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REVIEW ARTICLE

Genetic and molecular basis of cytoplasmic male sterility in maize

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ABSTRACT

Cytoplasmic male sterility is a maternally inherited trait that suppresses pollen production due to the interaction of nuclear and mitochondrial genomes. In maize three types of CMS systems, namely CMS-T, CMS-S and CMS-C, have been documented and are differentiated by the reaction to restorers, mitochondrial DNA restriction digest patterns, and complements of low molecular weight plasmids. CMS-T is restored fully by Rf-1 and Rf-2, CMS-S by Rf-3, and CMS-C by Rf-4. All restorer genes except Rf-2 restore fertility by affecting the transcript profile of CMS-associated locus. The sterility is caused by the disorganization of the tapetum and surrounding cell layers as a result of the expression of pollen specific genes. Even though such phenotypes are associated with gene dysfunction in mitochondria, the chloroplasts have emerged as ideal organs for engineering male sterility in crop plants. A number of systems such as *barnase-barstar* have been standardized in *Brassica*. Recently, polyhydroxy butyrate was identified as a potential candidate gene for engineering male sterility. Moreover, a broad group of proteins called PPR (pentatricopeptide repeat) proteins has also shown to hold great promise for engineering male sterility in crop plants as most of the restorers belong to this category. In maize one such protein, CRP-1, has been identified.

Key Words: *maize; cytoplasmic male sterility; T-urf 13; programmed cell death; barnase-barstar; PPR proteins*.

INTRODUCTION

Cytoplasmic male sterility (CMS), a maternally inherited trait that suppresses production of functional pollen grains, has been known for over 100 years and has been reported in over 150 plant species. The CMS phenotype is caused by incompatibility of nuclear and cytoplasmic genomes (Ruiz and Daniell, 2005) and represents a valuable tool in the production of hybrid seeds. Cytoplasmic control of male sterility has important economic and logistical advantages in hybrid seed production (Mackenzie et al., 2004). In maize

Rhoades (1933) described the first report of CMS. Being one of the important food crops, maize offers one of the obvious choices for exploitation of CMS for development of hybrid varieties and as such has been subjected to extensive genetic studies to ascertain the types of sterility.

Two models have been put forth for the possible origin of CMS cytoplasm in maize (Doebley and Sisco, 1989). Doebley and Sisco (1989) proposed that either it arose within maize due to a mutation in the mitochondrial genome or was the result of introgression of foreign cytoplasm. The CMS phenotype is thus essentially the result of incompatibility between foreign cytoplasm and the maize nuclear genome. They analyzed the chloroplast genomes of various CMS types in maize and those of *Z. parviglumis*, *Z. perrenis*, *Z. diploperrenis*, *Z. luxurians* and *Z. mexicana* (*Copandiro teosiute*). Chloroplast genomes of C and T types of cytoplasms were found to be similar to each other and *Z. parviglumis*, the teosinte which is thought to be the progenitor of maize. It implies that CMS in C & T cytoplasms arose by mutation within the maize mitochondria. The chloroplast genome of CMS S did not share any similarity with any maize accessions but was found in *Z. mexicana* (*Copanidro teosinte*). To confirm that CMS S and *Z. mexicana* possessed the same cytoplasm mitochondrial genome, both of them were analyzed and found to contain similar types of plasmids. Thus, it was concluded that CMS S originated from *Z. mexiciana* and was introgressed into domesticated maize through the Conico Norteno race of maize from Mexico, which also contains S cytoplasm. Doebley and Sisco (1989) further proposed that given this interpretation, Copanidro teosinte may also possess restorers for CMS-S and even be the source of restorers found in maize for S cytoplasm.

TYPES OF CMS IN MAIZE

There are four major types of cytoplasm, namely N (normal), C (Charrua), T (Texas) and S (USDA), which are classified according to differential sterility expression in response to restorer (Rf) genes (Laughnan, 1983), mitochondrial DNA restriction digest patterns (Pring and Levings, 1978), and compliments of low MW plasmids (Kemble and Bedrock, 1980). Even though testcrossing is the most conclusive method of categorizing maize cytoplasms, they are time-consuming and labor-intensive (Liu et al., 2002). In many plant species, mutations responsible for CMS have been shown to reside in mitochondrial DNA (Schnable and Wise, 1998). Therefore, various features of mitochondria, especially its genome structure and gene expression, have been used to distinguish major types of maize cytoplasm (Levings and Pring, 1977). Pring et al. (1977) were the first to show that mitochondrial preparations (not digested by restriction enzymes) from lines carrying S cytoplasm possessed unique low (molecular weight) MW plasmids not found in N, T or C cytoplasms. Later on Kemble et al. (1980) studied low MW mitochondria DNA from 31 separately discovered sources of cytoplasms. Unique banding patterns to each of four classes were identified which were in conformity to the classification of these sources done by Beckett (1971). A major advantage of this analysis was that no within-group heterogeneity was found as had been in earlier studies (Levings and Pring, 1977). Similarly, Forde et al. (1978) used SDS-PAGE to analyze mitochondrial translation products of CMS cytoplasm and detected additional or variant polypeptides in T and C cytoplasm. More recently, Liu et al. (2002) developed a rapid PCR assay of sterile cytoplasm in maize using three pairs of primers designed corresponding to chimeric regions of mt-DNA sequences. The primer sets used amplified unique 440 bp (CMS-T), 398 bp (CMS-C) and 799 bp (CMS-S) cytoplasm, whereas no unique fragment was amplified in N cytoplasm, possibly because no such fragment existed in N cytoplasm.

CMS-T

Rogers and Edwardson (1952) first discovered T-cytoplasm among Mexican OPV "Golden June" at the Texas Agricultural Experimental Station. It is characterized by failure of anther exertion and pollen abortion and has high proportion of binucleate cells (Roy and

Sarkar, 1991). For two decades (1950-1970) it was one of the chief methods of replacing detasseling for hybrid seed production. Nearly 85 % of hybrids grown in southern region and the corn belt of the United States of America carried T cytoplasm (Levings, 1993) as it provided a reliable and stable source of CMS for seed production. In 1970, a fungal disease known as Southern corn leaf blight epidemic caused by *Bipolaris maydis* severely blighted maize carrying CMS-T, but had a mild effect on maize carrying normal cytoplasm. This led to the discontinuation of use of CMS-T cytoplasm. The specific susceptibility of CMS-T maize to blight once again demonstrated the dangers of uniformity; although, in this case vulnerability was caused by cytoplasmic factors rather than nuclear.

The toxin of *Bipolaris maydis* (Bmt) is a functional analog of methomyl and is host specific to CMS-T. It inhibits mitochondrial respiration, uncouples oxidative phosphorylation, decreases calcium transport into mitochondria, increases membrane permeability of calcium and H+ and inhibits the malate dependent electron transport system. The specific virulence of *B. maydis* towards CMS-T maize was found to be due to mitochondrial gene *Turf 13*, which is also responsible for the CMS phenotype in CMS-T. It is a constitutively transcribed gene which produces a 13 kd polypetide (Williams et al., 1992). Such a polypeptide is not found in CMS -S, CMS -C or normal maize cytoplasm. *Turf 13* is a chimeric region gene which is a recombination product of 5' region of the *atp 6* gene and 3' region of the 265 ribosomal gene (*rrn 26*). Its transcription is presumably under the control of the *atp 6* promoter (Stamper et al., 1987). It is located in 3547-nucleotide mt DNA sequence that contains two open reading frames, one coding for *urf 13* and the other for *orf 221*, which codes for a 25 kd polypeptide consisting of 221 amino acids and is 77 nucleotides downstream of *urf 13* (Levings, 1990). The *orf 221* encodes a membrane bound protein that has been identified as ATP4 (Heazlewood et al., 2003).

The dominant nuclear genes Rf1 and Rf2 located on chromosome 3 and 9, respectively, restore full fertility in CMS-T maize, whereas, Rf 8 and Rf * partially restore fertility (Wise et al., 1999). Rf 1, and Rf 8 and Rf *are characterized by processing and concurrent reduction of *urf 13*-associated proteins (Wise et al., 1996). In fact, Kennel et al. (1987) observed that Rf 1 reduced the abundance of 13 kd *urf 13* protein by 80 %. However, the processing of *turf 13* RNA by Rf 1, Rf8 and Rf * occurs at different locations and the processing sites share some conserved sequences as well. In effect, the processing of *turf 13* RNA results in non-translation of the transcripts without any decrease in RNA abundance (Wise et al., 1999). In contrast, Rf 2 does not affect the expression levels of the *urf 13* gene and shares 75 % similarity with mammalian aldehyde dehydrogenase (Liu et al. 2001). The Rf1/Rf2 plants exhibited altered expressions of the *urf 13* gene while rf1/Rf2 does not, indicating that Rf1 is a constitutively expressed gene whereas Rf2 was a regulatory gene. In fact, Rf2 is the only restorer that does not effect the transcript profile or protein accumulation of the CMS-associated locus. It is a biochemical restorer that restores fertility by amelioration of residual metabolic defects caused by the downregulation of *turf 13* expression, especially the toxic effects of aldehydes. Some authors question whether it is a genuine restorer (Budar et al., 2003). There are two other examples wherein the restorers do not act at mRNA level, namely the *Fr* restorer of *Phaseolus* (Mackenzie and Chase, 1990) and Rfo restorer of ogura (Bellaoui et al., 1999). Rf2 can oxidize both aliphatic and aromatic aldehydes, even though the exact target substrate in anthers is yet to be identified (Liu and Schnable, 2002). Cui et al. (1996) were first to propose a working hypothesis for the role of Rf2 as nuclear restorer in CMS-T in maize, and proposed that Rf2 restored fertility by amelioration of mitochondrial lesions associated with the expression of *Turf 13* gene. Two routes carry out such amelioration. One is the metabolic route, whereby energy produced by oxidation of fatty acids by ALDH compensates for the possible energy deficit in tapetal cells. Alternatively, ALDH acts by detoxifying acetaldehyde. The interaction route involves interaction of ALDH with Urf-13 with a consequent reduction in its deleterious effects. The lack of mitochondrial ALDH activity, in fact, causes complete male sterility in T cytoplasm (Liu et al., 2001).

Rf2 is one of the extensively studied restorer genes that encodes an acetaldehyde dehydrogenase (EC. 1.2.1.3) and is expressed during microspore development (Tadege et al., 1999). It has a significant developmental role apart from fertility restoration, even in plants that do not carry T cytoplasm. Schnable and Wise (1998) proposed that Rf2 was very recently recruited to serve as a nuclear restorer, a hypothesis that gets supportive evidence from the fact that Rf2 does not encode ALDH mRNA and ALDH activity is not significantly higher in T cytoplasm than in N cytoplasm (Liu et al., 2001). Thus, in T cytoplasm the role of Rf2 is fertility restoration and in N cytoplasm it is anther development.

CMS-S

In view of the susceptibility of T-cytoplasm to *B. maydis*, other groups of cytoplasm conferring male sterility were sought. Jenkins of the USDA (Gracen et al., 1979) first discovered S-cytoplasm from a strain of Teopod maize. It is one of the most common sources of CMS and includes Ca, F, G, H, I, IA, J, K, L, F, M, ME, ML, MY, PS, R, SD, TA, VG and W cytoplasm sources (Gracen et al., 1979). In S cytoplasm, mitochondrial genomes are characterized by the presence of small, low MW plasmids S_1 and S_2. S_1 is 6.4 kbp and S_2 is 5.4 kbp long (Paillard et al., 1985). However, it has not been yet established as to whether these plasmids have any role in the CMS phenotype of S cytoplasmic types (Nair, 1993). These plasmids are structurally similar and share 1.7 kbp of conserved sequences. Unlike other sources, CMS-S is typified by spontaneous reversion to fertility either due to nuclear mutations or gene rearrangements. In such revertants, S1 and S2 plasmids were not detected. Pring et al. (1977) were first to hypothesize that unique DNA's (S_1 and S_2) are associated with the unstable nature of S cytoplasm group. These elements described as episomes by Laughnan and Gabay-Laughnan (1975) might be transposons, which when present on a chromosomal site confer stable fertility restoration and are associated with male sterility while at the cytoplasmic location. The reversion of sterility is influenced by cytoplasmic rather than nuclear genetic factors and loss of such factors (S_1 and S_2 plasmids) is correlated with cytoplasmic reversion from male sterility to male fertility (Levings et al., 1980). Escote et al. (1985), however, reported spontaneous cytoplasmic revertants not associated with the loss of S1 and S2 plasmids. Similarly, Small et al., (1988) found that no loss of DNA sequences was involved in CMS-S reversion. They further argued that nuclear rather than cytoplasmic genetic background affected the mitochondrial DNA reorganization associated with sterility reversion. A dominant nuclear gene Rf3 located on chromosome 2 restored the S-cytoplasmic sterility. The Rf3 gene action resulted in shorter transcripts of CMS -S associated *orf 355* and *orf 77* (*atp 9*) as well as smaller transcripts of *atp 6* and *cob* genes. The multiple RNA processing effects of Rf3 may be due to the action of same processing enzyme, conservation of processing sites, or that the Rf3 locus consists of 2-3 closely linked genes that process different RNA's. In addition to naturally occurring Rf alleles, almost 60 restoring alleles of S cytoplasm have been recovered as spontaneous nuclear mutations. While seven of such restorer alleles have been mapped to chromosome 2, others have been mapped to chromosomes 1, 3, 6 and 8 (Gabay-Laughnam et al., 2004). Most of these restorer alleles are not economical due to certain apparent limitations such as homozygous lethality and deleterious effects on endosperm development and have, therefore, been designated as *restorer of fertility lethal alleles* (Wen et al., 2003). There are, however, a number of exceptional homozygous restorers that are viable and have normal endosperm development. These have been reported from a number of races such as Tuxpeno, Oloton, Comiteco, and Tehua. Moreover, the lines belonging to *mexicana* (K-69-4 and K-69-6) and *parviglumis* (M046, M0106, 9477 and M063) also have viable homozygous alleles and a normal seed set.

CMS-C

CMS-C is one of the most attractive sources of male sterility for hybrid maize production and is resistant to *B. maydis*. It was first discovered by Beckett (1971) from Brazilian maize variety Charrua and includes RB, E1, EL, ES, BB and Bb sources (Pring et al., 1980). Earlier

works of Josephson et al. (1978) reported three or more nuclear genes responsible for its restoration, but that study involved partial restorers as well (Rf4, Rf5 and Rf6). Pour et al., (1981) and Newton (1983), however, reported no evidence of more than one dominant restorer Rf4 located on chromosome 8. The mitochondria of CMS-C are characterized by a unique 17.5 kd peptide in place of 15.5 kd membrane bound peptide in normal cytoplasm (Newton, 1989). Moreover, there are mutations in genes, namely *atp 6*, *atp 9* and *cosII*, resulting from rearrangements between positions of mitochondrial and chloroplast genes, which actually confer CMS phenotype. Like CMS -T, CMS-C is also specifically susceptible to race C of B maydis producing pm toxins (Liu et al., 1986). This toxin significantly enhances the activity of phenylalanine ammonia lyase.

MECHANISM OF CMS

The occurrence of male sterility in wild species is revealed by the coexistence of hermaphrodite and female (male sterile) plants in natural populations (gynodioecy). Most of the studies on such populations have led to the emergence of theoretical models based on the premise that CMS is a result of interaction between nuclear and cytoplasmic genomes. Gynodioecy is supposed to appear and be maintained in populations either due to female advantage or counter selection of nuclear fertility restorers (Budar et al., 2003). The genomic conflict theory (Cosmides and Tooby, 1981) provides for the interaction of cytoplasmic determinants (that prevent pollen production) and nuclear restorers (that restore fertility). The modes of inheritance of cytoplasmic (uniparental) and nuclear (biparental) factors generate a situation where selective interests are conflicting, resulting in differential resource allocation for pollen and seed production. Most of the CMS's were a result of an alloplasmic state, wherein an impaired mitochondrial activity occurred due to a conflict at genomic, proteomic or transcriptomic levels (Kaul, 1988).

Studies on the genetic and physiological factors underlying male sterility have revealed that significant differences exist between normal and sterile anthers (Nair, 1993). The tapetum, the innermost cell layer surrounding pollen grain, is a physiologically active site and nourishes the developing gametes. However, it degenerates later on and is not found as an organized tissue in mature anthers. The tapetum and other surrounding cell layers suffer early degeneration in sterile pollen as compared to fertile ones. An important question was: how does a mitochondrial dysfunction at the genetic and physiological level specifically alter pollen development (Levings, 1993)? Since mitochondria harbor genes governing vital biochemical pathways such as the electron transport system and encode enzyme systems and structural RNA's, how do such mutations specifically block pollen development, as mutations to any of them could be lethal?

One explanation could be that mitochondrial genes are expressed differently during anther development. Such differential gene expression may occur either by the interaction of CMS genes with some unknown factors in target tissue leading to localized developmental changes or interference of CMS gene products in the physiological machinery resulting in reduced respiration and ATP synthesis (Levings, 1993). Wallace (1989) suggested that minor impairments in mitochondrial function might selectively affect degeneration of specific cell types. Such deviations in expression were observed in all cell and tissue types and have also been reported in case of human cardiac dysrythmia (Wallace, 1988). Lee and Warmke (1979) observed a 10-fold increase in mitochondria/cell count in the tapetum during anther development. Such amplification indicates greater demand for energy. Sarria et al. (1998) observed that in *Phaseolus*, the CMS-associated gene product was degraded by proteases in mitochondria of vegetative cells. In CMS-T, the first sign of sterility is the rapid disorganization of tapetal cells soon after meiosis, which could be due to a mutant gene. Interestingly, such mitochondrial amplification has not been found in other maize tissues including the developing ear. *Urf 13* was proposed to be pivotal for affecting sterility in T-

cytoplasm sources. Flavell (1974) proposed that some anther-specific substances might interact with *urf 13* proteins to cause sterile phenotypes, because *urf 13* is expressed in other organs as well. The *urf 13* protein as such has also been found to be detrimental to cell viability, but such toxicity seems to be specifically mediated by membrane bound proteins for pollen abortion. Specific toxicity of *urf 13* protein to anther is not clearly understood. Levings (1993) proposed that it may be over-expressed in tapetal cells, a notion which gets boost from the fact that there are increased levels of mitochondrial biogenesis in tapetal cells during pollen formation. Alternately, the tapetal cells may be more sensitive to *urf 13* proteins. Iwabuchi et al. (1993) reported that CMS-Boro II is due to an abnormal version of the mitochondrial *atp 6* gene encoding aberrant mRNA transcripts in rice. Similarly, alterations in promoter regions of mitochondrial ATP synthase genes also cause sterility. Almost 12 mt-DNA regions associated with CMS have been found to involve ATP synthase subunit promoter regions and portions of coding sequences. Even if the chimeric gene itself does not involve an ATP synthase sequence, the ORF is often located in close proximity to an atp gene. In CMS-T maize, *orf 221* that is present downstream to *urf 13* encodes the Fo protein of ATP synthase (*atp 4*). Similarly, in CMS -S the *orf 77* is a chimeric gene downstream to *orf 355* and contains portions of coding sequences of *atp 9*. Cytotoxic gene products have been found to be associated with male sterility in sunflower (Nakai et al. 1995) and *Brasicaceae* (Duroc et al., 2005). In radish, Ogura CMS is controlled by two contranscribed reading frames *orf 138* and *Orf B*. While *Orf 138* is similar to most mitochondrial sterility-inducing genes, *orf B* codes subunit 8 of ATP- synthase complex (Gray et al., 1998).

In a recent study, Wang et al. (2006) characterized the role of mitochondrial open reading frames (ORF's) in male sterility in rice. They found that E. coli transformed with *Orf 79* established cytotoxicity of the *Orf 79* gene product. Later on, *Orf 79* was over-expressed in a normal, fertile rice line using the CaMV35S promoter and resulted in 750% pollen abortion, whereas female fertility was unaffected by such transgenic expression. They concluded that Qrf 79 encoded a cytotoxic peptide that caused CMS in rice. These and other studies have implicated different mechanisms of mitochondrial impairment in CMS phenotypes, but ultimately the programmed cell death of sporogenous tissue, especially the tapetum, causes pollen sterility. Mitochondria have been found to be involved in interpretation of cell death signals in both animals and plants. Recently Balk and Leaver (2001) showed that in CMS sunflower, the tapetal cells exhibited characteristic features of programmed cell death including the release of cytochrome c, cell condensation, and separation of chromatin into delineated masses and oligonucleosomal cleavage of nuclear DNA. Such characteristics are similar to apoptosis in mammals, except the condensation of nuclei and disintegration of cells into apoptotic bodies. In the case of maize, it was proposed that the *urf 13* gene caused male sterility by PCD, characterized by formation of pores in the mitochondrial membrane (Wu and Cheung, 2000). Holden and Sze (1987) proposed that in the presence of the Bm toxin of *Bipolais maydis*, the mitochondria carrying the *urf 13* gene experienced leakage of small ions and, consequently, loss of membrane potential. Tapetal degeneration has been found to be the common feature of such phenomenon in plants like petunia, wheat, sorghum, sunflower and maize (Laser and Lersten, 1972; Schnable and Wise, 1998). Frank and Barr (2003) generalized the term PCD for triggered and orderly death of cells with apoptosis as its specific subtype in animals.

ENGINEERING MALE STERILITY

The expression of a chimeric RNAase gene from *Bacillus amyloliquifaciens* in tobacco and rape heralded the beginning of engineering male sterility in crop plants. Mariani et al. (1990) constructed a transgenic rape line carrying the barnase gene from *B. amyloliquifaciens* in which barnase was selectively expressed in anther using anther-specific promoter. The expression of the barnase gene caused tapetal cell destruction causing pollen abortion.

Restoration of sterility was achieved by crossing sterile plants to transgenic plants containing the barstar gene from *B. amyloliquifaciens*, which is a barnase inhibitor gene. The F1 expressed both barnase and barstar genes and was fertile due to inhibition of cytotoxic activity of barnase. Similarly, in tobacco transgenic lines expressing *rol C* gense of T-DNA of *Agrobacterium rhizogens* were found to be male sterile (Schmulling et al., 1988). Over-expression of *rol C* genes also caused male sterility in other crops as well (Fladung, 1990). The restoration of *rol C* governed male sterility was achieved by crossing it with a transgenic line containing the role C-antisense gene (Schimulling et al., 1993). Goetz et al. (2001) used antisense repression of extracellular invertase gene Nin 88, which caused sterility. Nin 88 is an invertase that catalyses cleavage of sucrose in tapetal cells and developing pollen and maintains a carbohydrate supply for energy demands. They were able to achieve highly cell specific antisense repression of Nin 88 using Nin 88 promoter, thereby resulting in normal plant growth and development except for the failure to produce functional pollen.

Even though male sterility is strongly associated with gene dysfunction in mitochondria, chloroplasts have emerged as a more than ideal choice for engineering male sterility through the transgenic approach. Such an approach offers a number of advantages such as high levels of transgene expression, multi-gene cassette engineering, transgene containment viz., maternal inheritance, lack of gene silencing, position effect due to site-specific transgene integration, and lack of pleiotropic effects (Daniell, 2005). Moreover, such engineering strategy can enable the transfer of male sterility systems in certain recalcitrant crops such as cotton and soybean (Grevich and Daniell, 2005). It also overcomes the disadvantages of nuclear transformation, wherein the transformants segregate for fertility and sterility (Ruiz and Daniell, 2005).

Polyhydroxybutyrate (PHB) has been identified as a potential candidate gene system for engineering male sterility in crop plants. The synthesis of PHB is the result of three genes, namely PhaA (a-ketothiolase), PhaB (Acetoacetyl-coA-reductase) and PhaC (PHB synthase). The PHB causes abnormal development of the epidermis and endothecium with a broken tapetal layer. The first report of expression of Phab and PhaC genes *vis-a-vis* nuclear transformation came from Poirier et al. (1992), which yielded low levels of PHB. Nawrath et al. (1994) transformed Arabidopsis to reconstruct whole pathways by expression of all three genes by targeting their expression to chloroplasts and reported up to 14% PHB of leaf dry weight with no pleiotropic effects. In order to overcome pleiotropic effects of PHB, specifically due to PhaA gene encoding a-ketothiolase, Bohmert et al. (2002) analyzed transgene expression via nuclear transformation and reported decreased levels of a-ketothiolase but reduced transformation efficiency. A recent study on chloroplast engineering showed no pleiotropic effects and complete male sterility (Ruiz and Daniell, 2005). They used the PhaA gene from *Acinetobacter* driven by constitutive 16S rRNA promoter (*Prrn*) and transferred through the biolistic method. The important finding was that the expression of PhaA caused 100 % male sterility; the light illumination reverted the sterile lines back to fertile lines. They proposed that the PHB biosynthesis system was a viable system for engineering male sterility in crop plants. In crops like maize where hybrid seed production is based on nuclear restoration factors, PHB-mediated CMS and its subsequent restoration by continuous illumination offers a novel approach for creating transgenic male sterile plants. This will help save the time and resources required to backcross a male sterility-inducing cytoplasm into elite genetic backgrounds (Havey, 2004). Another major advantage of chloroplast engineering is the scope for diversification of CMS sources by introduction of different sterility-inducing factors into elite parental lines. This, therefore, prevents the risk of genetic uniformity of CMS sources as was encountered in CMS -T in maize.

Identification of potential candidate genes is imperative for engineering male sterility in crop plants. In this context, a broad group of proteins called PPR (Pentatricopeptide repeat) proteins hold great promise. The PPR protein family is a large group of proteins frequently

present in higher plants. They are characterized by tandem repeats of 35 amino acids, often arranged as a tandem array of 2-26 repeats per peptide (Small and Peeters, 2000), and are mainly targeted to mitochondria or chloroplasts. In Arabidopsis (450) and rice (600) such proteins have been identified and their closest homologues have been found in petunia (PPRS 92), maize (CRP1), radish (P67), and rice (PPR-8-1) (Fisk et al., 1999; Lahmy et al. 2000; Koizuka et al., 2003; Kazama and Toriyama, 2003; Wang et al., 2006) PPR proteins consist of two alpha-helices with the tandem repeats forming a supercoil with a central groove lined with hydrophilic side chains and positively charged bottom residues. Kotera et al. (2005) proposed that PPR proteins are basically involved in RNA editing in chloroplasts. In fact, the petunia Rf gene, which was the first restorer gene to be cloned (Bentolila and Hanson, 2001), was found to encode a PPR protein (PPR 592) that functions as an mRNA editing factor. Fertility restoration was followed by characterization of PPR proteins in other crops as well. An interesting observation in petunia was that non-restoring alleles carried a deletion in the promoter region of PPRs92 that reduced its tissue specificity. While the restoring allele is expressed in floral buds, the non-restoring allele encoding transcript is found in root tissues only.

Kazama and Toriyama (2003) proposed that the Rf-1 restorer of BT type or ms-boro type in rice (Kadowaki et al., 1990) was a PPR gene designated as PPR-8-1, encoding PPR motifs consisting of 18 repeats of 35 amino acids. This gene restored fertility by processing mRNA of the aberrant *atp 6* gene. The non-restoring allele had deletions in coding region. The sequence comparison of PPR-8-1 and petunia PPR-592 revealed 68% similarity between the first and thirteenth PPR. In Kosena radish, *orf 687* restored fertility by reducing the level of *Orf 125* protein that causes sterility. Genetic characterization of *Orf 687* (Koizuka et al., 2003) revealed that *Orf 687* had 16 repeated PPR motifs comprising 81.5 % of the protein sequence. Even though *Orf 687* shared only 27 % similarity, this was an important feature of the activity of PPR genes in regulating CMS-associated gene expression. Since most of the restorer genes seem to function by reducing the expression of CMS-inducing transcripts or proteins in mitochondria, PPR proteins are ideal candidate genes for use in engineering of male sterility in crop plants (Desioire et al., 2003). Another PPR protein with 16 repeats of 35 amino acids encoded by RF-1 restorer gene in insboro rice that is widely used in seed production of Japonica hybrids was cloned by Komori et al. (2004). The RF-1 PPR protein is a mitochondrial-targeted protein that reduces expression of CMS-associated mitochondrial genes. Sequences analysis revealed that the recessive allele had a deletion in coding region. Most of the PPR proteins characterized to date have striking similarity among themselves and with 20 odd Arabidopsis PPR proteins. It is very intriguing to speculate about the function of these PPR genes in Arabidopsis, which are similar to Rf-PPR genes, as CMS has never been described in this plant. However, a likely proposition may be that these genes have evolved as RNA processing genes and have been recruited for restoration of sterility arising out of aberrant mitochondrial genes.

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