Chapter 21
Male Sterility

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21.1 Introduction

The control of pollen fertility is central to the production of F1-hybrid seed in self-pollinating crops, and is potentially applicable to the containment of transgenes deployed in crop plants. Pollen sterility can be achieved through cytoplasmic male sterility (CMS) encoded by the plant mitochondrial genome, or through genic male sterility encoded by the nuclear genome. Both routes have been exploited in schemes for hybrid seed production. Recently, pollen sterility has been achieved through novel strategies involving nuclear or plastid transgenes. Here we review the applications of pollen sterility, and the genetic systems used for the control of pollen fertility.

21.2 Applications of Pollen Sterility

While F1-hybrid seed production has been the primary driving force behind strategies to achieve pollen sterility, the control of pollen function also plays a role in other valued phenotypes. Furthermore, the expanding plantings of transgenic crops have created interest in the use of pollen sterility to limit the flow of transgenes to nearby native plants and non-transgenic crops.

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21.2.1 Hybrid Seed Production

Hybrid vigor (or heterosis), uniformity, broad adaptation, and variety protection are desired features of crop plants that can be achieved through the use of F1-hybrid varieties (Duvick 1959, 2001; Perez-Prat and van Lookeren Campagne 2002; Virmani et al. 2003; Troyer 2006; Springer and Stupar 2007; Cheng et al. 2007). In the case of self-pollinating crops, uniform populations of pollen-sterile plants must be created, to then be cross pollinated for the production of F1-hybrid seed.

21.2.1.1 Hybrid Seeds via Cytoplasmic Male Sterility

In hybrid seed production, pollen sterility is often achieved through the use of CMS (Duvick 1959; Budar and Pelletier 2001; Havey 2004; Pelletier and Budar 2007; Cheng et al. 2007). Maternally inherited CMS results from genes in the plant mitochondrial genome (reviewed in Budar and Pelletier 2001; Hanson and Bentolila 2004; Chase 2007). These genes do not segregate, but expression of the CMS trait can be conveniently controlled by the presence or absence of nuclear restorer-of-fertility (restorer) alleles (reviewed in Schnable and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007; Pelletier and Budar 2007). Uniform populations of pollen-sterile plants are developed by crossing CMS plants with normal-cytoplasm, pollen-fertile plants that do not carry nuclear restorer alleles. The resulting pollen-sterile progeny produce F1-hybrid seed via cross fertilization with pollen-fertile plants grown nearby. Pollen fertility may or may not be restored in the F1 generation. If the F1 seed is grown to produce vegetative structures such as roots, tubers, or leaves, pollen fertility need not be restored in the F1 generation (Duvick 1959; Havey 2004). In the case of hybrid crops grown for a seed product, pollen fertility is achieved in the F1 generation via nuclear restorer alleles introduced in the previous generation (Duvick 1959; Havey 2004). Alternatively, pollen-sterile F1 hybrids can be grown along with male-fertile pollinators. In crops such as maize, F1-hybrid seed can be produced through the use of CMS or through hand emasculation (Duvick 1959). CMS (pollen-sterile) and normal-cytoplasm (pollen-fertile) versions of the same hybrid can be produced and blended for planting (Stamp et al. 2000; Weingartner et al. 2002a, b; Havey 2004).

In some cases, CMS confers a yield advantage independent of heterosis. Pollen-sterile CMS maize hybrids, fertilized with isonuclear pollen-fertile hybrids, yield more grain than do isonuclear pollen-fertile hybrids (Stamp et al. 2000). The Plus-Hybrid system combines CMS and a xenia effect to create a further yield advantage when pollen-sterile CMS hybrids are crossed with unrelated pollinators (Weingartner et al. 2002a, b). CMS lines of wheat out-yield isonuclear lines produced through use of chemical sterilizing agents (Adugna et al. 2004). Work in sorghum (Moran and Rooney 2003) and millet (Chandra-Shekara et al. 2007)

demonstrates genetic variation of male sterility-inducing cytoplasms with respect to yield.

CMS is used for hybrid seed production in many crops, including alfalfa, onion, sugar and table beets, oilseed and vegetable brassicas, maize, millet, rice, pepper, rye, sorghum, sunflower, and wheat (Duvick 1959; Havey 2004; Adugna et al. 2004; Cheng et al. 2007). T-cytoplasm maize carries an unusual CMS gene that also conditions susceptibility to the fungal pathogen *Bipolaris maydis* (reviewed in Pring and Lonsdale 1989; Wise et al. 1999). Although this caused concerns regarding the use of CMS for crop production, CMS genes are diverse and work by different mechanisms (Schnable and Wise 1998; Hanson and Bentolila 2004; Linke and Borner 2005; Chase 2007; Carlsson et al. 2008). Hence, undesirable phenotypes are not necessarily expected to result from every application of CMS in hybrid crop production.

21.2.1.2 Hybrid Seeds via Nuclear Male Sterility

In contrast to CMS, pollen sterility conditioned by dominant or recessive nuclear mutations is not true-breeding. The male-sterile plants must be propagated through pollination with wild-type, male-fertile plants. In some cases, this problem can be circumvented. Genic male-sterile leek plants are propagated asexually, and then cross pollinated to produce hybrid seed (Smith and Crowther 1995; Havey 2004). In China, considerable hybrid rice seed is developed through environmental genic male sterility systems. In photo-thermo genic male sterility, the seed parent line is amplified under environmental conditions that cause this line to be pollen-fertile (cool temperatures and shorter photoperiod). For the production of F1-hybrid seed, the seed parent is grown and cross-fertilized under environmental conditions that cause pollen sterility (warmer temperatures and longer day-length; Virmani et al. 2003; Cheng et al. 2007). Female parent propagation has been described as the key limiting factor for the production of hybrids in many crops (Perez-Prat and van Lookeren Campagne 2002). Asexual propagation is labor-intensive, and environmental factors are not always uniform enough to ensure complete pollen sterility. Novel strategies to address these challenges via the deployment of nuclear transgenes are described below.

21.2.2 Value-Added Traits

The control of pollen fertility has implications for plant improvement beyond hybrid seed production. Pollen sterility, combined with parthenocarpy, results in the production of desirable seedless fruits (Possingham 1998; Schijlen et al. 2007). Pollen is an allergen of significant importance (Goldberg et al. 1998; Mahillon et al. 2006), and pollination induces the rapid senescence of floral organs in many species

(Rubinstein 2000; Rogers 2006). Hence, ornamental species might be improved through control of pollen fertility (Daniell 2002).

21.2.3 Transgene Containment

The expansion of transgenic crops has raised concerns about the flow of transgenes to nearby cross-compatible crops and native plants (Daniell 2002; Chapman and Burke 2006; Lee and Natesan 2006; Brunner et al. 2007). Many factors influence transgene flow, including dispersal via seeds, pollen, or vegetative propagules (Brunner et al. 2007), and the fitness of the plants resulting from genetic exchange with the transgenic crop (Chapman and Burke 2006; Lee and Natesan 2006). No single strategy of containment will be 100% effective in any situation, and the best combined approaches must be determined on a case-by-case basis (Chapman and Burke 2006; Lee and Natesan 2006). Pollen sterility can make a significant contribution toward transgene containment (Brunner et al. 2007). This strategy is suited to species amenable to vegetative propagation, and has been applied to creeping bentgrass (Luo et al. 2005). The maize Plus-Hybrid system can also be adapted to limit transgene flow, through use of a pollen-sterile transgenic hybrid and a non-transgenic pollinator (Feil et al. 2003). Transgene containment in F1 hybrids of seed crops is also possible by crossing a homozygous male-sterile tobacco line, produced by metabolic engineering of glutamine, with a homozygous male-fertile line. The resulting heterozygous F1 plants produce 50% fertile pollen, and all of the fertile pollen is non-transgenic (Ribarits et al. 2007; see below).

21.3 Cytoplasmic Male Sterility Systems

CMS is observed in a wide array of plant species that exhibit diversity with respect to the causal gene and its phenotypic effects. Although the maternally inherited mitochondrial genome encodes CMS, nuclear restorer alleles regulate the expression of this trait (Schnable and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007).

21.3.1 CMS Genes

Most CMS genes are mosaic open reading frames (orfs) that include segments from normal mitochondrial gene coding and flanking sequences, and segments of unknown origin (Schnable and Wise 1998; Hanson and Bentolila 2004; Linke and

Borner 2005; Kubo and Newton 2008). These orfs, when fused to plant mitochondrial promoters or co-transcribed courtesy of upstream mitochondrial genes (Hanson and Bentolila 2004), become gain-of-function mutations. Recombination in plant mitochondrial genomes likely favors the creation of CMS genes, and places these in contexts that support gene expression (Sandhu et al. 2007; Kubo and Newton 2008). Functional copies of all essential mitochondrial genes are almost always retained in CMS mutants. Two examples of CMS involving essential mitochondrial respiratory genes, deletion of the NADH dehydrogenase subunit 7 gene in *Nicotiana sylvestris* (Pla et al. 2005), and truncation of the cytochrome oxidase subunit 2 gene in wild beet (Ducos et al. 2001), seem not to condition deleterious vegetative phenotypes. Perhaps alternative pathways of electron flow in plant mitochondria (Rasmusson et al. 2004; Rhoades and Subbaiah 2007; Noctor et al. 2007) compensate for the effects of these mutations.

21.3.2 CMS Phenotypes

CMS genes condition a wide array of reproductive abnormalities (Laser and Lersten 1972; Schnable and Wise 1998; Zubko 2004; Hanson and Bentolila 2004; Linke and Borner 2005; Chase 2007; Carlsson et al. 2008). In some cases, male reproductive organs (stamens) are transformed into petals or into female reproductive organs (carpels). Other CMS mutations condition degeneration of anthers and/or developing pollen.

21.3.2.1 Homeotic CMS

CMS involving the transformation of stamens to petals (petaloid) or stamens to carpels (carpeloid; Zubko 2004; Linke and Borner 2005; Carlsson et al. 2008) copies the phenotypes conditioned by mutations in nuclear floral organ identity genes (Coen and Meyerowitz 1991; Krizek and Fletcher 2005). In dicotyledonous plants, four whorls of plant floral organs (sepals, petals, stamens, and carpels) develop in response to spatial patterns established by three major classes of floral organ identity genes (A, B, and C). These encode MADS box transcription factors having distinct domains of expression that overlap, in part, to specify sepals (A alone), petals (A and B), stamens (B and C), and carpels (C alone). Decreased accumulation of B-class MADS box transcription factors occurs in several carpeloid CMS systems (Murai et al. 2002; Linke et al. 2003; Zubko 2004; Teixeira et al. 2005). Unusually, late expression of B-class genes is associated with abnormal anther and pollen development of CMS *B. napus* of the Napus (*nap*) type (Geddy et al. 2005). Nuclear MADs box genes are therefore targets of mitochondrial influence. Further study of homeotic CMS will enhance our understanding of retrograde (mitochondria-to-nucleus) signaling pathways in plants.

21.3.2.2 Degenerative CMS

In CMS sunflower, anther tissues exhibit features of apoptotic programmed cell death (PCD) observed in vertebrates, namely, the release of cytochrome *c* from the mitochondria, cleavage of nuclear DNA, and condensation of cytoplasm containing intact organelles (Balk and Leaver 2001). Declining ATP levels promote PCD in animal cells (Bras et al. 2005), and perhaps in sunflower, where the CMS gene encodes a protein related to mitochondrial ATP synthase subunit 8. Abundance of the ATP synthase complex is reduced in CMS compared to male-fertile sunflower (Sabar et al. 2003). The identity and targets of mitochondrial cell death signals in plants are not yet clear, as the caspase proteases, primary downstream target of mitochondria-signaled PCD in animals, are not conserved in plants (Jones 2000; Lam 2004; Logan 2006).

When ATP levels are insufficient to support PCD, cell death can occur by necrosis (Bras et al. 2005). In CMS-T maize plants, the tapetal cells lining the anther exhibit features of necrotic cell death, including the swelling and lysis of organelles and cells (Warmke and Lee 1977). In vegetative tissues, the CMS-T gene product (URF13) forms a mitochondrial pore upon exposure to a toxin produced by *Bipolaris maydis* (reviewed in Wise et al. 1999). In tapetal cells, this pore might form in the absence of toxin, leading to mitochondrial dysfunction and necrosis (Flavell 1974).

21.3.2.3 Male Specificity

In many cases, CMS gene products accumulate throughout the plant, but condition a phenotype only in male reproductive organs (Dewey et al. 1987; Wise et al. 1987; Nivison and Hanson 1989; Laver et al. 1991; Krishnasamy and Makaroff 1994). Warmke and Lee (1978) observed that a 20- to 40-fold increase in the number of mitochondria accompanies the normal development of tapetal cells and pollen within the anther. They proposed that CMS mutations compromise the energy status of tapetum and/or developing pollen. Energy considerations might explain both homeotic and degenerative CMS phenotypes, if the proper regulation of nuclear floral organ identity genes requires ATP-dependent proteolysis (Teixeira et al. 2005), and if declining levels of ATP trigger mitochondria-signaled PCD in anther tissues (Sabar et al. 2003). Alternatively, positive regulators of cell death produced by anthers or developing pollen (Flavell 1974) might induce or act synergistically with mitochondrial cell death signals, or negative regulators might repress these pathways in all but male reproductive organs. The large number of mitochondria in male reproductive organs (Warmke and Lee 1978) could amplify such mitochondrial signaling pathways.

21.3.3 Fertility Restoration

Expression of CMS can be suppressed by system-specific nuclear restorer alleles. The molecular cloning of several restorer genes identified pentatricopeptide repeat (PPR) protein coding genes in all but one case (reviewed in Schanble and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007).

21.3.3.1 Fertility Restoration Genetics

Nuclear fertility restoration systems can be sporophytic, acting in the diploid plant, or gametophytic, acting in the haploid pollen. In sporophytic restoration, all pollen produced by a CMS plant heterozygous for a dominant restoring allele (CMS *Rf*/*rf*) will function regardless of pollen genotype. In gametophytic restoration, only pollen carrying the restoring allele will function. Some fertility restoration systems require the complementary action of restoring alleles at two loci (Pring et al. 1999; Wise et al. 1999), and some CMS systems can be reversed through the action of two or more independent restoration systems (Zabala et al. 1997; Sarria et al. 1999; Wang et al. 2006).

21.3.3.2 Fertility Restoration Mechanisms

In many cases, mitochondria-encoded CMS gene products fail to accumulate in the presence of a nuclear restorer allele (Dewey et al. 1987; Nivison and Hanson 1989; Krishnasamy and Makaroff 1994; Moneger et al. 1994; Abad et al. 1995; Wang et al. 2006). The decreased abundance or internal cleavage of CMS transcripts accompanies this protein loss in some (Dewey et al. 1987; Pruitt and Hanson 1991; Moneger et al. 1994; Wang et al. 2006) but not all cases (Chase 1994; Krishnasamy and Makaroff 1994). It is not known whether these transcript effects are the basis for the protein phenotype, or a secondary consequence of failure to translate a CMS transcript.

21.3.3.3 Fertility Restoration Genes

Rf2 of CMS-T maize encodes a mitochondrial aldehyde dehydrogenase (Cui et al. 1996; Liu et al. 2001), and restores pollen fertility through a mechanism of metabolic compensation. All other restorer genes cloned to date encode PPR proteins (Bentolila et al. 2002; Kazama and Toriyama 2003; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Akagi et al. 2004; Komori et al. 2004; Klein et al. 2005; Wang et al. 2006), members of a highly expanded plant protein family (Lurin et al. 2004). PPR proteins are comprised largely of degenerate

35-amino acid repeats, which might confer sequence-specific RNA binding capability to mediate interactions between enzymes and RNA substrates (Lurin et al. 2004; Stern et al. 2004; Shikanai 2006; Delannoy et al. 2007). In Arabidopsis, 54% of the PPR proteins are predicted to be located in the mitochondria, and 19% are predicted to locate in the plastids (Lurin et al. 2004). PPR proteins function in the translation (Schmitz-Linneweber et al. 2005), editing (Kotera et al. 2005; Okuda et al. 2007), processing and splicing (Schmitz-Linneweber et al. 2006; Hattori et al. 2007) of plastid RNAs, and likely perform similar roles in the expression of normal plant mitochondrial genes. Restorer genes encoding PPR proteins might have evolved through duplication of genes encoding PPR proteins essential to normal mitochondrial gene expression, followed by divergence of mitochondrial targets (Touzet and Budar 2004; Geddy and Brown 2007).

21.3.4 Transgenic Approaches to CMS

Novel transgenic strategies have recently been applied to induce CMS. One strategy exploits genetic transformation of maternally inherited plastid genomes. Another modifies mitochondrial genome organization via nuclear genome transformation.

21.3.4.1 CMS via Plastid Transgenes

There is currently no technology for the genetic transformation of plant mitochondrial genomes (Pelletier and Budar 2007), but the genetic transformation of plastid genomes provides a route to engineering CMS. Ruiz and Daniell (2005) transformed the tobacco plastid genome with a bacterial gene (*phaA*) encoding the enzyme β-ketothiolase. In plants expressing a plastid *phaA* gene, tapetal cells undergo premature degeneration, anthers are misshapen, and pollen is collapsed. These plants are female-fertile, producing uniform pollen-sterile progenies after fertilization with wild-type pollen. β-ketothiolase might disrupt fatty acid metabolism critical to tapetal cell function and pollen development. Tapetal cell abnormalities often appear to be the basis of male sterility in mitochondrial CMS systems (reviewed in Hanson and Bentolila 2004; Chase 2007). Full restoration of male fertility has not been achieved for CMS conditioned by plastid-expressed *phaA*, but this example lays the groundwork for plastid-based CMS. While plastid transformation is not routine in all crops, advances have been made in some plants (Daniell et al. 2005; Okumura et al. 2006; C.W. Liu et al. 2007; Wurbs et al. 2007). It must be remembered, however, that plastid inheritance is paternal or bi-parental in a number of plant species (Hagemann 2004).

21.3.4.2 CMS via Nuclear Transgenes

The plant mitochondrial genome has a complex, multipartite structure that is under the control of nuclear genes (reviewed in Kubo and Newton 2008). The Arabidopsis nuclear gene *MSH1* encodes an organelle-targeted homolog of the *Escherichia coli* mismatch repair protein MutS. Mutations in *MSH1* condition mitochondrial genome rearrangements (Abdelnoor et al. 2003). The down-regulation of tobacco and tomato *MSH1* genes by an RNA interference (RNAi) transgene conditions mitochondrial genome rearrangements associated with male sterility. Male-sterile phenotypes are maternally inherited, and genetically stable following removal of the inducing RNAi construct via Mendelian segregation (Sandhu et al. 2007). This strategy potentially allows for the development of stable non-transgenic CMS systems in any plant amenable to genetic transformation. It remains to be seen whether conventional genetic screens will identify fertility restoration genes for these novel CMS systems.

21.4 Nuclear-Encoded Male Sterility Systems

21.4.1 Nuclear Male Sterility Genes

Natural nuclear mutations that impair pollen and anther development, but do not disturb female reproductive development, have been described in more than 175 plant species (Kaul 1988). Male-sterile mutants can be defined as structural (physical barriers preventing successful self-fertilization), sporogenous (pollen abortion), and functional (failure of anther dehiscence). In addition, various induced mutant approaches have been exploited to disrupt the complex network of developmental pathways involved in pollen development, anther dehiscence, and pollen germination (Chaudhury 1993; Williams 1995; Horner and Palmer 1995; McCormick 2004; X. Liu et al. 2007). Many such mutants have been evaluated for their adaptability with respect to F1-hybrid breeding, and are or have been used in breeding programs (Horner and Palmer 1995; Budar and Pelletier 2001; Perez-Prat and van Lookeren Campagne 2002; Atanassova 2007). They are referred to as genic male sterility (GMS) or nuclear male sterility (NMS) systems. Of practical importance is the environmental GMS found in rice and other crops (Ku et al. 2001; S. Li et al. 2007) that depends on stimuli like temperature or photoperiod, and has been used to develop hybrid rice varieties in the past.

Although nuclear male-sterile plants are valuable in elucidating pollen development, their application in breeding programs is limited, as no homozygous male-sterile populations can be produced (Williams 1995). In most systems, male-sterile plants must be propagated through pollination with male-fertile plants, which leads to segregation in the progeny and hampers the maintenance of the female (i.e., male-sterile) parent (Perez-Prat and van Lookeren Campagne 2002). In a few cases,

both a male sterility gene and a restorer gene are available, and three-line systems of F1-hybrid seed production have been applied to create 100% male-sterile populations (Lu et al. 2004), but the complexity of genotypes involved requires tremendous efforts. In contrast, transgenes offer virtually unlimited options to create male sterility traits, and several transgenic nuclear male sterility systems targeting pollen have been developed.

As many crop species are now amenable to nuclear transformation, genetically engineering nuclear male sterility is an important alternative to CMS and non-GMO NMS. To overcome the limitations of NMS, in particular the problem of segregation of the male sterility trait, numerous strategies have been devised to deploy and regulate nuclear genes for the purposes of creating and propagating uniformly pollen-sterile plant populations. Reversible male sterility (also called conditional male fertility) systems avoid the elimination of segregating male-fertile plants. Moreover, a novel technology adds the creation of homozygous male-sterile plants to reversible male sterility.

21.4.2 Genetically Engineered Nuclear Male Sterility

Due to the remarkable complexity of reproductive development (McCormick 2004; Scott et al. 2004), numerous approaches have been tried to induce nuclear male sterility. Male sterility phenotypes, i.e., abortion of pollen, can occur at different stages during pollen development, ranging from meiosis until dehiscence or pollen germination (Chaudhury 1993; Williams 1995; Perez-Prat and van Lookeren-Campagne 2002; Gleba et al. 2004). Both engineering natural plant genes and introducing genes from other sources, e.g., fungi and bacteria, have led to male sterility (Williams 1995; Budar and Pelletier 2001; Perez-Prat and van Lookeren-Campagne 2002; Gleba et al. 2004).

21.4.2.1 Restoration and Elimination of Segregating Male-Fertile Plants

The tapetum surrounds the developing pollen until first pollen mitosis, and plays a crucial role in the formation of microspores (Wilson and Yang 2004). Premature degeneration of the tapetal cells inevitably leads to pollen abortion. Severe defects in pollen development have been observed when toxic genes like *barnase* or *diphtheria toxin A* were transferred into plants under control of cell type-specific promoters (Mariani et al. 1990; Guerineau et al. 2003; Liu and Liu 2008). Similarly, when the tobacco-derived *TA29* promoter (Koltunow et al. 1990) was silenced in transgenic tobacco by RNAi, the tapetal cells degenerated and pollen aborted (Nawaz-ul-Rehman et al. 2007). Targeting the tapetum allowed for the development of the only commercialized transgenic male sterility, SeedLink^{TM}, used in canola and based on the work of Mariani et al. (1990). Pollen sterility resulting from the selective destruction of tapetal cells in transgenic tobacco and canola plants was

engineered by the tapetum-specific expression of a natural cytotoxic ribonuclease, the *barnase* gene of *Bacillus amyloliquefaciens* under control of the *TA29* promoter.

SeedLink™ sterility was reversed when the male-sterile plants were crossed with transgenic male-fertile plants expressing the barnase inhibitor barstar, which protected the tapetal cells from degradation via barnase-barstar protein complex formation (Mariani et al. 1992). To enhance the expression of the restorer (*barstar*) gene in male-sterile *TA29-barnase* lines of *Brassica juncea*, two promoters (*TA29* from tobacco, and *A9* from *Arabidopsis thaliana*) were used to express, and thereby extend, the temporal expression of two independent wild-type and codon-modified transcriptional units of *barstar* (Bisht et al. 2004). The two different versions of the *barstar* gene presumably also minimized co-suppression effects.

The *barnase*/*barstar* F1-hybrid technology was faced with the general problem inherent in NMS, i.e., segregation of male-sterile and male-fertile plants in maintenance breeding of the male-sterile line. Linking the *barnase* gene to the bialaphos resistance (*bar*; Reynaerts et al. 1993) or mutated acetolactate synthase (*ALS*; Ray et al. 2007) herbicide resistance genes allowed to eliminate male-fertile plants by herbicide treatments. Still, excess seeds needed to be sown to avoid yield penalties.

Another strategy to remove unwanted fertile plants in barnase-mediated NMS was a two-component system based on independent transgenic tomato lines carrying inactive partial barnase peptides (Burgess et al. 2002). Expression of partial peptides alone did not affect pollen viability, allowing for the maintenance of inbred parents in a homozygous state by selfing. When the inbreds were crossed, barnase activity was reconstituted by complementation, and destroyed pollen in the progeny. A recent variation on this theme was a system that combined the *barnase* gene with mutated *ALS* genes (Gils et al. 2008). Male sterility and linked herbicide resistance resulted from the functional complementation of split *barnase* and *ALS* fragments (respectively) fused to trans-splicing inteins, and located on two homologous chromosomes. This system acts, by design, only in heterozygous plants that combine all elements. Plants lacking one of the elements, e.g., after hybridization with a wild-type inbred line, are pollen-fertile.

Inactivation of transcription factors has frequently led to male sterility (e.g., Preston et al. 2004), but practical application has been discussed in only in a few cases. The transcription factor AtMYB103 is essential to tapetum development in Arabidopsis. An insertion mutant disrupting the *AtMYB103* gene led to premature vacuolation and degeneration of the tapetum, and complete disruption of pollen development (S.F. Li et al. 2007). Expression of the *MYB103* gene by a strong anther-specific promoter successfully restored pollen fertility. Based on the occurrence of *MYB103* homologs in important crop plants like rice, barley, and canola, the authors suggested the use of the system for F1-hybrid seed production. Over-expression of another transcription factor, MYB26, caused ectopic lignification that included the anther wall, causing failure of the anthers to dehisce (functional male sterility; Yang et al. 2007). Pollen was viable in the over-expression lines, and could be released mechanically from the anthers. Consequently, restoration was not required for the reversal of MYB26-mediated pollen sterility (Steiner-Lange et al. 2003; Yang et al. 2007).

21.4.2.2 Reversible Male Sterility

Pollen requires for its development nutrients and signals that are provided by the tapetum and other tissues of the anther. The nutritional requirements of developing pollen became evident when isolated microspores were able to develop into mature, fertile pollen under appropriate culture conditions (Benito Moreno et al. 1988; Tupý et al. 1991), and when pollen tube growth was enhanced upon addition of flavonols to in vitro matured pollen (Ylstra et al. 1992). Down-regulation or over-expression of enzymes involved in the synthesis of metabolites that are essential for pollen development offer numerous possibilities to engineer male sterility. Application of the missing nutrient should lead to restoration of pollen fertility (reversible male sterility).

Metabolic engineering of primary metabolites has targeted glutamine, carbohydrates, and pyruvate. The amino acid glutamine is essential for pollen development (Benito Moreno et al. 1988). Transferring into tobacco plants a gene driven by the tapetum-specific promoter *TA29* and encoding a dominant-negative version of glutamine synthetase (dnGS) resulted in male sterility (Ribarits et al. 2007). Spraying plants with glutamine restored male fertility. Anthers of the male-sterile lines contained viable microspores, and restoration was also achieved by isolating the microspores, maturing these in vitro in the presence of glutamine, and using the in vitro matured pollen for pollination of emasculated flowers in situ (Touraev and Heberle-Bors 1999). A similar strategy has been tried in tobacco plants. Here, knocking down an extracellular invertase gene by an anti-sense construct resulted in carbohydrate deficiency of pollen (Goetz et al. 2001), but no seeds were obtained after pollination with pollen matured in vitro in a sugar-containing medium. Down-regulation of pyruvate dehydrogenase was used to mimic CMS, and led to hypertrophy and vacuolation of the tapetal cells (Yui et al. 2003). However, no restoration strategies were devised in this case.

Male sterility resulting from disruption of flavonoid biosynthesis by anti-sense suppression, co-suppression, or mutations (Taylor and Jorgensen 1992; van der Meer et al. 1992; Napoli et al. 1999) was restored when flavonols were applied manually during pollination (Mo et al. 1992; Ylstra et al. 1994), or by expressing the key enzyme in flavonoid biosynthesis, chalcone synthase (Napoli et al. 1999). Exploiting the metabolic similarities of stilbene synthase and chalcone synthetase, tapetum-specific expression of grapevine stilbene synthase was shown to cause male sterility in tobacco, which could partially be reversed by application of 4-coumarate, resveratrol, and flavonols (Fischer et al. 1997). Jasmonic acid biosynthesis is also essential for pollen development and anther dehiscence, and suppression of several enzymes involved in this pathway has been shown to lead to male sterility that can be reversed by the application of linolenic or jasmonic acid (McConn and Browse 1996; Stintzi and Browse 2000; Ishiguro et al. 2001; Park et al. 2002).

Impaired reception and signal transduction of hormones in pollen, and reversal by exogenous application of growth regulators such as kinetin, gibberellic, or jasmonic acid have also been employed to engineer male sterility (Huang et al. 2003; Al-Ahmad and Gressel 2005). No restoration, however, has been reported for tobacco lines carrying an over-expressed ethylene receptor (Ishimaru et al. 2006).

21.4.2.3 Induced Male Sterility

An alternative to reversible male sterility is induced male sterility. The strategy described by Kriete et al. (1996) involved transgenic tobacco plants expressing the bacterial *argE* gene specifically in the tapetum. When these plants were treated with the non-toxic compound *N*-acetyl-phosphinothricin, it was converted into the herbicide phosphinotricin, thereby destroying the tapetal cells. F1-hybrid breeding based on induced male sterility employs two male-fertile inbred lines, one of which is sprayed with *N*-acetyl-phosphinothricin for F1-hybrid seed production.

21.4.2.4 Homozygous Nuclear Male-Sterile Lines

An F1-hybrid seed production system that combines reversible male sterility with a general strategy to produce homozygous male-sterile plants is based on the metabolic engineering of glutamine, as described above (Ribarits et al. 2007). To overcome segregation of the nuclear *TA29-dnGS* gene in maintenance breeding of the male-sterile inbred line, viable microspores contained in its anthers were recovered and used to produce doubled haploid plants through in vitro microspore culture in the presence of glutamine (Touraev and Heberle-Bors 1999). Being 100% homozygous, these doubled haploid plants did not segregate into male-sterile and male-fertile plants in the offspring, but produced only male-sterile plants. Doubled haploids are widely used in plant breeding, for example, in barley, wheat, and canola, not only in selection and combination breeding, but also as inbred lines in F1-hybrid breeding (Forster et al. 2007).

F1 hybrids produced by this method were male-sterile, and could be used for crops in which the vegetative parts of the plants are used. To make this technology applicable also for F1-hybrid breeding in seed crops, the microspore-specific *NTM19* promoter was employed to drive the *dnGS* gene. Transgenic plants containing one *NTM19-dnGS* locus were male-fertile, but 50% of the pollen (the pollen carrying *NTM19-dnGS*) was not viable. To achieve complete male sterility, viable microspores produced by these lines were isolated and cultured in vitro, in the presence of glutamine, to produce homozygous doubled haploid plants. Doubled haploid lines homozygous for the *NTM19-dnGS* gene were indeed male-sterile, and did not segregate into male-sterile and male-fertile plants in the offspring. To maintain the male-sterile line, plants were sprayed with glutamine, an innocuous chemical that restored pollen fertility. Crossing these lines with male-fertile wild-type lines produced heterozygous F1 lines that were male-fertile, due to the presence of 50% wild-type pollen.

In conclusion, this technology combined essential features of F1-hybrid breeding, i.e., a method to create inbred lines (doubled haploidy), a method to produce F1-hybrid seeds (male sterility), and a method to maintain nuclear male-sterile lines as homozygotes (fertility restoration by reversible sterility and doubled haploids).

21.5 Summary and Future Prospects

The past 15 years have seen a virtual explosion in attempts to engineer male sterility for the breeding of F1 hybrids. Breeders are today faced with a variety of options. Not all, however, apply to any given crop species, and some of the new published methods lack a complete strategy for F1-hybrid seed production. Given the choice between CMS and NMS, CMS provides the benefit that male-sterile lines can easily be propagated due to the maternal inheritance of mitochondrial and plastid genomes. CMS is, however, difficult and time-consuming to introduce in a new species, and even more so the corresponding restorer genes. NMS can more easily be introduced, particularly since today many crop species are amenable to nuclear transformation. In addition, the large number of genes expressed in pollen, and the availability of different molecular and genetic tools to engineer gene expression offer numerous possibilities to block pollen development, often in a conditional manner. However, NMS faces the problem of segregation during maintenance breeding. Yet, also for this problem recent developments have offered solutions. Given that most breeders today are familiar with GMOs and doubled haploids, F1-hybrid seed technologies that combine methods to create inbred lines by doubled haploidy, to produce F1-hybrid seeds by NMS, and to maintain nuclear male-sterile lines as homozygotes by fertility restoration through reversible sterility and doubled haploids seem to be a promising path to the future. Another is induced male sterility.

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