Thermo-sensitive Wild aborted Cytoplasmic Genic Male Sterility in Rice (*Oryza sativa* L.) having majority Panicles Completely Exserted

Gunendu Bikas Bhattacharya (PhD)
 Formerly Rice Breeder, Rice Research Station, Chinsurah- 712101, Government of West Bengal, India & Guest Faculty, Genetics and Plant Breeding Department, Institute of Agricultural Science, University of Calcutta, 51/2 Hazra Road, Calcutta-700 019, India.

**E-Mail:** acharya.g1234@gmail.com, gb.bhattacharya@yahoo.com **Tel:** +91 9836080300 / +91 9163193799

This paper reported a new male sterility system in rice, thermo-sensitive wild aborted cytoplasmic genic male sterility (TCMS-WA) having majority panicles completely exserted. True breeding TCMS-WA lines were developed from the self-progeny of three heterozygous CMS-WA lines (IR 58025A, IR 62829A, and PMS 3A). The plausible explanation for conserving heterozygosity within the genome of CMS-WA parents and its release was put forward. Presence of two different thermo-sensitive nuclear genes, one for inducing TCMS and the other for reverse TCMS character in CMS-WA parents appeared non-allelic and epistatic to the nuclear recessive ‘fr’ gene. Thermo-sensitive genes were found operative in two mutually exclusive environment-influenced genetic thresholds. TCMS-WA lines were superior to CMS-WA parents for homozygosity, panicle exsertion, and fertility-sterility alteration behavior. Genes controlling panicle exsertion were nuclear genes and independent of the cytoplasmic factor. Presence of three different alleles conferring panicle exsertion was postulated and their allelic relationship might be explained as the allele present in R-line was dominant to that in A-line and the allele present in A-line was dominant to that in B-line. Penetrance and expressivity of genes conferring panicle exsertion were elucidated. The major advantages of using TCMS-WA over TGMS for two-line hybrid development was discussed.

**Keywords:** Fertility-sterility alteration, Nuclear genes control panicle exsertion, Heterozygous CMS-WA parents, Homozygous TCMS-WA, Majority panicles completely exserted, Rice, Two-line hybrid.

**INTRODUCTION**

Hybrid rice has become a dream come true with the discovery of wild aborted cytoplasmic genic male sterility (CMS-WA) system and its successful use in three-line (CMS or A-line, maintainer or B-line, and restorer or R-line) hybrid breeding and seed production in commercial scale. In spite of this phenomenal success pioneered by China and then promoted by IRRI, a few deterrents like non-availability of genetically pure F1 hybrid seeds (Rai 2006), incomplete exsertion of panicles in A-line where 30 - 40% of the spikelets remain enclosed in the flag leaf sheath (Gangashetti *et al.* 2004), hazardous seed production technique (Lopez and Virmani, 2000) and instability of male sterility expression under certain weather conditions (Chang *et al.* 2016) etc. plagued the scientists and let them think of ways to overcome those. The landmark discovery of environment-sensitive genic male sterility (EGMS); that is, photoperiod–sensitive genic male sterility (PGMS) by the Chinese scientist, Prof. Shi Min Shong (1985) paved the foundation for two-line hybrid development eliminating B-line. Following the discovery of PGMS, two other kinds of EGMS designated, thermo-sensitive genic male sterility (TGMS) and photoperiod-thermo-sensitive genic male sterility (PTGMS) were also discovered. Fertility of the PGMS lines is mainly conditioned by day length duration while temperature is the main driving force affecting fertility of the TGMS. Fertility of the PTGMS lines is sensitive to both day-length and temperature (He *et al.* 2006). Hence, altogether three different types of EGMS can be distinguished based on differential photoperiod and temperature regime (Virmani 1996; He and Liu, 1998;
Virmani et al. (2003). China became successful in commercialization of two-line hybrid rice cultivation practically feasible using PGMS, PTGMS, and TGMS (Yuan 2003; Chang et al. 2016). Viraktamath and Virmani (2001) suggested that for India and other tropical countries where there is a large regional/seasonal temperature variation; TGMS would be useful for two-line hybrid breeding.

While investigating fertility-sterility transformation in response to change in temperature, Viraktamath and Virmani (2001) observed that indica TGMS lines become sterile when exposed to more than 8 hours at 32 °C and sudden interruption with lower temperature for a short period daily for a few days under the sterility inducing regime induced partial fertility. Furthermore, these authors suggested that for two-line hybrid development, a TGMS should possess critical sterility inducing temperature of 28°C and is also required to remain unaffected by a sudden temperature drop for a few hours daily for a few days. Hence, despite the discovery of TGMS, greater diversity in thermo-sensitive male sterility system in rice is required for the improvement and sustenance of two-line hybrid technology. Bhattacharya and Bose (2005 a, b) first reported the development of reverse thermo-sensitive wild aborted cytoplasmicgenic male sterility (rTCMS-WA) and improved CMS-WA having majority panicles completely exerted in rice. In those two earlier communications heterozygous genetic background of conventional CMS-WA lines was reported as self-population of this male sterility system produced a wide range of genetic variability in different plant characters. Following "Line Breeding" scheme, homozygous rTCMS-WA and improved CMS-WA lines having majority panicles completely exerted were developed and reported without providing any plausible cytogenetic explanation for conserving heterozygosity in the genome of parent CMS-WA lines and variability release.

This paper reports development of yet another type of true breeding thermo-sensitive wild aborted cytoplasmicgenic male sterility (TCMS-WA) having majority panicles completely exerted— a report not in record till date. Moreover, in this report attempt has been made to put forward the cytogenetic/genetic explanation of a number of important but underappreciated issues that are related to CMS-WA male sterility in rice. Finally, the advantages of using TCMS-WA lines, as a female parent, for two-line hybrid development in rice have been discussed.

**MATERIALS AND METHODS**

Self-seeds of three conventional CMS-WA lines; namely, IR 58025A, IR 62829A, and PMS 3A constituted the experimental materials for this investigation. In the wet season of the 1st year, approximately 5000 self-progeny of IR 58025A were grown at the experimental farm of Rice Research Station, Chinsurah (Latitude 22° 52 N’ and Longitude 88° 24 E’), West Bengal (India). Due to unavoidable circumstances, sowing was very late on 1st week of September (mentioned later as the late-wet season). However, 21 days old seedlings were transplanted in the main field. One seedling per hill was planted keeping 20 cm row to row and seedling to seedling distance. Other agronomic practices, as recommended for this zone, were followed to grow a healthy crop.

Instead of breeding true, this population displayed a wide array of genetic variation in different plant characters. At flowering, within this S0 population (henceforth generations will be mentioned as S1, S2, S3 and so on), plants showing white and/or yellowish white anthers with majority panicles completely exerted appeared. These plants were primarily selected and panicles were covered properly with butter paper bags to avoid outcrossing. When spikelet sterility was clearly recognized visually, plants that showed almost complete sterility (> 99% approx.) were finally selected. Near at maturity, these selected S0 single plants were transferred to the earthen pots in the nethouse and maintained there to study male sterility behavior in ratoons with the change of environments associated with the season. In this report, both male sterility and fertility are referred in relation to spikelet.

Earthen pots of 28 cm diameter and 30 cm height were filled with clay-loam soil keeping approximately 10 cm gape from the topmost-edge. Soils in these pots were fertilized with N, P2O5 and K2O (source of N as Urea, P2O5 as Single Super Phosphate and K2O as Muriate of Potash) @ 30:30:30 kg per ha. As supplementary fertilization, N as urea @ 10 g per pot was added at an interval of 25 days from the date of transferring selections in the pots. After fertilization, pots were flooded with water and soils were gently stirred. In the interim period between fertilization, pots were irrigated on alternate days. Need-based plant protection measures were taken to keep the plants healthy.

When the greenish color of unfilled sterile spikelets turned blackish; that is, at maturity, tillers were harvested carefully leaving no matured panicles; so that, new set of upcoming ratoon panicles could easily be recognized for assessing spikelet sterility/fertility. This process of harvesting was followed all through this study period. When ratoon-tillers started flowering in the warm environments of early-summer (2nd fortnights of February to March) and upcoming ratoons continued flowering till still warmer environments of mid- and late-summer (2nd fortnights of April to May), sterile S0 selections interacting with environments diverged into two groups: Group 1 that maintained high sterility (> 99% approx.) over environments and Group 2 selections that showed high sterility in the cold environments of December altered to high fertility (> 50% approx.) in the warm environments of
early-summer, and again reverted to high sterility in the still warmer environments of mid and late summer. Seeds from each selection were collected separately, thoroughly dried and stored for sowing in the next late-wet season.

Henceforth, generations were advanced pursuing “Line Breeding” scheme. In the 2nd year late-wet season, all single plant selections were grown in the field following plant to progeny row (s). Within progeny row of S₁ Group 1, selection was exercised for superior agronomic characters giving emphasis on complete exsertion of panicles, as there was no segregation for sterility trait. From the S₁ Group 2 progeny rows, segregants that showed high sterility, in field-planting, with majority panicles completely exserted were selected. Near at maturity, all selections were transferred to the pots in the nethouse and maintained as before. Selections in S₁ Group 1, in ratoons, maintained high sterility over seasons. Whereas selections in S₁ Group 2, in ratoons, separated into three types: i) selections resembling S₁ Group 1, ii) selections altered from high sterility to high fertility (>50% approx.) in the warm environments of early-summer and remained fertile during the still warmer environments of mid- and late-summer, forming S₁ Group 2 and iii) selections that showed high sterility in the cold environments of December but altered to high fertility in the warm environments of early-summer and converted again to high sterility in the still warmer environments of mid- and late-summer, forming S₁ Group 3. Seeds from each selection were collected, dried and stored for use in the next generation.

In the 3rd year late-wet season, selections were grown in the field as before. From S₂ Group 1 progeny row, plants showing majority panicles completely exserted and other desirable agronomic characters were selected as there was no segregation for sterility trait. Selections were transferred to and maintained in the pots in the nethouse so long plants remain healthy. Ratoons in potted S₂ Group 1 selections exhibited characters like that of S₁ Group 1. Within each progeny row of S₂ Group 2 and Group 3 selections, in field-planting, both sterile and fertile segregants appeared. Plants showing either high sterility or high fertility with majority panicles completely exserted were selected and transferred to the pots. In ratoons, sterile selections of S₂ Group 2 and Group 3 diverged into three types: i) like S₁ Group 1, ii) like S₁ Group 2 and iii) like S₁ Group 3 while fertile selections separated into two types: i) showing characters of S₂ Group 2 selections and ii) showing characters of S₂ Group 3 selections. Seeds from each selection were collected, dried and stored for use in the next generation.

In the 4th year late-wet season, selections of all three groups were grown in the field as before. In S₃ Group 1 progeny row, selection was practiced as for characters in S₂ Group 1 progeny row. In S₃ Group 2 and Group 3 progeny rows both highly sterile and highly fertile segregants with majority panicles completely exerted appeared and those having other superior agronomic characters were selected. All these selections in Group 1, 2 and 3 were transferred to the pots in the nethouse. In ratoons, S₃ Group 1 selections replicated characters of its previous generations. Potted sterile selections, in ratoons, belonged to S₃ Group 2 and Group 3 progeny rows each diverged into three groups: as stated above while in warm environments of early-summer and still warmer environments of mid- and late-summer, fertile selections separated into two types: i) selections maintained high fertility like that of S₂ Group 2 and ii) selections altered to high sterility in mid- and late-summer like that of S₂ Group 3 selections. Seeds from each single plant selection were collected for planting in the next generation.

Following the same procedure as above, breeding materials were advanced until selections became homozygous for characters of Group 1, Group 2 and Group 3 sterility with majority panicles completely exerted and other desirable agronomic characters.

While advancing generations pursuing “Line Breeding” scheme, segregating materials (S₅ through S₉ generations) were first grown in the field in the late-wet season as plant to progeny row (s) and plants that were selected from each progeny row, near at maturity, were transferred to and maintained in the pots so long plants remained healthy; that is, month of May. At maturity, tillers of each selection were harvested carefully leaving no matured panicle; so that, fertility/sterility of spikelets in a new set of upcoming ratoon-panicles could easily be recognized and assessed. From the second year onwards, all selections were deliberately sown late; that is, 1st. week of September; so that, selections get assured cold spell of winter at flowering for sterility expression during December at this location. Every time, panicles of plants that exhibited white and/or yellowish white anthers were covered properly with butter paper bags to avoid outcrossing. For selection within each progeny row (s) in field-planting, primary selection criteria were high sterility in S₀ to S₁ generation whereas both high sterility and high fertility in S₂ generation onwards along with the maximum number of completely exerted panicles. Other desirable agronomic characters were considered next. In the ratoon-crop, based on sustained high sterility or sterility-fertility transformation, lines were separated into three groups of sterility system such as almost complete sterility (> 99 % approx.) over the seasons for the group 1 selections while for the group 2 selections, high sterility in cold environments of December and alteration to high fertility in early-summer and maintaining the same till still warmer environments of mid- and late-summer. For Group 3 selections, high fertility in the cold environments of December to warm environments of early-summer and alteration to high sterility during still warmer environments of mid- and late-summer. Majority panicles completely exerted was the common important character for selection of all three groups of sterility system. For the present study, complete panicle exsertion
indicates when all potential spikelets of a panicle are exposed for outcrossing; that is, just exserted. It is apt to mention that in this report assessment of sterility/fertility percent, during selection, was based on visual observation. Finally, selections in group 1 were designated as improved wild aborted cytoplasmic genic male sterility (iCMS-WA), selections in group 2 as reverse thermo-sensitive wild aborted cytoplasmic genic male sterility (rTCMS-WA) and selections in group 3 as thermo-sensitive wild aborted cytoplasmic genic male sterility (TCMS-WA).

Since large spectrum of variability in plant characters within self-population of IR 58025A was observed, in the 2nd year of ongoing study, another two conventional CMS-WA lines; namely, IR 62829A and PMS 3A were sown in the 3rd week of June, as normal wet season crop, in time isolation and without B-line planting with an objective of getting self-seeds. In the 3rd year late-wet season, approximately 3000 plants from self-seeds of each of above two conventional CMS-WA lines were grown. All management practices remained the same as described for self-population of IR 58025A. Each of this self-population also exhibited genetic variability for three Groups of sterility characters; that is, iCMS-WA, rTCMS-WA, and TCMS-WA with majority panicles completely exserted. Accordingly, the same method for advancing generation and selection were followed to develop three homozygous male sterility systems as mentioned above in this section. In every generation, seeds from each selection were collected, dried thoroughly and stored for use in future.

In the initial generations (S1 to S3), 200 plants of each selection were grown in the field to form progeny rows. The only exception to this was progeny row of iCMS-WA due to the shortage of seed availability. The number of plants in ICMS-WA progeny row never exceeded 9. In S4 and S5 generations, for rTCMS-WA and TCMS-WA selections, plant number was reduced to 100 while this number was further reduced to 50 in the S6 generation. In the S6 generation, seeds from progeny rows showing similar characters for each of the three sterility systems were collected and bulked then stored after proper drying for use in future.

Meteorological data of 10 years, that included the period of this investigation, were used to get a preliminary knowledge about the environmental factors; that is, mean maximum air temperature (Tm), relative humidity (RH), and diurnal temperature (Td) during the sterile and fertile phase of TCMS-WA which is the focused sterility system of this report.

RESULTS AND DISCUSSION

A pilot study was conducted at RRS, Chinsurah in the dry season preceding wet season when this experiment was started. The objective of the pilot study was to identify whether any low-cost chemical spray, mainly hormones, has an increasing effect on panicle exsertion in CMS-WA line; IR 58025A. No effective low-cost chemical could be identified from this study to replace GA3. However, about 87.0 g seeds were obtained after harvest. This crop was time isolated from other rice fields, free from off-types and without B-line planting. Therefore, seeds produced on IR 58025A were logically considered as self-seeds. The said quantity of self-seeds was sown to raise the crop in the first year late-wet season of this experiment to observe whether it breeds true to produce IR 58025A population. If so, a new dimension; that is, the possibility of using such self-population, as the female parent, for two-line hybrid breeding and seed production would open.

Instead of breeding true, the progeny of this self-population displayed a wide array of genetic variability in different plant characters. Plausible explanations for conserving variability in the genome of conventional CMS-WA lines and the reason for its release have been provided later in this section. Within this S6 population, plants showing high sterility (> 99 % approx.) with majority panicles completely exserted appeared and were selected. Deviating from the standard breeding procedure; that is, backcrossing with B-line for maintaining CMS-WA sterility, as selections were initially believed to be, all selections were transferred to the earthen pots and maintained in the nethouse till the plants remained healthy; that is, the month of May to observe the consequences. The assumption was that any living organism including plants, in any severe crisis of completing lifecycle puts every effort to produce offspring/seed for perpetuating generation.

As the environments progressed from cold winter of December to warm environments of early-summer, most of the potted selections, in ratoons, transformed to high fertility (> 50 %) leaving a few as sterile as before. With the advent of still warmer environments of mid- and late-summer, a part of selections that transformed earlier to high fertility further altered to high sterility. Initial male sterile selections, while advancing following “Line Breeding” scheme from S1 to S6 generation, three distinctly different homozygous group of male sterility systems emerged. Entries that were selected in each group had majority panicles completely exserted and other desirable agronomic characters. Group 1 or improved CMS-WA (iCMS-WA) male sterility system is characterized by maintaining high sterility (> 99 % approx.) through cold environments of December, warm environments of early-summer and still warmer environments of mid- and late-summer. But varying number (0-2) of normal viable self-seed setting in panicles somewhere within the study period was observed in this group of selections. The word "improved" prefixed to the newly developed iCMS-WA sterility is for its improvement in character, "majority panicles completely exserted" that differentiates from its parent CMS-WA lines having all panicles partially
exserted. Group 2 selections that showed high sterility in the cold environments of December and converted to high fertility in the warm environments of early-summer and maintained fertility till still warmer environments of mid- and late-summer were designated as rTCMS-WA. While, the Group 3 or TCMS-WA sterility showed high fertility in the cold environments of December through warm environments of early-summer but altered to high sterility during still warmer environments of mid- and late-summer.

To observe whether results obtained from pure lining of selections derived from segregating self-population of IR 58025A is equally applicable for other conventional CMS-WA lines, self-progeny of IR 62829A and PMS 3A were advanced following the same "Line Breeding" scheme. Repetition of results validated the previous findings and helped to generate some important information on CMS-WA sterility system as discussed later in this section.

This method of growing breeding materials in the field provided opportunity for selection within segregating progeny rows for sterility, fertility, panicle exsertion and other desirable agronomic characters while transferring selections from the field to the pots and maintaining there as ratoon-crop over environments (seasons) allowed selections to diverge to two different thermo-sensitive sterility systems: rTCMS-WA and TCMS-WA. When former showed high sterility in the cold environments of December, the latter showed high fertility. Again, when former maintained fertility in the warmer environments of mid- and late-summer, the latter altered to high sterility. In other words, genes conditioning rTCMS and TCMS characters are operative in two mutually exclusive environment-influenced genetic thresholds. Taking sterility/fertility character expression, both in field-planting and in ratoons together, made it possible to identify three different type of male sterility systems; namely, iCMS-WA, rTCMS-WA, and TCMS-WA.

Since TCMS-WA sterility system is the main focus of this report, henceforth, discussion will be concentrated mainly on the said sterility. After S0 generations, the number of TCMS-WA lines, thus developed and finally retained, were 12, 17 and 9 from IR 58025A, IR 62829A, and PMS 3A respectively. All these TCMS-WA lines are homozygous for fertility-sterility alteration trait in presence of appropriate environmental threshold; panicle exsertion and other agronomic characters.

In rice, Ali et al. (2000) first reported the presence of fertility-sterility alteration behavior in conventional CMS-WA lines from their study under high and low temperature environments. Pollen shedding and self-fertility among CMS-WA lines, according to these workers, are due to the presence of a male fertility influencing gene sensitive to environments along with the nuclear ‘fr’ gene which induces cytoplasmic genic male sterility interacting with the cytoplasmic gene ‘ms’. They also mentioned the importance of conventional CMS-WA lines showing fertility-sterility alteration for producing two-line hybrids. But in their report, the heterozygous genetic background of these CMS-WA lines was not spelt out. Bhattacharya and Bose (2005 a, b) first reported the heterozygous genetic background of CMS-WA lines in their above two earlier communications. According to the present investigation self-fertility, often observed, in the conventional CMS-WA lines is due to the presence of two thermo-sensitive nuclear genes; one for rTCMS and the other for TCMS inducing character. These two thermo-sensitive genes are operative in two mutually exclusive environment-influenced genetic thresholds. Moreover, only for possessing fertility-sterility alteration character, a conventional CMS-WA line, as such, can never be useful for two-line hybrid breeding, unless it is made homozygous. Panicle exsertion is another important character. Conventional CMS-WA lines are having all panicles partially exserted. In the contrary, TCMS-WA lines are having majority panicles completely exserted. Hence, the present report altogether differs from that of Ali et al. (2000). This paper first reports the development of homozygous TCMS-WA lines with majority panicles completely exserted (Figure 1).

Figure 1 (a): TCMS-WA line in sterile phase having majority panicles completely exserted, (b) TCMS-WA line in fertile phase showing sterility-fertility alteration trait

The wide range of variability in plant characters was observed within self-progeny of parent CMS-WA lines. No cytological study could yet be done; therefore, it is beyond the scope at present, to explain the observed genetic variation from direct cytological evidence from rice. Nevertheless, how such a wide range of variability remained conserved within the genome of these CMS-WA lines and the cause for its release need a plausible explanation. Knight (1945) in his paper, “The theory and application of the backcross technique in cotton breeding” stated, “The essential value of backcrossing is that it provides a means of limiting the heterogeneity which would result from ‘straight’ crosses between two
types......" Hence, it seems likely that the variation observed resulted from altering the mating system from backcrossing to selfing which increased recombination through increasing chiasma frequency resulting, in turn, heterozygous-heterogeneous population, rather than due to any other cause. There is some evidence from studies on other self-pollinated plant species that low chiasma frequency is associated with outcrossing and high chiasma frequency with selfing (Grant 1958; Bodmer and Parsons 1962 and Allard 1963). Backcrossing with the B-line to develop and/or maintain A-line is a form of outcrossing. In such a situation, when a strong force (backcrossing in parent CMS-WA lines) remains active to restrict the exchange of chromatin material, it affects the release of the variability of progenies and of populations (Srivastava 1980). Sun and Rees (1964) also reported lower chiasma frequency in heterozygous F₁s among a set of Secale spp. and rye than that of their inbred parents. With the change of mating design favoring recombination; that is, selfing in the present case, a large array of variation appears to be released. Harinarayana and Murty (1971) compared the chiasma frequencies between the self-compatible and self-incompatible species of Brassica confirmed the observation of Grant (1958). These workers further reported that a cross-pollinating species will give rise to small but even flow of recombinants generation after generation. This finding is in good agreement with the appearance of so-called 'off-types' in low-frequency generation after generation which is actually segregants within backcross developed and/or maintained A-line population. In support of treating these so-called 'off-types' as segregants, the present author observed that when self-seeds of these so-called 'off-types' were planted that showed segregation for different plant characters within their progeny rows (detailed results will be communicated elsewhere). Harinarayana and Murty, in their above cited paper further reported that the self-pollinating species release their variability in periodical bursts alternating with generation when little recombination occurs and a change in the genetic background can also occur from inbreeding that substantially alters the recombination frequency which will be reflected in the rate of gene segregation and recombination. The restriction of recombination according to these workers, is a universal phenomenon related to the mating system. Hence, it is believed that with the withdrawal of restriction on recombination; that is, replacement of backcrossing by inbreeding encouraged recombination in the genome of CMS-WA lines. In the present case, it appears that 'burst' of variability release might have occurred which was reflected 'in the rate of gene segregation and recombination' resulting in wide range of variability release. However, this can be an interesting topic for future cytogenetic study in rice. Alternatively, heterozygosity in the conventional CMS-WA lines due to outcrossing from unknown pollen source is not tenable; since, mandatorily in a CMS-WA plot thorough rouging is completed prior to anthesis and seed production is done in isolation (natural or time or artificial isolation) from other rice fields.

Since backcrossing causes partial selfing when heterozygous A-lines are maintained by backcrossing with its respective homozygous B-line, according to the principle of genetics that would sporadically throw segregants. These segregants, by mistake, were always considered as "off-types" as a result of outcrossing from unknown pollen source and/or mixtures of B-line. Neither of these views is logically correct. In an A-line seed production programme, prior to harvesting seed parent (A-line), pollen parent (B-line) always is harvested leaving no residue of B-line. Although the pollen parent is harvested first, threshing of the seed parent is completed prior to pollen parent to avoid mixing of B-line seeds. All other precautions to avoid mixture are also meticulously followed as recommended (Virmani 1994). Hence, in a professionally managed A-line seed production programme, how B-line seeds get mixed every time in all years, locations and seasons with that of A-line are least convincing; moreover, when these so-called B-line mixtures are morphologically different from each other and also from true B-line plants. Ali et al. (2000) also reported, but in a conservative way, about the "inappropriateness" of so called "off-types" in CMS-WA population due to outcrossing from unknown pollen source or impurity of B-line. As stated previously, in this section, that when self-seeds of these so-called "off-types" were planted that segregated in different plant characters proving doubtless that these are segregants.

Bhattacharya and Bose (2005 a, b) in their two cited publications reported the development of true breeding rTCMS-WA and iCMS-WA sterility with majority panicles completely exerted from the segregating self-progeny of parent CMS-WA lines. In this communication a plausible cytogenetic explanation for the conservation of heterozygosity within the genome of parent CMS-WA lines and the cause for its release has been provided. Like the above two sterility systems, selfing is the driving force for throwing useful segregants like TCMS-WA, reported here. The appearance of such a wide range of variation within the self-progeny of parent CMS-WA population and success in selecting 3 different type of homozygous male sterility systems having majority panicles completely exerted, led the present author to infer conclusively that parent CMS-WA lines are genetically heterozygous. This further explains why in a CMS-WA seed production plot always plants of different morphology appear. And why production of genetically pure, good quality seeds of three-line hybrid is considered a "great challenge to the breeders" (Rai 2006).

In a two-line hybrid breeding and/or seed production programme, self-seeds of a homozygous female parent (PGMS or PTGMS or TGMS) is always used. Hence, the female line must be homozygous; otherwise quality hybrid seed production would be a mess. Moreover, complete exertion of panicles in a female parent is important for increased availability of potential spikelets for outcrossing (Virmani et al. 1980) and for reduced Fusarium Sheath rot
(FShR) disease incidence (Vinod et al., 1990; Biswas and Samajpati, 2000). In view of better exertion of panicles in three-line hybrid seed production using CMS-WA lines, spraying of GA3 is in practice (Dazhou 2007). Spraying GA3 may improve the situation to a certain extent but does not solve the problem. Moreover, GA3 is a costly chemical which significantly increases the cost of hybrid seeds (Virmani 1994) and also adversely affects the quality of hybrid seeds reducing dormancy and storage life (Honnaiah 2003). Hybrid rice breeders, therefore, are in constant search for a genetic alternative to the GA3 application (Khera et al. 2009). Nonetheless, rice breeders tend to employ the CMS-WA, preferentially, as it gives stable CMS lines (Das et al. 2010). About 85 percent of the A-lines used in commercial hybrid seed production still belong to the WA type (Yuan 2003). Therefore, development of homozygous TCMS-WA lines with majority panicles completely exserted would be a novel addition to the male sterility system for two-line hybrid breeding and genetically pure hybrid seed production in commercial scale.

From the present investigation, three different type of true breeding wild aborted cytoplasmic genic male sterility systems: i) iCMS-WA, ii) rTCMS-WA and iii) TCMS-WA with majority panicles completely exserted were developed from the segregating self-population of conventional CMS-WA parents. Hence, these three groups of male sterility system itself prove the existence of at least 3 different nuclear genes, one each responsible for conditioning iCMS-WA, rTCMS-WA, and TCMS-WA character. Of these genes, only the recessive 'fr' gene in iCMS-WA is well documented. The phenomenon of pollen abortion in rice-CMS is known for the incompatibilities between mitochondrial-nuclear genomic ('ms' - 'fr' genes) interactions (Fuji et al. 2010). But no information is yet available in rice about these two thermo-sensitive genes. However, when these two thermo-sensitive genes simultaneously exist along with recessive 'fr' gene, as in the heterozygous parents or in early generation heterozygous breeding materials, one is expressed depending upon the environmental threshold while expression of the other remains suppressed and this relationship is reciprocal. That is why, heterozygous parent lines and/or early generation heterozygous breeding materials showed sterility in the cold environments, alteration to fertility in the warm environments (rTCMS character) and again conversion to high sterility in the still warmer environments of mid- and late-summer (TCMS character) (Figure1b). This suggests a non-allelic relationship between these two thermo-sensitive nuclear genes. Since environment is the main driving force to trigger gene expression in such cases, it appears that in absence of proper environmental threshold, none of these two thermo-sensitive genes would produce its full phenotypic expression. Therefore, while working with such type of heterozygous conventional CMS-WA sterility, there is no wonder while working, different workers would report different degrees of male sterility and/or fertility in these lines.

Ali et al. (2000), evaluated self-fertility in 8 CMS lines, that included 5 conventional CMS-WA lines; namely, IR 58025A (25A), IR 58289A (29A), Pusa 33-16 A, Pusa 2-33-123A and Pusa 1-33-119A under natural field conditions and in the controlled growth chamber. Under natural low temperature at Katrain, these workers observed 49.0, 0.0, 50.7, 51.5 and 17.8 % spikelet fertility in 25A, 29A, Pusa 33-16 A, Pusa 2-33-123A and Pusa 1-33-119A respectively. To the contrary, in natural high temperature in Delhi, all these above lines showed 100 % sterility while 29A exhibited 100 % sterility in both the locations. Excepting 29A which showed 100 % sterility in the growth chamber (cool regime), all other lines showed a very high spikelet fertility which ranged from 79 to 86.8 %. Lara et al. (1992), evaluated 5 CMS-WA lines at 3 locations in the Philippines for pollen sterility, spikelet fertility, and other characters. Their results suggest that CMS-WA lines responded differently over locations for the expression of pollen sterility and spikelet fertility. Nghia et al. (1994), evaluated 13 CMS-WA lines at Vietnam and reported 2 and 5% spikelet fertility in 29A and 25A respectively in the wet season while other 11 lines showed 99-100% spikelet sterility. In the previous season at the same location, these workers observed 100% spikelet sterility in all the CMS-WA lines including 25A and 29A. Bhaumik et al. (2001), reported 14.1 and 12.9% pollen fertility in 25A and 29A respectively from West Bengal (India) in the dry season. Hence, pollen and/or spikelet fertility in CMS-WA is not due to genetic-breakdown of CMS-WA lines, rather it is due to the presence of two different thermo-sensitive nuclear genes along with recessive 'fr' gene. Each of these thermo-sensitive genes can only be operative in presence of its appropriate environmental threshold; otherwise, its expression remains suppressed showing 100% pollen and/or spikelet sterility in CMS-WA lines.

During advancing generations, it was observed that iCMS-WA segregants that were selected did not further segregate for its male sterility trait but segregated for other characters. Contrary to this, both rTCMS-WA and TCMS-WA selections each segregated further to iCMS-WA, rTCMS-WA, and TCMS-WA character. Hence, pollen and/or spikelet fertility in CMS-WA is not due to genetic-breakdown of CMS-WA lines, rather it is due to the presence of two different thermo-sensitive nuclear genes along with recessive 'fr' gene. Each of these thermo-sensitive genes can only be operative in presence of its appropriate environmental threshold; otherwise, its expression remains suppressed showing 100% pollen and/or spikelet sterility in CMS-WA lines.

In the hybrid literature, it is documented that partial exertion of panicles in CMS-WA sterility is conditioned by cytoplasmic factor rather than nuclear gene (Vinod et al. 1990; Gangashetti et al. 2004). Such being the case,
segregates like majority panicles completely exserted and all panicles partially exserted would have not been appeared within each heterozygous progeny rows of early generation segregating materials during the course of this investigation. Differentiating between nuclear gene and organelle gene inheritance of characters, Geiner et al. (2015), stated “Alleles of a nuclear gene always segregate during meiosis, with half of the gametes receiving one allele and half the other (‘Mendel’s first law’). Alleles of organelle genes may or may not segregate during meiosis; the mechanisms are the same as for vegetative segregation. Nuclear genes usually display Mendelian segregation. The non-Mendelian inheritance of organelle gene (s) is predominantly uniparental. Thus, organelle inheritance can be recognized as the reciprocal difference in sexual crosses. The virtual absence of recombination is among other features of organelle inheritance”. Birky (2001), while reviewing the inheritance of genes in mitochondria and chloroplasts reported, “Alleles of a nuclear gene always segregate during meiosis, with half of the gametes receiving one allele and half the other (‘Mendel’s first law’). Alleles of organelle genes may or may not segregate during meiosis; the mechanisms are the same as for vegetative segregation”. Both the above reports conformed to the nuclear gene control of panicle exsertion. Although, no reciprocal cross could be made but study of F2 population derived from selfing of F1 hybrids that included CMS-WA as female parent clearly displayed Mendelian segregation for panicle exsertion (data not presented). Hence, it can be concluded that panicle exsertion in conventional CMS-WA lines is conferred by nuclear genes and not by the cytoplasmic factor.

When a B-line having all panicles completely exserted, is crossed to an A-line having all panicles partially exserted, that always results an A-line with all panicles partially exserted. Hence, gene conditioning complete exsertion of panicles in B-line appears to be recessive to the gene conferring partial exsertion of panicles in A-line. Further, when a R-line with all panicles completely exserted, is crossed to an A-line that produces a F1 with completely exserted panicles suggesting gene responsible for panicle exsertion in R-line is dominant on the gene controlling panicle exsertion in A-line. From the above observations, the presence of three different alleles controlling panicle exsertion in A-, B- and R-line has been postulated and their allelic relationship appears as; gene present in R-line is dominant on that in A-line and gene operative in A-line is dominant on that in B-line. Mostly in all selections, irrespective of height, it was observed that instead of all panicles being completely exserted, a few were always left partially exserted. Even for complete exsertion, there is considerable variation in the degree of exsertion between plants as well as panicles of the same plant. This indicates incomplete penetrance and variable expressivity of the genes controlling panicle exsertion. Therefore, it appears that there is influence of environment and/or modifiers on the phenotypic expression of panicle exsertion.

For TCMS-WA lines at Chinsurah location, flowering in December and early-summer was observed as fertile phase while flowering in still warmer environments of mid- and late-summer was identified as the sterile phase. The 1st fortnight of February and the 1st fortnight of April, by and large, were the transition periods when varying degrees of fertility (< 50%) were observed and hence, was identified as the intermediate phase. A primary face investigation was attempted taking 10 years meteorological data maintained at this research station which covered the period of this investigation, to understand the environmental control for fertility-sterility alteration of TCMS-WA selections. It was observed that desired sterility level (> 99 % approx.) was achieved when at flowering average maximum air temperature (Ta) remained ≥ 32°C, diurnal temperature (Td) ≤ 2-3°C, and Relative Humidity (RH) around 65-67 %. In contrast, during the fertile phase, flowering time witnessed average maximum Ta ≤ 29°C, Td ≥ 9°C, and RH around 70-85 %. From these observations, it appears that high Ta with low RH are two important environmental variables that act as causal factors for inducing sterility. Further, as Td is influenced by RH, the role of Td cannot be ignored. While investigating the causal environmental factors affecting floret fertility in rice, Matsushima et al. (1982) and Matsui et al. (2007) reported that low RH in air associated with high Ta during anthesis increased floret sterility in rice. Brar (1982) mentioned the possible role of day/night temperature difference (Td) in inducing male sterility in sesame. Above studies also suggest that high Ta alone might not be the only environmental factor to affect reproductive physiology of TCMS-WA lines. However, a future study in controlled environments would provide precise information regarding environmental control on fertility-sterility alteration behavior of TCMS-WA sterility.

Major advantages of TCMS-WA lines over their CMS-WA parents are: 1) significant reduction in the cost of F1 hybrid seed production by switching over from three-line to two-line hybrid system, 2) simplification of hybrid seed production technique by eliminating the first stage; that is, maintenance of CMS-WA line by backcrossing with B-line, 3) homozygous genetic background which is essential for the production of genetically pure F1 hybrid seeds, 4) complete exsertion of majority panicles (Figure 1a) which in turn would a) increase the availability of potential spikelets for outcrossing, b) reduce incidence of FShR disease and c) withdrawal of GA3 application which will significantly reduce the cost of F1 hybrid seed production in commercial scale and also increase seed quality, and 5) most importantly, fertility-sterility alteration behavior — an obligatory trait (Figure 1b) for two-line hybrid breeding. Furthermore, 6) F1 hybrid between a homozygous female (TCMS-WA) and a homozygous pollinator (R-line) is expected to offer a higher magnitude of F1 heterosis.

The major limitation for TGMS is that sudden interruption with lower temperature even for a short period daily for a few days in their sterility inducing regime causes partial
fertility (Viraktamath and Virmani, 2001) which would jeopardize hybrid seed production. Like TGMS in TCMS-WA also, partial pollen fertility may appear in its sterile phase for the sudden onset of unfavorable environments. Nonetheless, unlike TGMS, the risk of self-seed setting on the TCMS-WA female would be least as fertile pollens in WA cytoplasm are expected to be less competitive than their counterpart in R-line carrying normal cytoplasm. Cheng et al. (2008) studied in vitro pollen germination and pollen tube growth in pistil of rice and reported that rice pollen starts to germinate at 2 minutes after pollination and the pollen tubes penetrate stigma into style in 5–10 minutes and only some pollen tubes reach embryo sac at 40 minutes after pollination. Fujii et al. (2010) reported pollen germination ability on the stigma of those CMS lines in rice that did not exhibit complete pollen dysfunction; namely, CW-CMS and W11-CMS lines which were classified as CMS-WA-like types (Huang et al., 2014). Fujii et al. observed that approximately 80% of pollens on the stigma germinated 6 hours after anthesis in wild-type T65 plants. In addition to pollen competition, pollen load size is another important factor for self-seed setting on a female parent. Matsui and Kagata (2003) observed that more than 20 fertile pollens are required on the stigma of a rice floret for successful self-fertilization. In the rightly selected target environments (location and time) for hybrid seed production in commercial scale where TCMS-WA lines will show 99-100 % spikelet sterility, the chance of such a large number of fertile pollen deposition on the stigma for self-fertilization would be least. Moreover, the difference in pollen germination time between fertile pollens in the normal cytoplasm as in TGMS and fertile pollens in the sterile cytoplasm is expected to render functional stability for male sterility to TCMS-WA lines even in unfavorable environments. Therefore, these TCMS-WA lines can successfully be used as a female parent for genetically pure two-line hybrid seed production at the target location. For seed production, TCMS-WA lines like their parents will need R-lines to use as pollinator unlike TGMS lines which are free from this restriction. Nevertheless, TCMS-WA lines would be the better choice than TGMS lines for two-line hybrid seed production considering their other merits.

CONCLUSION

This paper reports a new male sterility system in rice, TCMS-WA which were developed from the self-population of three CMS-WA lines; namely, IR 58025A, IR 62829A, and PMS 3A as these parent lines are heterozygous and produced a wide range of genetic variability for different plant characters. For two-line hybrid breeding and its genetically pure F₁ seed production in tropical countries, a suitable thermo-sensitive male sterile line would be required to use as a female parent. This male sterile line must be homozygous for all traits including fertility-sterility alteration. Moreover, the female parent must show genetic and/or functional stability for male sterility expression in its sterile phase even in sudden onset of unfavorable environments, like low temperature, persisting for a short period of time. Along with these characters, complete exsertion of panicles will offer a higher number of potential spikelets available for outcrossing without GA3 spray which would significantly reduce the cost of F₁ seed production, reduce the incidence of FShR disease and increase seed quality. Genes controlling panicle exsertion are nuclear genes. Presence of three different alleles conferring panicle exsertion have been postulated and their allelic relationship might be explained as the allele present in R-line is dominant to that in A-line and the allele present in A-line is dominant to that in B-line. Incomplete penetrance and variable expressivity of genes conferring panicle exsertion were elucidated. TCMS-WA lines are homozygous and exhibit high spikelet fertility (> 50% approx.) in their fertile phase during cold winter of December to warm environments of early-summer. And alter to high sterility (> 99 % approx.) in still warmer environments of mid- and late-summer (sterile phase) when average maximum Tₐ, RH, and Tₑ remain approximately ≥ 32°C, 65-67 % and ≤ 2-3°C respectively. Complete exsertion of majority panicles in TCMS-WA lines without GA3 spray and their maintenance without B-line would significantly reduce the cost of F1 hybrid seed production. Further, it is evident from the reports of Chen et al. (2001), Matsui, Kagata (2003) and Fujii et al. (2010) in rice that TCMS-WA lines would provide functional stability for sterility expression in its sterile phase compared to TGMS lines even if fertile pollens, to a limited percent, appear due to sudden onset of unfavorable environments. Hence, TCMS-WA lines would be a better option than TGMS lines for two-line hybrid breeding and seed production although these are not free of R-line like TGMS.

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CONFLICT OF INTEREST

There is no conflict of interest for this paper.
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