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REVIEW ARTICLE Agricultural Reviews, Volume Issue : ()

Next Generation Hybrid Seed Production Methods-Superior and Beneficial Biotechnological Approaches: A Review

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10.18805/ag.R-2437

ABSTRACT

Hybrid seed production technology requires cross pollination between parents of choice. Several physical and chemical techniques including manual or mechanical emasculation and male gametocide chemicals have been introduced each with respective disadvantages. Easy and quick technique such as environment sensitive male sterility is effected by environmental irregularities. Identification of commercial use of genetic means of male sterility systems like GMS and CGMS have brought revolutionary benefits to hybrid seed industry in many crops to feed an ever increasing human population. However, these strategies are complicated, laborious and require more cropping area for parental increase and maintenance including employment of sophisticated backcross breeding for line conversion and search for suitable restorers. The biotechnological methods (BMS) simplified the strategies each in its own approach, though having certain disadvantages too as for example, dual component Barnase/Barstar and Cystein protease/ Cystatin approach needed herbicidal spray. The next generation hybrid breeding systems were developed utilising GMS system under nuclear control avoiding search for suitable restorers and cytoplasm-nuclear interactions. The era of genomics assisted breeding has facilitated well characterised study of several crop genes including male fertility genes which is a never ending approach. In combination with transgenic and genome editing approaches, the next generation hybrid breeding systems such as “Seed Production Technology” (SPT), “Multi Control Sterility” (MCS) and “MGM-Maintainer approach” have provided easy and well defined mode of utilising male sterility for hybrid seed production bypassing all the complications of traditional techniques.

Key words: Biotechnological methods, Hybrid seed production, Male sterility, MGM-Maintainer approach, Next generation hybrid breeding.

Self-pollination as a breeding system favours constancy and fitness of genotypes. The breeding procedures employed for the naturally self-pollinated crops generally focus on development of homogeneous and homozygous populations usually as purelines by exploitation of existing variation via a phase of introduction, acclimatisation, selection, evaluation and release or by creation of variability by mutation and assimilation of variable allele forms through hybridization followed either by pedigree/mass/bulk selection or backcross breeding programmes all of which tends to utilise the additive gene effects and fix them. Cross pollinated crops on the other hand are highly heterozygous and heterogeneous species and possess specific heterozygous balance rendering respective considerable inbreeding depression upon inbreeding. The breeding procedures in these broadly focus on hybrid development exploiting overdominance and population improvement utilising additive gene effects. Inbred development, selection of appropriate parents and hybridization are well followed in both self and cross pollinating crops resulting in promising lines with favourable gene combinations. Mere selection of inbreds based on high per se performance is not indicative of high yielding hybrid upon crossing, though good indication of the same was previously reported in self-pollinating species (Busch *et al*., 1974; Hamblin and Evans, 1976) however, valuable additional information in the form of combining ability from appropriate mating designs is equally important. Commercial large scale exploitation of heterosis is strongly based on availability of suitable pollination control system such as male sterility. Male sterility was first utilised for hybrid seed production in Sorghum (Stephens, 1937) and onion (Jones and Emsweller, 1937). Male sterility is expressed in the form of malformation or absence of male sex organ in hermaphrodite flower, abnormal anther tissues, inability of pollen to mature, germinate or be released and no development of pollen. Detasseling technique is a predominant for hybrid breeding programme employed by breeder and commercial seed production in some crops. This method of vegetative part removal by mechanical detasseling results in reduction in nearly 40% inbred seed yield and causes detrimental effect (Wych, 1988). Where manual emasculation is not feasible as in case of rice, wheat, pearl millet and sorghum, male sterility via chemical or genetic means is an economical mode of hybrid seed production. In addition, the technique is labour intensive, time consuming and expensive.

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How to cite this article: Ikkurti, G. (2022). Next Generation Hybrid Seed Production Methods-Superior and Beneficial Biotechnological Approaches: A Review. Agricultural Reviews. DOI: 10.18805/ag.R-2437.

Submitted: 02-12-2021 **Accepted:** 28-10-2022 **Online:** 07-11-2022

Systems of male sterility

Numerous chemicals to be applied as foliar sprays were reported and introduced as potential gametocides. Chemical induction of male sterility was exploited after its first successful report on Zea mays L. (Moore, 1950). The technique is widely used in vegetable crops (Meer and Bennekom, 1973; Naveen *et al*., 2017; Yu *et al*., 2017) however, molecular mechanism and proteins involved are little know regarding CHA induced sterility. There has been limited industrial use of chemical hybridising agents due to disadvantages revolving around including environmental dependency, dose and duration specificity, residual effects, incomplete male sterility, logistical challenges and need for constant visual moitoring. Moreover, reduced seed yields were reported when used in excess (Newhouse *et al*., 1996). Among different types of male sterility systems reported, genetic male sterility is caused by nuclear genes alone (GMS). This type can also be artificially induced using mutagens like X-rays, Ethidium bromide and Neutrons (Kaul, 1988). The male sterile line expresses sterility in usually recessive homozygous state (ms ms) and heterozygote in recessive/wild type state (Ms ms) is used as a maintainer line. Sterile lines are maintained by crossing them with maintainer line and sterile plants are sorted for hybrid seed production where, they are crossed with homozygous fertile counterpart (Ms Ms) with diverse nuclear background. This heterozygous pattern makes it difficult to sort the GMS line making it laborious and time consuming. However, tight linkage with a molecular or morphological marker can ease the sorting-out job. In maize, seventeen GMS genes have been identified and cloned so far. All are recessive except ms44 (Fox *et al*., 2017). In addition, 62 putative maize orthologous genes have also been identified in Rice, Arabidopsis, wheat and barley combined. Information derived from cloned maize GMS genes have facilitated development of several biotechnology based male sterility system (Wan *et al*., 2019).

Further, the two line hybrid seed production system is 2nd generation GMS based hybrid technology comprising male sterile female and pollinator lines with application of environment sensitive GMS where male sterility is under genetic control (EGMS). The system functions in response to fluctuations in environmental conditions and seed production in easy and economical. Nong-ken 58s was the first reported photoperiod sensitive male sterile line that came in 1973 (Shi, 1985). The system requires 100% male sterility, clear and distinct fertility restoration and male sterility needs to last for more than 30 consecutive days. The system is further categorised as photoperiod sensitive (PGMS), temperature sensitive (TGMS) and photo-thermo sensitive (PTGMS). In rice under PGMS control, male sterility is induced in response to exposure to day length more than 13 hours and transformed to fertile plants when grown in day length less than 10 hours. Hybrid seed production is undertaken in longer day conditions. Temperature above 27°C in some rice lines, Annong S, Pei Ai 64 S and Hennong S, under TGMS control render sterility which is reversed when grown in below 24°C. This expression is reversed in certain other lines. The suitable tempersture regimes are used for hybrid seed production. The hybrid development in EGMS system is relatively simple as maintained line is not needed and pollinator can be chosen as any parent based on breeder's choice and analysis ensuring greater flexibility. Other advantages associated with EGMS system are that negative influence of male sterile cytoplasm is avoided and twice the area can be planted under commercial hybrid starting with equal female allocated area (Singh and Gopalkrishnan, 2003). However, this system is prone to unpredictable environmental conditions resulting in line maintaining problems sometimes leading to loss of a season and efforts.

Biotechnology based GMS hybrid seed production also called as next generation hybrid seed technology are the effective genetically engineered male sterility approaches with promising future impact in hybrid seed production. The approaches facilitate easy sorting on GMS and maintainer lines are covered further. Cytoplasmic genetic male sterility (CGMS) system comprises of male sterile line, maintainer line and restorer line as key components and was called as first generation hybrid seed technology (Fig 1). The sterility is under cytoplasmic gene and expression results as a function of interaction between cytoplasm and nucleus. The MS line is present with cytoplasmic male sterility gene and lacks nuclear fertility restorer gene. The restorer is homozygous dominant for male fertility restoring gene and restores fertility for CMS lines. The sterile lines are maintained by crossing with maintainer line that contains fertile cytoplasmic gene. Hybrid seed production through this system comprises of identification of suitable restoration lines with good combining ability and crossing with respective male sterile line. Thus the three line system gets

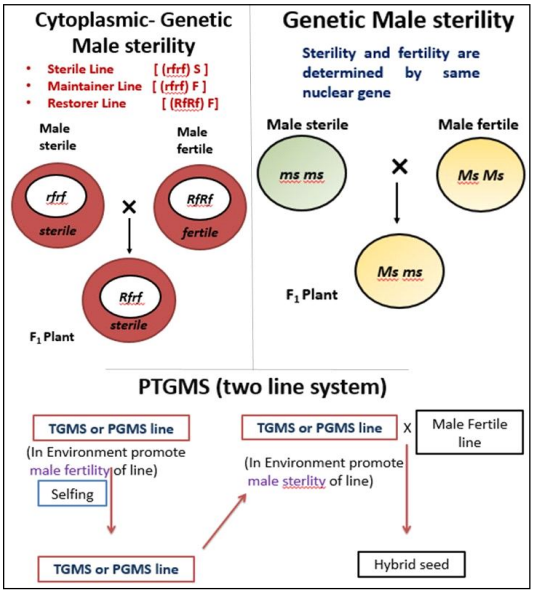

Fig 1: Male sterility systems, genetic and environmental.

complicated with several intrinsic problems with diversification of cytoplasmic lines, identification of suitable restorers which usually creates bottleneck where unreliable restorers are mostly found, breakdown of sterility in different genetic backgrounds and environments (Williams, 1995; Wu *et al*., 2016). The system is effective when suitable restorers are found, where unavailable the sterility is called as cytoplasmic male sterility (CMS). As CGMS system depends on diversification and identification of usually spontaneous male sterility genes, the method is not extensively applied in several crop species. Therefore, the next generation hybrid seed production technology was envisaged to exploit the biotechnological tools to generate MS lines and considerably generate sustainable hybrid seed production technology.

Dual component based male sterility utilising biotechnological approaches (BMS) were developed initially in tobacco, oilseed rape and rice such as Barnase/Barstar system, MYB103 chimeric system and Cystein/Cystatin system (Mariani *et al*., 1990; Li *et al*., 2007; Shukla *et al*., 2016). *Barnase/Barstar* system, first well-known BMS system developed in tobacco and Cystein/Cystatin system expresses in tapetal cell layer of anthers and causes male sterility under the action of *barnase* and Cystein protease genes, respectively both under *TA29* promoter. Crossing *TA29-barstar* lines with *barnase* lines leads to restoration of fertility as is similar with crossing with cystatin lines with Cystein protease carrying lines. However, the *Barnase/ Barstar* system system was linked to timely application of herbicide which removes transgenic plants facilitated by selectable marker phosphinothricin actyltransferase conferring tolerance to basta herbicide. This approach is not commercially favoured as the system requires timely application of herbicide adding to cost and labour and can't be applied in crops such as wheat with low multiplication factor, in addition, a mixture of transgenic and non transgenic seed is produced as hybrid seed and reversion to fertility was also observed (Banga and Raman, 1998). Another system of male sterility developed in Arabidopsis called as MYB103 chimeric system disrupts the development of tapetum and pollen by disrupting AtMYB103 by introducing AtMYB103-EAR chimeric repressor causing male sterility. Fertility is restored when the transgenic MS lines is crossed with restorer introduced with corresponding AtMYB103 gene driven by strong promoter.

Though these systems save the effort in identifying suitable restorers, however, the F_1 hybrid seed is a transgenic which is complicated when passing through regulations for commercialization. This minimizes the commercial application of transgenic approach for hybrid seed production. The next generation hybrid breeding is an approach that exploits complete benefits from transgenic technology while generating a non transgenic final commercial product. This technology is called as transgenic construct-driven non-transgenic seed system (Wan *et al*., 2019). The mapped as well as characterised male sterile mutants provide an excellent source of emasculation as in maize for hybrid seed production through construction of their respective male fertile counterparts.

Next generation hybrid breeding

One such is "Seed Production Technology" (SPT) from DuPont Pioneer (Wu *et al*., 2016). This hybridization system utilizes nuclear male sterility wherein, transgenic SPT maintainer is generated through transformation of male sterile mutant line. The team used *ms45* male sterile allele. The wild type *Ms45* allele was capable of complementing the male sterile phenotype in a single transformed copy when transformed into *ms45/ms45* plants. The system avoids search for suitable restorer/fertile allele counterpart and aims to generate all male sterile line during parent increase. The maintainer line is generated by transforming male sterile line with construct consisting of male fertile allele (Ms45), maize alpha-amylase gene for making transgene carrying pollen inviable and a screenable marker gene coding for DsRed2 protein to facilitate direct identification and removal of transgenic seeds from mixture. The construct of SPT is integrated in the maintainer line in a hemizygous form. This causes production of 50% fertile pollen grains (*ms45/ms45*) while the other 50% are deprived on starch due to action of alpha-amylase gene (*ms45/ms45; SPT/_*). The male sterile and maintainer lines are maintained or increased by crossing transgenic maintainer line with male sterile line and harvesting the progeny from male sterile line giving only male sterile progeny. Upon selfing transgenic maintainer line, the progeny consists of both male sterile and SPT maintainer lines which are selected based on seed colour and male sterile are discarded and SPT maintainer are retained as maintainer lines (Fig 2). SPT transgene is only inherited from female gametes of maintainer line, this facilitates inheritance of SPT in hemizygous state only on

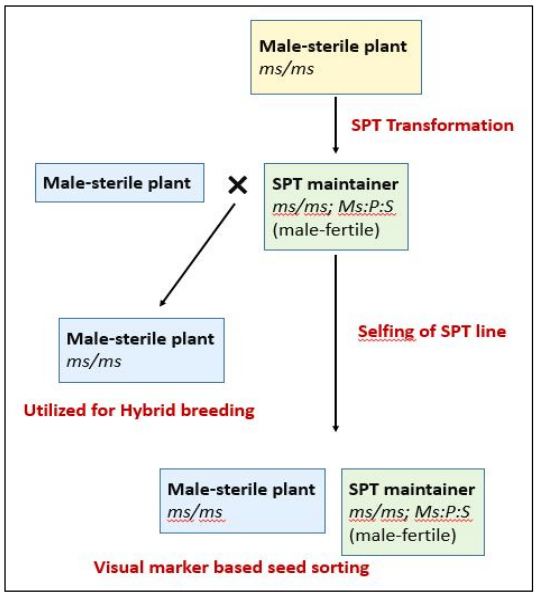

Fig 2: Propagation of SPT based system.

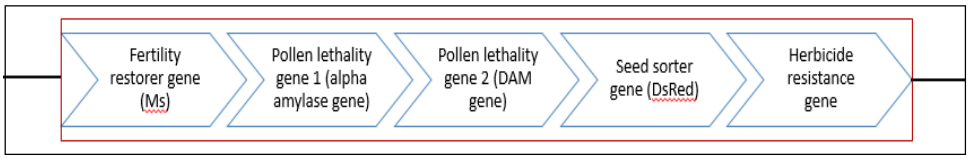

Fig 3: A representation of MCS transgenic construct.

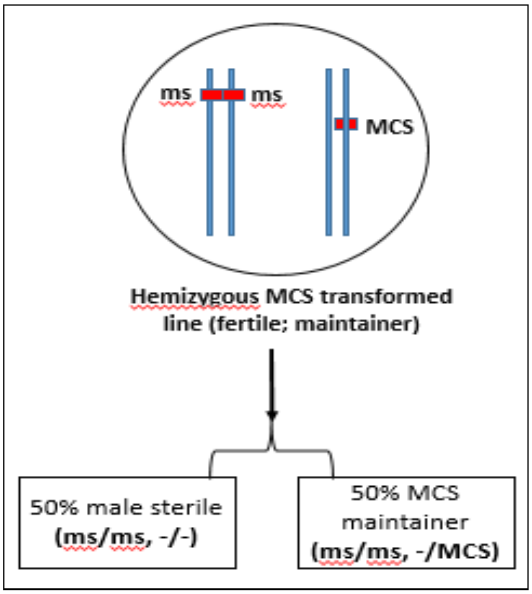

Fig 4: Multiplication of mainatainer line in MCS based strategy; MCS present in hemizygous state.

selfing. This system facilitates non transgenic male sterile line multiplied through transgenic approach to be crossed with any well combining male parent for hybrid seed production giving 100% non transgenic hybrid seed. The SPT system is associated with many advantages which include no need for detasseling, broad utilization in maize germplasm and increased hybrid seed purity. However, the transgene transmission rate through pollen was found to range from 0.002% to 0.518% when under single pollen disruption gene.

Therefore, to minimise the transgene transmission rate from maintainer pollen, a multi control sterility (MCS) system was developed base on maize *ms7* male sterility mutant (Zhang *et al*., 2018). The wild type male fertile gene *ZmMs7* was isolated and cloned leading to its utilization in GMS based hybrid seed production system. The MCS transgenic construct consisted of 5 components as functional modules which are, wild type ZmMs7, two pollen disruption modules as maize alpha-amylase gene under late pollen stage promoter PG47 and DNA adenine methylase gene *Dam*, screenable marker red fluorescent protein gene *DsRed2* facilitating sorting of transgene carrying maintainer red seeds from male sterile normal seeds and a herbicide resistant gene *Bar* capable of propagating transgene containing pure seeds only during maintainer multiplication (Fig 3). The two pollen disruption genes in the construct affects the transgenic pollen viability causing abnormal inviable pollen resulting in male sterile plants only when transgenic maintainer is crossed with male sterile parent. The maintainer line is increased by selfing the transgenic line resulting in the progeny with 50% transgenic male fertile maintainer line and 50% male sterile line selected and sorted in the harvested ear facilitated by *DsRed2* gene expression only in maintainer seed containing transgenic element in hemizygous state (Fig 4). Transgene transmissibility rate varied from 0.03% to 0.04% when under two pollen disruption elements and varied from 0.236% to 0.301% when under 0.23% to 0.30%, showing significantly reduced rates of transgenic pollen transmission when under two pollen disruption modules. Further, the herbicide resistance gene Bar facilitates selection and purity maintenance of transgenic maize maintainer lines by herbicide spray eliminating non transgenic plants. The MCS system is beneficial over CMS in the fact that MCS is under control of single nuclear male sterile recessive gene and any maize germplasm can be crossed as male in hybrid seed production programme avoiding search for any suitable restorers. Over SPT, the MCS is capable of providing 3 stages of checks preventing transgene flow and at the same time ensuring transgenic purity as only MCS maintainer line carries transgenic element, neither the sterile A line nor the hybrid seeds.

Hybrid breeding by genome editing

The current biotechnological advancement provided opportunities that precisely generate mutations in target genes as demonstrated in several crops (Chen *et al*., 2019; Dong *et al*., 2019). A next generation system of hybrid breeding was introduced, called as manipulated GMS maintainer (MGM), using CRISPR/Cas9 based gene knockout strategy and simultaneously providing with same nuclear male fertile exogenous element (Qi *et al*., 2020). Maize nuclear fertility gene *ZmMS26* was chosen and vector to develop GMS line was created using MS26 editor cassette consisting CRISPR/Cas9 system having sgRNA complementary to 5th exon while, maintainer vector (MGM) was generated to develop maintainer male fertile line having transgenic element with 3 functional modules consisting of fertile *ZmMS26* complementary DNA sequence, pollen disruption gene *ZmAA* and screenable marker gene *DsRed* as similar to SPT and MCS system (Fig 5). The MGM cassette was introduced into the tissue transformed with CRISPR editor cassette generating line homogyous with mutant male sterile *ms26/ms26* gene and hemizygous for *MGM/-* cassette. MGM transgenic plants were capable of producing viable pollen with *ms26/ms26*; -/- gene only while, the other set of pollen with *ms26/ms26*; *MGM/-* cassette are degerated or caused inviable as its presence inactivates

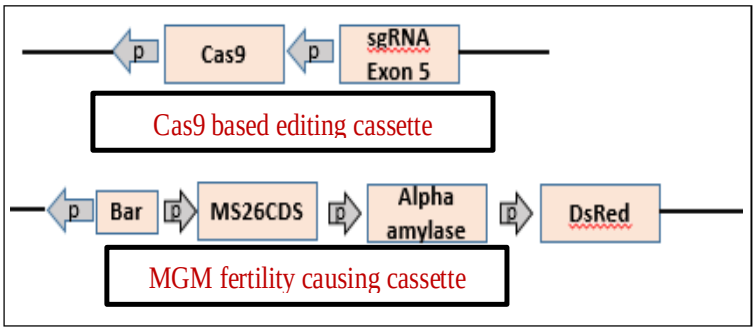

Fig 5: MGM based MS system vector cassette used for transformation.

	Gametes	
Male / **Female**	50% Sterile (ms26Edit; MGM)	50% Fertile (ms26Edit; -)
50% Fertile (ms26Edit; -)	No Progeny	GMS line (Normal colour seed) (ms26Edit/ms26Edit. -/-)
50% Fertile (ms26Edit; MGM)	No Progeny	MGM Maintainer (Red colour seed) (ms26Edit/ms26Edit. MGM/-)

Fig 6: Genotypes of gametes and sporophytes obtained upon selfing of MGM maintainer line.

the pollen however, the plant was capable of producing both kinds of gametes. Selfing of MGM transgenic plants produces progeny mixture of GMS sterile normal coloured seed free of transgenic element and MGM maintainer red fluorescent seed in 1:1 ratio (Fig 6). The maintainer are sorted out from GMS line using screenable marker. The system is developed where MGM maintainer produces both GMS line free of transgenic element and MGM maintainer itself with sortable seed through selfing. This reduces the effort as well as laborious backcross method of line conversion as practiced in SPT and MCS systems to convert GMS line into GMS maintainer. This knock-out male sterility method can be employed across any germplasm of crops species and across cloned male fertile genes generating GMS and its MGM maintainer line simultaneously.

Preventing genetic transmission of transgenic elements was achieved effectively through MCS approach. Genome editing based hybrid breeding can be made more effective by addition of extra functional modules of pollen disruption elements in transgenic construct. The methods and cheap, less laborious and easy to be implemented by any small scale laboratory with good expertise.

CONCLUSION

Overall, the biotechnology based next generation hybrid seed production provides opportunities to develop male sterile line from any germplasm irrespective of genotype and crop. The techniques involved are less laborious, easy-to-implement and less expensive in the age when biotechnological components are getting cheaper and high throughput with time moreover, overall land usage is reduced than conventional methods of parental development in hybrid breeding programmes. The methods quickly provide with male sterile and transgenic parents bypassing the cumbersome approach of restorer identification and maintainer line as well as male sterile line conversions. Moreover, the next generation hybrid breeding provides with non-transgenic hybrid seeds though from transgenic parental components thus escaping the complicated transgenic, standards, inspection and approval for final hybrid product. However, the method requires strict monitoring of transgene flow during maintainer and female line multiplication, though negligible.

Conflict of interest: None.

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