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REVIEW ARTICLE

ROLE OF GENETICALLY ENGINEERED SYSTEM OF MALE STERILITY IN HYBRID PRODUCTION OF VEGETABLES

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SUMMARY

Availability of cost effective mechanism/ method to produce large scale hybrid seed utilizing selected parental line is one of the important factors which ultimately determine the commercial viability of hybrid varieties. Manual emasculation increased cost of production, so use of various genetic mechanisms viz; male sterility, self incompatible, gynoccious lines, use of sex regulators and chemical hybridizing agents bases on relative importance in hybrid development in vegetable crops. Among these, genetic emasculation tools male sterility is commonly used for hybrid production. Recombinant DNA techniques have made it possible to engineer entirely new systems of male sterility by disturbing any or a number of developmental steps specifically required for the production of functional pollen within the microspore or for the development of any somatic tissues supporting the microspores. Although engineered male sterility systems are not currently in commercial use, except for possibly the barnase-barstar system, these are likely to have significant importance in future hybrid-breeding programs. The specific mechanisms causing male sterility in vegetables vary from species to species and are subject to influence by environment, and nuclear and cytoplasmic genes. Male sterility may be heritable or transient. It can be induced by anther culture, somatic cell culture, and somatic hybridization. However, the most significant development is the possibility of engineering male sterility by inserting cloned gene sequences which can disrupt any or more than one step during microsporogenesis. The male sterility was, in each case, heritable, associated with normal female fertility, and apparently maternal in its inheritance. Segregation of the transgene did not reverse the male sterile phenotype, producing stable, non transgenic male sterility. The reproducible transgenic induction of mitochondrial rearrangements in plants is unprecedented, providing a means to develop novel cytoplasmic male sterile lines for release as non-GMO or transgenic materials.

Keywords: *Abelmoschus esculentus*, Male hormonal profile, Sperm motility, Sperm count, Male Fertility.

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1. Introduction

Many of our modern-day vegetables are so-called "hybrids". These originate from parent plants that exhibit significant genetic differences. The aim of such hybrid-cultivation measures is to avoid the familiar negative effects of "in-breeding" (the cross-breeding of closely related organisms). Hybrids produce higher yields and are less susceptible to disease and environmental stress than non-hybrid varieties. A technical implication is encountered in the cultivation of hybrids is the fact that many plants bear both male and female reproductive organs which exhibit a tendency to auto gamy. In the production of hybrid seeds, therefore, efforts are made to use purely female – or more precisely "male-sterile" – plants for cultivation purposes. The pollen is transferred to the female organs manually. Male-sterile plants have been developed both by conventional cultivation procedures as well as by genetic modification. The manual removal of flowers or portions thereof and manual fertilization of female flowers by breeders are therefore no longer necessary. The genetic modification technique involves the introduction of genes that are active only in the male reproductive organs and that prevent the emergence of male flowers (or parts thereof). This results in purely female ("male-sterile") plants that can then be used for cultivation purposes and for the production of hybrid seeds. The production of hybrid seeds, the male-sterile plants of one parent is planted alongside unmodified plants of the other. The seeds developing in the male-sterile plants therefore can have developed only by crossing of two intended parents. An essential feature here is that hybrid plants those grow from the hybrid seeds go on to form pollen themselves to extent that the seed of hybrid plants indeed constitutes the crop to be harvested(leaves, stems, roots, etc.), the restoration of the pollen fertility is not absolutely necessary (e.g. sugar beet, animal-feed grasses). Male sterile plants don't produce pollen. That makes it easier to breed improved hybrids that yield and perform better, and to produce hybrid seed more economically. Sterility also helps ease concerns that genetically modified crops will spread their enhanced genetic characteristics, such as herbicide resistance, to wild plants. Scientists have long tried to develop male sterile plants through a variety of techniques, from tapping natural mutations to inducing sterility through radiation and chemical methods. And this characteristic can be unstable - some types of sterile plants can revert to fertility, which causes problems for growers. Sally Mackenzie (2003) a plant geneticist at University of Nebraska-Lincoln thinks she's found a genetic key to sterility. It promises to work for a wide range of crops and horticultural products. Scientists have long known that, in nature, changes in the cells' mitochondrial DNA cause the sterility mutation. Mackenzie and her team followed that genetic trail to recreate the mutation in the lab. They found a gene in the cell's nucleus that controls genetic changes in the mitochondria, which are the cell's energy producers and also contain DNA. By inserting foreign DNA into this gene, they turned it off, observed changes in the mitochondria and pinpointed which change actually triggers male sterility. They tracked down the gene in Arabidopsis, which they use as a model plant because its genetic code is known, but their findings have broad potential. Because all plants carry this gene that affects the mitochondria, these NU Institute of Agriculture and Natural Resources scientists can use their technique to trigger male sterility in others. Mackenzie now is growing transgenic tomatoes to search for additional male sterile. "The really cool thing about this is that once I induce a male sterile, it's stable," Mackenzie said. After removing the foreign DNA that caused the original genetic change, the plant remains sterile. But by eliminating the foreign DNA, the plant is no

longer considered transgenic. "That's the beauty of it," she says. "Nobody has to have any qualms about using GMO technology." Agriculture would benefit if this method of inducing male sterility proves successful. Mackenzie wants consumer to benefit, too. She's applying her findings to develop a sterile, seedless green bean that vegetable buyers should appreciate. Without seeds, the pod is tender and more easily digestible. Sterility also tricks the plant into producing three times the number of pods, increasing yields. While genetically modified crops have helped to reduce the need for agricultural pesticides, consumers have yet to benefit directly, she said. "If we hit the market with our male sterile and, at the same time, come up with our new seedless bean," said Mackenzie, "I think the consumer is going to say, 'this is nice engineering'."Researchers hope to work with an agribusiness to make sterile males commercially available in a variety of vegetable crops.

2. Manifestations of Male Sterility

a. Absence or malformation of male organs (stamens) in bisexual plants or no male flowers in dioecious plants.

b. Failure to develop normal microsporogenous tissue- anther.

c. Abnormal microsporogenesis--deformed or in viable pollen.

d. Abnormal pollen maturation; inability to germinate on compatible stigmata.

e. Non dehiscent anthers but viable pollen,- sporophytic control.

f. Barriers other than incompatibility preventing pollen from reaching ovule.

3. Kinds of Male Sterility

Genic male sterility:

It is also known as nuclear male sterility as controlled by nuclear genes whose action and expression are not influenced by the plasmon or cytoplasmic genes. Therefore, inheritance pattern and expression exhibit no reciprocal differences, low environmental impact, or nominal genomic influence. Hence, sterility is stable, reliable, and replicable. Predominantly, single recessive Mendelian genes control this type of sterility, through two to three recessive genes or control by one to two dominant genes (Kaul, 1986). Most of the naturally occurring or induced male sterile mutants are recessive in nature with few exceptions in cole vegetables (e.g. cabbage and broccoli) and genetically transformed male sterile lines. Certain mutants, which although produce functional pollen, pollen fail to self fertilize, either due to non-dehiscence of pollen or their special flower morphology e.g. positional sterility in tomato (Atanassova, 1999) and functional male sterility in eggplant (Phatak and Jaworski, l989). The occurrence of predominantly recessive male sterility clearly indicates that gms is the result of mutation in any gene(s) controlling microsporogenesis (pollen development), stamen development or microgatnetogenesis (male gamete development process).

3. Environmental sensitive genic male sterile (EGMS) Line

Certain GMS lines are conditional mutants, meaning thereby in a particular environment male sterile mutant plants turn into male fertile. Alter determination of critical environment (usually temperature or photoperiod) for sterility and fertility expression, such GMS mutants are classified under environmental sensitive genic male sterile (EGMS) lines. In vegetable crops, mostly temperature sensitive genic male sterile lines have been reported in cabbage, brussel sprout, broccoli and tomato (Kumar *et al.* 2000). From practical application view point, it is necessary to identify critical temperature or photoperiod for the fertility/ sterility expression in temperature and photoperiod sensitive genetic male sterility, respectively (Table-1).

Table 1. Environmental sensitive male sterility mutants

Vegetable	Mutant	Temperature
Cabbage	TGMS & PGMS	<10° C
Brussel sprout	TGMS	<10^0 C
Broccoli	TGMS	10°-11° C
Pepper	TGMS & TCMS	<25° /17°C
Carrot	TGMS	
Tomato	TGMS TCMS	< 30° C <18° C
Chive	TCMS	<24° C
Chinese cabbage	TCMS	Low temperature
Onion	TCMS	14-22° C

TGMS-Thermo sensitive genic male sterility, TCMS-Thermo sensitive cytoplasmic male sterility, PGMS- Photo sensitive genic male sterility

Like the spontaneously arisen genic male sterility mutations, majority of the induced male sterile described by Kumar et al., 2000 (Table 2). Although it appears possible to induce male sterility in any vegetable crop, being easier in diploids than in polyploids because of the genome multiplication and high gene interactions, induction following mutagenesis has been very limited and difficult, the main reason being the low frequency of male sterile, absence of marker genes, gradual diminution, and ultimate elimination of male sterile due to self-sterility.

Table 2. Cytoplasmic- nuclear male sterility in selected vegetable species

Vegetable spp.	Salient features of CMS
Tomato *(Lycopersicon esculentum)*	Sterile cytoplasm has been derived from the distinct species through protoplast fusion; restorer gene (Rf) is not available.
Pepper (*Capsicum spp.*)*	First reported in Indian accession; most of the cms lines are temperature sensitive; occurrence of Rf allele is common in small fruited (usually hot pepper) and rf in large fruited (usually sweet pepper) lines; RAPD markers linked to Rf gene have been identified.
Cole crops Cauliflower (*B. oleracea var. botrytis)**	In Cole crops, sterile cytoplasm derived from *B. nigra* and *Raphanus sativa*, Ogura type (Ogura, 1968); problem of seedling yellowing (at low temperature) associated with Ogura based cms lines of broccoli, cauliflower, cabbage, brussel sprout has been solved using protoplast fusion. Seed companies in France are utilizing Cybrids cms lines of cabbage and cauliflower; protoplast fusion has been utilized to transfer Ogura cytoplasm from broccoli from cabbage.
Cabbage (*B. O. var. capitata)**	
Brussel sprout (*B. o var. gammifera)*	
Brussel sprout (*var. gemmifera)**	
Onion (*Allium cepa)**	Two types of sterile cytoplasms viz; S and T have been reported: S- cytoplasm is most widely exploited.
Carrot (*Daucus carota)**	Two types (petaloid and brown anther) of male sterile lines are available; genetics of fertility restoration is complex because of structural variants of m DNA are numerous.
Radish (*Raphanus sativa)**	Sterile cytoplasm widely distributed in wild radish; occurrence of Rf allele is frequent in European and Chinese cultivars and rf in Japanese cultivars

* exploited at commercial scale

A perusal of Table -3 reveals that both physical and chemical mutagens, singly or in combination, induce male sterility. Sterility, irrespective of the ploidy level, in almost all these is conditioned by single recessive ms genes. Although y-rays and EMS treatments have induced male sterility in maximum species, this does not reflect the effectiveness, efficacy, or efficiency of these two mutagens, but indicates their wide usage. In fact, the use of other mutagens for inducing male sterility has been limited and many mutagens have

been tested neither on the same species nor on any single species. It is desirable to determine the male sterility induction potential of different doses of various potentials/chemical mutagens.

Table-3 Nuclear male sterility in selected vegetable species Source Kumar (2002) * exploited at commercial scale

Vegetable spp.	Salient features of gms
Tomato *(Lycopersicon esculentum)**	More than 55 recessive genes have been reported sl-2, ms-13 and ms-15 are temperature sensitive; ps-2 gene has been exploited at commercial scale: YAC clone containing ms- 14 gene has been cloned
Eggplant (*Solanum melongena*)	Monogenic recessive gene has been reported: monogenic recessive functional sterility available.
Pepper (*Capsicum spp.*)*	More than 2 recessive genes have been reported: MS-12 (ms-509 ms-10) Ms-3 genes are commercially utilized in India and Hungary respectively. The ms-10 gene is linked with taller plant height, erect growth and dark purple anther.
	Both recessive and dominant genes have been reported.
Cole crops Cauliflower (*B. O var. botrytis)*	Both recessive and dominant genes have been reported: RAPD marker linked to dominant gene has been identified and its use in hybrid seed production has been proposed.
Cabbage (*B. O. var. capitata)*	Recessive male sterile mutant has been reported.
Brussel sprout (*B. o var. gammifera)*	Six recessive non-allelic genes been reported: linkage of ms gene with bright green hypocotyls.
Broccoli (*var. italica)*	Recessive mutant been reported: linkage of ms gene with delayed green (dg) seedling marker gene.
Watermelon (*Citrullus lanatus*)	Five recessive non-allelic gene been reported: ms-1 is commercially utilized in India.
Muskmelon (*Cucumis melo*)*	Monogenic recessive gene has been reported: limited scope of utilization because of availability of gynoccious lines
Cucumber (*Cucumis sativa*)	Monogenic recessive gene has been reported: very limited scope of utilization because of availability of sex regulating mechanism using certain chemicals.
Summer squash (*Cucurbita pepo*)	Monogenic recessive gene has been reported Recessive male sterile mutant has been reported: not utilized at commercial scale because of the availability of cms lines as an alternative method.
Onion (*Allium cepa*)	Three recessive mutants been reported: commercially not utilized.
Carrot (*Daucus carota*)	
Radish (*Raphanus sativa*)	

Utilization of GMS

Since GMS is maintained through backcrossing in hybrid seed production field. 50% male fertile segregants (MSMS) need to be identified and removed before they shed pollen. In some gms lines, ms genes are tightly linked with the recessive phenotypic marker genes. Such marker genes, especially which expresses at seedling stage are good proposition for the identification of sterile/fertile plants at seedling stage. Hybrid seed production using EGMS line is more attractive because of the ease in seed multiplication of male sterile line. Seeds of EGMS line can be multiplied in an environment where it expresses male fertility trait while hybrid seeds can be produced in other environment, where it expresses male sterility. Because of more tedious maintenance process and non-availability of suitable marker gene among the vegetable crops. Utilization of GMS is restricted only in few vegetables (Table-3). The identification of fertilizing cytoplasm for specific nuclear male sterile gene , is an interesting research area, which upon success, may provide opportunity for most efficient utilization of gms lines, like cms line.

Cytoplasmic male sterility

Cytoplasmic male sterility is controlled solely by the specific cytoplasm genes (S-genes) whose action is not influenced by nuclear or other genes. Therefore, as long as the

cytoplasm of an individual has these specific genes, it is male sterile cytoplasm (S). In the absence of these genes, the cytoplasm is normal (N) or fertile (F). Therefore, irrespective of the nuclear genes, plants having the S-cytoplasm are male sterile. True cytoplasmic male sterile ideally should remain uninfluenced by nuclear genes; but such stable and true cytoplasmic male sterile are unknown as fertility restorer genes, and maintainer nuclear genes have been detected in all plants labeled CMS (Kaul, 1986).

Cytoplasmic Sterility

Compared to the large number of plants in which genic male sterility has been induced by mutagenic treatments, the number of plants in which genic cytoplasmic male sterility has been induced is exceedingly low. This is because, whereas predominantly the mutation of a single specific nuclear gene leads to genic male sterility, for gene-cytoplasmic male sterility the induction of two specific and either simultaneous or successive gene mutations, one at the nuclear and the other at the cytoplasmic level, are required. The probability of the occurrence of two such simultaneous mutations in specific genes is exceedingly low. In the species where gene-cytoplasmic male sterility has been induced , the nuclear fr genes were already present in the N-cytoplasm having the C gene. Therefore, in all cases, a cytoplasmic gene mutation has led to male sterility. Among all the chemical mutagens used, ethidium bromide has been successful in inducing the cytoplasmic mutation.

Utilization of CMS

The CMS system is the most commonly utilized male sterility to produce commercial hybrid seeds of several vegetables (Table-3) . The cms based hybrid development is often term as three lines method of hybrid breeding involving A line (male sterile S-rfrf), B line (maintainer: N-rfrf) and C or R line (restorer: S. or N-RfRf). As mentioned. cms line without restorer male parent can not be utilized in fruit producing vegetables (e.g. chilli), but it can be utilized in vegetables where vegetative part is of economic value (e.g. onion. Cole crops, radish. Leafy vegetables etc.). Besides several factor vulnerability of sterile cytoplasm to specific diseases is a major risk).

Mechanisms of male sterility (GMS and CMS)

In both GMS and CMS systems, male sterility is the consequence of breakdown of tightly regulated pollen development and fertilization processes at any of the pre- or pro-meiotic stages i.e. during meiotic process during the formation of tetrad. During the release of tetrad, at the vacuolate microspore stage at pollen dehiscence stage. Expression of male sterility trait is associated with a large number of morphological, physiological histological, biochemical and molecular changes of microsporogenesis and microgametogenesis.

Role of Tapetum

Tapetum cells are innermost layer of the anther wall that surrounds anther locule possessing sporogenous cells (developing pollen). These cells are involved in the transmission of nutrients/energy to sporogenous cells and associated with synthesis of callase enzyme. The most striking histological feature associated with the majority of male sterile plants (GMS and CMS) is persistence or premature breakdown of tapetum. Aberration in tapetum development leads to the failure of tapetum function, consequently failure of pollen development and, therefore expression of male sterility. Ever since the first report on abnormal developments of tapetum in male sterile plant. There is growing experimental evidence in favours of strong association between abnormal tapetum behavior and nuclear or Cytoplasmic male sterile plants. It has been analyzed constituents of C^{14} material, in male sterile microspores (pollen) and observed very less C^{14} material, although there was normal supply of this material in the tapetum. Therefore, they speculated disruption in the transfer of C^{14} material from tapetum to the developing microspores. Behavior of some

genetically transformed male sterile plants supports failure of tapetum development and its function leading to male sterility. In fact, knowledge on importance of tapetum has actually been utilized to develop gene construct that selectively destroys the tapetum and thereby induces male sterility in transformed plants. Several other experimental evidences see are also discussed under specific vegetables at appropriate places. Albeit alterations its tapetum form and function are considered as the primary causes of male sterility, there are male sterile lines in which both tapetum form and function are normal (Kaul, 1989).

Role of Callase

Callase is an enzyme required for breakdown of the callose that surrounds the PMCs. Thus helps in release of microspores (pollen) from tetrad after meiosis. Early or delayed callase activities have been found to be associated with male sterility. Mistiming of callase activity led to the pre mature or delay release of meiocytes and microspores, resulting in male sterility.

Role of Esterase

Esterase enzymes are believed to play role in the hydrolyses of sporopollenin, the polymer required for pollen formation. Decreased activity of esterase in male sterile plants has been observed in petunia. Hence it has been proposed that decreased activity of esterase has adverse effect on pollen development (Sawhney, 1997)

Role of Plant Growth Regulators (PGRs)

Endogenous PGRs play very important role in stamen and pollen development. Male sterility has been reported to be associated with change in a number of PGRs rather than any specific substance and perhaps it is the altered balance of PGRs that effects the pollen development process. Reduced level of Abscissic acid associate with seeds of GMS and CMS plants indicates that both kind of male sterility system probably involve some common pathways. On one hand exogenous supply of reduced substances in several male sterile has been found to restore fertility(tomato), on the other , in several cases expected fertility restoration could not be obtained (Sawhney, 1997)

Approaches for Development of Male Sterility

The specific mechanisms causing male sterility in plants vary from species to species and are subject to influence by environment, and nuclear and cytoplasmic genes. Male sterility may be permanent (heritable) or transient (CHAs). It can be induced by anther culture (Kaul, 1986), somatic cell culture (Mariana *et al.* 1990), and somatic hybridization. However, the most significant development is the possibility of engineering male sterility by inserting cloned gene sequences which can disrupt any or more than one step during microsporogenesis.

Commercial exploitation of hybrid vigour in hermaphrodite crops is facilitated by the availability of CMS lines to affect pollination control. As the CMS genotype results from alloplasmic substitutions or by harnessing the mitochondrial mutations, they are generally associated with yield penalty and imperfect fertility restoration in the hybrids. These imperfections in CMS-fertility restorer systems have stimulated considerable research effort to engineer new and perhaps more efficient male sterility systems (Kaul 1998; Perez-Prat and van Lookeren Campagne, 2002). Most of these efforts take advantage of the fact that microsporogenesis is a complex developmental process sensitive to mutations. Disruption of any of the steps of microsporogenesis or its premature termination results in male sterility. Male sterile plants have been produced by aberrant spatial or temporal expression of degrading enzymes or through inhibition of specific enzymes via antisense strategy. In the present write up an attempt has been made to review current realities and emerging possibilities in the arena of genetically engineered male sterility in crop plants.

A. Cell Cytotoxicity

1. Dominant Nuclear Male Sterility (Barnase-Barstar System)

Intensive studies revealed that the TA29 gene 5 region programmed the expression of the RNase] and barnase gene specifically to anther tapetal cells, which caused selective ablation of the tapetal cell layer that surrounds the pollen sac, presumably by hydrolyzing the tapetal cells. This disrupted the normal pollen formation and caused male sterility. Of these genes, barnase seemed more effective than RNase-T1 as only one copy of TA29-barnase was required to produce male sterile plants, where, in contrast, at least four copies of the TA29-RNaseTl gene were required for the same level of male sterility expression. Male sterile flowers showed noticeable reduction in length of stamen filament, petal size, reduction in bud diameter, ad tapetal cell content. Sterile anthers contained empty exiles.

To restore fertility, the barnase-specific RNase inhibitor, barstar, was used (Mariana *et al*. 1992). Upon crossing the genetically engineered male sterile plants with transgenic fertile plants carrying TA29-barstar gene, the F1 progeny showed co expression of both genes in the anthers of the male fertile plant. It was found that barstar gene is dominant to the barnase gene, and fertility restoration was due to the formation of tapetal cell-specific barnase and barstar complexes. Female fertility was not affected, and transformed plants had normal morphology. By coupling the TA29-RNase gene to a dominant herbicide resistance gene, uniform populations of male sterile plants could be produced. For this, the two genes coding for ribonucleases RNase and barnase were linked independently to the "bar" gene, which confers resistance to the herbicide bialophos (phosphinothricine or PPT), to obtain a marker for male sterility (Denis et al. 1993). The barnase-barstar male sterility-fertility restoration system is available in cauliflower and tomato (Banga and Raman 1998). Reversion to fertility from male sterile plants has been observed in some cases. It was found that RNase-T1-mediated male sterility is unstable at temperatures higher than 25°C. Jagannath et al. (2001) could overcome these problems by use of spacer DNA between barnase gene (driven by a tapetum specific promoter) and the CaMV 35S promoter-driven bar gene. This helped in insulating the tissue specific expression of the barnase gene over all developmental stages of transgenic plant. These newly developed male sterile lines, however, could not be restored by transgenic carrying wild type barstar. For that plants carrying modified barstar constructs were developed (Jagannath et al., 2002).

2. Male Sterility through Hormone Engineering

Drastic changes in endogenous levels of auxins have been demonstrated to cause male sterility in tomato (Sawhney 1997) and several other crops. Induction of male sterility by manipulating endogenous hormone levels (Spena et al. 1987). Such male sterile can be maintained by linking the gene for herbicide resistance to the male sterilizing "rol c". Male fertile segregants can be chemically rouged out by selective elimination in maintainer population.

3. Pollen Self-Destructive Engineered Male Sterility

Theoretically, it is feasible to genetically engineer plants having altered endogenous auxins indole acetic acid (IAA) levels with pollen exhibiting self-destructive mechanisms. To achieve this, McCormick *et al.* (1989) and Wood (1990) transformed plants with a chimeric gene consisting of pollen-specific promoter (LAT59) and a gene (fins2) that converts indole acetamide (IAM) into IAA. Although the detailed characterization of such a transgenic was not reported, it was argued that, if this system works, plants carrying the LAT59-fins2 gene when sprayed with IAM will selectively convert IAM into IAA at very high concentrations to kill the pollen and render the plants male sterile. Although this technology will avoid the problems of elimination of maintenance of male sterile and the restoration of fertility in F1 hybrids, the complications such as are associated with the use of chemical hybridizing agents would be there, except the

necessity of spraying gametocides/CHAs repeatedly at a specific growth stage and interval. Another possibility of inducing such male sterility is the transformation of plants with chimeric gene involving TA-29 promoter and coding region of β-glucuronidase (GUS). Such transformants, when sprayed with protoxins like sulfonyl urea or maleic hydrazide, cause male sterility through their breakdown in the tapetum by the β-glucuronidase enzyme. The transgenic plants not sprayed with protoxins remain fertile, so a fertility restoration system like TA29-barstar is not required. Shihshieh *et al.* (2003) used tissue-specific promoters expressing *CKX1* and *gai*, genes involved in oxidative cytokinin degradation and gibberellins (GA) signal transduction, respectively, to study the roles of cytokinin and GA in male organ development.

4. Male Sterility Using Patho genesis-Related Protein Genes

Pathogenesis-related (PR) protein β-1,3 glucanase (callase) is known to dissolve specific cell wall made of callase, a β-1,3-linked glucan between cellulose cell wall and plasma membrane and tetrads synthesized by microsporocyte. The β-1, 3 glucanase (callase) secreted by the tapetum helps to release free microspores into locular space by breaking down the callase wall. The genetic alteration of this mechanism in plants caused male sterility. Callase appearance and distribution was normal in male sterile transgenic plants up to prophase I, whereupon callase was prematurely degraded. Electron microscopy revealed a thick callase wall surrounding each microspore of the tetrad in fertile anthers, whereas it was clearly absent in sterile microspores. The premature dissolution of callase indicated that the modified glucanase is secreted from the tapetum and is active within the anther locule. Transgenic tobacco plants expressing the β-1, 3 glucanase under the control of CaMV 35S promoter showed normal fertility despite the expression of modified glucanase. This was attributed to either lower expression of the modified gene in the tapetum or inappropriate timing of the modified callase synthesis during the process of microsporogenesis. Stronger (Rf-WA-1) being located on chromosome 10. It has also been possible to identify 6 RAPD markers linked to another gene for fertility restoration (Rf-WA-3). Three of these mapped on chromosome1. These marker-aided selections for fertility-restoring ability.

B. Male Sterility through Modification of Biochemical Pathways

1. Flavonoids

Among the three major flower pigments, (flavonoids, carotenoids, and betalains), the flavonoids are the most common and most important. Apart from their role in color development, flavonoids are important in plant reproduction and defence-related mechanisms. In legumes, they may act as signal molecules in the interaction with nitrogen-fixing bacteria. The biochemical pathways of flavonoids synthesis have shown that these are produced as phenyl-propanoid based secondary metabolites via chalcone synthase (Chs). A large number of genes involved in the biochemical pathway of flavonoids synthesis have been cloned and characterized (Forkmann 1991). However, it was not concluded whether the anther box on its own directs the expression in the tapetum cells. It was argued that modules that confer organ specificity to the Chs or CaMV 35S promoter may act in concert with anther box. About 14% of transgenic plants showed a clear reduction of anther and pollen pigmentation, which confirmed the involvement of anther box in tapetum-specific expression. The copy number and orientation of the inserted anther box did not affect the antisense phenotype in a quantitative or a qualitative way.

2. Jasmonic acid

Jasmonic acid (JA), synthesized from linolenic acid (LA) through octadecanoid pathway (Weiler 1997), plays an important role in anther dehiscence and pollen maturation (Sanders et al. 2000; Stintzi and Browse 2000; Ishiguru et al. 2001). The triple *fad (fad3,fad7,fad8)* mutants lacking LA had

indehiscent anthers and hence showed functional male sterility (McConn and Browse 1996) Recently, Ishiguru et al. (2001) linked impaired dehiscence in Defective Anther Dehiscence I (DAD I) mutant (Sanders et al. 2000; Stintzi and Browse 1996) to mutation in DAD I gene that encoded a chloroplastic phospholipase AI to supply free LA at the initial step in JA biosynthesis, they further suggested that JA also regulated flower opening, anther dehiscence as well as pollen maturation. Exogenous application of JA reversed the impaired dehiscence. Of the 25 transgenic plants obtained, 3 showed delayed anther dehiscence and inviable pollen. Segregation of the transgenes in the selfed progeny was consistent with the Mendelian inheritance of two copies of the transgenes at different loci. The male sterile phenotype could be reversed by application of JA (0.5 µm) as well as LA:

Genetic male sterility based on Br DAD I gene has many advantages; most important being the ability to induce normal pollen production and dehiscence. This is critical for developing lines homozygous for the male sterilizing allele. Moreover, this system involves suppression of a single gene and floral morphology is not adversely affected. The stability of expression over a range of environmental conditions, however, remains to be investigated.

3. Carbohydrates

Carbohydrates play a critical role in the anther and pollen development by sustaining growth as well as signal pathways. Their transportation from photo synthetically active source tissues to developing sinks is regulated by extra cellular invertase. This class of invertase is encoded by small gene familiar with differential regulation and expression patterns. The extra cellular invertase *Nin* 88 of tobacco shows specific temporal and spatial expression patterns in developing anthers. At early stages of flower development, the invertase is present exclusively in the tapetum cell layers of the anthers followed by a distinct expression pattern during pollen development. The tissue specific antisense repression of *Nin* 88 under the control of corresponding promoter in plant caused male sterility by blocking pollen development during early stages of microsporogenesis (Goetz et al. 2001). Exogenous supply of carbohydrates in an in vitro maturation assay was able to partially overcome this block, thus opening up the possibility of maintaining this male sterility system. Fertility restoration essential for its use in hybrid seed production, can theoretically be achieved by crossing this GMS system with transgenic plants expressing distantly related invertase (may be from bacteria), which is not under the control of the antisense repression through a plant invertase. Alternatively introduction of a sucrose transporter could bypass the requirement for extra cellular sucrose cleavage. Male sterility caused by antisense expression of *Nin* 88 was not associated with any morphophysiological abnormality.

Transgenic induction of mitochondrial rearrangements for Cytoplasmic male sterility in crop plants.

Recently Ajay *et al*. (2007) found that stability of the mitochondrial genome is controlled by nuclear loci. In plants, nuclear genes suppress mitochondrial DNA rearrangements during development. One nuclear gene involved in this process is *Msh1*. *Msh1* appears to be involved in the suppression of illegitimate recombination in plant mitochondria. To test the hypothesis that *Msh1* disruption leads to the type of mitochondrial DNA rearrangements associated with naturally occurring cytoplasmic male sterility in plants, **a** transgenic approach for RNA was used to modulate expression of *Msh1* in tobacco and tomato. In both species, these experiments resulted in reproducible mitochondrial DNA rearrangements and a condition of male (pollen) sterility. The male sterility was, in each case, heritable, associated with normal female fertility, and apparently maternal in its inheritance. Segregation of the transgene did not reverse the male sterile phenotype, producing stable, non transgenic

male sterility. The reproducible transgenic induction of mitochondrial rearrangements in plants is unprecedented, providing a means to develop novel cytoplasmic male sterile lines for release as non-GMO or transgenic materials.

E. Engineering Cytoplasmic Male Sterility via the Chloroplast Genome

Naturally-occurring Cytoplasmic male sterility (CMS) has been known for over 100 years. CMS systems are used to produce commercial F1 hybrid lines. Ruiz and Daniel (2005) recently reported the first engineered cytoplasmic male sterility system in plants. They studied the effect of light regulation of the *phaA* gene coding for β-Ketothiolase engineered via the chloroplast genome. The *phaA* gene was efficiently expressed in all tissue types examined, including leaves, flowers, and anthers. The transgenic lines were normal except for the male sterile phenotype, lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Transgenic lines showed an accelerated pattern of anther development, affecting their maturation, and resulted in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium, and vacuolation decreased the inner space of the locule, affected pollen grain, and resulted in the irregular shape or collapsed phenotype. Reversibility of the male sterile phenotype was observed under continuous illumination, resulting in viable Pollen and a copious amount of seeds. This study offers a new tool for transgene containment for both nuclear and organelle genomes and provides an expedient mechanism for F1 hybrid seed production.

Advantages of the chloroplast transformation system

A chloroplast genetic engineering approach offers a number of attractive advantages, including high-level transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance, lack of gene silencing, position effect due to site-specific transgene integration, and lack of pleiotropic effects due to sub-cellular compartmentalization of transgene products. Genetically engineered cytoplasmic male sterility via the chloroplast genome may be used for the safe integration of foreign genes via the nuclear genome and in those rare cases in which plastid genomes are paternally or biparentally transmitted. Recently, plastid transformation was demonstrated in carrot with their ability to grow in very high saline conditions (up to 400 mμ sodium chloride). Additionally, plastid transformation of recalcitrant crops such as cotton and soybean allows the application of the cytoplasmic male sterile system to commercially important crops.

Havey (2004) documents the worldwide use of CMS to produce competitive hybrid cultivars. Major investments of time and resources are required to backcross a male-sterility-inducing cytoplasm into elite lines. These generations of backcrossing could be avoided by transformation of an organelle genome of the elite male-fertile inbred line to produce female inbred lines for hybrid seed production. Because the male-fertile parental and male-sterile transformed lines would be developed from the same inbred line, they should be highly uniform and possess the same nuclear genotype, excluding mutations and residual heterozygosis. Therefore, the male-fertile parental line becomes the maintainer line to seed-propagate the newly transformed male-sterile line. A few generations of seed increases would produce a CMS-maintainer pair for hybrid seed production. An additional advantage of organelle transformation would be the diversification of CMS sources used in commercial hybrid-seed production. Transformation of the chloroplast genome would allow breeders to introduce different male-sterility-inducing factors into superior inbred lines. Introduction of a male-sterility inducing transgenes into one of the organelle genomes of a higher plant would be a major breakthrough in the production of male-sterile inbred lines. This technique would be of great potential importance in the production of hybrid crops by avoiding generations of

backcrossing, an approach especially advantageous for crop plants with longer generation times. Moreover, transgenes that are engineered into annual crops could be introgressed into wild crops, persist in the environment, and thereby have negative ecological consequences. Therefore, it may be necessary to engineer a male sterility system that is 100% effective. For vegetable, fruit, or forage crops, restoration of male fertility in the hybrid is not necessary. This simplifies the production of hybrids because effort can be concentrated on maintainer line development, without concern over whether the pollinator restores male fertility in the hybrid. For crops with seeds as the economically important product, such as canola, sunflower, or maize, one or both of the hybrid's parents must bring in male-fertility restoration factors or the male-sterile hybrid seed must be blended with male-fertile hybrid seed. In currently available cytoplasmic male sterile lines, the nuclear genome controls various restoration factors and such factors are often located at multiple loci and are poorly understood. However, the authors report restoration of male fertility by changing conditions of illumination. Therefore, this is a novel approach for creating male sterile transgenic plants, which may help advance the field of plant biotechnology through effective transgenes containment.

Conclusions

Genetically engineered male sterility provides tremendous opportunities to the breeders for enforcing pollination control in hybrid seed production systems. On the other hand these systems have some disadvantages like availability of efficient gene construct, possible dispersion of transgene to other related species, availability of efficient transformation technique and very high initial investment. Much will, however, depend upon large scale availability of the desired constructs/genes as at present most of the genes are patented and not available to large number of breeders. Apart from barnase-barstar system, no other system has reached the commercial stage. Further researches and their subsequent commercialization are expected.

References

Ajay Pal S. Sandhu, Ricardo V. Abdelnoor and Sally A. Mackenzie. 2007 Biological Sciences/Agricultural Sciences. 104: 1766-1770

Atanassova, B. 1999. Euphytica. 107:13-21.

Banga, S. S and Raman, R 1998. Hybrid Cultivar Development. Co Pub. NPH, New Delhi and Springer Verlag, Germany. pp 185-191.

Forkmann, G. 1991. Plant Breed. 106 : 2 1-26.

Goetz, M., Godt, D.E., Guivarih, A., Kahmann, U., Chriqui, D. and Roitsch, T. 200l. Proceedings National Academy of Sciences 98(11): 6522-6527.

Havey MJ (2004) *Molecular Biology and Biotechnology of Plant Organelles*, Springer Publisher: The Netherlands, pp 617-628

Ishiguro, S., Kawai-Ode, A., Ueda, 3. Nishida, I. and Okada, K. 2001. Plant Cell 13: 2191-2209.

Jagannath, A., Bandyopadhyay, P., Arnmugam, N., Gupta, V., Burma, P.K. and Pental, D. 2001. Breed. 8: 11-23.

Jagannath, A., Arnmugam, N., Gupta, V., Pradhan, A., Burma, P.K. and Pental, D. 2002. Current Sci. 82 : 46-52.

Kaul, M.L.H. 1998. Hybrid Cultivar Development. Co-Pub. NPH, New Delhi and Springer- Verlag, Germany, pp 17-45.

Kumar, S., Kumar, Banerjee MK and Kalloo, G. 2000. Vegetable Sciences. 27: 1-24.

Kumar, S. 2002. Winter School on Recent Advances in Vegetable Production Technology. 3-23 December, 2002 at IIVR, Varanasi. pp 59- 69.

McComm, M. and Browse, J. 1996. Plant Cell 8: 403- 416.

Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J. and Goldberg, R.B. 1990. Nature 347: 737-741.

Mariani, C., Gossele, V., De Beuckeleer, M., De Block, M., Goldberg, R.13, Degreef, W. and Leemans, J. 1992. Nature 357: 384-387.

Mc Cormick, S., Twell, D., Wing, R., Ursine, V., Yamoguchi, 3. And Zarabell, S. 1989. Am. Soc. Plant. Physiology. Symp. Ser. 1: 128-135.

Perez-Prat, E. and van Lookeren Campagne, M.M. 2002. Trends in Plant Sciences 7: 199-203.

Phatak, SC and Jaworski CA. 1989. Hort. Science. 24: 1050

Ruiz, O and Daniell, H (2005) *Plant Physiology* 138, 1232-1246

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P. and Weiler, E.W. et al. 2000. Plant Cell 12: 1041-1061.

Sawhnëy, V.K. 1997. J. Exp. Bot. 25: 1004-1009.

Shihshieh Huang, R. Eric Cerny, Youlin Qi, Deepti Bhat, Carrie M. Aydt, Doris D.Hanson, Kathleen P.Malloy, and Linda A. Ness. *2003.* Plant Physiology. 131: 1-13.

Stintzi, A. and Browse, J. 2000. Proceeding of National Academy of Sciences USA 97: 10625-10630.

Spena, A., Schmulling, T., Konez, C. and Shell, J. 1987. EMBO. J. 6: 3891-3899.

Weiler, E.W. 1997. Nature. 84: 340-349.

Wood, M. 1990. Agric. Res 38: 22-23.