-responsive transcription factor 4 (Related to APETALA-2 protein 5) and ethylene-responsive protein, EIN2, EIL3 and AP2 domain containing protein RAP2.2 (*ETHYLENE RESPONSE FACTOR 66*), *and* AP2/ERF family protein, AP2 domain transcription factor *EREBP* (*ETHYLENE RESPONSE FACTOR 67*), *AP2/ERF* (*APETALA2*/ethylene-responsive factor) protein involved in the regulation of spikelet meristem determinancy and floral organ identity, ACC oxidase, EREBP-2 protein, ethylene responsive element binding protein 2 and Ethylene receptor-like protein, AP2/ERF family protein, ERF-associated EAR-motif-containing repressor required in the abiotic stress response and stress signalling and AP2-1 protein were down-regulated (Table 25).

Genes coding Tify domain containing protein involved in jasmonic acid synthesis and Jasmonate-induced protein were also down-regulated (Table 25).

Os06t0654600-00 (-1.683) gene encoding protein similar to BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 was down-regulated (Table 25).

Os03t0610900-01 (-1.217) gene coding serine/threonine protein kinase which is an abscisic acid (ABA)-activated protein kinase involved in the hyperosmotic stress response, ABA signal transduction and *Os08t0472000-01* (-1.562) gene coding bZIP transcription factor involved in the ABA-regulated transcription was down-regulated in the TGMS leaf in the sterility inducing condition (Table 25).

Table 25. Down-regulated genes related to biosynthesis and signalling of phytohormones under sterility in the TGMS leaves

Gene Name	Fold change	Description
Auxin		
Os02t0723400-02	-4.513	AUX/IAA family protein
Os01t0231000-03	-2.061	
Os01t0231000-01	-1.943	
Os07t0182400-01	-1.181	
Os12t0609600-01	-4.070	Auxin responsive SAUR protein
Os06t0702000-01	-1.255	
Os01t0768333-00	-3.968	
Os07t0475700-01	-3.591	
Os01t0221100-02	-3.051	GH3 auxin-responsive promoter family protein
Os01t0645400-01	-1.963	Flavin monooxygenase-like enzyme (YUCCA-like gene)
Os02t0445100-01	-1.532	Similar to auxin-induced SAUR-like protein
Os09t0457900-01	-3.314	AP2/ERF transcription factor, regulation of the internode elongation, tillering and panicle branching
Os02t0723400-01	-2.830	Similar to Isoform 2 of Auxin-responsive protein IAA8
Os01t0818000-02	-1.659	Auxin efflux carrier domain containing protein
Os01t0919800-01	-1.143	
Os05t0481900-01	-1.083	
Os09t0546900-01	-1.634	Similar to auxin induced protein
Os05t0563400-01	-1.438	Similar to auxin response factor 5
Os05t0447200-01	-1.606	Similar to auxin transporter-like protein 1
Os03t0347700-01	-1.130	Protein containing a Bric-a-Brac/Tramtrack/Broad (BTB) complex and an NPH3domain (BTBN), regulation of auxin transport
Os01t0924400-01	-1.081	Similar to Auxin-induced basic helix-loop-helix transcription factor
Gibberellic acid		
Os03t0760800-01	-4.092	A member of the GAST (gibberellin (GA)-Stimulated Transcript) family required in the control of seedling growth and alpha;-amylase expression and cell proliferation in meristems and panicles development
Os05t0560900-01	-3.800	Similar to gibberellin 2-beta-dioxygenase
Os03t0439500-01	-3.687	2OG-Fe(II) oxygenase domain containing protein
Os03t0439500-03	-1.806	
Os03t0856000-01	-1.067	
Os08t0560000-01	-2.870	Similar to gibberellin 20 oxidase 2
Os03t0607200-01	-1.943	Gibberellin regulated protein family protein
Os03t0856700-02	-1.097	GA 20-oxidase1, GA metabolism
Cytokinin		
Os04t0524300-01	-3.238	A-type response regulator, Cytokinin signalling
Os02t0830200-01	-1.579	
Os02t0557800-02	-1.195	
Os12t0454800-01	-1.651	Receptor-like serine/threonine kinase, Cytokinin signalling
Os05t0374200-00	-1.248	Similar to cytokinin dehydrogenase 2
Os04t0518800-01	-1.476	Cytokinin synthesis enzyme, cytokinin-activating enzyme
Os01t0197700-01	-1.001	Cytokinin oxidase/dehydrogenase, Regulation of grain production

Table 25. Down-regulated genes related to biosynthesis and signalling of phytohormones under sterility in the TGMS leaves (Continued)

Gene Name	Fold change	Description
Ethylene		
Os03t0727600-01	-5.782	ACC synthase enzyme, Ethylene biosynthesis
Os02t0764700-01	-3.262	Similar to Ethylene-responsive transcription factor 4 (Related to APETALA-2 protein 5)
Os03t0750000-01	-3.099	Similar to ethylene-responsive protein
Os03t0700800-01	-3.912	Similar to EIN2
Os08t0508700-01	-2.678	Similar to EIL3
Os03t0341000-01	-2.615	Similar to AP2 domain containing protein RAP2.2 (ETHYLENE RESPONSE FACTOR 66)
Os07t0674800-01	-2.565	Similar to AP2 domain transcription factor EREBP (ETHYLENE RESPONSE FACTOR 67)
Os05t0497200-01	-2.385	AP2/ERF (APETALA2/ethylene-responsive factor) protein which is involved in the regulation of spikelet meristem determinancy and floral organ identity
Os09t0451000-01	-1.623	ACC oxidase, Ethylene biosynthesis
Os05t0361700-01	-1.384	Similar to EREBP-2 protein
Os05t0361700-02	-1.312	Similar to ethylene responsive element binding protein
Os05t0155200-02	-1.004	Similar to Ethylene receptor-like protein
Os01t0797600-01	-1.571	AP2/ERF family protein, ERF-associated EAR-motif-containing repressor required in the abiotic stress response and stress signalling
Os02t0654700-01	-1.114	AP2/ERF family protein required in the abiotic stress response
Os11t0129700-01	-1.062	Similar to AP2-1 protein
Brassinosteroid		
Os06t0654600-00	-1.683	Similar to BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
Os03t0610900-01	-1.217	Serine/threonine protein kinase, abscisic acid (ABA)-activated protein kinase, hyperosmotic stress response, ABA signal transduction
Jasmonic acid		
Os10t0391400-01 *Os03t0181100-02* *Os03t0180900-01* *Os03t0181100-01*	-4.472 -2.265 -1.655 -1.831	Tify domain containing protein, Jasmonic acid synthesis
Os08t0472000-01	-1.562	bZIP transcription factor, ABA-regulated transcription
Os10t0132300-01	-1.121	Similar to Jasmonate-induced protein

4.4.2.2.4 Genes Regulating Flower Development

Os01t0944700-01 (-6.926), *Os01t0713200-01* (-1.879), *Os02t0200300-02* (-1.520), *Os01t0944800-00* (-1.501), *Os02t0200300-01* (-1.397) genes coding protein similar to β-1,3-glucanase precursor were down-regulated (Table 26). *Os03t0167600-01* (-3.977) gene reported to encode similar to Male sterility protein 2 was down-regulated. *Os01t0836600-01* (-3.535) gene producing ATP-binding cassette (ABC) transporter involved in the pollen wall formation, *Os02t0649300-01* (-3.376) gene encoding HD-ZIP I protein, transcription activator which regulates stress response and panicle development and *Os07t0549600-01* (-1.240) gene coding protein similar to *UNDEVELOPED TAPETUM 1* were down-regulated in the TGMS leaf maintained in the sterility inducing condition.

Genes encoding MADS-box transcription factor 15, transcription factor which is essential in the development of lodicules and stamens, phosphatase 2C, involved in abiotic stress response and early panicle development and transcription factor LAX PANICLE, EMF protein (*EMBRYONIC FLOWER 2A*), EARLY FLOWERING 4, K Homology domain containing protein, Nuclear RNA/DNA binding protein of the STAR (Signal Transduction and Activation of RNA) family which is involved in regulating flowering time control and pollenless3, pollen-specific protein SF21 and putative glucose-methanol-choline oxidoreductase which regulates tapetum degeneration, pollen exine formation and anther cuticle formation was also down-regulated in the TGMS leaf under sterility inducing condition.

Os02t0199800-01 (no pollen), *Os06t0678800-01* (pollen-specific protein NTP303 precursor), *Os02t0324400-01* (secreted protein with a CLE domain which maintains floral meristem and vegetative shoot apical meristem), *Os04t0488400-01* (FLOWERING LOCUS T protein), *Os06t0552900-00* (SP3D FLOWERING TIME LIKE GENE 12), *Os01t0218500-02* (FLOWERING LOCUS T (FT)-Like homolog), *Os01t0218500-01* (*ZCN14* (*FLOWERING TIME*

LIKE GENE 1) protein), *Os01t0202700-00* (Flowering locus T3) and *Os04t0282400-01* (FPF1 (Flowering-promoting factor 1-like protein) protein-like: RAA1) were down-regulated in the sterility inducing condition.

Os02t0610500-01 (-1.858) gene encoding CO-like protein containing two B-box zinc finger domains and one CCT domain which is a Constitutive flowering repressor, *Os04t0460600-02* (-1.648) gene producing NAC transcription factor involved in the regulation of flowering time, *Os04t0462500-02* (-1.083) gene encoding 14-3-3 protein, *Os10t0463400-01* (-1.668) gene coding B-type response regulator function as a floral inducer to promote short-day (SD) flowering pathway and gene *Os06t0498800-01* (-1.598) producing protein similar to MOTHER of FT and TF1 protein were down-regulated.

Genes encoding protein similar to Filamentous flower-like yabby protein, transcriptional regulator which is involved in the regulation of meristem activity and inflorescence development, MADS-domain-containing protein required in the sexual reproduction, EGG APPARATUS-1 protein (ZmEA1), RAFTIN1a protein and MADS box transcription factor required for pollen maturation was also down-regulated in the TGMS leaf under sterility inducing condition.

4.4.2.2.5 Pathways Up-Regulated and Down-Regulated in the TGMS Leaf under Sterility Inducing Condition

Pathways such as sucrose and starch degradation/metabolism, cytokinins degradation, cytokinin-O-glucoside biosynthesis, proline degradation and 4-hydroxyproline degradation were up-regulated in the TGMS plants maintained under sterility inducing condition (Table 27). On the other hand, pathways involved in IAA biosynthesis, gibberellin biosynthesis, ethylene biosynthesis, brassinosteroid biosynthesis, jasmonic acid biosynthesis and sucrose biosynthesis were down-regulated in the TGMS leaf maintained under sterility inducing condition (Table 27).

Table 26. Genes regulating flower development down-regulated under sterility in the TGMS leaves

Gene Name	Fold change	Description
Os01t0944700-01 *Os01t0713200-01* *Os02t0200300-02* *Os01t0944800-00* *Os02t0200300-01*	-6.926 -1.879 -1.520 -1.501 -1.397	Similar to β-1,3-glucanase precursor
Os06t0552900-00	-4.726	similar to SP3D FLOWERING TIME LIKE GENE 12
Os01t0218500-02	-4.054	FLOWERING LOCUS T (FT)-Like homolog which promotes flowering
Os03t0167600-01	-3.977	Similar to Male sterility protein 2
Os01t0836600-01	-3.535	ATP-binding cassette (ABC) transporter, Pollen wall formation
Os02t0649300-01	-3.376	HD-ZIP I protein, Transcription activator which regulates stress response and panicle development
Os07t0108900-01	-3.297	Similar to MADS-box transcription factor 15
Os03t0165900-02	-3.603	Similar to pollenless3
Os05t0196200-01	-2.676	Similar to pollen-specific protein SF21
Os01t0218500-01	-2.863	Similar to ZCN14 (FLOWERING TIME LIKE GENE 1) protein
Os01t0202700-00	-2.295	Similar to Flowering locus T3
Os04t0282400-01	-2.180	Similar to FPF1 (Flowering-promoting factor 1-like protein) protein-like (RAA1)
Os07t0549600-01	-1.240	Similar to Undeveloped tapetum 1
Os06t0712700-01	-1.944	Transcription factor, Development of lodicules and stamens
Os09t0325700-01	-1.722	Protein phosphatase 2C, abiotic stress response and early panicle development
Os01t0707500-01	-1.184	Similar to Transcription factor LAX PANICLE
Os07t0147550-00	-2.703	Similar to Photosystem II 10 kDa polypeptide, chloroplast
Os04t0413500-01	-1.425	Cell-wall invertase regulates carbon partitioning during early grain filling
Os04t0162100-02	-2.353	Similar to EMF protein (EMBRYONIC FLOWER 2A)
Os11t0621500-01	-1.394	Similar to EARLY FLOWERING 4
Os03t0815700-01	-1.297	K Homology domain containing protein Nuclear RNA/DNA binding protein of the STAR (Signal Transduction and Activation of RNA) family which is involved in regulating flowering time control
Os10t0524500-01	-1.517	Putative glucose-methanol-choline oxidoreductase, Regulates tapetum degeneration, pollen exine formation and anther cuticle formation
Os02t0199800-01	-1.213	Similar to no pollen
Os06t0678800-01	-1.013	similar to pollen-specific protein NTP303 precursor
Os02t0324400-01	-1.002	Secreted protein with a CLE domain maintains floral meristem and vegetative shoot apical meristem
Os04t0488400-01	-3.240	Similar to FLOWERING LOCUS T protein

Table 26. Genes regulating flower development down-regulated under sterility in the TGMS leaves (Continued)

Gene Name	Fold change	Description
Os02t0610500-01	-1.858	CO-like protein containing two B-box zinc finger domains and one CCT domain, Constitutive flowering repressor
Os04t0460600-02	-1.648	NAC transcription factor, Regulation of flowering time
Os04t0462500-02	-1.083	14-3-3 protein, Florigen receptor, Regulate flowering
Os10t0463400-01	-1.668	B-type response regulator function as a floral inducer to promote short-day (SD) flowering pathway
Os06t0498800-01	-1.598	Similar to MOTHER of FT and TF1 protein
Os12t0621100-02	-1.237	Similar to Filamentous flower-like yabby protein
Os10t0478000-01	-2.567	Transcriptional regulator which is involved in the regulation of meristem activity and inflorescence development
Os03t0215400-01	-2.241	MADS-domain-containing protein required in the sexual reproduction
Os07t0605400-01	-1.874	Similar to EGG APPARATUS-1 protein (ZmEA1)
Os08t0496800-01	-0.867	RAFTIN1a protein
Os06t0223300-01	-0.415	MADS box transcription factor required for pollen maturation

Table 27. Pathways up-regulated and down-regulated in the TGMS leaf under sterility inducing condition

Pathway	Genes	No of genes
Up-regulated pathways		
Sucrose degradation I	*OS01G0894300, OS12G0133800, OS01G0940100, OS01G0894300*	4
Cytokinins degradation	*OS01G0940000*	1
Proline degradation II	*OS10G0550900,OS04G0540600,OS06G0592400,OS01G0591300,OS01G0591000, OS10G0550900, OS04G0540600,OS06G0592400, OS01G0591300, OS01G0591000*	10
Cytokineins-O-glucoside biosynthesis	*OS04G0206500,OS01G0735300,OS05G0526900,OS06G0593200,OS07G0503200,OS02G0242100,OS02G0206100,OS03G0841600,OS04G0206700,OS01G0638000,OS02G0755900,OS07G0510500,OS01G0805400,OS01G0179600,OS01G0176200,OS02G0188000,OS05G0526800,OS07G0148200,OS01G0597800,OS03G0757100,OS11G0599200,OS07G0241800,OS01G0805500,OS08G0489100,OS01G0735500,OS01G0869400,OS01G0734800,OS09G0423600,OS07G0490100,OS04G0204100*	30

Table 27. Pathways up-regulated and down-regulated in the TGMS leaf under sterility inducing condition (Continued)

Pathway	Genes	No of genes
Starch degradation	*OS06G0367100,OS12G0133800,OS04G0164900,OS09G0569200,OS01G0940100,OS07G0543100,OS09G0469400,OS10G0465700*	8
4-hydroxyproline degradation	*OS04G0540600,OS06G0592400,OS01G0591300,OS01G0591000*	4
Down-regulated pathways		
Ethylene biosynthesis	*OS06G0345200,OS01G0178000,OS03G0727600*	3
Brassinosteroid biosynthesis	*OS01G0645400*	1
IAA biosynthesis	*OS12G0559200,OS03G0767000,OS03G0438100,OS01G0186900,OS05G0183900,OS02G0194700*	6
Jasmonic acid biosynthesis	*OS04G0518400,OS12G0559200,OS06G0729000,OS03G0767000,OS07G0604000,OS03G0438100,OS04G0485300,OS10G0329300,OS03G0416500,OS03G0856700,OS03G0264400,OS02G0139700,OS06G0110000,OS07G0190000,OS07G0197100,OS07G0622200,OS06G0246500,OS01G0159400,OS06G0499900,OS02G0278700,OS05G0408900,OS02G0194700,OS03G0727600*	23
Gibberellin biosynthesis	*OS03G0856700*	1
Sucrose biosynthesis	*OS03G0268400,OS02G0184400,OS02G0831500,OS06G0476200*	4

4.4.2.3 Genes Up-regulated in the Sterile Panicle of TGMS Line under Sterility Inducing Condition Compared to Fertile Panicle

4.4.2.3.1 Genes Encoding Abiotic Stress Proteins

Abiotic stress genes encoding Hsp 82, Hsp 80, 22.7 kDa class IV heat shock protein precursor, Hsp 81-1, Hsp 40 protein, HSP70B (ATP binding), Hsp 20-like chaperone domain containing protein, Hsp 26, Class I low-molecular-weight heat shock protein 17.9, Class II small heat shock protein, 16.9 kDa class I heat shock protein 1, Hsp DnaJ, Hsp 101, Hsp 70 and small heat shock-like protein was up-regulated (Table 28). *Os06t0565200-00* (putative heat stress transcription factor A-6a), *Os09t0455200-01* (heat shock factor (HSF)-type: DNA-binding domain containing protein), *Os03t0267000-00* (low molecular mass heat shock protein Oshsp18.0), *Os02t0217900-00* (Cytosolic class II small heat shock protein 4) and *Os02t0537400-01* (heat shock protein) was up-regulated. Peroxidase 24 precursor, Peroxidase, Peroxidase P7 (TP7), Class III peroxidase GvPx2b, peroxidase 11 precursor, Class III peroxidase 13, Class III peroxidase 44, Class III peroxidase 53, Class III peroxidase and plant peroxidase domain containing protein, superoxide dismutase, copper/zinc binding domain containing protein and Proline-rich protein APG-like, proline-rich family protein, stress regulated protein and hydroxyproline-rich glycoprotein DZ-HRGP precursor, STT3A (*STAUROSPORIN AND TEMPERATURE SENSITIVE 3-LIKE A*; oligosaccharyl transferase, Actin depolymerizing factor, Actin-binding protein required in abiotic stress response) and Pollen Ole e 1 allergen and extensin domain containing protein encoding genes were also up-regulated in the TGMS panicle maintained under sterility inducing condition.

4.4.2.3.2 Genes Related to Calcium Signalling

Calcium binding protein, calmodulin related genes *Os11t0586200-00*, *Os10t0180800-02*, *Os08t0360300-00*, *Os01t0810300-01*, *Os09t0569300-01* and *Os03t0636700-00* and *Os11t0136600-00* and *Os12t0133500-00* (Calcium-dependent protein kinase) were also up-regulated (Table 29).

Table 28. Genes encoding abiotic stress proteins up-regulated in the sterile panicle of TGMS line

Gene Name	Fold change	Description
Os04t0107900-01	6.990	Similar to Heat shock protein 82
Os04t0107900-03 *Os04t0107900-04*	5.597 4.545	Similar to Hsp 80
Os04t0445100-01	4.283	Similar to 22.7 kDa class IV heat shock protein precursor
Os04t0107900-02	3.186	Heat shock protein 81-1 (HSP81-1)
Os03t0222700-01	2.932	Hsp40 protein
Os01t0688900-00	2.809	Similar to HSP70B (ATP binding)
Os09t0345500-00 *Os10t0159700-01* *Os10t0437700-01* *Os01t0587500-00* *Os01t0588000-01* *Os12t0165200-01*	2.613 2.174 1.768 1.505 1.409 1.288	HSP20-like chaperone domain containing protein
Os03t0245800-02	2.226	Similar to Heat shock protein 26
Os03t0266300-01 *Os11t0244200-01*	2.197 2.921	Class I low-molecular-weight heat shock protein 17.9
Os01t0184100-01	2.109	Class II small Hsp involved in regulating heat tolerance
Os01t0136200-01 *Os01t0136100-01*	1.573 1.367	16.9 kDa class I heat shock protein 1
Os01t0157800-01	1.519	Heat shock protein DnaJ
Os02t0297132-00	1.487	Similar to heat-shock protein 101
Os03t0277300-01 *Os11t0187500-01* *Os03t0218500-01*	1.373 1.011 1.419	Hsp 70
Os12t0624100-01	1.337	Similar to Small heat shock-like protein
Os06t0565200-00	2.605	Putative heat stress transcription factor A-6a
Os09t0455200-01	1.017	Heat shock factor (HSF)-type, DNA-binding domain
Os03t0267000-00	1.119	Low molecular mass heat shock protein Oshsp18.0
Os02t0217900-00	1.405	Similar to Cytosolic class II small heat shock protein 4
Os02t0537400-01	1.421	Similar to Heat shock protein
Os12t0530100-01	3.132	Similar to Peroxidase 24 precursor (Atperox P24; ATP47)
Os04t0688200-01 *Os02t0161800-01* *Os01t0326000-01* *Os07t0626700-01*	5.050 1.717 2.386 1.600	Peroxidase
Os03t0683800-01	1.103	Similar to Proline-rich protein APG-like
Os02t0556800-01 *Os04t0612500-02*	1.153 2.175	Similar to proline-rich family protein
Os06t0547400-01	1.244	Similar to Peroxidase P7 (TP7)
Os04t0573200-03	1.284	Superoxide dismutase, copper/zinc binding domain
Os05t0135200-01	1.291	Plant peroxidase domain containing protein
Os01t0382700-01	1.307	Similar to stress regulated protein
Os06t0546500-01	1.461	Similar to Class III peroxidase GvPx2b

Gene Name	Fold change	Description
Os06t0274800-01	1.563	Similar to Peroxidase 11 precursor (Atperox P11)
Os03t0245300-01	1.895	Similar to Hydroxyproline-rich glycoprotein DZ-HRGP
Os01t0326000-02	2.093	Similar to Class III peroxidase 13
Os07t0638600-00	2.651	Similar to Class III peroxidase 44
Os04t0134800-01	2.752	Similar to Class III peroxidase 53
Os01t0294500-00	2.810	Similar to Class III peroxidase 9
Os05t0519900-01	1.637	Similar to STT3A (STAUROSPORIN AND TEMPERATURE SENSITIVE 3-LIKE A); oligosaccharyl transferase
Os03t0820500-01	1.931	Actin depolymerizing factor, Actin-binding protein, Abiotic stress response

Table 29. Genes related to calcium signalling up-regulated in the sterile panicle of TGMS line

Gene Name	Fold change	Description
Os11t0586200-00	2.931	Similar to Calmodulin
Os10t0180800-02	1.041	Similar to Calcium binding EGF domain containing protein
Os08t0360300-00	1.046	Calmodulin binding protein-like domain containing protein
Os11t0136600-00 *Os12t0133500-00*	1.286 1.756	Similar to Calcium-dependent protein kinase
Os01t0810300-01	1.445	Similar to Calmodulin-like protein
Os03t0636700-00	1.710	IQ calmodulin-binding region domain containing protein

4.4.2.3.3 Genes Related to Biosynthesis and Signalling of Phytohormones

Genes associated with phytohormones were up-regulated in the TGMS panicle under sterility inducing condition (Table 30). Genes encoding Auxin transporter-like protein 3, Auxin induced protein, Auxin responsive SAUR protein family protein, IAA6, Aux/IAA_ARF_dimerisation domain containing protein, Auxin transport protein REH1, Auxin induced protein, PIN1-like auxin transport protein, Auxin response factor 14, Auxin-responsive protein IAA31 (Indoleacetic acid-induced protein 31), Auxin-responsive protein IAA20, AUX1-like protein, auxin transporter-like protein 3, Bric-a-Brac/Tramtrack/Broad (BTB) complex and an NPH3domain (BTBN), gene required in the regulation of shoot gravitropism, tiller angle and regulation of polar auxin transport, indole-3-acetate beta-glucosyltransferase and indole-3-acetic acid amido synthetase were up-regulated in the TGMS panicle under sterility inducing condition.

Gibberellin responsive 1, GA 3-beta-hydroxylase1, 2OG-Fe(II) oxygenase domain containing protein, gibberellin regulated protein family protein coding genes and transcription factor, DNA-binding intermediate protein for SLR1 involved in the modulation of gibberellin signalling pathway were up-regulated in the TGMS panicle under sterility inducing condition. *Os04t0194400-00* (2.845) gene encoding enzyme similar to cytokinin-O-glucosyltransferase 3 was also up-regulated in the TGMS panicle.

Regarding Ethylene genes, *Os09t0490200-01* (Ethylene signal transcription factor), *Os09t0571700-00* (Ethylene-responsive transcription factor ERF096), *Os11t0242300-00* (AP2 domain containing protein), *Os05t0196600-00* (1-aminocyclopropane-1-carboxylic acid synthase), *Os02t0820900-01* and *Os02t0820900-02* (Ethylene receptor-like protein 2) and *Os02t0574800-01* (Ethylene insensitive 3 domain containing protein) was up-regulated in the TGMS sterile panicle compared to fertile panicle.

Os07t0686100-01 (Abscissic acid responsive elements-binding factor), *Os01t0859300-01* (bZIP transcription factor involved in stress response and ABA

signal transduction and regulation of plant fertility) and *Os08t0467500-02* (Abscisic acid and stress inducible: A22) gene was up-regulated in the sterile panicle. *Os02t0762600-01* (1.071) gene coding for SG1 (SHORT GRAIN1)-related protein involved in Brassinosteroid signalling regulating seed and panicle development was also up-regulated in the TGMS panicle under sterility inducing condition.

4.4.2.3.4 Genes Regulating Flower Development

Genes coding CONSTANS-like protein CO9, Anther-specific proline-rich protein (APG), TA9 protein, ATP-binding cassette (ABC) transporter involved in pollen wall formation, member of the family of multiple C2 domain and transmembrane region proteins (MCTPs) which mediates flowering time and regulates florigen transport, secretory fasciclin glycoprotein involved in regulation of anther development and pollen formation, programmed cell death protein 2 was up-regulated (Table 31). COM1/SAE2 homolog which involves in homologous chromosome synapsis and recombination in meiosis, member of the synaptonemal complex (SC: OmpH/coiled-coil motif-containing protein involved in the regulation of meiotic double-strand break (DSB) formation and SC assembly, meiotic recombination), sugar carrier protein C, Aluminum-activated malate transporter required in the maintenance of panicle size and grain yield and Radc1 (aspartic protease 25, rice anther down-regulated by chilling 1) was up-regulated in the TGMS sterile panicle (Table 30).

Os08t0509600-01 (2.103) gene producing squamosa promter-binding-like transcription activator involved in the regulation of branching in panicles and vegetative shoots, *Os05t0560100-01* (pollen-specific kinase partner protein), *Os12t0152000-00* (3.064) gene coding protein similar to terminal flower 1-like protein, *Os04t0111301-00* (clavata-like kinase), *Os10t0374450-00* (3.232) gene coding protein similar to barren inflorescence2-like serine/threonine protein kinase and *Os12t0614600-01* (serine/threonine protein kinase domain containing protein) was also up-regulated in the TGMS sterile panicle.

Table 30. Up-regulated genes related to biosynthesis and signalling of phytohormones in the sterile panicle of TGMS line

Gene Name	Fold change	Description
Os10t0147400-03	1.548	Similar to Auxin transporter-like protein 3
Os09t0545700-01 *Os11t0247000-02* *Os09t0547100-01* *Os11t0247000-01*	2.696 1.182 1.169 1.135	Similar to Auxin induced protein
Os04t0517900-01 *Os09t0545500-00* *Os07t0475700-01* *Os08t0118500-00* *Os09t0437400-00*	2.394 2.222 2.111 1.794 1.600	Auxin responsive SAUR protein family protein
Os03t0633800-01	1.979	Similar to IAA6
Os11t0221000-01	1.669	Aux/IAA_ARF_dimerisation domain containing protein
Os06t0232300-01	1.812	Similar to Auxin transport protein REH1
Os08t0550700-00	1.668	Similar to Auxin induced protein
Os11t0137000-01	1.532	Similar to PIN1-like auxin transport protein
Os05t0515400-01	1.391	Similar to Auxin response factor 14
Os01t0286900-01	1.342	Similar to Auxin-responsive protein IAA31 (Indoleacetic acid-induced protein 31)
Os06t0166500-01	1.170	Similar to Auxin-responsive protein IAA20
Os03t0244600-01	1.113	Similar to AUX1-like protein
Os03t0244600-02	1.072	Similar to auxin transporter-like protein 3
Os03t0347700-01	1.082	Protein containing a Bric-a-Brac/Tramtrack/Broad (BTB) complex and an NPH3domain (BTBN), Regulation of auxin transport
Os11t0490600-01	2.787	Regulation of shoot gravitropism, tiller angle and regulation of polar auxin transport
Os01t0805500-00	1.261	Similar to indole-3-acetate beta-glucosyltransferase
Os07t0576100-00	2.233	Similar to indole-3-acetic acid amido synthetase
Os01t0571166-00	1.956	Similar to gibberellin responsive1
Os05t0178100-01	1.901	Protein GA 3-beta-hydroxylase1, GA metabolism
Os10t0558200-00 *Os01t0351800-01*	1.861 1.324	2OG-Fe(II) oxygenase domain containing protein
Os07t0592000-01	1.304	Gibberellin regulated protein family protein
Os02t0643200-01	1.016	Transcription factor, DNA-binding intermediate protein for SLR1, Modulation of gibberellin signaling pathway
Os04t0194400-00	2.845	Similar to cytokinin-O-glucosyltransferase 3
Os09t0490200-01	2.334	Similar to Ethylene signal transcription factor
Os09t0571700-00	2.165	Similar to Ethylene-responsive transcription factor ERF096
Os11t0242300-00	1.723	Similar to AP2 domain containing protein
Os05t0196600-00	1.371	Similar to 1-aminocyclopropane-1-carboxylic acid synthase
Os02t0820900-01 *Os02t0820900-02*	1.227 1.007	Similar to Ethylene receptor-like protein 2
Os02t0574800-01	2.669	Ethylene insensitive 3 domain containing protein
Os07t0686100-01	1.318	Similar to Abscissic acid responsive elements-binding factor
Os08t0467500-02	1.046	Abscisic acid and stress inducible (A22)
Os02t0762600-01	1.071	SG1 (SHORT GRAIN1)-related protein, Brassinosteroid signalling regulating seed and panicle development

Table 31. Genes regulating flower development up-regulated in the sterile panicle of TGMS line

Gene Name	Fold change	Description
Os03t0139500-01	4.432	Similar to CONSTANS-like protein CO9
Os09t0540400-01	3.666	Similar to Anther-specific proline-rich protein (APG)
Os05t0566900-01	3.498	Similar to TA9 protein
Os10t0546100-01	3.104	Pollen Ole e 1 allergen and extensin domain containing protein
Os12t0152000-00	3.064	Similar to Terminal flower 1-like protein
Os10t0374450-00	3.232	Similar to Barren inflorescence2-like serine/threonine protein kinase
Os12t0614600-01	2.762	Serine/threonine protein kinase domain containing protein
Os01t0836600-01	1.373	ATP-binding cassette (ABC) transporter involved in pollen wall formation
Os06t0614000-01	1.372	A member of the family of multiple C2 domain and transmembrane region proteins (MCTPs), Mediates flowering time and regulates florigen transport
Os02t0491300-01	1.317	Secretory fasciclin glycoprotein involved in regulation of anther development and pollen formation
Os01t0859300-01	1.219	bZIP transcription factor, Stress response and ABA signal transduction and regulation of plant fertility
Os01t0578200-00	1.937	Similar to programmed cell death protein 2
Os06t0613400-01	2.881	COM1/SAE2 homolog, Homologous chromosome synapsis and recombination in meiosis
Os05t0251400-01	1.237	A member of the synaptonemal complex (SC), OmpH/coiled-coil motif-containing protein involved in the regulation of meiotic double-strand break (DSB) formation and SC assembly, Meiotic recombination
Os08t0509600-01	2.103	Squamosa promter-binding-like transcription activator involved in the regulation of branching in panicles and vegetative shoots
Os05t0560100-01	1.440	Similar to Pollen-specific kinase partner protein
Os04t0111301-00	1.919	Similar to Clavata-like kinase

4.4.2.4 Genes Down-regulated in the Sterile Panicle of TGMS Line under Sterility Inducing Condition Compared to Fertile Panicle

4.4.2.4.1 Genes Encoding Abiotic Stress Proteins

Genes encoding Hsp40, Hsp 70, Hsp20, Hsp 70 kDa protein 4, low molecular weight heat shock protein precursor (Mitochondrial small heat shock protein 22), Chaperone protein dnaJ 10, Heat shock protein STI (Stress inducible protein) (GmSTI), heat shock transcription factor 31 and protein similar to non-cell-autonomous heat shock cognate protein 70 was down-regulated (Table 32). *Os03t0745000-02* and *Os03t0745000-01* (heat stress transcription factor A-2a), *Os08t0546800-01* (heat stress transcription factor B-2b), *Os04t0568700-00* and *Os09t0456800-01* (heat stress transcription factor Spl7: Heat shock factor RHSF10), *Os03t0161900-01* (isoform 2 of heat stress transcription factor A-2d) and *Os09t0526600-01* and *Os09t0526600-03* (Isoform 2 of Heat stress transcription factor B-2c) was down-regulated in the TGMS sterile panicle.

Peroxidase 1, peroxidase domain containing protein, hydroxyproline-rich glycoprotein family protein, peroxidase 56m, Peroxidase BP 1 precursor, Class III peroxidase 33, Peroxidase 2, Peroxidase 72 precursor (Atperox P72) (PRXR8) (ATP6a), Peroxidase P7 (TP7), proline transporter and L-ascorbate oxidase, TFIIIA-type zinc finger protein which is a transcription activator involved in the abiotic stress tolerance, R-R-type MYB-like transcription factor required in the response to drought stress during reproductive development, signal transduction response regulator, receiver region domain containing protein and pollen Ole e 1 allergen/extensin domain containing protein was also down-regulated in the TGMS panicle under sterility inducing condition.

4.4.2.4.2 Genes Related to Calcium Signalling

Calcium associated genes such as *Os07t0501700-01*, *Os02t0518000-01* and *Os01t0934100-01* genes coding C2 calcium-dependent membrane targeting domain containing protein was down-regulated in the TGMS panicle (Table 33).

Calmodulin related genes like *Os12t0603800-00*, *Os02t0832000-01*, *Os08t0540400-01* (-2.132), *Os02t0126400-02* (-1.071), *Os04t0584600-01* (-1.054), *Os01t0955100-01*, *Os09t0459600-01*, *Os01t0949500-01* Calmodulin, *Os10t0420200-01*, *Os05t0541100-01* and *Os01t0570800-01*, *Os03t0319300-01*, *Os11t0151002-00* and *Os06t0256300-01* were down-regulated in the TGMS panicle. *Os05t0585500-01* (Calcium-dependent protein kinase), *Os05t0223000-01* (TCH2 (TOUCH 2); calcium ion binding) and *Os07t0681400-01* (Calcium-binding protein CAST) were also down-regulated in the TGMS panicle maintained under sterility inducing condition.

4.4.2.4.3 Genes Related to Biosynthesis and Signalling of Phytohormones

Genes encoding Tryptophan aminotransferase in the IAA biosynthesis, Auxin responsive SAUR protein family protein, Auxin response factor 13, AUX/IAA protein family protein, Auxin-induced basic helix-loop-helix transcription factor, Auxin response factor 1, Auxin-induced protein X15, auxin transporter-like protein 1, Auxin-responsive protein IAA18 (Indole acetic acid-induced protein 18), Auxin-responsive protein IAA26 (Indole acetic acid-induced prote in 26: Phytochrome-associated protein 1) were down-regulated in the sterile panicle of TGMS line (Table 34).

Gibberellic acid genes such as *Os06t0176300-00*, *Os02t0630300-01*, *Os04t0182200-01*, *Os04t0667400-01*, *Os10t0559500-01* and *Os03t0439500-01* (2OG-Fe(II) oxygenase domain containing protein), *Os07t0160100-02* (YABBY family transcription factor involved in the feedback regulation of gibberellin biosynthesis which regulates stamen and carpel development), *Os01t0177400-01* (GA 3 beta-hydroxylase2) and *Os01t0209700-01* (GA 2-oxidase 5) was also down-regulated in the sterile panicle.

Table 32. Genes encoding abiotic stress proteins down-regulated in the sterile panicle of TGMS line under sterility

Gene Name	Fold change	Description
Os03t0745000-02 *Os03t0745000-01*	-5.852 -4.980	Heat stress transcription factor A-2a
Os05t0428600-01 *Os03t0276500-01* *Os01t0180800-03*	-3.269 -2.278 -1.078	Similar to Heat shock protein 70
Os08t0546800-01	-3.850	Protein similar to Heat stress transcription factor B-2b
Os07t0443500-00 *Os04t0675400-01* *Os06t0195800-01* *Os05t0562300-01*	-3.568 -1.657 -1.025 -2.250	Molecular chaperone, heat shock protein, Hsp40, DnaJ domain containing protein
Os04t0568700-00 *Os09t0456800-01*	-3.639 -1.094	Similar to Heat stress transcription factor Spl7 (Heat shock factor RHSF10)
Os03t0161900-01	-3.638	Similar to Isoform 2 of Heat stress transcription factor A-2d
Os02t0711300-01	-2.100	Heat shock protein Hsp20 domain containing protein
Os09t0526600-03 *Os09t0526600-01*	-1.795 -1.839	Similar to Isoform 2 of Heat stress transcription factor B-2c
Os06t0212900-01 *Os01t0180800-01*	-1.238 -1.163	Heat shock protein Hsp70 family protein
Os01t0180800-02	-1.169	Similar to heat shock 70 kDa protein 4
Os02t0758000-01	-1.141	Similar to Low molecular weight heat shock protein precursor (Mitochondrial small heat shock protein 22)
Os01t0702450-00	-1.753	Similar to Chaperone protein dnaJ 10
Os06t0163000-03	-1.453	Similar to Heat shock protein STI (Stress inducible protein) (GmSTI)
Os02t0527300-01	-1.424	Similar to Heat shock transcription factor 31
Os03t0821100-01	-1.094	Similar to Non-cell-autonomous heat shock cognate protein 70
Os03t0368300-01 *Os03t0121200-01*	-2.336 -1.886	Similar to Peroxidase 1
Os01t0378100-01 *Os01t0787000-01*	-2.215 -1.895	Plant peroxidase domain containing protein
Os02t0515200-02	-5.259	Similar to hydroxyproline-rich glycoprotein family protein
Os01t0584100-01	-2.133	Similar to hydroxyproline-rich glycoprotein family protein
Os01t0787000-02	-2.030	Similar to peroxidase 56
Os01t0963000-04	-1.678	Similar to Peroxidase BP 1 precursor
Os07t0510900-01	-1.645	Similar to L-ascorbate oxidase
Os03t0121200-03	-1.300	Similar to Class III peroxidase 33
Os03t0434800-00	-1.245	Similar to Peroxidase 2
Os01t0543100-01	-1.203	Similar to Peroxidase 72 precursor (Atperox P72) (PRXR8)
Os02t0236600-01	-1.064	Peroxidase P7 (TP7)
Os01t0621200-00	-1.035	Similar to proline transporter
Os05t0181200-01	-5.616	Similar to Phytochrome P450-like protein
Os03t0820300-01	-1.721	TFIIIA-type zinc finger protein which is a transcription activator involved in the abiotic stress tolerance
Os03t0417700-01	-1.669	Similar to Phytochrome P450
Os05t0442400-01	-1.213	R-R-type MYB-like transcription factor required in the response to drought stress during reproductive development

Table 33. Genes related to calcium signalling down-regulated in the sterile panicle of TGMS line under sterility

Gene Name	Fold change	Description
Os07t0501700-01 *Os02t0518000-01* *Os01t0934100-01*	-2.621 -1.406 -1.239	C2 calcium-dependent membrane targeting domain containing protein
Os12t0603800-00	-2.574	Similar to Calmodulin NtCaM13
Os02t0832000-01	-2.259	Similar to Calmodulin-like-domain protein kinase CPK2
Os08t0540400-01 *Os02t0126400-02* *Os04t0584600-01* *Os05t0585500-01*	-2.132 -1.071 -1.054 -1.288	Similar to Calcium-dependent protein kinase
Os01t0955100-01	-2.006	Similar to Calmodulin-like protein (Fragment)
Os05t0223000-01	-1.811	Similar to TCH2 (TOUCH 2); calcium ion binding
Os07t0681400-01	-1.505	Similar to Calcium-binding protein CAST
Os09t0459600-01	-1.380)	Similar to Calmodulin-binding protein phosphatase
Os01t0949500-01	-1.314	Similar to Calmodulin (CaM)
Os10t0420200-01 *Os05t0541100-01* *Os01t0570800-01*	-1.292 -1.161 -1.165	IQ calmodulin-binding region domain containing protein
Os03t0319300-01	-1.108	Calmodulin involved in signalling of thermotolerance
Os11t0151002-00	-1.064	Similar to Calmodulin binding protein
Os06t0256300-01	-1.027	Similar to Calmodulin-binding heat-shock protein

Table 34. Down-regulated genes related to biosynthesis and signalling of phytohormones in the sterile panicle of TGMS line under sterility

Gene Name	Fold change	Description
Auxin		
Os05t0169300-01	-2.761	Tryptophan aminotransferase in the IAA biosynthesis required in the grain development
Os04t0662400-01 *Os01t0768333-00* *Os12t0626200-01*	-2.323 -2.281 -1.589	Auxin responsive SAUR protein family protein
Os04t0690600-01	-1.961	Similar to Auxin response factor 13
Os02t0723400-02 *Os03t0797800-01*	-1.852 -1.060	AUX/IAA protein family protein
Os04t0526000-01	-1.666	Similar to Auxin-induced basic helix-loop-helix transcription factor
Os06t0196700-01	-1.377	Similar to Auxin response factor 1
Os04t0537100-01	-1.314	Similar to Auxin-induced protein X15
Os01t0856500-04	-1.133	Similar to auxin transporter-like protein 1
Os02t0228900-01	-1.068	Similar to Auxin-responsive protein IAA18 (Indole acetic acid-induced protein 18)
Os01t0190300-01	-1.042	Similar to Auxin-responsive protein IAA26 (Phytochrome-associated protein 1)

Table 34. Down-regulated genes related to phytohormones (Continued)

Gene Name	Fold change	Description
Gibberellic acid		
Os06t0176300-00 *Os02t0630300-01* *Os04t0182200-01* *Os04t0667400-01* *Os10t0559500-01* *Os03t0439500-01*	-4.296 -2.305 -1.181 -1.116 -1.102 -1.031	2OG-Fe(II) oxygenase domain containing protein
Os01t0209700-01	-2.088	Similar to GA 2-oxidase 5
Os07t0160100-02	-1.625	YABBY family transcription factor: feedback regulation of GA biosynthesis which regulates stamen and carpel development
Os01t0177400-01	-1.188	GA 3 beta-hydroxylase2 mediates GA metabolism
Ethylene		
Os08t0474000-01	-4.135	Similar to AP2 domain containing protein RAP2.6
Os02t0781300-00	-3.692	Similar to AP2/ERF domain-containing transcription factor
Os02t0764700-01	-3.648	Similar to Ethylene-responsive transcription factor 4 related to APETALA-2 protein 5
Os07t0674800-01	-2.788	Similar to AP2 domain transcription factor EREBP
Os02t0655200-01	-2.655	Similar to Ap25
Os03t0727600-01	-2.410	ACC synthase enzyme involved in the ethylene biosynthesis
Os01t0580500-01	-1.648	ACC oxidase involved in the ethylene biosynthesis
Os06t0592500-01	-1.517	Similar to Ethylene-responsive transcriptional coactivator
Os02t0531600-01	-1.512	TFL1/CEN ortholog, Phosphatidylethanolamine-binding protein, PEBP family protein which control the phase transition of meristem and control flowering time
Os05t0361700-02	-1.186	Similar to ethylene responsive element binding protein 2
Os02t0771600-01	-1.056	Similar to 1-aminocyclopropane-1-carboxylate oxidase
Jasmonic acid		
Os10t0391400-01 Os03t0181100-01 *Os03t0180900-01* *Os03t0181100-02* *Os04t0395800-01* *Os10t0392400-01*	-3.845 -2.382 -2.266 -2.261 -2.152 -1.687	Tify domain containing protein
Os03t0225900-01	-2.025	Allene oxide synthase (CYP74A2) involved in the biosynthesis of jasmonic acid
Os03t0767000-01	-1.760	Allene oxide synthase (CYP74A1) involved the biosynthesis of jasmonic acid
Cytokinin		
Os11t0143300-02 *Os12t0139400-01* *Os02t0830200-01* *Os02t0631700-01*	-1.350 -1.264 -1.261 -1.228	A-type response regulator in the cytokinin signalling
Os02t0796500-03	-1.661	B-type response regulator in the cytokinin signalling
Os01t0187600-01	-1.279	Similar to Cytokinin dehydrogenase 1
Brassinosteroid		
Os10t0533150-00 *Os01t0323000-02*	-1.235 -1.066	Similar to BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
Os08t0562500-01	-1.220	BAHD acyltransferase-like protein involved in the control of grain size, leaf angle and regulation of brassinosteroid homeostasis
Abscissic acid		
Os02t0703600-01	-1.085	Similar to Abscisic acid 8'-hydroxylase 1

Os11t0143300-02, *Os12t0139400-01*, *Os02t0830200-01*, *Os02t0631700-01*, *Os02t0557800-01* and *Os11t0143300-01* (A-type response regulator in the cytokinin signalling), *Os02t0796500-03* (B-type response regulator involved in the cytokinin signalling) and *Os01t0187600-01* (Cytokinin dehydrogenase 1) were down-regulated.

Os08t0474000-01 (-4.135) gene coding protein similar to AP2 domain containing protein RAP2.6, *Os02t0781300-00* (-3.692) gene encoding protein similar to AP2/ERF domain-containing transcription factor, *Os02t0764700-01* (-3.648) gene coding protein similar to Ethylene-responsive transcription factor 4 related to APETALA-2 protein 5, *Os07t0674800-01* (-2.788) gene encoding protein similar to AP2 domain transcription factor EREBP, *Os02t0655200-01* (-2.655) producing protein similar to Ap25, *Os03t0727600-01* (-2.410) gene encoding ACC synthase enzyme and *Os01t0580500-01* (-1.648) encoding enzyme ACC oxidase was down-regulated.

Os06t0592500-01 (Ethylene-responsive transcriptional coactivator), *Os02t0531600-01* (TFL1/CEN ortholog, Phosphatidylethanolamine-binding protein, PEBP family protein which controls the phase transition of meristem and control flowering time), *Os05t0361700-02* (ethylene responsive element binding protein 2) and *Os02t0771600-01* (1-aminocyclopropane-1-carboxylate oxidase) was down-regulated in the panicle maintained in the sterility inducing condition.

Genes coding Tify domain containing protein, Allene oxide synthase (CYP74A2) involved in the biosynthesis of jasmonic acid and Allene oxide synthase (CYP74A1) were down-regulated in the sterile panicle. Brassinosteroid related *Os10t0533150-00* and *Os01t0323000-02* (BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1), *Os08t0562500-01* (BAHD acyltransferase-like protein involved in the control of grain size, leaf angle and regulation of brassinosteroid homeostasis) and *Os10t0533150-00*, *Os01t0323000-02* and *Os08t0562500-01* brassinosteroid associated genes were down-regulated.

Os02t0703600-01 (-1.085) gene encoding protein similar to Abscisic acid 8'-hydroxylase 1 was also down-regulated in the sterile panicle of TGMS line.

4.4.2.4.4 Genes Regulating Flower Development

Os06t0607700-01 (-10.727) gene encoding ATP-binding cassette (ABC) transporter which is involved in the post-meiotic anther and pollen development was down-regulated significantly in the panicle in the sterility inducing condition (Table 35). A Protease inhibitor, lipid transfer protein (LTP) encoding gene involved in postmeiotic anther development, *Os11t0582500-01* (-9.844) was also down-regulated. *Os10t0524500-01* (-8.300) gene coding enzyme putative glucose-methanol-choline oxidoreductase which regulates tapetum degeneration, pollen exine formation and anther cuticle formation, *Os10t0484800-01* (-7.737) gene which produces an orthologue of *Arabidopsis* PKSA/LAP6 involved in the regulation of pollen exine formation, *Os03t0263600-01* (-6.006) gene encoding atypical strictosidine synthase involved in the regulation of anther development and pollen wall formation and *Os05t0181200-01* (-5.616) and *Os03t0417700-01* (-1.669) gene encoding protein similar to Phytochrome P450-like protein was down-regulated in the sterile panicle.

Os03t0167600-01 (-5.869: Male sterility protein 2), *Os08t0131100-01* (-4.852: Cytochrome P450 hydroxylase which regulates anther cuticle and pollen exine development), *Os04t0310800-01* (-4.232 Fatty acyl-CoA synthetase which controls pollen exine formation, anther development and sporopollenin synthesis), *Os02t0621300-01* (-3.404: homologous protein of CER1, very-long-chain (VLC) alkane biosynthesis involved in the regulation of anther development and plastids differentiation), *Os03t0581400-00* and *Os01t0728100-02* (anther-specific proline-rich protein APG) were also down-regulated. *Os06t0113800-00* (Kinetechore protein-like), *Os01t0594300-02*, *Os01t0180000-01* and *Os08t0108300-01* (pistil-specific extensin-like protein family protein), *Os01t0900400-01* (putative triacylglycerol (TAG) lipase), *Os10t0494300-01* (ATP binding cassette G

transporter) and *Os01t0309100-01* (anther-specific protein SF18) was down-regulated in the panicle.

Genes encoding RAFTIN1a protein (RAFTIN1a anther protein), Beta-1, 3-glucanase-like protein, pollenless3, basic helix-loop-helix transcription factor which is the regulator of tapetal programmed cell death during the male reproductive development, pectate lyase-like protein involved in pollen development, phosphatase 2C required in abiotic stress response and regulated early panicle development, WRKY transcription factor which is involved in the control of flowering time and plant height, F-box-containing protein important in male meiotic DNA double-strand break repair and controls meiotic progression, pollen-specific phospholipase, Patatin like phospholipase A which is vital in maintaining pollen viability, FLOWERING LOCUS T protein, AGAMOUS homolog, autonomous floral activator and Filamentous flower-like yabby protein were also down-regulated in the TGMS panicle maintained under sterility inducing condition.

Other down-regulated genes include *Os01t0883100-01* (PISTILLATA-like MADS box protein), *Os02t0682200-01* (MADS-box transcription factor involved in the regulation of floral organ identity and meristem fate), *Os04t0411400-01* (Terminal flower 1-like protein), *Os07t0667300-01* (CONSTANS-like transcriptional activator involved in the negative regulation of flowering), *Os05t0494600-01* (circadian clock coupling factor ZGT), *Os09t0480900-01* (Anther-specific protein), *Os01t0889400-01* (LOB (LATERAL ORGAN BOUNDARIES) domain-containing protein involved in the regulation of empty-glume identity, floral organ number control and female gametophyte development) and *Os04t0168400-01* (embryogenesis transmembrane protein) was down-regulated.

Wax synthase domain containing protein encoding gene, *Os01t0651500-00* (-9.110) was down-regulated in the panicle under sterility inducing condition. Sucrose transporter, cell-wall invertase enzyme involved in the carbon

partitioning during early grain filling, sugar/inositol transporter domain containing protein, Apoptosis regulator Bcl-2 protein, BAG domain containing protein, Polygalacturonase, DnaJ domain containing protein (ER-resident J-protein 3A, *THERMOSENSITIVE MALE STERILE 1*), R2R3-type MYB transcription factor (*Carbon Starved Anther*) responsible for sugar partitioning into anther and *UDPase* involved in pollen mother cell meiosis and pollen development was also down-regulated in the TGMS panicle under sterility inducing condition.

4.4.2.4.5 Pathways Up-Regulated and Down Regulated in the TGMS Panicle under Sterility Inducing Condition

Pathways such as ethylene biosynthesis, tryptophan biosynthesis, cytokinin degradation, cytokinin-O-glucoside biosynthesis (23 genes), starch and sucrose metabolism/degradation (7 genes), 9-N-glucoside biosynthesis (23 genes), 4-hydroxyproline degradation and proline degradation were up-regulated in the TGMS sterile panicle (Table 37). Pathways like cellulose biosynthesis, brassinosteroid biosynthesis (16 genes), IAA biosynthesis, gibberellin biosynthesis, jasmonic acid biosynthesis, starch and sucrose biosynthesis, fatty acid biosynthesis, removal of superoxide radicals, phospholipid biosynthesis, biosynthesis of unsaturated fatty acids, carbon fixation in photosynthetic organisms, glycolysis/gluconeogenesis, photosynthesis, circadian rhythm were down-regulated in the TGMS panicle maintained under sterility inducing condition (Table 37).

Table 35. Down-regulated genes regulating flower development in the sterile panicle of TGMS line under sterility

Gene Name	Fold change	Description
Os06t0607700-01 *Os03t0742300-0*	-10.73 -3.447	ATP-binding cassette (ABC) transporter which is involved in the post-meiotic anther and pollen development
Os11t0582500-01	-9.844	Protease inhibitor, lipid transfer protein (LTP) encoding gene involved in postmeiotic anther development
Os01t0651500-00	-9.110	Wax synthase domain containing protein
Os10t0484800-01	-7.737	An Orthologue of Arabidopsis PKSA/LAP6 involved in the regulation of pollen exine formation
Os03t0263600-01	-6.006	Atypical strictosidine synthase involved in the regulation of anther development and pollen wall formation
Os02t0576600-01 *Os02t0827200-01*	-6.684 -1.043	Sucrose transporter
Os03t0167600-01	-5.869	Similar to Male sterility protein 2
Os08t0131100-01	-4.852	Cytochrome P450 hydroxylase which regulates anther cuticle and pollen exine development
Os04t0310800-01	-4.232	Fatty acyl-CoA synthetase which controls pollen exine formation, anther development and sporopollenin synthesis
Os02t0621300-01	-3.404	Homologous protein of CER1, Very-long-chain (VLC) alkane biosynthesis involved in the regulation of anther development and plastids differentiation
Os01t0900400-01	-2.312	Putative triacylglycerol (TAG) lipase, Phospholipase A1 involved in the specification of empty-glume identity, regulation of spikelet development and control of endogenous jasmonic acid (JA) biosynthesis
Os10t0494300-01	-2.270	ATP binding cassette G transporter which controls male reproduction and anther cuticle development
Os03t0581400-00 *Os01t0728100-02*	-1.971 -1.547	Similar to anther-specific proline-rich protein APG
Os04t0413500-01	-2.011	Cell-wall invertase enzyme involved in the carbon partitioning during early grain filling
Os04t0511400-02	-1.017	Sugar/inositol transporter domain containing protein
Os01t0594300-02 *Os08t0108300-01*	-1.689 -1.303	Pistil-specific extensin-like protein family protein
Os01t0309100-01	-1.005	Similar to anther-specific protein SF18
Os08t0496800-01	-2.165	Similar to RAFTIN1a protein (RAFTIN1a anther protein)
Os03t0792800-01 *Os05t0399200-01*	-2.049 -1.282	Beta-1, 3-glucanase-like protein
Os05t0495900-01	-1.728	Similar to Beta-1, 3-glucanase precursor
Os03t0165900-02	-1.937	Similar to pollenless3
Os04t0599300-01	-1.931	Basic helix-loop-helix transcription factor which is the regulator of tapetal programmed cell death during the male reproductive development

Table 35. Down-regulated genes regulating flower development in the sterile panicle of TGMS line under sterility (Continued)

Gene Name	Fold change	Description
Os02t0120500-01	-1.594	Basic helix-loop-helix (bHLH) transcription factor essential in tapetum development and degeneration
Os02t0214400-01	-1.793	Pectate lyase-like protein involved in pollen development
Os09t0325700-01	-1.226	Protein phosphatase 2C required in abiotic stress response and regulated early panicle development
Os01t0626400-01	-1.209	WRKY transcription factor which is involved in the control of flowering time and plant height
Os04t0464966-01	-1.209	F-box-containing protein important in male meiotic DNA double-strand break repair and controls meiotic progression
Os03t0393900-01	-1.185	Pollen-specific phospholipase, Patatin like phospholipase A which is vital in maintaining pollen viability
Os04t0488400-01	-2.204	Similar to FLOWERING LOCUS T protein
Os01t0886200-01	-1.657	Similar to AGAMOUS homolog
Os11t0547000-01	-1.526	Autonomous floral activator essential in the promotion of flowering
Os12t0621100-01 *Os12t0621100-02*	-1.494 -1.436	Similar to Filamentous flower-like yabby protein
Os01t0883100-01	-1.278	Similar to PISTILLATA-like MADS box protein was down regulated. PISTILLATA genes control organ determination in the second and third whorls of flower
Os02t0682200-01	-1.253	MADS-box transcription factor involved in the regulation of floral organ identity and meristem fate
Os04t0411400-01	-1.520	Similar to Terminal flower 1-like protein
Os07t0667300-01	-1.214	CONSTANS-like transcriptional activator involved in the negative regulation of flowering
Os05t0494600-01	-1.161	Similar to circadian clock coupling factor ZGT
Os09t0480900-01	-1.403	Similar to Anther-specific protein
Os01t0889400-01	-1.152	LOB (LATERAL ORGAN BOUNDARIES) domain-containing protein involved in the regulation of empty-glume identity, floral organ number control and female gametophyte development
Os04t0168400-01	-2.312	Similar to Embryogenesis transmembrane protein
Os06t0611500-01	-2.204	similar to Polygalacturonase
Os02t0120500-01	-1.594	basic helix-loop-helix (bHLH) transcription factor needed for the tapetum development and degeneration
Os03t0293000-01	-0.324	similar to DnaJ domain containing protein (ER-resident J-protein 3A, *THERMOSENSITIVE MALE STERILE 1*)
Os01t0274800-01	-0.875	R2R3-type MYB transcription factor (Carbon Starved Anther) responsible for sugar partitioning into anther
Os09t0553200-01	-0.467	UDPase involved in pollen mother cell meiosis and pollen development

Table 36. Pathways up-regulated and down regulated in the TGMS panicle under sterility inducing condition

Pathway	Genes	No of genes
Up-regulated pathways		
Ethylene biosynthesis	*OS05G0196600,OS02G0302200*	2
Cytokinins degradation	*OS01G0197700*	1
Proline degradation	*OS02G0730000, OS02G0730000*	2
Sucrose degradation	*OS02G0831500,OS06G0476200*	2
Cytokinins-O-glucoside biosynthesis	*OS03G0814500,OS02G0207100,OS05G0526900,OS04G0324000,OS06G0282400,OS02G0242550,OS09G0330000,OS01G0805500,OS08G0489100,OS05G0493600,OS10G0162980,OS04G0272700,OS01G0176100,OS02G0206700,OS01G0734800,OS04G0314100,OS01G0176200,OS03G0757600,OS05G0526800,OS05G0215300,OS10G0178500,OS04G0204100,OS06G0343600*	23
Tryptophan biosynthesis	*OS03G0797000,OS06G0614000*	2
Starch degradation	*OS06G0675700,OS01G0847300,OS06G0476200*	3
Cytokinins 9-N-glucoside biosynthesis	*OS03G0814500,OS02G0207100,OS05G0526900,OS04G0324000,OS06G0282400,OS02G0242550,OS09G0330000,OS01G0805500,OS08G0489100,OS05G0493600,OS10G0162980,OS04G0272700,OS01G0176100,OS02G0206700,OS01G0734800,OS04G0314100,OS01G0176200,OS03G0757600,OS05G0526800,OS05G0215300,OS10G0178500,OS04G0204100,OS06G0343600*	23
4-hydroxyproline degradation I	*OS02G0730000*	1
Cytokinins 7-N-glucoside biosynthesis	*OS03G0814500,OS02G0207100,OS05G0526900,OS04G0324000,OS06G0282400,OS02G0242550,OS09G0330000,OS01G0805500,OS08G0489100,OS05G0493600,OS10G0162980,OS04G0272700,OS01G0176100,OS02G0206700,OS01G0734800,OS04G0314100,OS01G0176200,OS03G0757600,OS05G0526800,OS05G0215300,OS10G0178500,OS04G0204100,OS06G0343600*	23
Starch and sucrose metabolism	*OS09G0530200,OS08G0345800,OS06G0675700,OS02G0831500,OS06G0232200,OS02G0744800,OS06G0676700*	7
Down-regulated pathways		
Cellulose biosynthesis	*OS06G0256900,OS01G0312800,OS01G0942300*	3
Brassinosteroid biosynthesis	*OS04G0630900,OS04G0630300,OS08G0277200,OS01G0237750,OS10G0576900,OS04G0618200,OS02G0180700,OS08G0515900,OS04G0630100,OS09G0526700,OS02G0791500,OS01G0150000,OS01G0237500,OS07G0602000,OS01G0837300,OS01G0127500*	16

Table 37. Pathways up-regulated and down regulated in the TGMS panicle under sterility inducing condition (Continued)

Pathway	Genes	No of genes
Fatty acid biosynthesis	*OS04G0445700,OS06G0143400,OS07G0616200*	3
Removal of superoxide radicals	*OS02G0115700*	1
IAA biosynthesis	*OS11G0170000, OS11G0170000*	2
Phospholipid biosynthesis	*OS12G0121300,OS01G0895200,OS06G0492000*	3
Jasmonic acid biosynthesis	*OS03G0767000,OS03G0438100,OS03G0738600,OS01G0582600,OS03G0179900,OS08G0508800,OS01G0186900,OS03G0700700,OS04G0670500,OS05G0183900,OS08G0509100,OS04G0447100*	12
Biosynthesis of unsaturated fatty acids	*OS03G0290300,OS10G0457600,OS04G0271200,OS01G0150000,OS07G0416900*	5
Carbon fixation in photosynthetic organisms	*OS10G0503500,OS12G0292400,OS04G0234600,OS05G0186300*	4
Fatty acid biosynthesis	*OS10G0484800,OS02G0205500,OS03G0220100,OS07G0616200*	4
Glycolysis / Gluconeogenesis	*OS05G0469800,OS11G0210300,OS11G0138600,OS07G0197100,OS06G0499900,OS04G0458600,OS05G0469600,OS03G0381000,OS02G0575800*	9
Photosynthesis	*OS07G0513000*	1
Starch biosynthesis	*OS06G0229800,OS09G0469400*	2
Gibberellin biosynthesis	*OS01G0177400*	1
Sucrose biosynthesis	*OS07G0616800,OS11G0236100*	2
Circadian rhythm - plant	*OS11G0547000*	1

DISCUSSION

5. DISCUSSION

The demand for rice to sustain the self sufficiency is increasing rapidly and more rice has to be produced from currently available limited resources. Hybrid rice technology utilizing two line breeding method is the only proven technique to accelerate the rice production since hybrids can produce 20-30% higher yield than conventional varieties and they can perform better under stress conditions. Although many rice hybrids have been released in India using CMS system, they are with white slender grain. It is not suitable to the state of Kerala since the people prefer to consume red/bold grain. At this circumstances, it is essential to develop red rice hybrids suitable to the state of Kerala and TGMS system is the best option. With this objective, stable TGMS line EC720903 was imported from IRRI, Philippines and used as the donor plant for transferring male sterile gene to popular red rice variety of Kerala, Jyothi. The sterile F_2 plants obtained were backcrossed to recover maximum red rice character.

The TGMS trait is transferrable to any background and can be utilized to produce red rice hybrids suitable for commercial cultivation in the state of Kerala since the trait is controlled by recessive nuclear gene (Borkakati and Virmani, 1996; Virmani *et al.*, 2003) and temperature is the determining factor for expression of sterility/fertility in TGMS lines (Viraktamath and Virmani, 2001; Latha *et al.*, 2004; Chandirakala *et al.*, 2008; Latha and Thiyagarajan, 2010). TGMS lines used in two line breeding programme are sensitive to certain temperature when it is exposed during a specific stage of floral development. TGMS lines regains partial fertility even with mild variations in temperature during the thermosensitive phase.

The present study 'Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was conducted for evaluating the environmental conditions required for complete male sterility of TGMS plants and to manipulate the male sterility by using plant growth regulators. The study also envisaged to understand the molecular mechanism associated with TGMS system. The results obtained from the study are discussed in this chapter.

5.1 EVALUATION OF CLIMATIC CONDITIONS FOR TGMS SYSTEM

The critical limit of temperature for inducing fertility and sterility is specific for different TGMS lines (Lopez and Virmani, 2000; Viraktamath and Virmani, 2001; Santosh, 2003; Lee *et al.*, 2005; RamaKrishna *et al.*, 2006; Chandirakala *et al.*, 2008; Thiyagarajan *et al.*, 2010; Salgotra *et al.*, 2012; Wongpatsa *et al.*, 2014; Manonmani *et al.*, 2016; Kumar *et al.*, 2016). Therefore, each TGMS line should be characterized for determining its CST and critical thermosensitive stage. Moreover the success of hybrid rice technology depends on the stability in sterility/fetility expression (Virmani *et al.*, 2003; Lu *et al.*, 1994). The critical thermosensitive stage for fertility alteration in the TGMS line varies from 5-15 days after panicle initiation or 15 to 25 days before heading (Virmani *et al.*, 1997; Viraktamath and Virmani, 2001; Kalaiyarasi and Vaidyanathan, 2003; Santosh, 2003; Pham *et al.*, 2004a; Sanchez and Virmani, 2005; Salgotra *et al.*, 2012; Rekha *et al.*, 2017). The critical thermosensitive stage of the stable TGMS line EC720903 was 15-22 days before flowering with a sterility inducing mean temperature of 26.9°C (Celine *et al.*, 2014).

In this context the newly developed TGMS line in the red rice background was evaluated to identify the ideal season for hybridization and seed multiplication. The plants were raised under two different growing conditions in the open field and inside the Rain Out Shelter (ROS) for twelve months as a preliminary step in the exploitation of TGMS red rice line for commercialization.

Analysis of weather data revealed that the mean temperature during the critical thermosensitive stage *i.e.*, 15-22 days before flowering, of all the monthly sown TGMS red rice were above the critical sterility inducing average temperature of 26.9°C under both conditions (Table 37; Fig. 16). The maximum and mean temperature recorded throughout the crop period inside the rain out shelter was more compared to ambient temperature whereas the relative humidity observed was lower inside the rain out shelter. The variations in the weather parameters may be due to physical properties of UV stabilized covering material, which traps the long wave radiations. Pandey *et al.* (2005) and Dhandare *et al.* (2008) observed high temperature under UV stabilized sheets when compared to open field. Similar findings were reported by Hirama *et al.*

(2003), Shahak *et al.* (2008) and Pooja and Hakkim (2017). The relative humidity recorded inside the rainshelter was lower compared to open field. This is in consistent with the results obtained by Parvej *et al.* (2010) that RH under UV stabilized structure was always 5-10 per cent lower than open condition. Besides temperature, relative humidity and sunshine hours were also found to influence fertility alteration as reported by Latha *et al.* (2004), Chandirakala *et al.* (2008) and Latha and Thiyagarajan (2010).

The pollen sterility and spikelet sterility were monitored under two growing conditions. The newly developed TGMS line on red rice background exhibited stable sterility throughout the study period at both the experimental conditions (Table 7). Anatomical studies on anther revealed that it was pollen free which is a specific character of TGMS line EC720903 to produce pollen free anthers during complete sterility period (Plate 5, 6). The same line EC720903 displayed acceptable seed setting percentage (>80%) in the high altitude zone where the mean temperature is below the critical sterility inducing average temperature. TGMS line Annong S-1 produced empty (pollen free) anthers when grown under high temperature conditions (Huang *et al.*, 2014). According to Lu *et al.* (1994), the TGMS lines with more than 30 per cent self-seed set under low temperature condition can be considered as promising lines for commercial exploitation. Spikelet sterility at the time of maturity was observed and was completely sterile. The average temperature prevailed during the critical thermosensitive period of monthly sown TGMS red rice line was above the sterility inducing average temperature of 26.9 °C and caused complete pollen and spikelet sterility. The TGMS lines will be sterile when exposed to CST during its thermosensitive stage (Virmani *et al.*, 1997; Lopez and Virmani, 2000; Celine *et al.*, 2014; Wongpatsa *et al.*, 2014; Rekha *et al.*, 2017).

Characterization of TGMS lines for morphological as well as phenological traits is a requirement to commercially exploit TGMS lines (Virmani *et al.*, 1997; Kalaiyarasi and Vaidyanathan, 2002). The morphological characters such as plant height and number of productive tillers were higher for the rain out shelter grown TGMS red rice plants. The modified micro-climate inside the rain out shelter favoured the growth of TGMS lines compared to open field condition. It was noted that the light intensity measured inside rain out shelter was 48.14 % less compared to open field. The low light

intensity observed inside the rain out shelter caused increased plant height of TGMS red rice line (Appendix III). The weather condition during the growth period of TGMS rice in open condition was coincided with low light intensity and low temperature which may be the reason for increased number of productive tillers. Smith (2000), Kittas *et al.* (2006) and Li *et al.* (2014) also reported increased plant height under low light conditions. The increase in plant height may also be related to high temperature inside the rain out shelter. Oh-e *et al.* (2007) stated that the increase in plant height was steeper under high temperature compared to ambient condition. This is also in agreement with the findings of Krishnan *et al.* (2011) in which the plant height of rice increased with the rise of temperature within the range of 30 to 35^{0}C.

Early flowering was also observed in the rain out shelter grown crops. Earliness in flowering compared to open field crops may be due to high temperature prevailed inside the rain out shelter. In general, low temperature delays and high temperature hastens heading in rice (Yoshida, 1973; Krishnan *et al.*, 2011). Higher maximum and minimum temperature are more conducive for early flowering in rice varieties as observed by Sridevi and Chellamuthu (2015).

The seed production and propagation of TGMS lines in two line system of hybridization is not difficult as that of three line system since maintainer line to multiply sterile lines are not required. However sterility/fertility alterations of sensitive phases of TGMS lines for different areas need to be determined, so that the appropraite locations for seed multiplication and hybrid seed production can be identified. A crop calendar was generated considering the weather data for the Thiruvananthapuram district to schedule the hybrid seed production (Fig. 17). Crop calendar depicts the ideal season for hybrid seed production and seed multiplication of TGMS lines. The weather data observed in the present study suggests that September, October and November (second crop season) sowing should be avoided for hybrid seed production in Vellayani location. There is a chance for partial sterility because the difference between CST and mean temperature during the second crop sowing was only marginal (Table 36). Crop calendar can also be developed for each district for scheduling hybrid seed production based on the weather data. It is also evident that the first crop season (April-May to September-October) and third crop season (December-January to March-April) are ideal

for hybrid seed production. At the same time the average temperature recorded during the critical thermosensitive period of all the monthly sown crops inside the rain out shelter were considerably above the CST of 26.9^{0}C. Thus hybrid rice seed production can be assured round the year inside the rain out shelter. Hence this low cost structure which is covered with a UV stabilized transparent sheet can be used for commercial hybrid seed production using TGMS lines throughout the year.

Thirty year mean temperature data recorded in fourteen districts of Kerala were analysed to find out the suitable season for hybrid seed production and seed multiplication of TGMS lines (Fig. 18). It is suggested that third crop season is ideal for hybrid seed production in all the districts of Kerala except high altitudes of Idukki and Wayanad. Moreover the first and second crop season should be avoided for hybrid seed production since there exists only narrow difference between the mean temperature recorded and CST. The hybrid seeds produced in the third crop season can be utilized for sowing in the first and second crop season. In Wayanad and Idukki, average temperature was always below the CST, hence seed multiplication can be done throughout the year.

The TGMS red rice line is performing similar to the donor parent in red rice background also, hence confirms the transfer of male sterile gene. Therefore the TGMS line can be recommended to the state of Kerala as a female parent for the development of suitable rice hybrids. Third crop season is ideal for hybrid seed production in all the districts. In high altitude regions, seed multiplication can be done throughout the year. Additionally year round commercial hybrid seed production can be guarenteed using the low cost rain out shelters.

Table 37. Weather parameters at College of Agriculture, Vellayani during the critical thermosensitive stages (15-22 days before flowering) of monthly sown TGMS red rice

Month of sowing	Open field						Rain out shelter				
	Minimum Temperature (°C)	Maximum Temperature (°C)	Mean Temperature (°C)	*RH (%)	Sunshine hours (h)	Rainfall (mm)	Minimum Temperature (°C)	Maximum Temperature (°C)	Mean Temperature (°C)	*RH (%)	Rainfall (mm)
May	24.9	32.0	28.5	84.0	9.1	8.6	24.1	37.4	30.8	77	0.0
June	24.4	31.8	28.1	84.8	7.1	13.3	23.8	39.1	31.4	75.3	0.0
July	25.0	31.5	28.3	87.3	7.9	15.7	24.3	35.4	29.8	74.3	0.0
August	24.7	31.0	27.8	91.7	6.6	13.3	24.2	36.8	30.5	74.8	0.0
September	23.9	30.9	27.4	87.5	5.6	16.5	24.4	31.8	28.1	75.4	0.0
October	22.3	31.7	27.0	83.5	9.3	9.3	21.5	38.4	29.9	61.3	0.0
November	22.8	31.8	27.3	83.6	8.6	8.6	21.7	38.5	30.1	55.5	0.0
December	23.6	32.9	28.2	83.8	9.3	9.3	22.3	39.3	30.8	49	0.0
January	25.7	34.3	30.0	80.5	9.3	1.1	23.9	39.4	31.6	57	0.0
February	26.0	34.0	30.0	79.9	7.9	6.1	24.0	42.3	33.1	60.9	0.0
March	24.9	31.2	28.1	89.0	3.9	15.6	23.5	40.7	32.1	68.8	0.0
April	24.5	31.5	28.0	83.4	7.5	5.1	23.7	40.7	32.2	64.5	0.0

*RH: Relative humidity

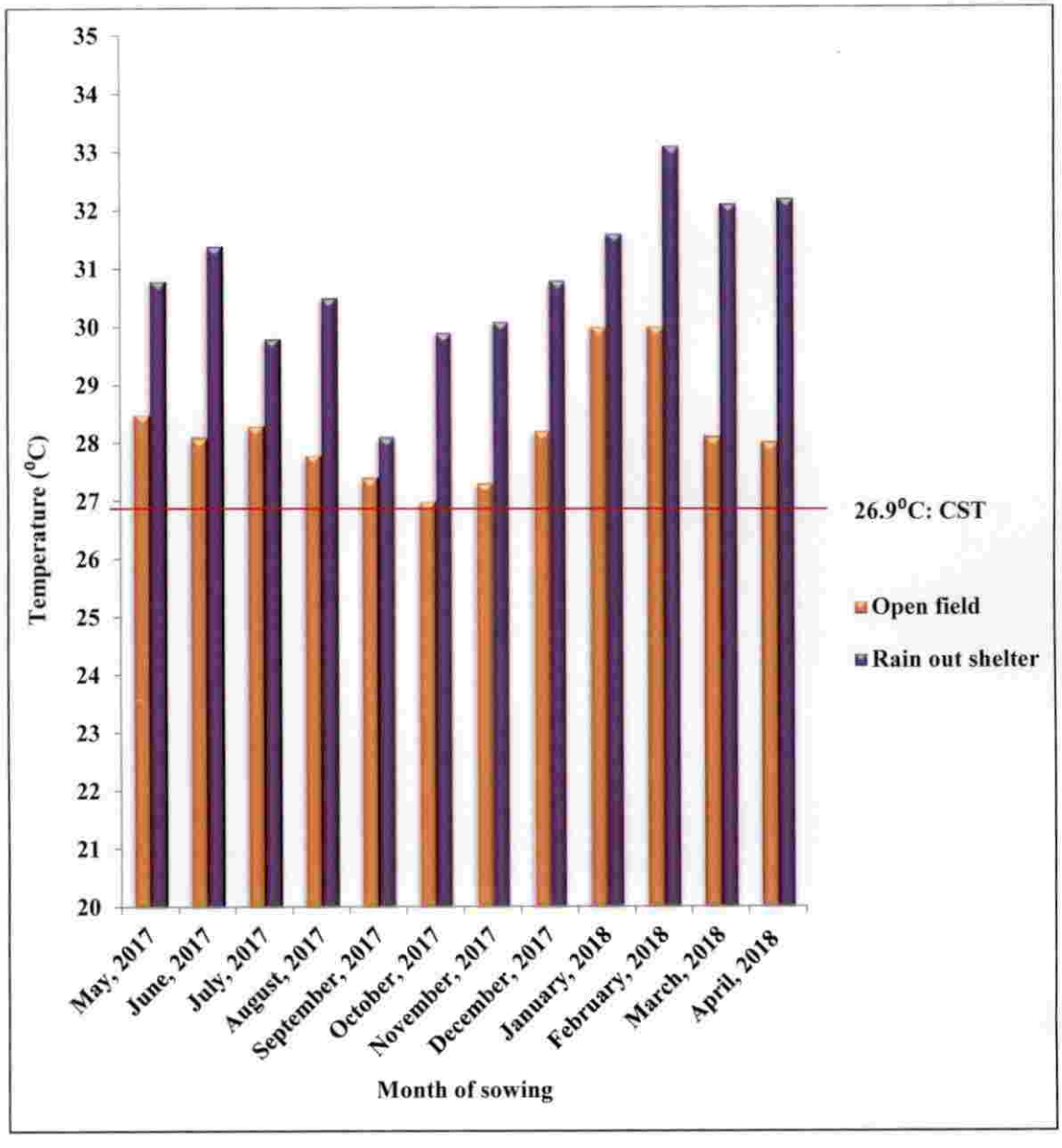
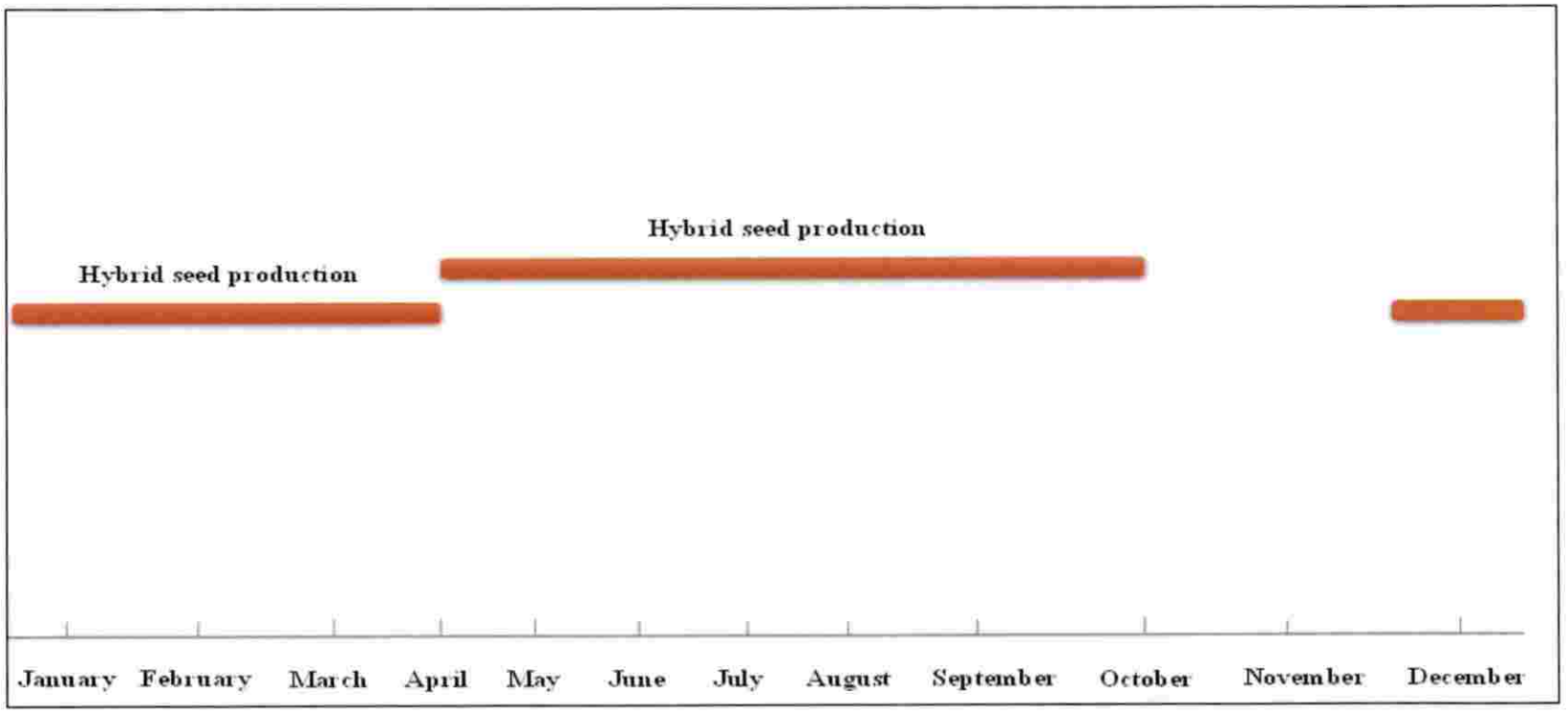


Fig. 16 Mean temperature (^{0}C) during the critical thermosensitive stage of TGMS plants in the open field and inside the rain out shelter at College of Agriculture, Vellayani



First crop season (April-May to September-October) and third crop season (December-January to March-April) ideal for hybrid seed production

Fig. 17 Crop calendar showing ideal season for hybrid rice seed production using TGMS lines at Vellayani location

	January	February	March	April	May	June	July	August	September	October	November	December
Thiruvananthapuram	27.0	28.0	28.50	29.00	28.50	27.50	27.00	27.0	27.50	27.5	27.00	27.00
Kollam	27.0	28.0	29.50	29.50	29.00	27.00	26.50	27.0	27.50	27.0	27.00	27.00
Pathanamthitta	27.0	29.0	30.00	28.00	27.50	26.50	26.00	26.0	26.00	26.0	26.50	25.50
Alappuzha	27.5	28.0	29.50	29.50	29.00	27.00	26.50	26.5	27.00	27.5	28.00	27.50
Kottayam	27.0	26.0	29.50	29.00	28.50	26.50	26.50	26.5	27.00	27.0	27.50	27.50
Idukki	19.5	24.5	23.50	24.00	23.50	21.00	19.50	20.5	21.50	21.5	20.50	19.50
Ernakulam	27.5	28.0	29.00	29.00	28.50	27.00	26.50	26.5	27.00	27.5	27.50	27.50
Thrissur	27.0	28.5	29.50	30.00	29.00	26.50	26.00	26.5	26.50	27.0	27.50	26.50
Palakkad	28.0	29.0	30.50	31.00	29.50	26.50	26.00	26.5	27.00	27.5	28.00	27.50
Malappuram	28.0	29.0	29.50	29.50	30.00	27.00	26.00	27.0	27.50	28.0	28.00	28.00
Wayanad	21.5	22.0	24.50	24.50	24.50	22.50	21.00	22.0	22.50	22.0	21.50	21.00
Kozhikode	27.0	28.0	29.50	29.50	29.00	27.00	26.00	26.0	27.00	27.5	27.50	27.00
Kannur	28.0	29.0	30.50	30.50	30.50	27.50	26.50	27.0	27.50	28.0	28.50	28.50
Kasargode	26.5	27.0	28.00	29.50	29.50	27.00	26.00	26.5	26.50	27.0	27.00	26.50

Ideal for hybrid seed production (third crop season: December-January to March-April)

Fig. 18 Crop calendar depicting the crop seasons suitable for hybridization based on thirty year mean temperature data of fourteen districts of Kerala

5.2 EVALUATION OF PLANT GROWTH REGULATORS FOR INDUCING COMPLETE MALE STERILITY

Low temperature interference during the critical thermosensitive stage can alter the male sterility/fertility behaviour in TGMS lines. Even slight variation in the mean temperature can induce partial fertility in TGMS lines, since the sterility expression is regulated by temperature and becomes difficult to maintain the hybrid seed quality. The environmental casualities such as sudden depression of temperature due to rainfall even in summer months necessitates the development of techniques to maintain the male sterility expression. Thus it is essential to find out an alternate strategy to manipulate sterility/fertility expression. Hence we have evaluated the potential of plant growth regulators for inducing male sterility in TGMS rice since they have a major role in plant reproductive development. The plant growth regulators, ethrel, maleic hydrazide and salicylic acid were selected based on their role played in the anther and pollen development in rice. With this objective, the TGMS line EC720903 was sown at RARS, Ambalavayal, a high altitude zone where the mean temperature is below CST throughout the year which is congenial for the suppression of male sterility gene expression (Appendix IV). Moreover high altitude zone is ideal for seed multiplication and the same line showed an acceptable seed setting percentage of more than 80%.

The stages between panicle initiation and flowering especially 15 to 22 days before anthesis is considered as critical thermosensitive stage in TGMS rice (Borkakati and Virmani, 1997,; Virmani *et al.*, 1997; Viraktamath and Virmani, 2001; Pham *et al.*, 2004a; Sanchez and Virmani, 2005; Sharma and Sharma, 2005; Celine *et al.*, 2014; Rekha *et al.*, 2017). More precisely the panicle developmental stages from stamen and pistil primordial differentiation to meiotic division of pollen mother cell have been described as most critical stages for TGMS lines (Borkakati and Virmani, 1997).

Thus based on the thermosensitive stage of TGMS lines it was decided to apply three plant growth regulators *viz.*, ethrel, maleic hydrazide and salicylic acid

as foliar spray at panicle initiation stage and two weeks after panicle initiation stage to asses the pollen sterility. Initiation of panicle, which is the start of reproductive phase, was confirmed by split opening the primary tiller longitudinally from base and identified through microscope (Plate 4).

The three growth regulators *viz*., ethrel, maleic hydrazide and salicylic acid evaluated at different concentrations were capable of enhancing pollen sterility in all the treated TGMS rice. The pollen sterility percentage was significantly high in maleic hydrazide (1000 mg L^{-1}) treated plants which were on par with salicylic acid (600 mg L^{-1}), ethrel (400 mg L^{-1} and 800 mg L^{-1}) and maleic hydrazide (800 mg L^{-1}). Control plants recorded 19.92% pollen sterility. There was 319.13 per cent increase in pollen sterility in the maleic hydrazide 1000 mg L^{-1} applied plants compared to control.

The phytohormone auxin plays an important role in anther and pollen development. It has also been reported that tapetum layer supplies the developing pollen grains with indole acetic acid (Aloni *et al*., 2006) and decreased auxin levels in the tapetum results in reduced pollen development. The hindrance of auxin flow in anther filaments resulted in shortened filaments with defected pollen grains and suppressed pollen mitosis processes (Feng *et al*., 2006). The growth regulator maleic hydrazide is an antiauxin and is capable of inducing male sterility in plants. Earlier studies have reported maleic hydrazide as inhibitor of mitosis at higher concentrations resulting in growth retardation (Patil and Bhat, 1992). In the present study, maleic hydrazide produced significantly higher percentage of pollen sterility (83.49%) compared to all other growth regulators evaluated. Similar results were reported by Praba and Thangaraj (2005) and Vijayalakshmi and Bangarusamy (2006) in TGMS rice and observed 94.4% and 81.31% pollen sterility respectively by maleic hydrazide treatment. MH treatment induced 84.33 % pollen sterility in okra (Deepak, 2007) and suppressed pollen viability in green foxtail millet (*Setaria viridis* (L.) P. Beauv. (Rizal *et al*., 2015).

The phenolic compound salicylic acid exhibits thermogenic properties and activates the alternate oxidase pathway which increases the inflorescence temperature up to 14^{0}C (Raskin *et al.*, 1989) and consequently induces metabolic heat which would cause pollen sterility in plants. In this study, the external application of salicylic acid at three different concentrations induced 62-82% of pollen sterility in rice. Earlier reports of Praba and Thangaraj (2005) and Vijayalakshmi and Bangarusamy (2006) in TGMS rice agrees with these findings. Salicylic acid mediated PCD of cells may also be suggested as the reason for pollen sterility. Salicylic acid induced oxidative stress resulting in the PCD of cells has also been reported (Mazel and Levine, 2001; Kovacs *et al.*, 2016).

Several reports suggested that high amount of ethylene released from ethrel was associated with induction of femaleness through the suppression of staminal buds. In this study also application of ethrel caused more than 75% pollen sterility. Rowell and Miller (1971) in wheat, Law and Stoskopf (1973) in barley, Parmar *et al.* (1979), Virmani *et al.* (1997), Praba and Thangaraj (2005) and Khan *et al.* (2011) in rice, Chakraborty and Devakumar (2006) in wheat and Amelework *et al.* (2016) in sorghum also reported gametocidal property of ethylene.

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents, protein and phenol content in the plant growth regulator treated TGMS plants were observed inorder to find out any adverse effects of these PGRs on the primary and secondary metabolism. The results revealed that the plant growth regulators evaluated were not inhibitory to the primary metabolism of TGMS lines. The protein and phenol contents estimated in the leaf sample reduced from fifteen days after panicle initiation to the time of flowering. The protein content in all the treatments were reduced at the time of flowering except maleic hydrazide 600 mg L^{-1} and 800 mg L^{-1} compared to fifteen days after panicle initiation. at low concentration, MH cause a reduction in cell elongation. But at specified concentration is suboptimal to cause an inhibition in protein synthesis. The inhibitory role of MH on the cell division might be effective even at this concentration might have caused male sterility (Brian and Hemming, 2008).

During the vegetative phase sufficient sink is there to partition the photosynthates produced by the source whereas in the reproductive phase major portion of the photosynthates may be transferred to the developing reproductive organs. Leaves are the main source of photosynthates and other metabolites during the grain formation and filling period in rice (Yoshida, 1981). The leaves are considered as the primary phloem loading source and flag leaf is mainly involved in the phloem transport during grain filling stage in rice (Eom *et al.*, 2012). Thus the protein and phenols content at the time of flowering may get lowered in the leaf.

Hence the compounds such as ethrel, salicylic acid and MH are capable of inducing pollen sterility in TGMS rice. Spraying of MH (1000 mg L^{-1}) two times at the time of panicle initiation and fifteen days after panicle initiation is most effective. Therefore application of efficient plant growth regulators can be exploited for sterility regulation in TGMS system for commercial hybrid rice seed production especially when there is lowering of temperature during the thermosensitive stage. The potential of plant growth regulators can be utilized in the plains so that the TGMS lines can be rescued from environmental casualities.

5.3 EVALUATING THE POTENTIAL OF USING TGMS LINE AS FEMALE PARENT

The discovery of male sterility in rice has put forth the beginning of hybrid rice development and provided new paths in exploiting heterosis in rice. The two line hybrids are reported to exhibit high proportion of heterosis (>10%) than CMS hybrids and can be utilized for developing suitable rice hybrids. The TGMS lines which shows acceptable level of pollen fertility under low temperature condition and stable sterility under high temperature condition can be well exploited as TGMS donor for generating new TGMS lines on specific backgrounds (Lu *et al.*, 1994; Lopez and Virmani, 2000). The stable sterile line EC720903 shows acceptable seed setting percentage (80%) in the high altitude zone where the mean temperature is below the critical sterility inducing temperature and the same line exhibits complete sterility in the plains was used for hybridization. The pollen

sterility of TGMS lines were monitored throughout the study period to ensure the sterility to eliminate self seed setting.

Thus for evaluating the potential of using TGMS line as a female parent in Kerala condition, the TGMS line EC720903 was hybridized with two rice varieties of Kerala, Aiswarya and Swetha using proximal hybridization method. The varieties were selected based on their grain colour to develop the hybrid on red and white background. Aiswarya is with red, long bold grain and Swetha is with white, short bold grain. The long duration rice variety Swetha was sown one week prior to EC720903 based on the difference in the number of days taken to 50% flowering between male and female parents inorder to obtain synchronization (Virmani *et al.*, 2003).

The main objective of hybridization experiment using TGMS line was to determine its crossability as a female parent. The seed setting percentage and spikelet sterility percentage recorded on EC720903 when hybridized with Aiswarya and Swetha was found to be significantly different. The seed setting percentage was 40.07% for the cross involving Swetha as male parent whereas it was only 30.18% for Aiswarya and EC720903.

Any TGMS lines with more than 30% outcrossing ability can be used as female parent with potential pollen donors for the successful production of two-line rice hybrids (Virmani *et al.*, 2003; Arasakesary *et al.*, 2015).

The synchronization of the flowering time of TGMS lines and the pollen parents determines the crossing rate as mentioned by RamaKrishna *et al.* (2006) and determine the success rate of two line breeding programme. They also specified the difference in the duration of flowering in different TGMS lines which varied from three to four days in TGMS line DRR 1S to four to seven days in another TGMS line IR 73834-21S which confirms the present findings. According to Arasakesary *et al.* (2015) the outcrossing potential ranged from 24% to 40% in different TGMS lines. Ealier reports of Zhang *et al.* (2017) also support the present results. However, the staggered flowering behaviour of TGMS line

EC720903 was not seen in the backcrossed BC_1F_2 plants in Jyothi background which was used in first experiment. Since the outcrossing potential of TGMS line EC720903 is in the satisfactory range, it indicates the suitability of TGMS line as a female parent for the development of hybrids in the state of Kerala. Among the two rice varieties, Swetha can be used as a better male parent than Aiswarya.

The F_1 hybrids developed were evaluated for its agronomic as well as physiological performances. Evaluation of F_1 hybrids developed from TGMS line for its performance is a prerequisite for finding out its potential to use as a female parent (Virmani *et al.*, 1997; Kalaiyarasi and Vaidyanathan, 2003). The phenological characterization revealed better performance of F_1 hybrids than their respective male parents. The F_1 progenies of Aiswarya and Swetha flowered early and attained 50% flowering faster than parents which is a desirable character for a hybrid. The male parent Aiswarya, which is medium duration, took 96.50 days for first flowering and 99.25 days to 50% flowering. Swetha, long duration rice variety, attained its first flowering and 50% flowering at 98.38 days and 101.25 days respectively, while the female parent TGMS EC720903 had taken only 90.75 days for flowering. The F_1 progenies achieved physiological maturity faster than male and female parents which is also a desirable trait as it contributes to better performance. Virmani *et al.* (1981), Kalaiyarasi *et al.* (2002), Santosh (2003) and Shukla and Pandey (2008) reported earliness in the hybrids derived from TGMS lines.

The plant height and total dry matter production were found to be significantly more for the F_1 progenies compared to their respective male and female parents. The photosynthetic rate of F_1 progenies also showed positive heterosis over parents at the time of panicle initiation. There noticed a 28% reduction in the photosynthetic rate in the leaves during flowering in the F_1 progenies compared to the panicle initiation stage indicating partitioning of photosynthates to sink (developing seed). In contrast, the photosynthetic rate of TGMS line EC720903 increased by 10% from panicle initiation to flowering due to lack of sufficient sink since anther or pollen development is affected.

Similarly the F_1 progenies exhibited heterosis on transpiration rate and stomatal conductance at panicle initiation and the transpiration rate decreased towards flowering except in TGMS line. The leaf temperature was in the range of 26.88 °C to 27.84 °C (Fig. 18). The total soluble protein content in male parents was higher than their respective F_1 progenies at panicle initiation (Table 15). Improved performance on morphological as well as physiological characters of F_1 hybrids were described by Kalaiyarasi *et al.* (2002), Santosh (2003) and Pham *et al.* (2004a). Cuong *et al.* (2014) reported that the dry matter accumulation was maintained in F_1 hybrids developed from TGMS line 103 which was in agreement with the present result.

The major focus of F_1 evaluation was to observe whether sterility is seen in the F_1 hybrids. The TGMS trait is controlled by recessive nuclear gene (*tms* gene) hence in the F_1s (heterozygote), the *tms* trait will not be expressed. The F_1s developed from Aiswarya and Swetha did not show sterility during flowering and their seed setting percentage was in the acceptable range (more than 80%) for F_1 progenies of Swetha and more than 70% for F_1 progenies of Aiswarya). The seed setting percentage of F_1 hybrids were better than their parents, Aiswarya, Swetha and the TGMS line which is consistant with the findings of Shankar *et al.* (2007) were F_1 of all the crosses with TGMS lines were fertile with high pollen and spikelet fertility of more than 70%. The evaluation of yield and yield attributes of F_1 hybrids confirmed the potential of this TGMS line using as a female parent and F_1 progenies performed better. Similar results were reported by Kalaiyarasi *et al.* (2002), Sundar, 2003; Pham *et al.* (2004a), Dhivyapriya and Kalaiyarasi (2014), Sasikala *et al.* (2015) and Zhang *et al.* (2017).

Considering the stable sterility throughout the study period, preferred CST, satisfactory seed setting percentage, physiological and agronomic acceptability and outcrossing potential, the TGMS line is a better option for developing suitable hybrids in any background. Hence the present study suggests that the TGMS line EC720903 can be recommended as a female parent for the development of hybrids suitable to the state of Kerala.

5.4 ANALYSING THE MOLECULAR MECHANISM OF MALE STERILITY IN TGMS LINES

The male sterility behaviour in TGMS lines are controlled by a single nuclear recessive gene or a pair of nuclear recessive genes that are responsive to temperature and is heritable (Borkakati and Virmani, 1996; Virmani *et al.*, 2003). TGMS lines when exposed to high temperature at certain stage of floral development *i.e,* between panicle initiation and flowering are male-sterile but convert to male-fertile under low temperatures. Hence, they can self-pollinate under low temperature conditions and can be multiplied. Thus it is quite interesting to understand the molecular mechanism underlying male fertility/sterility alterations in the TGMS line.

5.4.1 Protein Profiling of TGMS Line

In the present study, the protein profiling of TGMS line under sterility inducing and fertility inducing conditions were observed (Plate 8). Variation in the expressions of proteins were observed in leaf and panicle collected at ten days after panicle initiation from fertility and sterility conditions. In the leaf of TGMS plants grown under fertility inducing condition, increased expression of proteins at 25-35 kDa and 35-48 kDa were noticed whereas under sterility inducing condition increased expression was observed at 245 kDa. In the panicle under fertility inducing condition, increased intensity of proteins at 17 kDa was observed. Protein between 48-63 kDa under sterility inducing condition was observed with increased intensity. Under fertility inducing condition, presence of protein band of 245 kDa was observed in the panicle. Senthil *et al.* (2003) reported that under fertile conditions, in TGMS leaf an increased expression of 64, 54 and 24 kDa proteins and in anther tissues an enhanced expression of 48, 38 and 22 kDa proteins were observed. Most of the proteins differentially expressed were closely associated with energy metabolism, protein biosynthesis, cell wall formation and stress responses (Xiao *et al.*, 2009; Song *et al.*, 2015; Wang *et al.*, 2019). Therefore in the present study also differential expression of proteins

associated with pollen development might have caused male sterility in the TGMS line.

5.4.2 Microarray Gene Expression Analysis

The microarray gene expression analysis was done to understand the molecular mechanism associated with TGMS system. TGMS plants were maintained in sterility inducing and fertility inducing conditions simultaneously and young panicles and leaves were collected from both conditions at ten days after panicle initiation. The gene expression study using microarray technique revealed differences in gene expression under sterility and fertility inducing conditions. In the TGMS line, 4205 genes were up-regulated and 3559 genes were down-regulated in the leaves under sterility inducing condition compared to fertile condition. Whereas sterility induced up-regulation of 4867 genes and down-regulation of 2979 genes in sterile panicles compared to fertile panicles. Regarding up-regulated genes, 41.4% was specifically up-regulated in leaf and 23% up-regulated in the panicle and 44.2% was specifically down-regulated in the leaf and 30.9% was specifically down-regulated in the panicle (Fig. 19). Here we considered significant genes which were up-regulated or down-regulated in the TGMS leaf and panicle under sterility inducing condition which would have resulted in the development of pollen free anthers in the TGMS line.

5.4.2.1 Differential Expression of Genes Encoding Abiotic Stress Proteins

In the present study, genes encoding heat shock proteins and abiotic stress proteins were differentially expressed in the TGMS leaf and panicle under sterility inducing condition compared to fertility inducing condition. Heat shock proteins are ubiquitious proteins found in plants involved in stress response. They provide thermo-tolerance to plants, act as molecular chaperons, stabilize proteins and assists in protein refolding under elevated temperature (Park and Seo, 2015; Kumar *et al.*, 2017). High temperature prevailed in the sterility inducing condition caused the increased expression of Hsp genes in leaf and panicle. However down-regulation of significant Hsp genes was also observed in the leaf and panicle

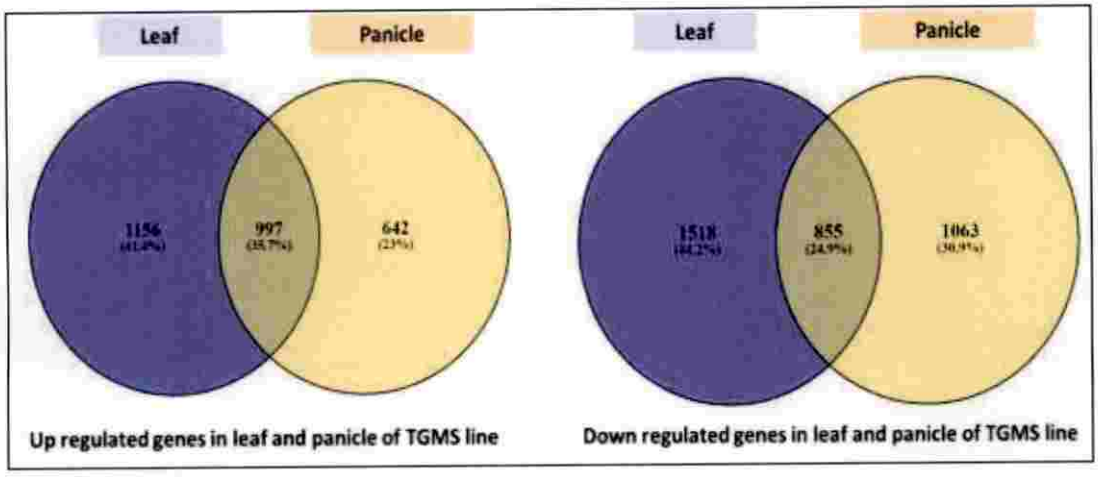


Fig. 19 Comparison of differentially expressed genes

under sterility condition. Hsp genes such as *Os05t0519700-03* coding Hsp 101 and *Os05t0519700-01* and *Os06t0682900-01* genes encoding heat shock protein needed for long-term acquired thermotolerance were down-regulated in the leaf which may increase the susceptibility to high temperature. Hsp 101 is essential for thermotolerance in rice that confers long-term acquired thermotolerance (Queitsch *et al.*, 2000; Lin *et al.*, 2014). Down-regulation of other specific Hsp genes in the leaf and panicle decreased the tolerance to high temperature which may lead to male sterility since pollen development is highly sensitive to high temperature (Rieu *et al.*, 2017).

The abiotic stress related genes, *Os10t0154700-01* and *Os09t0571400-01* encoding Cyclophilin protein which plays a regulatory role in the heat shock response was up-regulated in the leaf. Cyclophilin D (mitochondrial cyclophilin) is an integral part of the mitochondrial permeability transition complex and has a crucial role in mechanisms of cell death (Andreeva *et al.*, 1999; Trivedi *et al.*, 2012; Kumari *et al.*, 2013). Moreover, the gene encoding the enzyme proline oxidase which degrades the well-known osmolyte proline was also up-regulated under sterility. Proline accumulation in the cell improves its stress tolerance by stabilizing membranes, proteins detoxifying Reactive Oxygen Species and also it acts as a signalling molecule (Hayat *et al.*, 2012; Hossain *et al.*, 2014). More recently it has been reported that proline biosynthetic genes *Pyrroline-5-Carboxylate Synthetase* 1 and 2 were strongly expressed in *Arabidopsis* developing microspores and pollen grains (Mattioli *et al.*, 2018) which again confirms the requirement of proline for functional pollen development (Biancucci *et al.*, 2015).

Whereas the genes encoding pollen Ole e 1 allergen protein, proline transporter, sugar transporter protein, enzymes such as Peroxidase, Sucrose synthase coding genes were down-regulated in the TGMS leaf under sterility inducing condition. Pollen allergen proteins, proline and the enzyme Peroxidase are involved in the abiotic stress responses and their down-regulation may decrease the resistance to high temperature. The pollen allergens are involved in

stress responses and cell wall metabolism during pollen development (Chen *et al.*, 2016). Carbohydrate supply from leaf (source) to anthers (sink) is required for pollen development and maturation (Zhang *et al.*, 2013). Mutation in *Carbon Starved Anther* (*CSA*), key regulator of *OsMST8*, a monosaccharide transporter, decreased the amount of sugar accumulation in the anther resulting in male sterility. Since the genes for sugar transporter protein and the enzyme sucrose synthase were down-regulated in the TGMS leaf, limiting the sugar supply to the developing pollen grain which caused the production of pollen free anthers in the present study. Chen *et al.* (2007), Xiao *et al.* (2009), Pan *et al.* (2014), Pearce *et al.* (2015), Song *et al.* (2015), Liu *et al.* (2018) and Wang *et al.* (2019) also reported that carbohydrate metabolism caused male sterility in TGMS lines.

In the panicle of TGMS plants which were maintained in the sterility inducing condition, down-regulation of genes encoding several heat stress transcription factors, enzymes ascorbate oxidase and peroxidase and proline were also observed which might have caused male sterility in TGMS line. Similar results were reported in TGMS line AnnongS-1 where decrease in abundance of proteins such as heat shock protein, chaperons, peroxidase and superoxide dismutase and other stress regulating factors weakened the ability to resist high temperature resulting in abortive pollen grains under high temperature conditions (Wang *et al.*, 2019). The role of proline on pollen fertility has been reported in the model plant *Arabidopsis.* Any disruption of proline synthesis in developing microspores and pollen grains caused pollen abortion during gametophyte development (Funck *et al.*, 2012; Mattioli *et al.*, 2012; Biancucci *et al.*, 2015).

Thus, down-regulation of important abiotic stress encoding genes might have resulted In male sterility in the TGMS line.

5.4.2.2 Differential Expression of Genes Related to Calcium Signalling

Calcium is a widely accepted second messenger regulating signalling mechanism in plants. Down-regulation of calcium associated genes and calcium binding protein encoding genes in leaf and panicle caused male sterility in TGMS

line. Differential distribution of calcium were reported in sterile and fertile anthers of PGMS rice which agrees the present results (Tian *et al.*, 1998). Calcium precipitation observed were found to be less abundant in sterile anther tapetal cells, surface of their ubish bodies and on the microspores than in fertile anthers. In addition sterile grains had an incomplete exine. A strong peak of cytosolic Ca^{2+} in microspores and young pollen grains was confirmed recently in rapeseed (*Brassica napus* L.) (Rivas-Sendra *et al.*, 2017). Spatial and temporal redistribution of calcium was also reported in tobacco (Ge *et al.*, 2007). During microsporogenesis, calcium accumulation was observed around the callose wall and vacuoles of tapetum. However fewer calcium precipitations were observed in the young microspores. A Ca^{2+} binding protein, *Defective in Exine Formation 1* (*OsDEX1*) is required for tapetum function and pollen formation in rice which again explains the importance of calcium in pollen development (Yu *et al.*, 2016).

C2 calcium-dependent membrane targeting domain containing protein was down-regulated in the present study. Another functional C2-domain calcium dependent phospholipid-binding protein *OsPBP1* was identified in rice and reported to be required for pollen fertility (Yang *et al.*, 2008). Moreover down regulation of calcium related genes may intercept the signal transduction from leaf to panicle for proper pollen development which may lead to male sterility in the present case.

5.4.2.3 Differential Expression of Genes Related to Biosynthesis and Signalling of Phytohormones

Phytohormones play a significant role in male reproductive development since they are involved in different developmental processes. The plant hormone auxin is an important regulator of pollen development (Aloni *et al.*, 2006; Cheng *et al.*, 2006; Cecchetti *et al.*, 2008; Hirano *et al.*, 2008; Yang *et al.*, 2017; Song *et al.*, 2018). Auxin accumulation was reported in the developing anther and pollen (Feng *et al.*, 2006). Auxin related genes were differentially regulated in the TGMS leaf and panicle under sterility inducing condition. The genes encoding

enzyme similar to indole-3-acetic acid (IAA)-amido synthetase and indole-3-acetate beta-glucosyltransferase which catalyses the formation of auxin conjugate, bound form of auxin, was up regulated in the leaf and panicle under sterile condition. Auxin conjugates decreased level of physiologically active form of auxin inside the TGMS plant which may lead to abnormalities in pollen development. Aux/IAA proteins are short-lived transcriptional factors that function as repressors of early auxin response genes at low auxin concentrations (Tiwari *et al.*, 2001; Zhang and Peer, 2017). Genes coding protein similar to auxin-responsive protein (*Aux/IAA*) were also up-regulated in both leaf and panicle of TGMS line in the sterility inducing condition.

Whereas auxin biosynthesis gene, *Os01t0645400-01* coding Flavin monooxygenase-like enzyme (*YUCCA*-like gene) was down-regulated in the leaf under sterility inducing condition. The *YUC* genes are chiefly expressed in meristems, young primordia, vascular tissues and reproductive organs, thecas, tapetum, endothecium and procambium (Cheng *et al.*, 2006; Cecchetti *et al.*, 2008). On the other hand gene encoding tryptophan aminotransferase involved in the IAA biosynthesis required in the grain development was down-regulated in the panicle. Genes producing auxin responsive SAUR protein was down-regulated in both leaf and panicle. *SMALL AUXIN UP RNA*s (*SAUR*s) are the largest family of early auxin response genes found in plants (Markakis *et al.*, 2013; Ren and Gray, 2015; Xu *et al.*, 2017a). Furthermore, auxin efflux carrier, auxin induced protein and auxin transport protein coding genes were down-regulated in the leaf and auxin response factor, auxin transporter and auxin-induced protein encoding genes were down-regulated in the panicle which might have caused pollen sterility in TGMS line. Auxin content in plants are regulated by its biosynthesis, conjugation and degradation (Korasick *et al.*, 2013; Zhao, 2014; Armengot *et al.*, 2016; Zhang and Peer, 2017; Wang *et al.*, 2018). Hence any imbalance in auxin homeostasis can affect normal pollen development in rice.

Regarding genes associated with gibberellic acid, GA2-oxidase2 and GA2-oxidase 9 were up-regulated in the leaf under sterility inducing condition. These

enzymes reduce endogenous levels of bioactive GA (Lo *et al.*, 2008; Lo *et al.*, 2017). Ectopic expression of GA2-oxidase1 inhibited the development of reproductive organs in rice (Sakamoto *et al.*, 2001). In case of panicle, the gene encoding enzyme GA 3-beta-hydroxylase1 which regulates GA metabolism was up-regulated. Decrease in bioactive GA levels can cause irregularities in pollen development since GA is essential for normal pollen development in rice (Chhun *et al.*, 2007; Hirano *et al.*, 2008; Aya *et al.*, 2009; Sakata *et al.*, 2014; Kwon and Paek, 2016). *Os03t0760800-01* gene encoding a member of the GAST (GA-Stimulated Transcript) family required in the cell proliferation in meristems and panicles development was highly (-4.092) down-regulated in the leaf. Two GAST genes of rice, *OsGASR1* and *OsGASR2*, in which *OsGASR1* was strongly expressed in florets while *OsGASR2* was expressed in both florets and leaves (Furukawa *et al.*, 2006; Lee *et al.*, 2017). *Os08t0560000-01* gene producing gibberellin 20 oxidase 2 key oxidase enzyme required in the biosynthesis of gibberellin was also down-regulated. In the panicle, YABBY family transcription factor which regulates stamen and carpel development (Jang *et al.*, 2004; Dai *et al.*, 2007; Tanaka *et al.*, 2017) encoding gene was down-regulated. Therefore down-regulation of these genes caused male sterility in the present TGMS line.

Cytokinin dehydrogenase and Cytokinin oxidase enzyme encoding genes were up-regulated in the leaf. Cytokinin dehydrogenase/oxidase catalyzes the oxidation of cytokinins (Schmulling *et al.*, 2003; Frebort *et al.*, 2011). Whereas in the panicle, enzyme cytokinin-O-glucosyltransferase, helps in the formation of cytokinin glycoconjugates (Smehilova *et al.*, 2016), encoding gene was up-regulated. The down-regulated genes in the leaf and panicle include those which related to cytokinin synthesis, cytokinin-activation and cytokinin signalling. This differential expression of genes caused reduction in cytokinin content and signalling which resulted in male sterility. Huang *et al.* (2003), Hirano *et al.* (2008), Kinoshita-Tsujimura and Kakimoto (2011), Ding (2017) and Kurusu *et al.* (2017) described the importance of cytokinin in pollen development and observed male sterility in cytokinin deficient plants.

Genes related to ethylene biosynthesis including ACC oxidase enzyme, ethylene-responsive transcriptional coactivator and ethylene-responsive methionine synthase was up-regulated in the leaf. Ethylene signal transcription factor, ethylene-responsive transcription factor, 1-aminocyclopropane-1-carboxylic acid synthase involved in ethylene biosynthesis and ethylene receptor-like protein was up-regulated in the panicle. *APETALA2*/ethylene-responsive factor required in the abiotic stress response, regulation of spikelet meristem determinancy and floral organ identity and ERF-associated EAR-motif-containing repressor required in the abiotic stress response and stress signalling were down-regulated in the leaf. *AP2/ERFs* are considered as key regulator of different stress responses and survival during stress conditions (Xie *et al.*, 2019). On the other hand, phosphatidylethanolamine-binding protein, PEBP family proteins which control the phase transition of meristem and control flowering time was down-regulated in the panicle. Ethylene is required in the PCD (Takada *et al.*, 2006; Hobo *et al.*, 2008; Hirano *et al.*, 2008). Moreover, high amount of ethylene was also associated with femaleness through the suppression of staminal buds. In *Arabidopsis* also high ethylene content reduced pollen fertility (Lin *et al.*, 2009). Here in this study also high ethylene content developed through the up-regulation of ethylene biosynthesis genes may induce male sterility.

Abscissic acid deactivation enzymes at the later stages of tapetal development may have a significant role in promoting PCD in tapetum cells (Fath *et al.*, 2000). In the present study, abscisic acid responsive elements-binding factor encoding gene was up-regulated in the leaf and panicle, which was reported in the stress response (Hossain *et al.*, 2010). ABA activated protein kinase involved in the hyperosmotic stress response (Yang *et al.*, 2011) was down-regulated in the leaf and Abscisic acid 8'-hydroxylase 1 coding gene was down-regulated in the panicle.

Brassinosteroids are isolated from *Brassica napus* pollen (Grove *et al.*, 1979) and is essential for microspore development, maintaining viability and sporopollenin synthesis (Hirano *et al.*, 2008). In addition, brassinosteroids

promotes pollen development in rice by directly promoting the expression of *Carbon Starved Anther* (Zhu *et al.*, 2015). Brassinosteroid insensitive 1-associated receptor kinase encoding gene was down-regulated in the leaf and gene which involved in the regulation of brassinosteroid homeostasis was down-regulated in the panicle resulting in pollen sterility. *Brassinosteroid Insensitive 1* is a plasma membrane localized receptor kinase which senses the plant hormone brassinosteroid (Phukan *et al.*, 2017; Xie *et al.*, 2019).

Jasmonic acid also plays a major part in pollen maturation, elongation of anther filament and the production of functional pollen grains. Jasmonic acid deficient mutants were male sterile as reported by Stintzi and Browse (2000), Thines *et al.* (2013), Cai *et al.* (2014) and Liu *et al.* (2015). Jasmonic acid related Tify domain protein encoding gene involved in jasmonic acid synthesis and *Os10t0132300-01* gene producing similar to Jasmonate-induced protein were down-regulated in the leaf. In rice, most of the *OsTIFY* genes were mainly expressed in leaf and were responsive to jasmonic acid and abiotic stresses (Ye *et al.*, 2009). In panicle also genes coding Tify domain protein and Allene oxide synthase involved in the biosynthesis of jasmonic acid were down-regulated in the panicle. Thus the down-regulation of jasmonic acid biosynthesis genes might also have contributed to pollen sterility in this study.

Maintenance of proper hormonal balance is a prerequisite for the development of functional pollen grains. Therefore, change in the phytohormone level inside the leaf and panicle in the present study caused the production of pollen free anthers in the TGMS line.

5.4.2.3 Differential Expression of Genes Associated with Male Sterility in the Leaf of TGMS Line

Interestingly, significant flowering related genes were differentially regulated in the leaf and panicle under sterility inducing condition. In leaf, gene encoding Nuclear ribonuclease Z having RNase Z^{S1} function was up regulated. Differential expression of this gene was observed under sterility inducing

condition and it had a role in thermosensitive splicing of mRNAs in TGMS line which can lead to male sterility as reported by Zhou *et al.* (2014). Membrane-bound NAC-like transcription factor coding gene which acts as transcriptional repressor involved in the suppression of flowering was up-regulated. Gene *Os01t0578200-00* encoding programmed cell death protein 2 was up-regulated. Programmed cell death protein controls selective destruction of individual cells, tissues or organs and it may lead to destruction of cells required for the normal pollen development or involved in signalling from leaf to panicle. Gene encoding phytochrome A was up-regulated in the leaf. Phytochrome A was reported as a thermo receptor in *Arabidopsis* (Jung *et al.*, 2016; Song *et al.*, 2017), thus TGMS leaf had sensed the temperature variation for the expression of their male sterility genes to cause complete male sterility in the TGMS line in the present study.

More importantly, genes coding β-1,3-glucanase precursor was down-regulated in the leaf. β-1,3-glucanase degrades callose deposition which is crucial in normal pollen development. The microspores were released from the tetrads by the degradation of callose wall secreted by tapetal cells (Zhang and Wilson, 2009; Lin *et al.*, 2017) and silencing of glucanase encoding gene resulted in the degeneration of microspores, consequently caused male sterility (Wan *et al.*, 2011). In callose synthase mutant of *Arabidopsis*, *cals5*, normal callose deposition was disrupted in meiocytes, tetrads, microspores and mature pollen, subsequently pollen exine wall was not formed and resulted in male sterility (Dong *et al.*, 2005; Shi *et al.*, 2016).

ATP-binding cassette (ABC) transporter encoding gene involved in the pollen wall formation was down-regulated in the leaf. Previous study showed that plasma membrane localized ATP-binding cassette (ABC) transporter, *OsABCG3*, was expressed mainly in anther when exine started to form and reported to transport the tapetum-produced materials essential for pollen wall formation (Chang *et al.*, 2018). Two ATP binding cassette, *OsABCG26* and *OsABCG15* together regulate male reproduction in rice (Quilichini *et al.*, 2010; Niu *et al.*, 2013; Zhao *et al.*, 2015; Zhao *et al.*, 2016). *OsABCG26* is mainly responsible for

the transport of lipid molecules from tapetal cells to anther wall layers whereas *OsABCG15* is responsible for the export of sporopollenin precursors from the tapetal cells to anther locules for pollen exine development. The mutant phenotypes of ABCG transporter genes showed degeneration of microspores. Therefore in the present study also down-regulation of transporter encoding gene might have caused a decrease in the translocation of materials required for pollen wall formation and exine synthesis and resulted in male sterility.

Gene *Os03t0167600-01* reported to encode Male sterility protein 2 was down-regulated. Male sterility protein is a transcriptional activator required for post-meiotic pollen development, maturation and development of pollen components and pollen wall especially exine formation in *Arabidopsis* (Aart *et al.*, 1997; Pearce *et al.*, 2015). It controls the tapetal development by regulating tapetal PCD. *Os07t0108900-01* coding MADS-box transcription factor 15 was also down-regulated. MADS-box transcription factor 15 is expressed in the floral meristem at very early stage of the spikelet development (Kyozuka *et al.*, 2000). It is also expressed in lemmas, paleas and lodicules from early to late stage of flower development. Another MADS-box transcription factor, MADS6 was reported to play a key role in specifying flower development by interacting with other floral homeotic genes in rice (Li *et al.*, 2011b).

Gene producing transcription factor which is essential in the development of lodicules and stamens (Li *et al.*, 2011b) and *POLLENLESS3* were down-regulated. *POLLENLESS3* protein, expressed within anther cells undergoing meiosis, is essential for microspore and pollen grain production, regulation of cell division after meiosis I and II to assist exit from meiosis and transition to G1 phase of cell division (Sanders *et al.*, 1999). Mutant phenotype showed meiotic abnormalities in cell layers surrounding the anther locules.

Genes coding pollen-specific protein SF21 and putative glucose-methanol-choline oxidoreductase which regulates tapetum degeneration, pollen exine formation and anther cuticle formation was down-regulated in the present study.

Presence of SF21 was observed in tobacco pollen grains (Kraeuter-Canham *et al.*, 2001). Down-regulation of *HTH1* gene coding glucose-methanol-choline oxidoreductase, involved in the biosynthesis of long-chain α-ω-dicarboxylic fatty acids for cutin biosynthesis, required for anther development and pollen fertility in rice was reported to produce defective anther wall and aborted pollen (Xu *et al.*, 2017b). *HTH1* is similar to *HOTHEAD* (*HTH*) in *Arabidopsis thaliana* (Krolikowski *et al.*, 2003).

No pollen, pollen-specific protein *NTP303* precursor, 14-3-3 protein, a florigen receptor in the shoot apical meristem which regulate flowering, filamentous flower-like yabby protein encoding genes were also down-regulated. No pollen mutant showed smaller anthers with a smooth cuticle surface, abnormal ubisch bodies and aborted pollen grains with irregular exine (Liu *et al.*, 2017). Down-regulation of *NTP303* precursor in tobacco resulted in male sterility (Groot *et al.*, 2004). *NTP303* was reported to be seventh most abundant transcript in mature tobacco pollen (Weterings *et al.*, 1995; Scarpin *et al.*, 2017; Hafidh *et al.*, 2018). *YABBY* genes are involved in differentiation and patterning of floral organs (Yang *et al.*, 2016). *FLOWERING LOCUS T* (*FT*) homologue Hd3a5 in rice interacts with 14-3-3 proteins in the shoot apex along with (*Os*)*FD1* transcription factor, forms 'florigen activation complex' (FAC) and activates the transcription of *OsMADS15* for flowering (Taoka *et al.*, 2011). Additionally, gene encoding transcriptional regulator which is involved in the regulation of meristem activity and inflorescence development and RAFTIN1a, an anther specific protein was also down-regulated in the leaf resulting in male sterility. RAFTIN is needed for the late phase of pollen development in rice and the mutant plants exhibited pollen collapse and reduced viability (Wang *et al.*, 2003).

5.4.2.4 Differential Expression of Genes Associated with Male Sterility in the Panicle of TGMS Line

In case of panicle, genes encoding CONSTANS-like protein CO9 (fold change: 4.432) and Anther-specific proline-rich protein (3.666) were up-regulated

in the panicle. Overexpression of *OsCOL9* and *OsCOL16* delayed the flowering time by suppressing the early heading date 1 (Ehd1) pathway in rice (Liu *et al.*, 2016; Wu *et al.*, 2017). APG is found in sporophytic and gametophytic cell types in the anther in male fertile plants and expressed in male gametogenesis during microspore development (Roberts *et al.*, 1993). Its higher expression is found during microspore mitosis with a dramatic decline during pollen maturation. Similar to leaf, gene encoding programmed cell death protein 2 was up-regulated in the panicle.

Gene *Os06t0607700-01* encoding ATP-binding cassette (ABC) transporter which is involved in the post-meiotic anther and pollen development was significantly (-10.727) down-regulated in the panicle. ABC transporters are involved in the transportation of materials essential for pollen wall formation (Quilichini *et al.*, 2010; Niu *et al.*, 2013; Zhao *et al.*, 2015; Zhao *et al.*, 2016; Chang *et al.*, 2018). Other significantly down-regulated genes encode a protease inhibitor (-9.844), lipid transfer protein (LTP) encoding gene involved in postmeiotic anther development (*Os11t0582500-01* -9.844) and putative glucose-methanol-choline oxidoreductase (-8.300) which regulates tapetum degeneration, pollen exine formation and anther cuticle formation. Glucose-methanol-choline oxidoreductase is involved in the biosynthesis of fatty acids for cutin biosynthesis. In rice, down-regulation of *HTH1* gene coding glucose-methanol-choline oxidoreductase was reported to produce defective anther wall and aborted pollen (Xu *et al.*, 2017b). *HTH1* was highly expressed in epidermal cells of anthers.

An orthologue of *Arabidopsis* PKSA/LAP6 (-7.737) gene involved in the regulation of pollen exine formation was down-regulated. Rice *OsLAP6/OsPKS1*, an orthologue of *Arabidopsis PKSA/LAP6* (*LAP6: Less Adhesive Pollen 6; PKSA: Polyketide Synthase A*), was reported to be critical for proper pollen exine formation by involving in sporopollenin synthesis (Kim *et al.*, 2010; Pearce *et al.*, 2015; Zou *et al.*, 2017a; Zou *et al.*, 2018). It is localized to the endoplasmic reticulum and mainly expressed in tapetum. Mutant plants were completely male sterile due to defects in pollen exine formation. *OsTKPR1*, a Tetraketide α Pyrone

Reductase described in rice, accumulated in the anther tapetum and microspores and was involved in sporopollenin synthesis (Pearce *et al.*, 2015; Xu *et al.*, 2019). Loss of function of *OsTKPR1* resulted in complete male sterility by delayed tapetum degradation, reduced anther cuticular lipids and damage in Ubisch body, pollen exine formation and aborted pollen grains.

Atypical strictosidine synthase gene (-6.006) involved in the regulation of anther development and pollen wall formation was down-regulated in the panicle. Strictosidine synthase (STR) plays a significant role in the biosynthesis of terpenoid indole alkaloids. An atypical strictosidine synthase found in rice, *OsSTRL2*, expressed high in tapetal cells and microspores, plays key role in anther development and pollen wall formation (Zou *et al.*, 2017b).

Phytochrome P450-like protein and *Os08t0131100-01* (-4.852) gene coding Cytochrome P450 hydroxylase which regulates anther cuticle and pollen exine development was also down-regulated. Phytochrome P450 belongs to cytochrome P450 family. Cytochrome P450 fatty acid hydroxylase, encoding heme thiolate monooxygenases was reported to be involved in sporopollenin biosynthesis and hence essential for male fertility in rice (Morant *et al.*, 2003; Li and Zhang, 2010; Bak *et al.*, 2011; Niu *et al.*, 2013; Yang *et al.*, 2014; Pearce *et al.*, 2015).

Male sterility protein 2 encoding gene (fold change: -5.869) required for anther and post-meiotic pollen development, maturation, exine formation and tapetal PCD (Aart *et al.*, 1997; Pearce *et al.*, 2015) was down-regulated in the panicle. Fatty acyl-CoA synthetase coding gene which controls pollen exine formation, anther development and sporopollenin synthesis was down-regulated. *OsACOS12*, an acyl-CoA synthetase5 found in rice, which is an orthologue of *Arabidopsis ACOS5* (Souza *et al.*, 2009), is required for lipid metabolism for sporopollenin and cuticle biosynthesis (Li *et al.*, 2016; Yang *et al.*, 2017). *OsACOS12* protein is expressed in tapetal cells and microspores and moreover mutants exhibited defective pollen exine resulting in male sterility without any pollen grains. Additionally, *DPW2* (*DEFECTIVE POLLEN WALL2*), a

cytoplasmically localized BAHD acyltransferase regulates the biosynthesis of anther cuticle and pollen wall and the mutants were male sterile with an abnormal anther cuticle and defective pollen wall (Xu *et al.*, 2017).

Homologous protein of CER1, very-long-chain (VLC) alkane biosynthesis involved in the regulation of anther development and plastids differentiation was down-regulated. *ECERIFERUM1* (*CER1*) identified in *Arabidopsis* is considered as the core component of VLC alkane biosynthesis (Lai *et al.*, 2007). Rice *OsCER1* protein, homologous protein of CER1, was accumulated highly in the tapetum (stage 10) and bicellular pollen (stage 11) and the down-regulation led to male sterility (Ni *et al.*, 2018). Furthermore, down-regulation caused delayed tapetal PCD and irregular development of plastids in the tapetal cells.

Anther-specific proline-rich protein APG was down-regulated in the panicle. Anther-specific proline-rich protein APG is found in sporophytic and gametophytic cell types in the anther, only in male fertile plants and is expressed in male gametogenesis, during microspore development (Roberts *et al.*, 1993). Higher expression is found during microspore mitosis with a dramatic decline during pollen maturation. Gene encoding putative triacylglycerol (TAG) lipase, Phospholipase A1 involved in the specification of empty-glume identity, regulation of spikelet development and control of endogenous jasmonic acid (JA) biosynthesis was down-regulated.

Lipid transfer proteins (LTPs) are small secretory proteins with distinct lipid-binding structures for lipid transport (Huang *et al.*, 2013). The lipid transfer proteins, type III LTPs in *Arabidopsis* (Huang *et al.*, 2013) and OsLTPg25 encoded by *OsC6*, in rice (Zhang *et al.*, 2010; Salminen *et al.*, 2016) were expressed in tapetal cells and microspores during the post-meiotic stages 9-11 of anther development. They transport lipid precursors for pollen exine synthesis (Ariizumi and Toriyama, 2011). Silencing of *OsC6* resulted in degenerated tapetal cells, irregular and shrunken microspores and reduced pollen fertility. *Arabidopsis*

monoacylglycerol lipase (MAGL) genes, *AtMAGL4*, *AtMAGL10*, *AtMAGL11* and *AtMAGL13* were predominantly expressed in pollen (Kim *et al.*, 2016).

ATP binding cassette G transporter, controls male reproduction and anther cuticle development by regulating the transport of materials produced by the tapetal cells for pollen wall formation (Chang *et al.*, 2018; Quilichini *et al.*, 2010; Niu *et al.*, 2013; Zhao *et al.*, 2015; Zhao *et al.*, 2016) was down-regulated. Anther-specific protein SF18 was down-regulated in the panicle. Anther-specific protein SF18 regulate the formation of anther-specific cell wall protein (Evrard *et al.*, 1991) which could contribute to the cell wall architecture of epidermal anther cells via intermolecular disulfide bridges.

Decreased level of RAFTIN1a anther protein, β-1,3-glucanase and pollenless3 protein were observed in the panicle. RAFTIN1 is an anther specific protein required in the late phase of pollen development (Wang *et al.*, 2003). β-1,3-glucanase removes callose deposition in tetrads which is vital for nourishment for normal pollen development (Zhang and Wilson, 2009; Lin *et al.*, 2017; Wan *et al.*, 2011; Dong *et al.*, 2005; Shi *et al.*, 2016). pollenless3 protein is essential for the meiosis during pollen development (Sanders *et al.*, 1999).

Basic helix-loop-helix (bHLH) transcription factor required in the regulation of tapetal programmed cell death and in tapetum development and degeneration was down-regulated. Rice basic helix-loop-helix protein, *TDR INTERACTING PROTEIN2* (*TIP2*), plays a vital role in the meristematic differentiation during early anther development and the mutants exhibited undifferentiated three anther walls and aborted tapetal PCD led to complete male sterility (Fu *et al.*, 2014; Ko *et al.*, 2014). *TIP2* encodes a bHLH transcription factor which promotes tapetal PCD and callose degeneration. *TDR* (TAPETUM DEGENERATION RETARDATION) (Li *et al.*, 2006), *EAT1* (*ETERNAL TAPETUM 1*) (Ji *et al.*, 2013) and *UDT1* (Jung *et al.*, 2005) genes encoding putative basic helix-loop-helix, specifically expressed in the tapetum regulates tapetum development and degeneration. Furthermore, *bHLH142* identified in rice coordinates carbohydrate

and lipid metabolism, cell wall modification, ROS homeostasis and PCD during anther development (Ranjan *et al.*, 2017).

Pectate lyase-like protein involved in pollen development and F-box-containing protein important in male meiotic DNA double-strand break repair and controls meiotic progression was down-regulated. F-box gene namely *MEIOTIC F-BOX* (*MOF*) necessary for male meiotic progress was reported in rice and it was mainly active during leptotene to pachytene stage of prophase I of meiosis (He *et al.*, 2016). Pollen-specific phospholipase, Patatin like phospholipase A which is vital in maintaining pollen viability, autonomous floral activator essential in the promotion of flowering (Zhang *et al.*, 2010; Salminen *et al.*, 2016; Kim *et al.*, 2016) and Filamentous flower-like yabby protein were down-regulated. *OsPBP1* encodes a calcium dependent phospholipid-binding protein which is essential for pollen viability (Yang *et al.*, 2008). YABBY proteins are well known to regulate flower development.

The *PISTILLATA-like* MADS box protein coding gene was down-regulated in the panicle. *PISTILLATA* genes control organ determination in the second and third whorls of flower *i.e.*, petals and stamens (Kater *et al.*, 2006; Taiz and Zeiger, 2010). Gene which produce MADS-box transcription factor involved in the regulation of floral organ identity and meristem fate (Kyozuka *et al.*, 2000; Kater *et al.*, 2006; Li *et al.*, 2011b), anther-specific protein, *LOB* (*LATERAL ORGAN BOUNDARIES*) domain-containing protein involved in the regulation of empty-glume identity, floral organ number control and female gametophyte development was down-regulated. *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) proteins were found in *Arabidopsis*. *AtLBD10* interacts with *AtLBD27* to control pollen development in *Arabidopsis* by regulating asymmetric division of microspores to produce bicellular vegetative and germ cells resulting in the formation of mature tricellular pollen (Kim *et al.*, 2015). Alterations in floral organs due to ectopic expression of *LOB* caused male sterility in *Arabidopsis* (Shuai *et al.*, 2002).

Wax synthase protein and sucrose transporter encoding genes were also down-regulated in the TGMS panicle maintained in the sterility inducing condition. Wax is an important constituent of anther wall. *WAX-DEFICIENT ANTHER1* (*WDA1*) mutants were male sterile due to defective pollen exine formation (Jung *et al.*, 2006; Li and Zhang, 2010). Chen *et al.* (2007), Xiao *et al.* (2009), Pan *et al.* (2014), Pearce *et al.* (2015), Song *et al.* (2015), Liu *et al.* (2018) and Wang *et al.* (2019) reported increased carbohydrate metabolism caused male sterility in TGMS lines. Gene coding Polygalacturonase enzyme was down-regulated. The enzyme polygalacturonase is involved in the timely degradation of pectins found in the pollen mother cell wall. Two Polygalacturonases namely, *QUARTET2* (*QRT2*), *QRT3*, *ADPG1* (*ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1*) and *ADPG2* were required for pollen tetrad separation during pollen development (Xiao *et al.*, 2014).

Gene encoding protein similar to DnaJ domain containing protein (ER-resident J-protein 3A, *THERMOSENSITIVE MALE STERILE 1*) was down-regulated. *TMS1* is required for thermotolerance of pollen tubes in *Arabidopsis* by functioning as a co-molecular chaperone (Yang *et al.*, 2009). Gene encoding R2R3-type MYB transcription factor (*Carbon Starved Anther*) and *UDPase* was also down-regulated in the panicle. *Carbon Starved Anther* and *UDPase* are responsible for sugar partitioning into anther (Zhang *et al.*, 2013; Pan *et al.*, 2014).

5.4.2.5 Differentially Regulated Pathways Associated with Male Sterility in the TGMS Line

Pathways such as sucrose and starch degradation as well as metabolism, sucrose biosynthesis cytokinins degradation, proline degradation were up-regulated in leaf of TGMS plants maintained under sterility inducing condition. Pathways involved in the biosynthesis of IAA, gibberellin, brassinosteroid and jasmonic acid biosynthesis, were down-regulated in the leaf. In panicle, pathways such as ethylene biosynthesis, cytokinins degradation, starch and sucrose

metabolism and proline degradation were up-regulated in the TGMS sterile panicle. Pathways like cellulose, starch and sucrose biosynthesis, IAA, gibberellin, brassinosteroid and jasmonic acid biosynthesis, removal of superoxide radicals, phospholipid biosynthesis II, biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, glycolysis/gluconeogenesis, photosynthesis, circadian rhythm were down-regulated in the panicle. Endo *et al.* (2004), Chen *et al.* (2007), Dai *et al.* (2007), Xiao *et al.* (2009), Zhang *et al.* (2013), Chueasiri *et al.* (2014), Pan *et al.* (2014), Pearce *et al.* (2015), Song *et al.* (2015), Li *et al.* (2015), Muiller and Rieu (2016) and Wang *et al.* (2019) reported that degradation of carbohydrate, hormone, fatty acids and stress protein caused deformities in anther and pollen development.

Therefore in the present study, occurrence of pollen free anthers in the TGMS line during sterility inducing condition is mainly due to the down-regulation of Hsp genes and abiotic stress related transcription factors in the leaf and panicle which increased the susceptibility to high temperature stress in the TGMS line. Signalling mechanism for regulating gene expression for proper pollen development from leaf to panicle interrupted due to down-regulation of calcium associated genes. Moreover, maintaining phytohormone homeostasis is also mandatory for pollen development, maturation, pollen viability, pollen exine, sporopollenin synthesis and controlling PCD. Thus imbalance in bioactive hormone content due to up-regulation of genes associated with hormone metabolism and down-regulation of biosynthesis genes which caused the production of pollen free anthers.

Significant down-regulation of genes encoding ABC transporter proteins involved in the material transport from tapetum to microspore for exine and sporopollenin synthesis, lipid transfer protein and strictosidine synthase, glucose-methanol-choline oxidoreductase, male sterility protein 2, wax synthase and β-1,3-glucanase in the panicle which led to male sterility in the TGMS line (Fig. 20). Furthermore, down-regulation of hormone biosynthesis in TGMS plants and

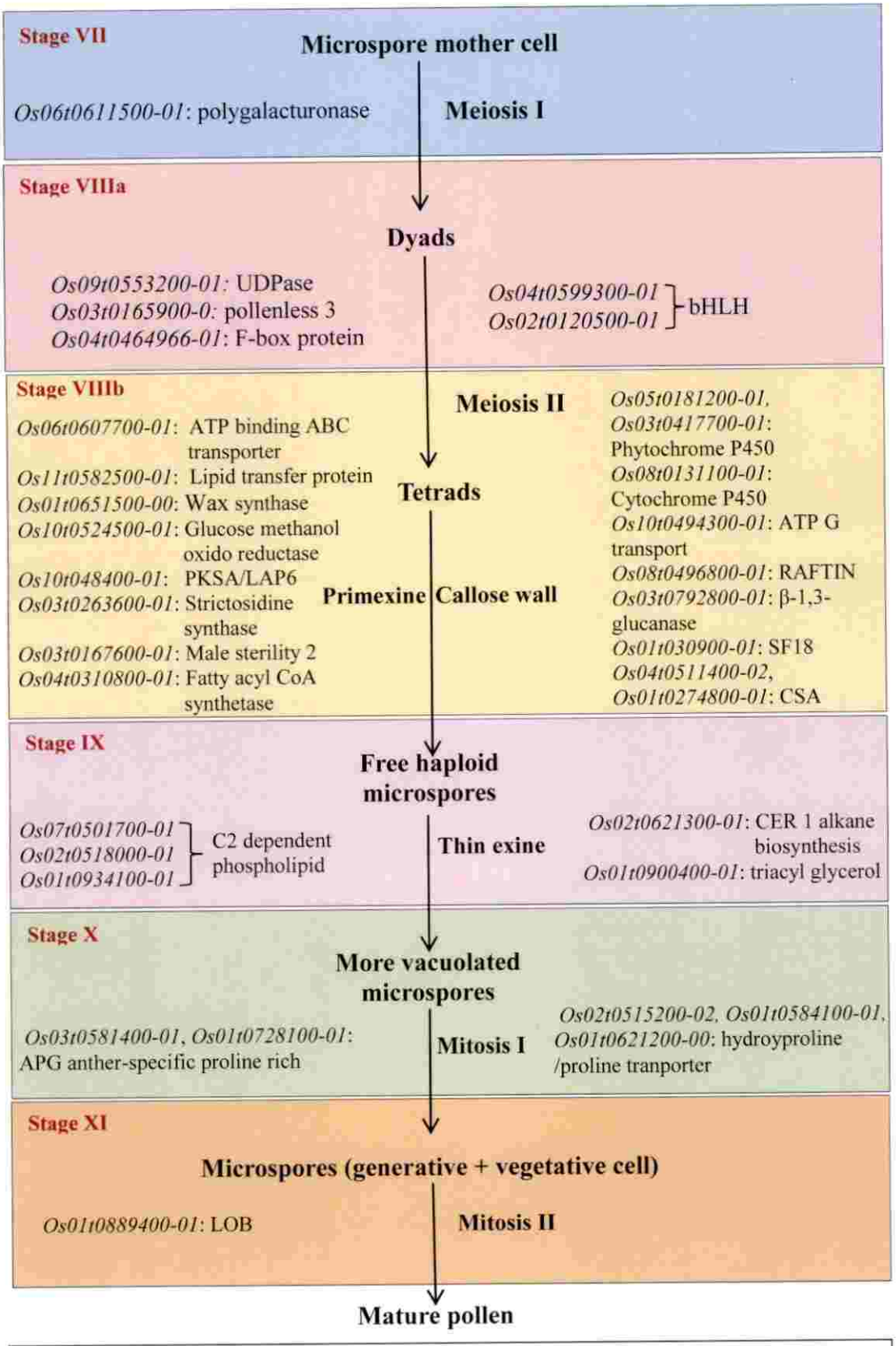


Fig. 20 Down-regulated genes in panicle during the most sensitive stage of pollen development in the TGMS line exposed to high temperature

carbohydrate supply to the developing pollen grain might have resulted in the production of pollen free anthers in the present study.

Hence occurrence of pollen free anthers in the TGMS line during sterility inducing condition is mainly due to the down-regulation of genes encoding ABC transporter proteins, lipid transfer protein and strictosidine synthase, glucose-methanol-choline oxidoreductase, male sterility protein 2, wax synthase, β-1,3-glucanase and pollenless3.

SUMMARY

6. SUMMARY

Rice is the staple food of people around the world. The area under rice cultivation is declining day by day imparting risk to food security. Hybrid rice production is an ideal technology to meet the demand of ever growing population since hybrids are able to produce 20-30% higher yield over conventional high yielding varieties. The phenomenon of male sterility is exploited in the production of rice hybrids. The most widely accepted system of hybridization method, the three line system of hybrid rice production, utilizes cytoplasmic male sterility (CMS). CMS system requires maintaining restorer lines for restoring fertility of male sterile line which is a major limiting factor and is difficult to transfer to interested line.

Two line breeding method exploiting thermosensitive genic male sterility (TGMS) system has emerged as an alternate strategy to overcome the problems associated with CMS system. Moreover TGMS hybrids produce nearly ten per cent more yield than CMS hybrids. TGMS system is suitable in tropical countries where significant variations in temperature are available between season and between altitudes. TGMS lines are sensitive to the temperature for the expression of their male sterility gene(s). TGMS lines maintain sterility when temperature is above critical sterility temperature (CST) and became fertile if the temperature is below CST. Male sterility in TGMS is governed by single recessive nuclear gene which is transferable to any rice line of interest. Around hundred hybrids were released in India using CMS system for commercial rice cultivation however, they are not suitable to the state of Kerala, since they are with white slender grain and people in Kerala prefer to consume red bold rice.

To achieve this objective, stable TGMS line EC720903, imported from IRRI, Philippines was used as the donor plant for transferring male sterile gene to popular red rice variety of Kerala, Jyothi at the Department of Plant Physiology, College of Agriculture, Vellayani. The sterile F_2 plants obtained were backcrossed to recover red rice character. Hence the present study entitled 'Physiological

approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was proposed to evaluate the environmental conditions required for complete male sterility of TGMS plants and to manipulate the male sterility by using plant growth regulators and also to understand the molecular mechanism associated with TGMS system.

Newly developed TGMS red rice line was evaluated under open field and inside the rain out shelter for twelve months. Result revealed that the average temperature prevailed during the critical thermosensitive period of monthly sown TGMS red rice line was above the sterility inducing average temperature of 26.9°C and caused complete pollen and spikelet sterility. Anatomical studies of anther showed that it was pollen free which is a specific character preffered for TGMS line. It was observed that the critical thermosensitive stage of TGMS line on red rice background was 15-22 days before flowering with the CST of 26.9°C.

The modified micro-climate inside the rain out shelter favoured the growth of TGMS plants. The TGMS plants inside rain out shelter performed better on morphological characters such as plant height and number of productive tillers compared to open field grown plants. Early flowering was observed in the rain out shelter grown crops than open field which may be due to high temperature prevailed inside the rain out shelter. Hence this low cost structure which is covered with a UV stabilized transparent sheet can be used for commercial hybrid seed production using TGMS lines throughout the year.

The crop calendar generated from the weather data of Vellayani location suggests that first crop season (April-May to September-October) and third crop season (December-January to March-April) are ideal for hybrid seed production. Thirty year mean temperature data recorded in the fourteen districts of Kerala recommends that third crop season can be utilized for the hybrid seed production in all the districts of Kerala except in high altitudes. In high altitude districts, Wayanad and Idukki, the average temperature was always below the CST hence seed multiplication can be done throughout the year. Thus the newly developed

TGMS line can be recommended to the state of Kerala as a female parent for the development of suitable rice hybrids. Location specific crop calendar can be prepared based on the weather data for commercial hybrid rice seed production.

The plant growth regulators *viz.*, ethrel, salicylic acid and maleic hydrazide were capable of enhancing pollen sterility in all the treated TGMS rice. Maleic hydrazide produced significantly higher percentage of pollen sterility (83.49%) compared to all other growth regulators evaluated. The growth regulator maleic hydrazide, which is an antiauxin, was capable of inhibiting mitosis at higher concentrations and induced male sterility in TGMS plants. The external application of salicylic acid at three different concentrations induced 62-82% of pollen sterility in rice. The thermogenic properties of salicylic acid along with induction of PCD of cells might have caused pollen sterility in TGMS line. Application of ethrel caused more than 75% pollen sterility. High amount of ethylene was associated with induction of femaleness through the suppression of staminal buds which may be the reason for ethrel induced pollen sterility in TGMS rice. It was also observed that the evaluated plant growth regulators applied were not inhibitory to the primary metabolism of TGMS lines. Hence spraying of MH (1000 mg L^{-1}) two times at the time of panicle initiation and fifteen days after panicle initiation is more effective followed by salicylic acid 600 mg L^{-1}. Moreover PGRs can also be utilized to protect TGMS lines from environmental casualities such as lowering of temperature during critical thermosensitive stage due to unexpected rainfall in commercial hybrid seed production programmes. The dosage of above PGRs may be identified for inducing 100% sterility for quality hybrid seed production.

The TGMS line was crossed with one white rice variety, Swetha and red rice variety, Aiswarya. The results revealed that the outcrossing potential of TGMS line was in the acceptable range. F_1 hybrids were evaluated for its performance, earliness in flowering and attainment of physiological maturity. The plant height and total dry matter production were found to be significantly more for the F_1 progenies compared to their respective male and female parents.

Physiological characters such as photosynthetic rate, transpiration rate, stomatal conductance, leaf temperature and the protein content were also found to be better for F_1 hybrids. The evaluation of yield and yield attributes of F_1 progenies confirmed the potential of using TGMS line as a female parent. Hence the present study suggests that the TGMS line can be recommended as a female parent for the development of hybrids in any background which is suitable to the state of Kerala. The new TGMS line can be recommended due to the stable sterility throughout the study period, preferred CST, satisfactory seed setting percentage, physiological and agronomic acceptability and preferred outcrossing potential.

Differential expressions of proteins were observed in leaf and panicle tissues collected at ten days after panicle initiation from fertility and sterility inducing conditions. Enhanced expression of proteins were observed at 25-35 kDa and 35-48 kDa maintained in the fertility inducing condition whereas increased expression was observed at 245 kDa under sterility inducing condition in TGMS leaf. In the panicle which was grown under fertility inducing condition, increased expression at 17 kDa protein was observed and whereas it was between 48-63 kDa under sterility inducing condition. The protein band observed at 245 kDa in the panicle maintained at fertility inducing condition was found to be absent under sterility inducing condition in the panicle. These differentially expressed proteins will have a role on pollen development and might have caused pollen sterility in the TGMS line.

Microarray gene expression analysis revealed that 4205 genes were up-regulated and 3559 genes were down-regulated in the TGMS leaves under sterility condition. In sterile panicles, 4867 genes were up-regulated and 2979 genes were down-regulated compared to fertile panicles. Down-regulation of Hsp genes, abiotic stress related transcription factors, interruption in signalling mechanism for regulating pollen development due to down-regulation of calcium associated genes and imbalance in phytohormone homeostasis in the leaf and panicle were associated with the male sterility in the TGMS line. Moreover, significant down-regulation of genes encoding ABC transporter proteins required in the transfer of

precursors for exine and sporopollenin synthesis from tapetum to microspore, lipid transfer protein, strictosidine synthase and glucose-methanol-choline oxidoreductase required for postmeiotic phase of anther development, male sterility protein 2, wax synthase and β-1,3-glucanase in the panicle maintained under sterility inducing condition also influenced male sterility. It was observed that carbohydrate synthesis and transport and hormone biosynthesis were down-regulated under sterility resulted in the production of pollen free anthers in the TGMS line.

Hence the TGMS line can be recommended to the state of Kerala as a female parent for the development of suitable red rice hybrids because of its stable sterility. Hybrid seed production can be done in a particular location if the temperature goes above 27^0C in a particular season. Application of MH (1000 mg L^{-1}) at the time of panicle initiation and fifteen days after panicle initiation is more effective in sterility manipulation when there is drop in temperature. Occurrence of pollen free anthers in the TGMS line during sterility inducing condition is mainly due to the down-regulation of genes encoding ABC transporter proteins, lipid transfer protein and strictosidine synthase, glucose-methanol-choline oxidoreductase, male sterility protein 2, wax synthase and β-1,3-glucanase.

Therefore, in future, the TGMS line should be evaluated in multi locations for assessing suitability and stability. Potential of point mutation in genes associated with male sterility to introduce TGMS trait in conventional rice varieties need to be explored. Information on temperature sensing by plants is quite interesting. Therefore identification of thermo receptors present in TGMS line is necessary for understanding the mechanism of temperature perception and related gene expression. The newly developed stable TGMS line on red rice background may be utilized for developing commercial rice hybrids suitable to the state of Kerala.

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APPENDICES

Appendix I Weather parameters prevailed during the crop growth period (June, 2017 to May, 2018) in the open field condition at College of Agriculture, Vellayani

Standard week	Temperature (^{0}C)			Average Relative Humidity (%)	Sunshine hours (h)	Rainfall (mm)
	Minimum	Maximum	Average			
23	24.60	30.80	27.70	89.80	7.50	5.20
24	25.20	31.70	28.50	83.60	8.30	3.80
25	24.40	32.20	28.30	84.20	8.80	9.50
26	23.70	31.10	27.40	90.60	5.90	20.50
27	24.60	31.70	28.10	83.50	8.90	12.70
28	24.50	31.20	27.90	84.10	7.90	2.50
29	24.60	31.20	27.90	84.70	8.40	5.50
30	25.00	32.20	28.60	81.90	9.30	0.00
31	25.00	32.30	28.70	84.90	8.80	7.20
32	24.50	31.30	27.90	84.20	9.70	9.30
33	24.70	31.10	27.90	86.30	7.70	7.10
34	24.60	30.80	27.70	86.20	6.70	15.30
35	24.40	31.50	27.90	82.70	7.90	4.70
36	24.60	32.30	28.50	84.70	7.90	28.70
37	24.20	31.50	27.80	85.10	7.00	5.10
38	24.40	30.40	27.40	88.00	7.10	15.60
39	24.90	31.60	28.30	86.40	8.70	14.00
40	25.10	31.70	28.40	85.20	6.70	15.80
41	24.80	31.40	28.10	89.40	7.30	17.20
42	24.60	30.70	27.70	92.40	5.60	8.02
43	24.90	31.00	28.00	90.50	8.00	21.70
44	24.80	30.60	27.70	90.70	5.20	5.30
45	24.40	30.60	27.50	90.90	5.30	14.90
46	24.10	31.60	27.90	84.20	8.20	0.00
47	23.90	31.10	27.50	87.40	4.80	22.70
48	22.50	29.50	26.00	94.60	1.10	41.20
49	23.20	31.30	27.30	86.30	8.00	9.40
50	24.10	31.40	27.80	87.00	5.70	0.90
51	23.80	32.30	28.00	84.20	8.80	0.00
52	23.20	32.80	28.00	83.50	9.30	0.00
1	22.10	31.80	27.00	83.30	9.20	0.00
2	21.70	31.30	26.50	86.60	8.20	0.00
3	21.60	32.20	26.90	83.50	8.80	0.00
4	21.50	31.70	26.60	82.40	7.70	0.00
5	22.80	31.70	27.20	82.60	9.10	0.00
6	24.20	32.40	28.30	84.50	9.00	0.00
7	23.70	32.60	28.10	84.70	9.10	0.00
8	23.10	32.50	27.80	85.00	9.10	0.00
9	24.10	33.50	28.80	82.10	9.60	0.00
10	24.10	32.90	28.50	83.90	8.90	0.00
11	24.50	33.30	28.90	84.40	7.90	3.00
12	25.10	33.70	29.40	82.50	9.00	5.30
13	25.60	33.90	29.80	81.20	9.30	0.00
14	25.70	34.40	30.10	80.50	9.40	1.10
15	25.70	33.60	29.70	81.50	8.20	13.20

Appendix I Weather parameters prevailed during the crop growth period (June, 2017 to May, 2018) in the open field condition at College of Agriculture, Vellayani (Continued)

16	25.90	33.30	29.60	83.30	7.30	1.50
17	26.30	34.30	30.30	79.20	9.10	4.30
18	26.10	34.70	30.40	77.90	8.80	2.00
19	25.70	33.20	29.50	82.30	6.10	6.80
20	24.80	32.20	28.50	82.10	4.80	27.30
21	24.80	32.20	28.50	86.30	3.30	10.70
22	25.10	31.50	28.30	86.90	3.90	13.60
23	24.70	30.60	27.60	91.20	4.00	18.10
24	25.10	31.20	28.10	87.20	5.80	9.10
25	24.60	31.00	27.80	88.10	6.20	14.30
26	24.40	31.50	27.90	85.50	6.60	25.20
27	24.70	31.60	28.10	81.00	7.90	10.20
28	23.00	29.60	26.30	89.60	2.80	9.90
29	23.50	30.40	27.00	85.10	5.50	56.30
30	23.60	31.40	27.50	81.30	6.80	4.40
31	23.90	29.50	26.70	85.60	4.60	34.05
32	23.30	30.30	26.80	88.10	2.40	26.80
33	22.60	29.10	25.80	92.40	2.50	34.20
34	24.00	31.00	27.50	83.00	8.30	2.80

Appendix II Weather parameters prevailed during the crop growth period (June, 2017 to May, 2018) inside the rain out shelter at College of Agriculture, Vellayani

Standard week	**Temperature (^{0}C)**			**Relative Humidity (Average) (%)**	**Sunshine hours (h)**
	Minimum	**Maximum**	**Average**		
23	24.80	37.00	30.90	72.50	7.50
24	25.00	38.00	31.50	73.00	8.30
25	24.00	37.00	30.50	72.00	8.80
26	24.50	37.00	30.75	73.50	5.90
27	24.00	35.50	29.75	79.50	8.90
28	24.00	35.00	29.50	76.50	7.90
29	24.50	34.00	29.25	80.00	8.40
30	24.00	35.50	29.75	78.50	9.30
31	24.00	42.00	33.00	77.50	8.80
32	24.00	38.00	31.00	72.00	9.70
33	24.00	35.00	29.50	73.50	7.70
34	25.00	35.50	30.25	75.50	6.70
35	24.50	41.00	32.75	75.50	7.90
36	23.00	42.00	32.50	76.00	7.90
37	24.00	32.50	32.50	75.00	7.00
38	23.50	41.00	28.00	74.50	7.10
39	23.50	32.00	32.25	73.50	8.70
40	25.00	34.50	28.50	73.50	6.70
41	24.00	34.00	29.25	73.50	7.30
42	24.50	34.00	29.25	76.50	5.60
43	24.00	42.00	33.00	74.50	8.00
44	25.00	34.00	29.50	75.50	5.20

Appendix II Weather parameters prevailed during the crop growth period (June, 2017 to May, 2018) inside the rain out shelter at College of Agriculture, Vellayani (Continued)

45	24.00	32.00	28.00	75.50	5.30	0
46	24.50	31.30	27.90	75.00	8.20	0
47	24.00	30.00	27.00	75.50	4.80	0
48	21.00	31.70	26.35	70.50	1.10	0
49	23.50	38.50	31.00	70.00	8.00	0
50	23.50	39.00	31.25	74.50	5.70	0
51	23.50	37.50	30.50	59.50	8.80	0
52	20.00	40.00	30.00	53.00	9.30	0
1	20.00	37.50	28.75	55.00	9.20	0
2	22.50	38.50	30.50	77.50	8.20	0
3	20.00	38.50	29.25	57.00	8.80	0
4	20.50	39.00	29.75	62.00	7.70	0
5	21.00	38.00	29.50	52.00	9.10	0
6	22.50	37.00	29.75	53.50	9.00	0
7	21.00	39.50	30.75	46.00	9.10	0
8	22.50	41.50	32.00	47.50	9.10	0
9	22.00	41.00	31.50	52.00	9.60	0
10	24.00	41.50	32.75	59.50	8.90	0
11	24.00	41.50	32.75	58.00	7.90	0
12	24.00	39.00	31.50	56.50	9.00	0
13	24.00	38.00	31.00	56.50	9.30	0
14	23.50	39.00	31.25	57.00	9.40	0
15	22.00	44.00	33.00	59.50	8.20	0
16	24.00	44.00	34.00	57.00	7.30	0
17	25.00	41.00	33.00	55.50	9.10	0
18	25.00	40.00	32.50	71.50	8.80	0
19	24.50	41.00	32.75	69.00	6.10	0
20	22.50	43.00	32.75	70.50	4.80	0
21	23.00	38.00	30.50	68.00	3.30	0
22	25.00	41.00	33.00	68.00	3.90	0
23	24.50	41.50	33.00	67.50	4.00	0
24	23.50	40.00	31.75	69.00	5.80	0
25	23.50	40.00	31.75	62.00	6.20	0
26	24.00	42.00	33.00	62.50	6.60	0
27	24.00	43.00	33.50	62.00	7.90	0
28	23.50	38.00	30.75	66.50	2.80	0
29	24.00	38.00	31.00	66.00	5.50	0
30	24.00	42.00	33.00	63.00	6.80	0
31	24.50	37.00	30.75	65.50	4.60	0
32	24.00	38.00	31.00	66.50	2.40	0
33	24.50	38.00	31.25	65.50	2.50	0
34	24.00	39.50	31.75	66.00	8.30	0

Appendix III Light intensity (Klux) prevailed during the crop growth period (June, 2017 to May, 2018) inside the rain out shelter at College of Agriculture, Vellayani

Standard week	Open field	Rain Out Shelter
23	105. 2	44.9
24	110.3	70. 2
25	85.1	26.4
26	109.7	41.6
27	124.6	75.9
28	90.7	72.8
29	105.4	54.8
30	125.5	51.6
31	115. 2	70.8
32	92.1	55.9
33	134.3	38.4
34	117.4	78.5
35	97.5	45.9
36	100.7	31.3
37	97.5	47.5
38	90.7	40.7
39	87.2	37.2
40	110.7	60.7
41	97.6	47.6
42	109.7	59.7
43	110.3	60.3
44	77.7	37.7
45	124.6	54.6
46	128.4	68.4
47	97.5	47.5
48	65.7	35.7
49	108.3	58.3
50	125.4	65.4
51	105.4	55.4
52	125.5	65.5
1	104.4	68.5
2	95. 2	72.1
3	100.3	70.6
4	98.1	68.6
5	105.3	70.1
6	121.4	72.3
7	109.3	70.7
8	95.1	70.3
9	113.6	69.1
10	107.4	68. 2
11	124.1	71. 2
12	97.3	65.6
13	100.5	62.4
14	98.0	61.1
15	121.6	70. 2
16	105. 2	65.5
17	103.1	62.1
18	115.2	55.2
19	92.1	42.1
20	84.1	34.1

Appendix III Light intensity (Klux) prevailed during the crop growth period (June, 2017 to May, 2018) inside the rain out shelter at College of Agriculture, Vellayani (Continued)

21	90.5	40.5
22	92.4	42.4
23	87.4	37.4
24	94.2	44.2
25	101.4	51.4
26	103.8	53.8
27	134.3	84.3
28	94.2	44.2
29	94.5	44.5
30	97.9	47.9
31	78.4	38.4
32	97.4	47.4
33	84.2	34.2
34	117.4	57.4

Appendix IV Weather data of RARS, Ambalavayal during the crop period (June, 2017 to November, 2017)

Standard week	Temperature (^{0}C)			Relative Humidity (Average) (%)	Sunshine hours (h)	Rainfall (mm)
	Maximum	Minimum	Average			
22	27.1	19.6	23.3	78.64	4.0	10.4
23	26.4	19.6	23.0	89.21	1.5	18.0
24	24.8	19.8	22.3	90.21	0.9	36.6
25	26.8	19.5	23.2	85.36	3.7	28.6
26	24.4	19.3	21.9	91.29	1.3	116.4
27	24.7	19.4	22.0	95.14	1.3	3.7
28	25.5	18.9	22.2	94.29	2.2	15.6
29	23.8	18.9	21.4	91.36	1.6	126.2
30	25.4	19.2	22.3	85.79	2.8	22.4
31	25.5	19.3	22.4	86.21	3.8	23.6
32	25.7	19.2	22.4	84.50	3.8	60.4
33	24.0	18.7	21.4	86.29	2.0	28.8
34	24.7	19.1	21.9	91.93	0.8	93.6
35	26.6	19.1	22.9	89.79	1.4	128.2
36	27.7	18.8	23.3	87.86	4.8	134.2
37	26.6	20.2	23.4	90.93	2.6	28.0
38	24.3	18.5	21.4	85.29	2.5	136.2
39	26.0	19.6	22.8	91.50	2.8	66.0
40	25.1	21.8	23.5	91.07	1.5	33.4
41	26.0	19.2	22.6	92.21	2.7	35.4
42	26.1	18.9	22.5	85.21	1.9	5.2
43	27.0	17.6	22.3	85.07	4.7	55.6
44	27.6	19.1	23.3	72.14	6.3	0.0
45	26.7	18.4	22.5	82.86	4.5	22.4
46	26.9	17.6	22.2	78.50	5.3	0.0
47	27.9	18.6	23.3	79.64	5.2	45.6
48	26.6	18.5	22.5	83.50	4.9	4.0

PHYSIOLOGICAL APPROACHES FOR MANIPULATING MALE STERILITY IN THERMOSENSITIVE GENIC MALE STERILE SYSTEM FOR HYBRID RICE SEED PRODUCTION

by

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ABSTRACT OF THE THESIS

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Abstract

Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production

Thermosensitive Genic Male Sterile (TGMS) plants which are male sterile above critical sterility temperature (CST) and male fertile below CST can be utilized as an efficient system for developing hybrid rice suitable to the state of Kerala. In this context, a study on 'Physiological approaches for manipulating male sterility in thermosensitive genic male sterile system for hybrid rice seed production' was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani during 2015 to 2018 to evaluate the environmental conditions required for complete male sterility of TGMS plants and to manipulate the male sterility by using plant growth regulators and also to understand the molecular mechanism associated with TGMS system.

The male sterile trait of stable TGMS line EC720903 from IRRI, Philippines, was transferred to red rice variety of Kerala, Jyothi. The CST of EC720903 was determined as 26.9^{0}C and the critical thermosensitive phase was identified as 15-22 days before flowering. The seeds of BC_1F_2 plants were used for the experiment. The seeds were sown in pots in the open field and Rain Out Shelter (ROS) at monthly interval from June, 2017 to May, 2018 to evaluate the environmental conditions required for complete male sterility. The newly developed TGMS line exhibited complete pollen and spikelet sterility throughout the study period at both the experimental conditions since the average temperature prevailed during the critical thermosensitive period of TGMS red rice line was above the CST of 26.9^{0}C. Anatomical studies of anther also showed that the TGMS lines were pollen free which is a preferred character of an ideal TGMS plant. The TGMS plants inside ROS had higher plant height, number of productive tillers and they flowered early compared to open field. Hence this low cost structure which is covered with a UV stabilized transparent sheet can be used for commercial hybrid seed production using TGMS lines throughout the year.

To maintain the sterility expression during the critical stages, the potential of plant growth regulators (PGR), were evaluated at RARS, Ambalavayal. Three PGRs, ethrel (400 mg L^{-1}, 800 mg L^{-1} and 1200 mg L^{-1}), salicylic acid (400 mg L^{-1}, 600 mg L^{-1} and 800 mg L^{-1}) and maleic hydrazide (MH: 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}) were applied as foliar spray at two stages *viz*., panicle initiation and two weeks after panicle initiation. The PGRs were capable of enhancing pollen sterility in all the treated TGMS plants. MH induced significantly higher percentage of pollen sterility (83.49%) compared control plants (19.92%). The external application of salicylic acid at three different concentrations induced 62-82% of pollen sterility in rice and ethrel caused more than 75% pollen sterility. It was also observed that the PGRs applied were not inhibitory to the primary metabolism of TGMS lines. Hence spraying of MH (1000 mg L^{-1}) two times at the time of panicle initiation and fifteen days after panicle initiation can be recommended to maintain male sterility.

The TGMS line was hybridized with two rice varieties as pollen parents, Aiswarya (red) and Swetha (white) using proximal hybridization to evaluate the potential of using TGMS line as a female parent in Kerala condition. Seed setting was significantly high for the cross involving Swetha (40.07%). The F_1 progenies obtained from two crosses along with parents and female parent EC720903 were sown in pots during April-May to September-October. The F_1 progenies attained early flowering and physiological maturity. The protein content at panicle initiation stage was also high. Plant height, photosynthetic rate, transpiration rate and stomatal conductance at panicle initiation stage of F_1 progenies of Swetha were significantly higher. The F_1 plants did not show sterility and the seed setting percentage was in the acceptable range. Complete spikelet sterility was exhibited by the TGMS line since temperature was higher than CST.

Molecular characterization of TGMS plants maintained at sterility inducing and fertility inducing conditions was done using samples collected from leaf and young panicle at ten days after panicle initiation. Protein profiling with SDS-Poly Acrylamide Gel Electrophoresis showed an enhanced expression of proteins at 25-35 kDa, 35-48 kDa and 245 kDa under sterility inducing condition. In the panicle,

increased expression at 17 kDa and presence of protein band at 245 kDa was observed at fertility and it was between 48-63 kDa under sterility inducing condition.

Microarray gene expression analysis of TGMS leaf and panicle revealed that the genes encoding proteins similar to programmed cell death protein 2, Nuclear ribonuclease Z, hormone degradation and conjugation were up regulated and genes encoding proteins similar to male sterility protein 2, pollenless3, pollen-specific protein SF21 and no pollen were down regulated in the TGMS leaf under sterility inducing condition. The genes involved in hormone degradation and conjugation and programmed cell death protein 2 and genes encoding ABC transporter proteins required for exine and sporopollenin synthesis, lipid transfer protein and wax synthase, sucrose transporter, male sterility protein 2, β-1,3-glucanase and tapetal programmed cell death were down-regulated in the panicle. Pathways involved in the IAA, GA, brassinosteroid and jasmonic acid biosynthesis and carbohydrate synthesis and transport was down-regulated during sterility inducing condition in the TGMS line leaf and panicle.

The TGMS line can be recommended to the state of Kerala as a female parent for the development of suitable red rice hybrids because of its stable sterility. If the temperature of a particular location goes above 27^0C in a particular season that period can be used for hybrid seed production. Application of MH (1000 mg L^{-1}) at the time of panicle initiation and fifteen days after panicle initiation is more effective in sterility manipulation when there is drop in temperature. Occurrence of pollen free anthers in the TGMS line during sterility inducing condition is mainly due to the down-regulation of genes encoding ABC transporter proteins, lipid transfer protein and strictosidine synthase, glucose-methanol-choline oxidoreductase, male sterility protein 2, wax synthase and β-1,3-glucanase.