**Advances in male sterility system: Genetic & molecular concepts and breeding strategies**

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**Introduction**

The world's population is predicted to grow by 25% by the midpoint of the twenty-first century, reaching a staggering 10 billion individuals. This surge in population poses a significant threat to global food security. Due to the limited amount of freshwater and agricultural land, modern agricultural technologies must be used to produce more sustainably. Global crop production has significantly benefited economically from the use of heterosis. Hybrid cultivation dominates the production of major crops, with hybrid cultivars accounting for over half the global output of rapeseed, sunflower, sorghum, maize, and rice. As a result, hybrid breeding makes significant contributions to the global food supply. Rice, pearl millet, sorghum, maize, barley and rapeseed are making incredible contributions to the hybrid breeding system. The characteristics seen in hybrid plants include increased uniformity, improved abiotic and biotic stress tolerance and better adaptability.

A well-known condition in higher plants known as **male sterility**, is a situation where the male reproductive parts of a plant are either absent, aborted or non-functional, resulting in their inability to participate in the natural sexual reproduction process, is known as male sterility (Saxena and Hingane, 2015). Male sterility in plant is considered as typical example of maternal inheritance because it is transmitted through female only. It is caused by barrier of tapetal layer, improper timing of callase activity, abnormal micro-sporogenesis, involvement of esterase, absence or malformation of male organs (stamens). Joseph Gottlieb Kolreuter, in the 18th century, was the first to record the presence of naturally occurring plants with damaged anthers within specific populations. Later, Jones & Emsweller (1937) and Stephens & Holland (1937) proved male sterility for the development of hybrid onion and sorghum seeds, respectively. Any irregularities observed in development at any stage of pollen grain or microsporogenesis release may give rise to disability. In addition to cytoplasmic male sterility (CMS) and genetic male sterility (GMS), male sterility has been reported in around **610 plant species**. CMS is caused by the interaction between nuclear and cytoplasmic genomes, while GMS is solely controlled by nuclear genes. CMS has been documented in over **200 plant species**. Effective use of CMS has made easier to incorporate the desired characters into hybrids. Male sterile plants are critical for hybrid breeding and the study of stamen/pollen development and cytoplasmic-nuclear interactions (Chen and Liu, 2014).

CMS is associated with a typical mitochondrial **genes**/**open reading frames** (ORFs) (Chase and Gabay-Laughnan, 2004; Hanson and Bentolila, 2004). Nuclear encoded genes which restored the male fertility, termed as **restorer-of-fertility (*Rf*)** factor, have the significant agronomic benefit for producing hybrid seeds, frequently used in crop production. Fertility is restored by a series of *Rf* genes (*Rf1*, *Rf1a*, *Rf1b*, *Rf2*, *Rf3*, *Rf4*, *Rf5*, *Rf6, Rf17 etc.*) encoded in the nucleus. Nine *Rf* genes have been identified across seven plant species, including maize, Petunia, radish, *Brassica*, rice, sorghum and sugar beet. The first isolated restorer gene for CMS-T maize is *Rf2*.

Most *Rf* genes encode PPR (pentatricopeptide repeat) proteins that process mitochondrial mRNA. PPR proteins are abundant in the nucleus, with around 450 and 650 in *Arabidopsis* and rice, respectively, primarily targeting mitochondria and plastids. Male sterility arises from cytoplasmic disfunction, which is suppressed by nuclear restorer genes to restore fertility (Eckardt, 2006).

**Three-line breeding system/CMS system**

CMS-based hybrid seed production involves a three-line system: - **CMS lines (A-line)** - have male-sterile cytoplasm and lack functional *Rf* genes. **Maintainer lines (B-line)** - have normal cytoplasm but similar nuclear genome as A-line, used to propagate A-line. **Restorer lines (R-line)** - have functional *Rf* genes, used as male parent to produce fertile F1 hybrids by restoring fertility in the CMS A-line. Three lines are involved so it is known as **Three-line breeding system** (**Figure 1**).

 **Figure 1: Three-line breeding system**

**Association of mitochondrial genes in CMS**

CMS and fertility restoration **provide insights into the molecular genetic interactions between plant mitochondria and nucleus**. Studies on CMS associated mitochondrial genes and their genomic features shed light on the origin and spread of these genes. Analyses of CMS phenotypes and restorer genes have yielded insights into signalling pathways and the role of nuclear genes in mitochondrial gene expression.

Numerous metabolic pathways are carried out by mitochondria that are essential to higher eukaryotic life. It includes the pathways *like.*, tricarboxylic acid cycle (TCA), respiratory electron transfer and ATP synthesis. An **open reading frame (ORF)** refers to a continuous DNA sequence between a start and stop codon that has the potential to be transcribed and translated into a protein. ORFs require an uninterrupted sequence starting from the start codon, with a length that is a multiple of three nucleotides, extending to the stop codon within the same reading frame. Open refers to the fact that the **road is open** for ribosome to read continuously triplet after triplet untilribosome meets this stop codon.

**At least 14 mitochondrial genes** responsible for CMS have been identified as open reading frames (ORFs) that include segments from mitochondrial gene coding and gene flanking sequences, as well as sequences of unknown origin. (Chase and Gabay-Laughnan, 2004; Hanson and Bentolila, 2004). The ***Cox1*, *atp8* and *atp6*** mitochondrial genes are commonly implicated in the development of CMS genes. Recombination occurs in genome of mitochondria which is responsible for the formation of ORFs and their placement downstream of sequences that support gene expression. **ORFs are expressed** **either through direct fusion with mitochondrial promoter sequences** or by co-transcription with upstream mitochondrial genes (Chase, 2007).

**CMS associated genes in various crops species**

Several CMS cytoplasm was recovered from the breeding lines. Many putative mitochondrial ORFs have been found in rice. CMS-WA and CMS-RT102, with *WA352* and its variant *orf352,* respectively, consist of *orf284, orf224*, *orf288* three segments in the mitochondrial ORFs and a short sequence of unknown origin (SUO). CMS-CW with CW-*orf307* include two segments: *orf288* and a SUO. The CMS-BT line with *orf79* from *indica* rice Boro II and CMS-HL with *orfH79* from wild rice Hong-Lian both encode small proteins featuring a B-*atp6* N-terminus akin to *cox1*, while the rest of their sequences remain unidentified ("Investigation of B-*atp6-orfH79* distributing in Chinese populations of *Oryza rufipogon* and analysis of its chimeric structure", 2022) (Kazama *et al.*, 2016). In Sorghum, CMS-A3 is linked to *orf107*, which similarly encodes an *atp9* protein at its N-terminus, paralleling the rice *orf79* (Kazama *et al.*, 2016). In Wheat, the CMS-AP line, combining *Triticum aestivum* nuclear genome with *Triticum timopheevii* cytoplasm, is associated with *orf256*, where the initial 11 amino acids share significant similarity with *cox1* (Li *et al.*, 2022)

In the dicot species, the CMS genes are presented with *atp8* sequences. The Brassica CMS-Ogu and CMS-Kos genes, *orf138* and *orf125*, which are derived from the radish species, encode proteins analogous to *atp8*. The Brassica CMS-Pol and CMS-Nap genes, *orf224* and *orf222*, which encode membrane proteins exhibiting 79% sequence similarity, encompass an atp8-derived sequence along with a SUO (Singh and Brown, 1991). The *atp8* sequences are also identified in *orf522* of sunflower CMS-PET1 and *orfB-cms* in carrot CMS-Petaloid, both of which contain an additional SUO. Numerous other CMS genes harbor *atp6* sequences of varying lengths, such as *atp6-C* in maize CMS-C and *preSatp6* in sugar beet CMS-Owen. Consequently, various recognized CMS genes (such as *orf125* and *orf138* in radish associated with CMS-Kos and its variants in *Brassica* corresponding to CMS-Ogu, respectively) and the mutated *cox2* in sugar beet CMS-G are characterized as nonchimeric genes that consist of sequences sourced from single origins **(Table 1)**.

**Table 1: Different crop species with CMS lines and their associated genes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sr. No** | **Crop species** | **CMS line** | **Associated genes** |
| 1 | *Brassica* | CMS-Ogu | *orf138* binds with *atp8-like* proteins. |
| CMS-Pol | *orf224* with 2 segments *like*, *atp8* & short sequence of unknown origin (SUO). |
| CMS-Nap | *orf222* similar with *orf224.* |
| 2 | Carrot | CMS-Petaloid | *orfB-cms* with *atp8* andSUO. |
| 3 | Maize | CMS-T | *orf221* with *atp4* and 5’ UTR having *atp6* encodes with *urf13.* |
| CMS-S | *orf355* and *orf77* with 2 segments of *atp4* & *atp6* and both ORFs combinedly known R sequence. |
| CMS-C | *atp6-*Cinvolving the short sequence of unknown origin (SUO) and *atp6.* |
| 4 | Rice  | CMS-WA | WA352 with *orf284, orf224*, *orf288* three segments in ORFs and a SUO. |
| CMS-RT102 | *orf352* is variant of *WA352*. |
| CMS-CW | *CW-orf307* having *orf288* and SUO. |
| CMS-BT | *orf79* encode small proteins B-*atp6* at N terminus similar with the *cox1* and the remaining SUO. |
| CMS-HL | *orfH79* is similar with *orf79.* |
| 5 | Radish | CMS-Don | *orf463* binds with *cox1* and SUO. |
| CMS-Kos | *orf125* binds with *atp8-like* proteins. |
| 6 | Sorghum | CMS-A3 | *orf107* with *atp9* protein at the N terminus and other remaining portions just similar to rice *orf79*. |
| 7 | Sugar beet | CMS-Owen | *preSatp6* encodes with *Satp6.* |
| I-12CMS (3) | *orf129* with *cox2* and SUO. |
| 8 | Sunflower | CMS-Baso/PET1 | *orf522* with involving *atp8,* SUO & *atp9* segments. |
| 9 | Wheat  | CMS-AP | *orf256* encodes the *cox1* |

***Abbreviations:*** *CMS-Ogu: Ogura type, CMS-Pol: Polima type, CMS-Nap: Brassica napus type of CMS in brassica species; CMS-T: Texas type, CMS-C: Two dominant genes are involved (Rf4)(Rf5) & both restore the fertility independently, CMS-S: Reliance on single dominant gene (Rf3)(located on chromosome 2) and less stable, more environmental sensitive then CMS-C in maize; CMS-WA: Wild abortive type, CMS-RT102: RT102 type, CMS-CW: Chinese wild type, CMS-BT: Chinsurah Boro type, CMS-HL: Hong Lian type of CMS in rice; CMS-Don: First identified in Japanese radish and associated with CMS Ogura type, CMS-Kos: Specifically line derived from the Kosena cultivar which has been introduced to fertility restoring genes in breeding programme of radish; CMS-Owen: Owen cytoplasm which provide genetic basis for male sterility in sugar beet; CMS-Baso: Derived from the Baso cytoplasm in sunflower.*

**Genetic and molecular basis of male sterility**

The various models explained the sterility that exhibits in plant, the important models are discussed below:

**1) The cytotoxicity model:**

Contains CMS proteins, which cause immediately **cell death** in sterile plants. The cytotoxicity of CMS proteins was assessed using transgenic expression of CMS genes in prokaryotic and eukaryotic cultured cell systems. The CMS protein normally has a molecular weight between 10 and 35 kDa with transmembrane proteins and contains hydrophobic regions, which are characteristics of cytotoxic proteins. In very simple term, CMS proteins cause mitochondrial disfunction in the anther's saprophytic or gametophytic cells, leading to male abortion (Levings, 1993). *URF13*, the first CMS protein identified in maize CMS-T, is toxic to many eukaryotic cells and *Escherichia coli*. (Korth *et al.*, 1991; Korth and Levings, 1993).

**2) The energy deficiency model:**

The mitochondrial electron transfer chain (mtETC) is the primary mechanism through which plant cells produce biological energy (ATP) during **respiration**. Studies on sporophytic and gametophytic cells in plant anthers have shown that these cells have **higher energy demands compared to other plant tissues**. According to the energy deficiency model, CMS (cytoplasmic male sterility) proteins interfere with the energy production necessary for the development of male reproductive organs. The structure of CMS proteins provides the molecular basis for this model.

Several CMS proteins, such as *preSatp6* in CMS-Owen of sugar beet, *orf138* in CMS-Ogu of *Brassica*, *orf79* in CMS-BT of rice, *orfH78* in CMS-HL of rice, and *URF13* in CMS-T of maize, are mitochondrial transmembrane proteins. These proteins associate with the inner mitochondrial membrane, disrupting the proton gradient and impairing ATP production (Rhoads *et al.*, 1995). CMS genes are linked to essential mitochondrial genes involved in respiration, including *nad3, nad5* and *nad7* for **complex I**; *cox1* and *cox2* for **complex IV** and *atp1*, *atp4*, *atp6*, *atp8* and *atp9* for **complex V**. CMS proteins compete with the normal function of the mtETC, underscoring the connection between respiratory pathways and CMS.

**3) The aberrant programmed cell death model:**

**Apoptosis**, a cellular process involving nuclear DNA fragmentation, is regulated by signals originating from the mitochondria. Programmed cell death (PCD) plays a critical role in various plant processes, such as seed germination, root tip elongation, senescence and organ development. One key event in plant PCD is the release of **cytochrome *c* from the mitochondria into the cytosol,** which plays a major role in triggering cell death (Liu *et al.*, 1996).

In the formation of male gametophytes within anthers, the interaction between sporophytic and gametophytic cells is essential. Controlled PCD is particularly important for the degradation of the tapetum, the innermost cell layer of the anther wall, which supports pollen development (Ma H., 2005). Proper function of the tapetum relies on the timely initiation and progression of PCD. **Any premature or delayed PCD can result in male sterility** (Ji *et al.,* 2013). For instance, in sunflower CMS-PET1 cytoplasm, premature PCD of tapetal cells has been observed and is associated with the release of cytochrome *c* from the mitochondria to the cytosol (Balk and Leaver, 2001).

**4) The retrograde regulation model:**

Retrograde means reverse, this regulation is the general term used for mitochondrial signalling. During retrograde signalling, **they are sent to the nucleus instead of signals leaving the nucleus**. MADS-box transcription factors play a crucial role in the development of male and female gametophytes, as well as in embryo, seed, root, flower and fruit development. However, their expression is suppressed in CMS (cytoplasmic male sterile) lines. For instance, in carpeloid CMS lines of carrots, the expression of MADS-box genes responsible for controlling whorls 2 and 3 in the flower is downregulated. This suggests that retrograde signalling from the mitochondria influences the expression of these nuclear MADS-box genes, leading to organ conversion in carpeloid CMS (Linke *et al*., 2003). Furthermore, increased expression of retrograde-regulated male sterility (RMS) genes inhibits pollen germination, ultimately causing male sterility.

**Fertility Restoration**

The expression related with CMS genes can be suppressed or counteracted by the products of specific restorer genes, thereby allowing pollen fertility. Fertility-restoring alleles are giving information related to genetic crosses involving a male-sterile seed parent and a restorer parent of a different nuclear genotype. Restoration pattern is divided into two parts: **sporophytic (anther wall) and gametophytic (microspore)** restorer. In which sporophytic restorer carried out in either sporophytic tissue or before the meiosis. Gametophytic restorer is done after meiosis in microspores or pollen grains. Both follow the **different transmission pattern**.

A diploid plant having a male-sterile cytoplasm and being heterozygous in nature for a restorer gives rise to two different pollen grain classes: those that carry the restorer and those that do not. Both types of genotypic classes of gametes will be functional in the case of **sporophytic restorers.** A plant with a heterozygous nature for a **gametophytic restorer** that only gametes carry the restorer allele will be functional. For example, Sorghum fertility-restoring genes *Rf1* and *Rf2* for CMS A1 (Klein *et al.,* 2005; Jordan *et al*., 2010), *Rf5* (Jordan *et al*., 2011) and *Rf6* (Praveen *et al*., 2015) for CMS A2 are **sporophytic** fertility restorer genes, and *Rf3* and *Rf4* function as **gametophytic**-restorers for CMS A3. Another well-known example is that the S-cytoplasm of maize is characterized as a CMS system that is restored gametophytically.

**Why restoration is essential?**

* Cytoplasmic male sterility (CMS) is a maternally inherited trait typically **linked to the mitochondrial genome**.
* Male sterility alone is insufficient for hybrid seed production; fertility must be restored in the F1 generation to ensure successful hybrid seed development
* In plants, nuclear genes known as restorers of fertility (*Rf*) play a key role in **restoring pollen fertility**.
* At the time of pollen development, ***Rf* genes either block or compensate for the mitochondrial dysfunctions** that cause male sterility. Therefore, the presence of Rf genes is essential for hybrid seed production, as male sterility alone will not fulfill the requirement without fertility restoration.

**Genetic basis of fertility restoration**

The diversity in fertility restoration patterns in CMS plants has contributed to an increase in the number of restorer genes. Typically, one or two major restorer loci are responsible for the full restoration of fertility. However, in some cases, full restoration requires the **coordinated action** of several genes, where some genes may only have minor effects on fertility. For instance, a **single gene restores fertility** in the Al male-sterile cytoplasm of sorghum (Murthy and Gangadhar, 1990). On the other hand, in T-cytoplasm maize, PET-cytoplasm sunflower, and T-cytoplasm onion, two unlinked restorer genes are necessary for complete restoration (Schnable and Wise, 1998). **Duplicate restorer loci** are found in many systems. For example, in maize, the T-cytoplasm restorer *Rf1* is the major gene, with *Rf8* serving as a partial substitute. Similar cases are seen in PET1-cytoplasm sunflower, T-cytoplasm onion, and CMS in Phaseolus. These overlapping functions could result from the duplication of gene functions or indicate that multiple mechanisms are involved in fertility restoration. **The first restorer gene, *Rf2*, was identified in maize**. In the CMS-T type, the *Rf2* allele is required in combination with the *Rf1* allele (which is not linked to *Rf2*) for male fertility restoration. *Rf2* encodes a functional mitochondrial aldehyde dehydrogenase, suggesting that fertility restoration occurs through metabolic compensation for the effects of a mitochondrial CMS-inducing gene. CMS systems and their *Rf* genes are found in a wide range of crops, including maize, rice, sunflower, *brassica*, radish, sorghum, wheat, common bean, carrot and sugar beet (Table 2).

A series of *Rf* genes belong to the pentatricopeptide-repeat (PPR) protein family, one of the **largest protein families** in land plants. PPR proteins are key regulators of mitochondrial gene expression in plants and are found mainly in eukaryotes. They are localized in mitochondria or chloroplasts, where they modulate gene expression at the RNA level. **PPR motifs consist** of degenerate 35-amino-acid sequences arranged in tandem arrays, with 2-27 repeats per protein (Saha *et al*., 2007) **(Figure 2)**. Functional studies have revealed the roles of PPR proteins in RNA processing, embryogenesis, fertility restoration in CMS plants and plant development.

**COO‑**

**NH2**

**2-27 PPR repeats in tandem 35 amino acid per repeat unit**

**Variable length organelle targeting sequence**

**C terminal optional motif**

**Figure 2: PPR protein structure arrangement**

**Table 2: Cytoplasmic male sterility (CMS)/restorer (*Rf*) gene systems in major crops**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Crop species** | **CMS type** | ***Rf* locus** | **Protein property** | **References** |
| *B. napus* | CMS-Ogu (S) | *Rfo* (P) | PPR Protein | Brown *et al*., 2003Uyttewaal *et al*., 2008 |
| CMS-Pol (S) | *Rfp* (R) | Unknown | Singh and Brown., 1991 |
| CMS-Nap (S) | *Rfn* (R) | Unknown | L’Homme *et al*., 1997 |
| *B. juncea* | CMS-Hau (S) | *UK* | Unknown | Jing *et al*., 2012 |
| CMS-*orf220* | *UK* | Unknown | Yang *et al.,* 2010 |
| *B. tournefortii* | CMS-Tour (S) | *Unknown* (P) | Unknown | Landgren *et al.*, 1996 |
| *Daucus carota* | CMS-Petaloid | *Unknown* (R) | Unknown | Nakajima *et al.*, 2001 |
| *Proteus vulgaris* | CMS-Sprite(S) | *Fr* (G)*, Fr2* (P) | Unknown | Abad *et al.*, 1995 |
| *Zea mays* | CMS-T (S) | *Rf1* (R) | Unknown | Dill *et al*., 1997 |
| CMS-S (G) | *Rf2* (M) | Unknown | Zabala *et al*., 1997 |
| CMS-C (S) | *Rf3* (R) | Unknown | Dewey *et al.*, 1991 |
| *Raphanus sativus* | CMS-Kos (S) | *Rfk1* (P) | PPR Protein | Iwabuchi *et al.,* 1999 |
| CMS-Don (S) | *Rfd1* (P) | Unknown | Park *et al*., 2013 |
| *Oryza sativa* | CMS-BT (G) | *Rf1a* (R)*, Rf1b* (R) | PPR Protein | Akagi *et al.*, 2004 Komori *et al*., 2004Wang *et al*., 2006 |
| CMS-HL (G) | *Rf5 (Rf1a)* (R) | PPR Protein | Wang *et al*., 2013 |
| CMS-LD (G) | *Rf2* (P) | Glycine-rich protein | Itabashi *et al*., 2011 Itabashi *et al*., 2009 |
| CMS-CW (G) | *Rf17* (P) | Acyl-carrier protein synthase | Fujii and Toriyama, 2009 |
| CMS-WA (S) | *Rf3* (P)*, Rf4* (R) | unknown | Zhang *et al*., 1997 Zhang *et al*., 2002 |
| CMS-RT120 | *Rf102* | unknown | Okazaki *et al*., 2013 |
| CMS-RT98 | *Unknown* | unknown | Igarashi *et al*., 2013 |
| *Sorghum bicolor* | CMS-A3 (G) | *Rf3* (R) | unknown | Tang *et al*., 1996 |
| CMS-A1 (G) | *Rf1, Rf2* | PPR protein | Klein *et al.,* 2005 |
| *Beta vulgaris* | CMS-Owen | *Rf1* (P) | Peptide | Matsuhira *et al*., 2012 |
| I-12 CMS-(3) | *UK*(P) | unknown | Yamamoto *et al*., 2008 |
| CMS-G | *RfG1*, *RfG2* | unknown | Ducos *et al.*, 2001 |
| *Helianthus annuus* | CMS-PET1 (G) | *Rf1* (R) | unknown | Horn *et al.,* 2003 |
| *Triticum aestivum* | CMS-AP | *Unknown* | unknown | Song and Hedgcoth, 1994 |

**Mechanism of fertility restoration**

Cytoplasmic male sterility (CMS) in plants is caused by specific mitochondrial proteins that disrupt normal pollen development. During pollen formation, fertility restorer (*Rf*) genes mitigate or compensate for these mitochondrial dysfunctions, which are phenotypically expressed. This fertility restoration is driven by interactions between nuclear and mitochondrial genes **(Figure 3)**. Mitochondrial-sorting gene (MSG) products, along with RF proteins and tissue-specific regulatory factors (TSRFs), are encoded in the nucleus and target the mitochondria, regulating them through components of the mitochondrial electron transfer chain (mtETC) complexes. At the translational or

post-translational level, TSRFs modulate the male organ-specific accumulation of CMS proteins, contributing to male sterility. These CMS proteins interact with mtETC subunits, altering their function or ATP synthesis, which in turn generates retrograde signals that initiate aberrant programmed cell death (PCD) in the tapetum or microspores. Fertility restoration by RF proteins can occur through multiple mechanisms, including genomic, post-transcriptional, translational or post-translational and metabolic pathways.



**Figure 3: Generalized mechanism of CMS/*Rf* System (Modified from Chen and Liu, 2014)**

1. **Restoration at Genomic Level**

The mitochondrial genome exhibits remarkable dynamism and frequently experiences alterations in both the structural configuration and the copy number of mitochondrial DNA entities. In certain cytoplasmic male sterility (CMS) plants, an occurrence of spontaneous fertility reversion transpires, wherein sub-stoichiometric shifting pertains to the relative copy numbers of specific sub-genomic entities that harbor CMS genes. The restoration of pollen fertility may be concomitant with the excision of an mt-DNA fragment from the mitochondrial genome. For example, mitochondrial PVS sequence alterations are induced by CMS Sprite in the *Phaseolus vulgaris* species. This represents the inaugural instance of *Rf* gene restoration at the genomic scale mediated through sub-stoichiometric shifting. The existence of the dominant nuclear gene *Fr* produced cut in the PVS mitochondrial genomic sequence. Consequently, CMS-associated mitochondrial DNA molecules transition into a normative state, with the nuclear gene *Fr* being present in the progeny of the ensuing generation, thereby indicating the fertility of that generation (Xu *et al*., 2022).

1. **Restoration at the** **Post-transcriptional Level**

Extensive expression and sequencing studies have provided valuable insights into the genetic basis of cytoplasmic male sterility (CMS) in various crop species. These analyses have shed light on the CMS-associated transcripts and the critical role of restorer-of-fertility (*Rf*) gene products in their processing. The CMS-associated transcripts undergo complex post-transcriptional modifications mediated by the *Rf* gene products. These mechanisms include RNA editing, splicing, and cleavage. The RNA editing process involves the **conversion of cytidine (C) residues to uridine (U) at specific sites within the RNA sequences,** particularly in the mitochondrial genomes of plant organelles. Furthermore, the CMS-associated transcripts may undergo RNA exo/endonucleolytic cleavage. This process can occur within the coding regions of multicistronic transcripts and/or the intercistronic (spacer) sequences. For example, Four C-to-U editing sites are present in the *orf107* of **CMS-A3 of sorghum**. If the plant is sterile than site 1 and 2 edited frequently and infrequently respectively. *Rf3* gene is required in the action of site 3 and 4 approximately 80% and 60% effectiveness. *orf107* is degraded in the plants which has *Rf3* is present (Tang *et al*., 1999). In **CMS-T of maize** restore the fertility by *Rf1* and *Rf2* gene. CMS-T is associated with *urf13-orf221* dicistronic transcript is processed with *Rf1* and reduced the abundance of cleaved *urf13* RNA fragment (Kennell and Pring, 1989). In **CMS-BT rice**, cleaves the *B-atp6-orf79* dicistronic transcripts by *Rf1a* and *B-atp6* is available at 5' untranslated region, but in some case *B-atp6-orf79* transcripts is degraded by *Rf1b*. If the restoring plant exhibits the presence of *Rf1a* and *Rf1b*, then *Rf1a* provides critical insights regarding the epistatic influence on the cleavage of the transcripts. The cleaved *orf79* RNA fragment is devoid of its ribosome-binding site, resulting in a lack of translation (Wang *et al*., 2006). A comparable mechanism occurs in the *atp6-orfH79* of **CMS-HL** (Yi *et al*., 2002). The *Rf1a* gene in CMS-BT and its analogous gene *Rf5* in CMS-HL demonstrate similar cleavage patterns; however, it is noteworthy that *Rf1a* directly interacts with the *B-atp6-orf79* mRNA (Kazama *et al*., 2008), while *Rf5* necessitates the glycine-rich protein *GRP162*, which functions as an adaptor in the restoration of fertility complexes and binds to the *atp6-orfH79* mRNA for cleavage (Hu *et al*., 2012). *Rf4* in **CMS-WA** is responsible for the degradation of the *rpl5-WA352* dicistronic transcripts and the *WA352* monocistronic transcripts, subsequently diminishing the abundance of these transcripts by nearly 20% in the *Rf4*-restored plants. (Luo *et al*., 2013).

1. **Restoration at the Translational or Post-translational Level**

In certain crop plant species exhibiting cytoplasmic male sterility (CMS) systems, the **dimensions and quantities of transcripts associated with CMS remain constant**. During this period, mechanisms for the restoration of fertility at the translational or post-translational level are activated. An illustrative example is *Rf4* of **CMS-C** in *Zea mays*, which does not alter the steady-state concentration of *atp6-C* mRNA; consequently, it is evident that restoration may occur at the protein level (Dewey *et al*., 1991). Similarly, *Fr2* of **CMS-Sprite i**n *Phaseolus vulgaris* does not influence the PVS transcript but significantly enhances the accumulation of *orf239* protein (Sarria *et al*., 1998). The **CMS-Ogu** system in Brassica and Raphanus, which is restored by *Rfo* and encodes PPR-B (also referred to as *Orf687*), demonstrates that while the levels of *orf138* mRNA remain unchanged, there is a suppression of the accumulated *orf138* protein. PPR-B and *orf138* mRNA frequently interact, thereby inhibiting the translation of *orf138* (Uyttewaal *et al.*, 2008).

1. **Restoration at the Metabolic Level**

Some *Rf* gene encodes for the production of enzymes. This **enzyme converts harmful molecules into non-harmful molecules** thus, thus restoring fertility by eliminating the harmful effects of such harmful molecules. For example, *Rf2* of CMS-T in maize, which encodes an enzyme classified as aldehyde dehydrogenase. This enzyme is pivotal in the metabolic pathways of fatty acids and amino acids, as well as in the detoxification processes of alcohols and other toxins, achieved through the modification of aldehyde-induced cellular and tissue damage. The *RF2* protein facilitates the oxidation of at least three distinct aldehydes. Notably, the presence of *Rf2* does not alter the transcripts of *urf13-orf221* or the URF13 protein; thus, it is postulated that *RF2* could mitigate the detrimental effects induced by URF13, thereby reinstating CMS-T by neutralizing harmful molecules (Liu *et al*., 2001).

**Transformation of agronomically optimal genotypes into male sterility: concepts and breeding methodologies**

**Attributes of Agronomically Ideal Genotypes**

The term agronomically ideal genotype refers to a plant variety or cultivar that exhibits specific characteristics and traits optimized for increasing production, efficiency and sustainability in agriculture (Khush, 1999). In addition to pests, diseases, environmental stresses and market demands, these genotypes are converted to meet the needs of farmers (Tester and Langridge, 2010). Depending on the crop species, growing conditions and specific breeding objectives, agronomically ideal genotypes may have different characteristics. However, some common attributes include:

* **Yield Potential:** Agronomically ideal genotypes typically produce high yields when grown under optimal conditions. Farmers rely on this trait to maximize production and profitability.
* **Disease Resistance:** To reduce crop losses and minimize pesticide use, crops must resist pathogens such as fungi, bacteria, viruses and nematodes.
* **Pest Resistance:** Crops with pest resistance are protected from damage and require fewer insecticides to thrive, making them environmentally sustainable.
* **Abiotic Stress Tolerance:** It might be resistant to several abiotic factors, including heat, salinity, cold and drought, ensuring constant yields even in unfavorable environmental conditions.
* **Adaptability:** Adaptability to diverse agro-climatic conditions and cropping systems enables farmers to grow these genotypes across different regions and environments.
* **Quality Traits:** Quality attributes such as nutritional content, taste, texture and shelf-life are important for meeting consumer preferences and market demands.
* **Efficient Resource Use:** Genotypes that utilize water, nutrients and other resources efficiently contribute to sustainable agriculture by reducing resource wastage and environmental impact.
* **Early Maturity:** Early maturing genotypes allow for shorter cropping cycles, enabling farmers to grow multiple crops within a single growing season or to adapt to shorter growing seasons.
* **Uniformity and Stability:** Uniformity in traits across plants and stability in performance over different seasons and environments are desirable characteristics for ensuring consistent and reliable crop production.
* **Ease of Management:** Genotypes that are easy to manage, harvest and process contribute to labor efficiency and cost-effectiveness for farmers.

**Challenges in introducing male sterility into agronomically ideal genotypes**

Introducing male sterility into agronomically ideal genotypes presents several challenges due to the complex genetics and physiological mechanisms involved (Varshney *et al.,* 2012). Male sterility is often utilized in hybrid seed production, which facilitates the production of high-yielding hybrid cultivars. However, incorporating male sterility into ideal genotypes can be challenging due to issues such as genetic instability, linkage drag, and difficulty maintaining agronomic performance. Here are some challenges described below:

* **Genetic Stability and Segregation:** Maintaining genetic stability while introducing male sterility genes into agronomically ideal genotypes is crucial to avoid unintended changes in important agronomic traits. Ensuring proper segregation of male sterility traits in subsequent generations is essential for stable performance.
* **Linkage Drag:** Male sterility genes may be linked with undesirable traits, leading to linkage drag and compromising the overall agronomic performance of the genotype. Overcoming linkage drag requires precise genetic manipulation and selection strategies to uncouple male sterility from undesirable traits.
* **Maintaining Agronomic Performance:** Introducing male sterility should not compromise the overall agronomic performance of the genotype, including yield potential, stress tolerance and quality traits. Maintaining or even enhancing agronomic performance while incorporating male sterility requires careful selection and breeding strategies.
* **Environmental Sensitivity:** Male sterility may be sensitive to environmental factors such as temperature, photoperiod and nutrient availability, leading to variable expression and reduced efficiency under different growing conditions (Reynolds and Langridge, 2016). Developing male sterile lines with stable and robust performance across diverse environments is a significant challenge.
* **Transgene Flow and Biosafety Concerns:** Introducing male sterility through genetic modification raises concerns about transgene flow and potential environmental impacts. Addressing biosafety concerns and regulatory requirements is essential to ensure the safe deployment of male sterile genotypes in agricultural systems.

Overcoming these challenges requires interdisciplinary research efforts integrating genetics, genomics, breeding and biotechnology to develop male sterile genotypes with improved stability, performance and environmental safety.

**Concepts of Conversion into Male Sterile Genotypes**

Converting crops into male sterile genotypes is a critical step in hybrid seed production, enabling the efficient production of high-yielding hybrid cultivars. Several approaches have been developed to induce male sterility, including genetic modification, chemical treatments and cytoplasmic male sterility (CMS). Here are some concepts of conversion into male sterile genotypes:

1. **Genetic Modification:**

Genetic modification involves the insertion of male sterility genes or disrupting genes essential for pollen development using techniques such as CRISPR-Cas9. This approach allows precise manipulation of the plant genome to induce male sterility while maintaining agronomic performance.

By using sequence-specific nucleases, genome editing technology allows for extremely precise modifications to the host plant's genome, including only a few base pair alterations in the target gene sequence. When implemented properly, this technique produces transgene-free plants and minimizes many regulatory authorities' concerns about biosafety. Zinc Finger Nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system represent prominent methodologies in the field of genome editing (Cong *et al*., 2013; Cox *et al*., 2015; Zhang *et al.,* 2013). Because CRISPR technology is simpler and less demanding technologically than the previous two technologies, researchers are more attracted to using it for gene/genome editing.

Technological advancements in gene and genome editing can precisely enhance genetic characteristics and accelerate breeding cycles by minimizing the duration of breeding processes. A significant advantage of employing such technologies is the generation of male sterile plant varieties. For instance, Zhou *et al*. (2016) implemented CRISPR/Cas9-mediated knockout of the TMS5 gene to develop "transgene-clean" thermo-sensitive genic male sterile (TGMS) rice lines (Zhou *et al*., 2016). The production of genome-edited TGMS lines within a single year underscores the efficacy of genome editing in streamlining the breeding process. A comparable approach was employed to disrupt the *ZmTMS5* gene, which is a homolog of the rice *TMS5*, thereby producing TGMS lines in maize (Li *et al*., 2017). Furthermore, Li *et al*. (2016) utilized CRISPR/Cas9 to alter the carbon starved anther (CSA) gene, resulting in the development of a photoperiod-sensitive genic male sterile (PGMS) japonica rice 9522 line. These genome-edited specimens exhibited male sterility under short-day conditions (10.5 hour photoperiod). However, a degree of male fertility was observed under long-day conditions (14 hour photoperiod). Conversely, it was also observed that CSA gene editing in the japonica variety Kongyu-131 exhibited sensitivity to both temperature and photoperiod, indicating that various alleles may affect rice plant fertility in response to specific environmental factors across diverse genetic backgrounds.

okada *et al*. (2019) used the CRISPR/Cas9 technology to expediently knockout the fertility-associated gene Ms1, thereby generating male-sterile wheat cultivars intended for commercial utilization. The induction of male sterility in tomato plants was accomplished through CRISPR/Cas9-targeted mutations of the stamen-specific gene SlSTR1, as demonstrated by Du *et al*. (2020). In the species of maize, Arabidopsis and rice, the complete knockout of the *ZmMs7* gene utilizing CRISPR/Cas9 resulted in male sterility, as reported by An *et al*. (2020).

Xu *et al*. (2020) observed mutations in both pollen and embryo sacs in rice plants, which stemmed from CRISPR/Cas9-mediated alterations in the *OsROS1* gene. Zhang *et al*. (2020) implemented CRISPR/Cas9-mediated modifications in the *LpDMC1* gene of ryegrass, a gene integral to meiosis, which led to the generation of entirely male-sterile individuals. Furthermore, a CRISPR/Cas9-mediated mutation of the soybean homolog of the ABORTED MICROSPORES (AMS) gene, GmAMS1, was found to induce male sterility, whereas GmAMS2 did not exhibit such effects, indicating a significant role of GmAMS1 in the development of the tapetum (Chen *et al.* 2021). The above examples demonstrate the technology's ability to quickly and easily create male sterile lines by deleting certain genes involved in anther development and fertility restoration, among other genes. Gene editing systems can edit multiple genes simultaneously by employing numerous domains in the vector to target many genes in a single operation. With this preference, the technique is ideal for creating stable enough materials to manipulate targets. As a result, the technology might start to be used by the researchers regularly. In this direction, Singh *et al.* (2018) showed that the CRISPR/Cas9 system allowed for the quick creation of male sterile bread wheat, *Triticum aestivum* L., through triple homozygous mutations of the *Ms45* gene. Li *et al.* (2020) employed a similar methodology to produce a triple mutant of the wheat *TaNP1* gene, which resulted in male sterility. Liu *et al.* (2022) used a different study to illustrate the multi-gene editing technique by introducing numerous mutations in homologous genes affecting maize male fertility and pollen maturation. The findings showed that total male sterility requires a triple homozygous gene mutation of *ZmTGA9-1/-2/-3*. However, double-gene mutants of ZmDFR1/2 and single-gene mutants of ZmACOS5-2 also displayed male sterility.

Additionally, the restoration of male fertility was demonstrated by eliminating genes linked to CMS. For instance, male fertility was successfully restored through the knockout of *orf79* in boro rice and *orf125* in the Kosena-type cytoplasmic male sterility (CMS) of rapeseed, utilizing mitochondria-targeted TALENs (Kazama *et al*., 2019). A comparable technique was applied to remove *orf312* in the Tadukanta-type CMS (TAA) of rice, resulting in the restoration of fertility (Takatsuka *et al*., 2022). The various systems developed *via* genetic engineering methodologies are summarized in **Table** **3** and the associated phenomenon is illustrated in **Figure 4.**



**Figure 4:Schematic depiction of genetically engineered male sterility and fertility restoration systems for hybrid variety development (Modified from Gautam *et al.,* 2023)**

Using a genetic modification technology, Male sterile transgenic plants are produced by introducing gene sequences specific to the male reproductive system that inhibit or disrupt pollen generation or anther development. This process generates exclusively in female plants, which are suitable for hybrid seed production. The isolation, cloning and characterization of anther or pollen-specific genes and promoter sequences permitted the construction of transgenic male sterility systems. According to Kumar *et al.* (2000), these genes are expressed in gametophytic pollen as well as sporophytic cells and tissues that support pollen formation, such as the tapetum, filament and anther wall. However, the sterile source which can be discovered by protoplast fusion, synthetic mutation and natural selection, is absent from most crops. Rapid advancements in plant genetic engineering have made it possible to create male sterile materials quickly.

**Barnase-Barstar system (Abolition-restoration system):** This method creates transgenic male sterile plants by disrupting (abolishing; as the word abolition) the pollen production process using foreign trans-gene constructs. These transgenes usually encode cytotoxic chemicals *like* lipase, protease and RNAase that harm the integrity of cells. Male sterility results from the expulsion of gametophytic and sporophytic cells due to the expression of particular genes in developing pollen or supporting tissues (such as tapetum cells), which are triggered by tissue-specific promoters. Another transgene is used to restore pollen fertility in order to reverse this effect.

This system is best shown by the first transgenic male sterility mechanism, known as the Barnase-Barstar system, which Mariani and his colleagues created in tobacco and rapeseed, among other species. The chimeric RNAase gene, referred to as Barnase, incorporates a **tapetum-specific promoter (TA29)**, which was employed to engineer transformed plant specimens. Since the altered plants tapetum cells produce the cytotoxic enzyme Barnase, the tapetum cells are developed and pollen was monitored, creating transgenic male sterile plants (Mariani *et al.,* 1990). It is not possible to achieve homozygosity of the Barnase gene in cases involving male sterile plants and maintenance issues continue to be major barriers. A generation of 50% hemizygous (Barnase) male sterile F1s is created when transgenic male sterile plants are crossed with conventional plants. In agricultural contexts where the fruits or seeds have economic value, these male sterile plants are not suited for production. Studies showed that the use of pollen from another transgenic plant with the Barstar gene and the TA29 promoter could potentially recover the fertility of F1s from transgenic male sterile plants (dominant). In F1 plants, the primary inhibitor of the cytotoxic effects linked to chimeric RNAase is the Barstar gene product. Within the tapetum cells, the transcript of the Barstar gene forms complexes with the chimeric RNAase produced from the TA29-Barstar gene (Mariani *et al*., 1992). A transgenic construct incorporating a Barnase: herbicide resistance gene linkage has been developed to mitigate the maintenance challenges. In these situations, hemizygous male sterile plants can interbreed with their normal sister plants because the **herbicide resistance gene (HER2)** is genetically connected to the male sterility gene (Barnase). Half of the sterile segregants will survive if herbicide is applied to the progeny, while half of the fertile segregants will be eliminated.

|  |  |
| --- | --- |
| **Table 3: List of genetically engineered male sterile system** |  |
| **Gene for male sterility** | **Gene function** | **Promoter used** | **Salient features of male sterile plant** | **Source of gene** | **Plant** | **References**  |
| Barnase | Ribonuclease gene | TA29 | Tapetal cell layerdestruction, no pollenformation and restoredthrough barstar | *Bacillus amyloliquefaciens* | Tobacco, oilseed, riceetc. | Kumar and Purty (2023);Mariani *et al*. (1990, 1992) |
| Cysteine protease | Protease | TA29 | Stamen length reduced, pollen was shrunken and deformed, Restored through Cystatin | *Arachis diogoi* | Tobacco, *Brassica* | Gautam *et al*. (2019);Shukla *et al*. (2014, 2016) |
| *BECLIN1* | Autophagy | TA29 and A9 | Tapetal degeneration, non-viable pollen, Conditional male sterile system | *Arabidopsis* | Tobacco | Singh *et al*. (2015) |
| *RIP* | Ribosome inactivatingprotein | TA29 | Tapetal tissue of the anther degenerated completely | *Dianthus chinensis* L | Tobacco | Cho *et al*. (2001) |
| *AtBI-1* | *Arabidopsis thaliana* ortholog of Baxinhibitor-1 | Osg6B promoter andLTP12 promoter | Tapetum degenerationand pollen abortion | *Arabidopsis* | Tobacco | Kawanabe *et al.* (2006) |
| *BoCysP1* and *BoCP3* | Cysteine proteasesinvolved in programmed cell death | A3 or A9 promoter | Anther were swollen andexcessively vacuolated | *Brassica oleracea* and*B. rapa* | *Arabidopsis* | *Konagaya et al.* (2008) |
| Cm-ETR1/H69A | Melon ethylene receptorgene | CaMV 35S (the cauliflower mosaic virus) | Abnormal stamen development | Melon | Tobacco | Takada *et al.* (2005) |
| *PbTM6a* and *PbTM6b* | B-class MADS-box | CaMV 35S | Decreased fertility of pollen grains | *Pyrus betulifolia* | Tomato | Zhang *et al*. (2023a, b) |
| MYC5-SRDX chimericrepressor | Transcription factor in JAhormone | MYC5 promoter andCaMV 35S | Defective stamen filament elongation | *Arabidopsis* | *Arabidopsis* | *Figueroa and Browse* (2015) |
| No Pollen 1 (*OsNP1*) | Glucose–methanol–choline oxidoreductase | CaMV 35S | Complete male sterility | *Oryza sativa* | Rice | Chang *et al*. (2016) |
| *BnaC.MAGL8.a* | Monoacylglycerol lipase | BnA9 promoter andCaMV35S promoter | Impaired pollen development | *Brassica napus* | *Arabidopsis* | *Gao et al. (2019)* |
| *PsEND1* | A pea anther-specific gene expressed inanther primordium | PsEND1 | Male sterility | *Pisum sativum* | *Arabidopsis*, Tobacco, | *Roque et al. (2019)* |

1. **Chemical Induction:**

(Source: Gautam *et al.,* 2023)

The process of chemically causing male sterility involves the use of chemicals or hormones that interfere with pollen formation or function. Certain compounds, such as male gametocides and gibberellin biosynthesis inhibitors, can specifically block pollen production, making males sterile. Male sterility in plants is caused by chemicals called **chemical hybridizing agents (CHA)**. It was found in 1950 that some substances, including maleic hydrazide, may result in selective male sterility in maize (Moore, 1950; Naylor, 1950). Despite these disadvantages, it was recognized that there might be some advantages, especially in the time required to identify hybrids that are commercially viable. The reason for this is that chemical techniques for male sterility induction can do away with the time-consuming procedures usually needed to create male sterile and restoration lines.

**Site and mode of action of CHA:** The most important general feature that the literature has illustrated is that the previously identified compounds (e.g., ethephon, FW-450, PPX 3778, RH-531) can result in a range of specific effects that differ based on how treatment time and dosage interact. Among the general impacts are the following: (McRae, 1985): (a) Early disruption of meiosis and early stoppage of the next developmental stage (b) Microspores have thin walls, a deformed shape and are not viable due to disruptions in exine production (c) Abnormalities in the microspore vacuoles, reduced starch deposition and ongoing tapetum (d) While anthers are normal, pollen is not viable. Although there is pollen and it is viable, anthers either do not dehisce at all or do so slowly.

**Mechanisms of male sterility**

**(1) Cytological changes:** The microsporogenesis process's pre- and post-meiotic stages could ultimately break down abnormalities can occur at various stages of pollen development, including the mature or nearly mature pollen stage, the vacuolate microspore stage, during meiosis in tetrad formation, or at the release of tetrads when callose dissolves.

**(2) Biochemical changes:** A few biochemical alterations, including changes in the structure and number of proteins, amino acids and anther-developing enzymes, are linked to male sterility. According to Kaul (1988), it has been linked to lower levels of proline, leucine, isoleucine, phenylalanine & valine and increased levels of aspartic acid, glycine and arginine. Significantly affected **proline levels** are in male sterile anthers. In comparison to fertile anthers, adult male sterile anthers have only one-eighth the proline concentration, according to Kakihara *et al.* (1988). Male sterile plants have less soluble protein and fewer polypeptide bands in their anthers. Mutant stamens lacked certain polypeptides that were synthesized in normal stamens.

1. **Cytoplasmic Male Sterility (CMS):**

Cytoplasmic Male Sterility (CMS) results from the interaction between nuclear and cytoplasmic genomes, leading to the cessation of pollen formation. It is often associated with the expression of chimeric open reading frames in the mitochondrial genome and the rearrangement of mitochondrial DNA. (Rahman *et al.,* 2024). Since the male sterility was express by mt gene and After fertilization, the mitochondria typically get removed from the pollen; cytoplasmic male sterility is a characteristic that mothers inherit. The cytoplasm of a zygote primarily comes from an egg cell, hence plants that are male-sterile would inevitably give birth to male-sterile progeny. CMS can be easily passed on to a strain by using that strain as a pollinator (recurrent parent) in the backcross program's following generations. The male sterile line would have a nuclear genotype that is nearly identical to the recurrent pollinator strain thanks to six to seven backcrosses.

1. **Environmental Manipulation:**

Environmental factors such as temperature, photoperiod and nutrient availability can affect pollen development and fertility. The term Environment Sensitive Genetic Male Sterility (EGMS) refers to the induction of male sterility in crops by manipulating these environmental conditions during critical stages of pollen production.

Certain genetic lines of male sterility are considered conditional mutants, meaning that the expression of male sterility is dependent on a specific set of environmental conditions. If these environmental requirements are not met, the male sterile plants can revert to being male fertile. These genetically male sterile (GMS) mutants are further categorized as either Temperature-sensitive Genic Male Sterile (TGMS) lines or Photoperiod-sensitive Genic Male Sterile (PGMS) lines, depending on the crucial environmental factor, typically temperature or photoperiod, that triggers the switch between sterility and fertility. Temperature-sensitive EGMS (Environmentally-sensitive Genic Male Sterility) lines have been observed in various vegetable crops, such as tomato, carrot, cabbage, Brussels sprouts, broccoli and peppers (both sweet pepper and chili). According to Kumar *et al.,* 2000, many of these lines were initially recognized as typical genic male sterile lines, without the understanding of their environmental sensitivity.

EGMS lines are currently the most successful method for creating hybrid seeds, despite the fact that they were formerly believed to be incredibly impracticable due to the instability issue. Practically speaking, nevertheless, for temperature- and photoperiod-sensitive genetic male sterility, respectively, it is crucial to ascertain the critical temperature or photoperiod for the manifestation of fertility/sterility.

1. **Epigenetic Regulation:**

Pollen development-related gene expression patterns can be regulated by epigenetic alterations such DNA methylation and histone modifications (Wan *et al.,* 2021). Choosing targets at epigenetic regulators provides a viable approach to causing male sterility in crops.

1. **Utilization of marker gene:**

In the seedling stage a recessive gene was expressed, which controls the non-lobbing leaf trait in watermelon, can be utilized as a marker gene. This approach simplifies and reduces the cost of hybrid seed generation. (Whitaker and Davis, 1962). Seeds from the non-lobed lines are the only ones that can be gathered. The inbred lines of lobed and non-lobed can be sown in alternating rows. When they are still seedlings, it is easy to identify the F1 hybrids with lobed leaves. However, since only approximately one-third of the seedlings will be F1 hybrids on average, roughly 6–8 seeds/hill may be sowed.

A marker gene for purple stem pigmentation in cabbage was proposed by Swarup and Gill (1964) to help identify F1 hybrid seedlings before transplantation. To create F1 hybrid seeds, brussels sprouts is crossed with a marker recessive gene for glossy foliage (North and Priestley, 1962). Johnson (1966) recommended adding a recessive marker gene to the Brussels sprout A and B lines and proposed a partial chlorosis trait for this reason in addition to the glossy foliage.

In a novel method for producing hybrid onions, Davis (1966) used an inbred line with brown seeds to show how the colour of the brown seed coat is linked to male sterility. A single recessive gene determines the brown seed coat's colour. Both the brown-seeded male sterile line and the black-seeded pollen parent line can yield hybrid seeds. The male sterile line will be used to extract black hybrid seeds. This process is also useful for rouging off types that are present in male sterile seed parents and male fertile pollen parents.

**Conversion Breeding and Principles of Introducing Male Sterility Traits**

A conversion breeding technique aims at inducing desirable characteristics, such as male sterility, in elite germplasm or cultivars. To restore the genetic heritage of the recurrent parent and introgress the desired trait, this method usually entails several rounds of backcrossing and selection (Xu *et al.,* 1997). Male sterility is an essential characteristic in the generation of hybrid seeds, which makes it possible to produce high-yielding hybrid cultivars with better uniformity and vigour.

Introducing male sterility traits into breeding lines involves several key principles to ensure successful conversion and maintain agronomic performance:

* **Identification of Male Sterility Sources:** Identifying and characterizing male sterile lines or sources with stable and heritable male sterility traits is the first step in conversion breeding.
* **Marker-Assisted Selection (MAS):** Effective selection and introgression of male sterility features during backcrossing are made possible by the use of molecular markers associated with male sterility genes. Photoperiod-sensitive male sterile japonica rice has been successfully bred for enhanced cross-compatibility with indica rice using marker-assisted selection (MAS) (Liangming *et al.,* 2010). The limited fertility of rice hybrids between Japonica and Indica has prevented breeders from taking advantage of the significant heterotic potential of these hybrids. Allelic interactions at a few loci can cause hybrid sterility, which can be overcome by simple introgression at the main loci of sterility. They present a male sterility gene from CV that is photoperiod-sensitive. Combining the yellow leaf gene from line Yellow249 (indica) with Lunhui 422S (indica) produced the elite japonica cv. Zhendao 88 through backcrossing assisted by markers. The microsatellite markers RM276, RM455, RM141 and RM185 were used in that order to tag the fertility genes S5, S8, S7 and S9 The male sterile plant Line 509S is photoperiod-sensitive and true-breeding; its morphology is similar to that of the japonica type. According to genotypic research, 92% of the DNA in line 509S is from Japan. Hybrids resulting from the cross-pollination of indica types and line 509S exhibit a high degree of heterosis, but hybrids involving japonica types and the line suffer from hybrid sterility. These results suggest that a useful strategy for utilizing the inter-subspecies heterosis in rice is segment replacement on fertility loci based on available information and marker-assisted selection.
* **Backcrossing and Recurrent Selection:** Backcrossing to the recurrent parent is essential to recover its genetic background while incorporating male sterility traits. Recurrent selection further enhances converted lines' performance through iterative selection and recombination cycles.
* **Genetic Mapping and Gene Discovery:** Genetic mapping and gene discovery are crucial for understanding male sterility in crops, enabling the identification of candidate genes for targeted introgression. Recent studies have focused on fine mapping male sterility genes, such as ms-3 in cucumber (Han *et al.,* 2018) and MS-cd1 in *Brassica oleracea* (Zhang *et al.,* 2011), revealing significant insights into their genetic architecture.
* **Evaluation of Agronomic Performance:** Converted lines must undergo precise evaluation for agronomic performance, including yield potential, stress tolerance and quality traits, to ensure that male sterility introduction does not compromise overall performance.
* **Hybrid Seed Production:** Male sterile lines are employed in the production of hybrid seeds to capitalize on hybrid vigor, thereby enhancing yield and ensuring uniformity in commercial agricultural products.

These principles guide the successful introduction of male sterility traits through conversion breeding, facilitating the development of hybrid cultivars with improved performance and productivity.

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