Chapter 14
Engineered Male Sterility

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14.1 Introduction

The agricultural exploitation of hybrid crop varieties has enabled enormous increases in food productivity through increased uniformity and hybrid vigour. Because of hybrid vigour, or heterosis, these crops are characterized by an increased resistance to disease and enhanced performance in different environments when comparing the heterozygous hybrid progeny (called F1 hybrids) to the homozygous parents (Lefort-Buson et al. 1987). Heterotic hybrid varieties in major crops, such as cotton, maize, and rice, exhibit >20% yield advantage over conventional varieties under the same cultivation conditions. The increased vigour, uniformity, and yield of F1 hybrids has been exploited in most crops for which the pollination system allows for an economical and convenient cross hybridization (Basra 2000).

In hybrid seed production, one line is designated as the female parent and the other as the male parent. The production of hybrid seeds requires a pollination control system to prevent unwanted self-pollination of the female line, which can be a great challenge, particularly for crop species with hermaphrodite flowers. Many methods exist to prevent the self-pollination of the seed parent (female line) during hybrid seed production: the application of male-specific gametocides, such as mitomycin and streptomycin (Jan and Rutger 1988); some inter- and intra-specific crosses (Hanson and Conde 1985); the mechanical removal of male flowers or anthers, chemical treatment, i.e. the patented chemical *hybridising agent*, Croisor, and use of genetic cytoplasmic or nucleus-encoded male sterility. Generally, naturally occurring genetically male sterile plants maintain fully normal female

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functions. The phenotypic characteristics of male sterility are diverse, including the complete absence of male organs, the abortion of pollen at any step of its development, a failure to develop normal sporogenous tissues, the absence of stamen dehiscence, or an inability of mature pollen to germinate on compatible stigma.

The generation of male sterility, mainly nucleus-encoded, is the basis of new, reliable, and cost-effective pollination control systems for genetic engineering that have been developed during the past decade. The propagation of male-sterile female parent lines is an important aspect for the successful application of these systems in large-scale hybrid seed production. Engineered male sterility has also been discussed in a number of recent reviews (Khan 2005; Takada et al. 2005; Chase 2006; Stockmeyer and Kempken 2006; Pelletier and Budar 2007).

14.2 Natural Male Sterility Systems in Plants

In order to prevent the self-pollination of female lines, pollen fertility must be controlled to permit fertilization only by pollen from the male parent. A simple way to establish a female line for hybrid seed production is to identify or create a line that is unable to produce viable pollen, similar to some lines of maize (Laughnan and Gabay-Laughnan 1983) or rice (Kadowaki et al. 1988). Therefore, this type of male-sterile line is unable to self-pollinate and seed formation is dependent upon pollen from the male line.

14.2.1 Cytoplasmic Male Sterility

The mitochondrion serves essential functions as the centre of energy metabolism in developing eukaryotic organisms. Pollen development in plants appears to be particularly influenced by mitochondrial function. Rearrangements of mitochondrial DNA that lead to unique chimeric genes sometimes result in an inability of the plant to produce fertile pollen (Fig. 14.1). This process, known as cytoplasmic male sterility (CMS), is particularly useful for the production of hybrid varieties for increased crop productivity and has been extensively reviewed (Schnable and Wise 1998; Kempken and Pring 1999; Linke and Börner 2005; Chase 2007). The association of CMS with abnormal mitochondrial gene expression has been established in many plant species, including maize (Levings 1990), petunia (Bino 1985), and sorghum (Pring et al. 1995; Xu et al. 1995a). It is thought that a disruption in pollen development is a consequence of mitochondrial dysfunction resulting from chimeric genes. The incorporation of the derived proteins into the mitochondrial membrane or multi-protein enzyme complexes may lead to the impairment of mitochondrial function. However, it has only been possible in a few cases to artificially introduce CMS by expressing CMS-associated chimeric genes, thus proving them to be causative agents of CMS (Hernould et al. 1993b; Gómez-Casati

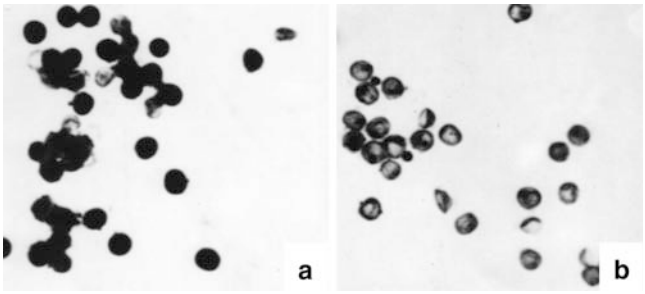

Fig. 14.1 Fertile and sterile sorghum pollen. Iodine–potassium stain of sorghum pollen from fertile and sterile lines. **A** Dark-stained fertile pollen indicating starch production. **B** Unstained pollen from the sterile line

et al. 2002), because these attempts often fail (Stockmeyer et al. 2007). However, there are ways to engineer CMS, e.g. expression of the beta-ketothiolase from *Acinetobacter* in tobacco plastids conditions maternally inherited male sterility (Chase 2006; Pelletier and Budar 2007). A unique feature of CMS is that the expression of the trait is influenced by nuclear fertility restorer genes (Schnable and Wise 1998; Kempken and Pring 1999). Nuclear restorer genes can suppress the effect of the sterile cytoplasm and restore fertility in the next generation. A number of restorer genes have been shown to encode pentatricopeptide repeat (PPR) proteins (Brown et al. 2003; Desloire et al. 2003; Kazama and Toriyama 2003; Akagi et al. 2004; Wang et al. 2006). The PPR proteins are a large family of 500–600 members in higher plants (Small and Peeters 2000).

Cytoplasmic male sterility has been utilized in some important crops, such as sunflower, rice (Chap. 22), oilseed rape (Chap. 21), and sorghum, to prevent unwanted pollinations, but CMS mutants and restorer systems are not available for all agricultural crops. In some cases, CMS has been associated with increased disease susceptibility. For example, the susceptibility of T-cytoplasm in maize to race T of the southern corn leaf blight (*Bipolaris maydis*) led to an epidemic in the United States in 1970 (Wise et al. 1987). Cytoplasmic male sterility is only transmitted maternally and all progeny are sterile. These CMS lines must be maintained by repeated crossings to a sister line, the maintainer line, which is genetically identical except for possessing normal cytoplasm and is male-fertile. The maintainer thus carries the recessive restorer alleles. Fertility restoration is essential in crops, such as corn or sunflower, where seeds are harvested.

14.2.2 Nuclear Male Sterility

Anther and pollen development and fertilization processes have been the subjects of much investigation (Goldberg et al. 1993). Many nuclear genes involved in pollen

development have been identified as mutants that lead to pollen abortion and male sterility. This nuclear, or genic, male sterility is useful for hybrid seed production, but it has limitations because female parental lines are heterozygous and their offspring segregate into fertile and sterile plants in a 1:1 ratio. Nuclear male sterility in plants includes both spontaneous natural and engineered sterility. Spontaneous mutations leading to nuclear male sterility commonly occur at a high frequency. Such mutations can be easily induced by chemical mutagens or ionising radiation. Nuclear male sterility is usually controlled by a pair of recessive genes. Generally, these recessive mutations affect a large number of functions and proteins that are, for example, involved in male meiosis (Glover et al. 1998). In many crops, nuclear male sterility does not permit the effective production of a population with 100% male-sterile plants. This fact seriously limits its use in hybrid seed production (see also Chap. 6).

14.3 Methods of Producing Male-Sterile Plants

Many different strategies have been reported for the production of male-sterile plants by interfering with the development and metabolism of the tapetum (van de Meer et al. 1992; Hernould et al. 1998) or pollen (Worrall et al. 1992) in transgenic plants since the first transgenic male sterility system was described. Male sterility is further induced by using sense or antisense suppression to inhibit essential genes (Xu et al. 1995b; Luo et al. 2000) or by expressing aberrant mitochondrial gene products (Hernould et al. 1993a; He et al. 1996; Gómez-Casati et al. 2002). However, all of the available strategies have drawbacks, such as metabolic or general development interference or being restricted to specific species. Thus, a universal and dominant male sterility system with efficient effects on pollen growth, and offering the possibility to efficiently restore fertility, would be a great advantage for the production of hybrid seeds.

14.3.1 The Selective Destruction of Tissues Important for the Production of Functional Pollen

One way to achieve male sterility systems, is the use of a gene which encodes a protein that is able to disrupt cell function, for example a ribonuclease that destroys the RNA of the tapetal cells (Mariani et al. 1990; Mariani et al. 1992; Burgess et al. 2002). A well known example of this kind is the Barnase/Barstar system shown in Fig. 14.2. Using the PsEND1 promoter is a novel method of producing genetically engineered male-sterile plants by early anther ablation (Roque et al. 2007). The PsEND1 promoter belongs to an anther-specific gene from pea that confers very early gene expression in anther primordium cells. The authors fused this promoter to the barnase gene.

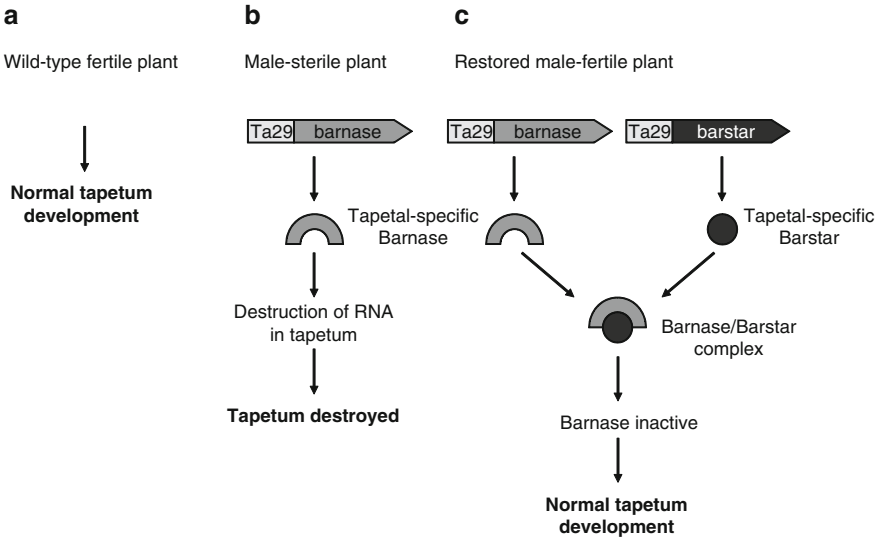

Fig. 14.2 The Barnase/Barstar system. (**a**) Normal tapetum development in the wild-type plant. (**b**) Tapetal-specific promoter Ta29 drives expression of the barnase gene, leading to male-sterile plants. (**c**) Barnase inactivated by barstar inhibitor, resulting in restored male fertility. Based on data from Mariani et al. (1990, 1992)

Another way to introduce male sterility is the use of diphtheria toxin A-chain (Koltunow et al. 1990), which is expressed in a tissue-specific manner. The tapetum serves as a good target for these expression strategies because it plays a critical secretion role in the process of pollen formation. In some of these systems, sterility or fertility can be chemically regulated. For example, inducible sterility can be obtained through the expression of a gene encoding a protein that catalyses the conversion of a pro-herbicide into a toxic herbicide only in male reproductive tissues. In transgenic *Nicotiana tabacum* plants, male sterility was introduced by tapetum-specific deacetylation of the externally applied non-toxic compound N-acetyl-L-phosphinothricin (N-ac-Pt) (Kriete et al. 1996). Transgenic tobacco plants expressing *argE* from *Escherichia coli* under the control of the tapetum-specific tobacco TA29 promoter were produced. The gene product of *argE* represents an N-acetyl-L-ornithine deacetylase, which removes the acetyl group from N-ac-Pt, resulting in the cytotoxic compound L-phosphinothricin (Pt, glufosinate). The application of N-ac-Pt leads to empty anthers, resulting in male-sterile plants. Another example of tissue-specific cell ablation is the use of a bacterial phosphonate monoester hydrolase as a conditional lethal gene (Dotson et al. 1996).

In *Arabidopsis thaliana*, *pehA* from *Burkholderia caryophilli*, a glyphosate metabolizing bacterium, has been expressed using a tapetum-specific promoter. The treatment of transgenic plants with the protoxin glyceryl phosphate leads to male sterility because of the hydrolysis to glyphosate, a potent herbicide inhibiting the biosynthesis of aromatic amino acids. Another example for such

chemical control is the inducible expression of a male-sterility gene by the application of a chemical (Mariani et al. 1990; Goff et al. 1999). In order to induce fertility, the expression of a fertility restorer gene that can complement the sterility, or a male sterility gene repressor, can be chemically controlled (Cigan and Albertsen 2000).

An alternative method for fertility restoration has been suggested by Luo et al. (2000). They used a site-specific recombination system, FLP/FRT from yeast, to restore fertility in *Arabidopsis* plants that were male-sterile due to the antisense expression of the pollen- and tapetum-specific *bcpl* (Mariani et al. 1992) restored the fertility of male-sterile plants generated through the use of the bacterial extracellular ribonuclease Barnase (Paddon et al. 1989) by expressing a specific inhibitor of Barnase, called Barstar (see Fig. 14.2).

Ethylene controls many physiological and developmental processes in plants, including fruit and flower development. Ethylene exerts its effects through the ethylene receptor, which has been isolated in a variety of plant species. The overexpression of mutated melon ethylene receptor genes affects pollen development and induces a male-sterile phenotype in transgenic plants. The inducible male sterility system using mutated ethylene receptor genes could be a possible strategy for preventing pollen dispersal from these plants, thereby reducing the potential impact associated with transgenic plants. The system has been tested in tobacco and lettuce (*Lactuca sativa*; Takada et al. 2005; Ma et al. 2006; Takada et al. 2006; Takada et al. 2007).

Yet another, though quite unusual, approach is based on the nuclear expression of the mitochondrial *atp9* from wheat (see also Sect. 14.3.3). In plant mitochondria, the *atp9* transcript is subject to RNA editing. This editing process is believed to be essential for the function of the encoded peptide. To obtain male-sterile plants, the unedited sequence is fused to a mitochondrial targeting sequence and expressed under control of three different promoters in *A. thaliana*. Indeed male-sterile plants have been obtained (Hernould et al. 1993b; Gómez-Casati et al. 2002).

14.3.2 Changing the Levels of Metabolites Needed for the Production of Viable Pollen

Another approach to induce male sterility in plants is the metabolic engineering of the carbohydrate supply. Carbohydrates are important for anther and pollen development. The extracellular invertase Nin88 mediates the phloem unloading of carbohydrates via an apoplastic pathway. Tissue-specific antisense repression of *nin88* in tobacco causes male sterility because early stages of pollen development are blocked (Goetz et al. 2001). McConn and Browse (1996) demonstrated that *Arabidopsis* triple mutants that contained negligible levels of trienoic fatty acids, such as jasmonate, were male-sterile and produced no seed. In that case, the fertility could be restored through the exogenous application of jasmonate.

14.3.3 *Engineering Cytoplasmic Male-Sterile Plants*

Several efforts are being made to generate engineered CMS plants (Chase 2006; Pelletier and Budar 2007). A quite promising approach was described by Ruiz and Daniell (2005) and reviewed by Khan (2005). Their approach has three advantages: (i) pollination and subsequent self-fertilisation is artificially suppressed, (ii) the trait is based on a cytoplasmic trait that cannot be transmitted via the pollen, and (iii) it allows for the selective restoration of male fertility, at least to some extent. This approach is based on inserting *phaA*, a gene that encodes β-ketothiolase, from the bacterium *Acinetobacter* into the chloroplast genome under control of the chloroplast *psbA* promoter. In transgenic tobacco plants, the enzyme accumulates in the leaves and anthers, altering the course of fatty acid synthesis (Fig. 14.3). By modifying lipid metabolism, pollen development is strongly impaired (Ruiz and Daniell 2005). The expression of β-ketothiolase also accelerates anther development and causes the pollen grains to collapse, leading to male sterility. Fertility restoration was achieved to some extent by growing the plants under continuous light. This effect is due to the light-sensitive gene expression controlled by the *psbA* promoter. Under these conditions, acetyl-CoA carboxylase gains the upper hand, thereby restoring normal fatty acid metabolism (Ruiz and Daniell 2005). However, restoration is only partial and the procedure does not appear to be applicable to field conditions.

14.4 Strategies for the Multiplication of Male-Sterile Lines

Although the described systems have provided important information about anther and pollen development, and ways to interfere with it, their potential use for commercial hybrid seed production is often limited because of the lack of

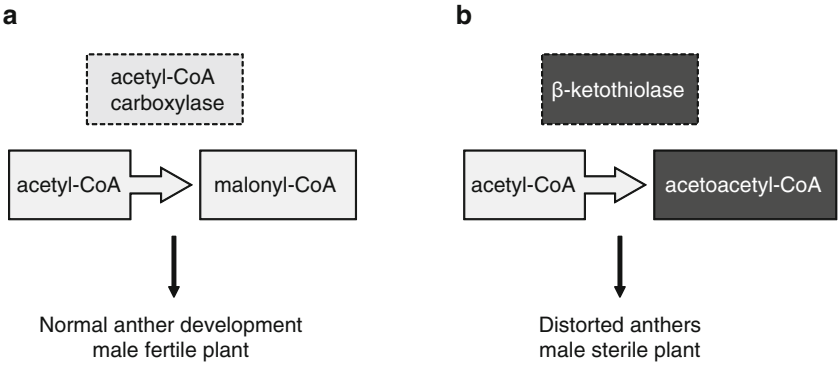

Fig. 14.3 Engineering male sterility with β-ketothiolase. (**a**) In chloroplasts, acetyl-CoA is normally converted by acetyl-CoA carboxylase to yield malonyl-CoA. (**b**) In transgenic plants expressing high amounts of β-ketothiolase, this enzyme out-competes acetyl-CoA carboxylase converting acetyl-CoA into acetoactyl-CoA. As a consequence, anther development is impaired. Based on data from Ruiz and Daniell (2005)

cost-effective and efficient methods to multiply the engineered male-sterile plants (for an overview of multiplication strategies, see Perez-Prat and van Lookeren Campagne 2002).

14.4.1 Herbicide Application for Selection of Male-Sterile Plants

One strategy for the propagation of male-sterile plants is to combine a gene conferring dominant male sterility with an herbicide resistance gene (Denis et al. 1993). After crossing the heterozygous male-sterile plants with a wild-type line of the same genetic background, the male-sterile progeny can be selected by herbicide application. It is important to eliminate all of the fertile plants to prevent any self-pollination, as this could lead to impure hybrid seeds (see Chap. 6).

14.4.2 Reversible Male Sterility

One approach for multiplying male-sterile plants is to produce plants that are conditionally fertile. During female parent multiplication, male-sterile plants are treated with a fertility-restoring chemical and can self-fertilize. For the production of hybrid seeds, chemical application is not required and the plants remain sterile. This system has some advantages over the selection of male-sterile plants by herbicide application. For example, the chemical has to be used during female parent multiplication and not during hybrid seed production and can be applied to a smaller acreage.

Based on conditional male fertility, several pollination control systems have been described. An example of the regulation of male fertility is that the manipulation of hormones in male reproductive tissues (Huang et al. 2003) induced male-sterile plants through tissue-specific expression of the *CKXl* genes and *gai,* which are involved in oxidative cytokinin degradation and gibberellin signal transduction. In this dominant male-sterility system, the male-sterile phenotype is achieved in transgenic plants that are homozygous for the transgene, and it is reversible by exogenous hormone application.

Alternatively, fertility can be induced by environmental conditions. In thermo-sensitive genetic male-sterile (TGMS) and photoperiod-sensitive genetic male-sterile (PGMS) mutants of rice, male sterility is influenced by temperature and photoperiod length (He et al. 1999; Dong et al. 2000). The temperature just after panicle initiation is the most critical in the expression of fertility and sterility. Most rice TGMS lines are male fertile at temperatures less than 25°C and sterile at higher temperatures (Sun et al. 1989). The seeds from TGMS lines are multiplied by selfing when exposed to the right temperature at the critical growth stage. The PGMS lines are fertile under the conditions of a natural short day and are male-sterile under long-day conditions. In this system, the male-sterile female line can be propagated by growing it under the environmental conditions that restore

fertility. This approach requires no restorer lines and no chemical treatment. However, controlled environmental conditions are needed to avoid the plants being constantly challenged by unfavourable fluctuations in their environment. Other conditional male fertility systems are based on repressing the male sterility gene or the inducible expression of a fertility restorer gene that complements the defect (Cigan and Albertsen 2000). Recently, a combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen was established (Ribarits et al. 2007).

14.4.3 Use of Maintainer Lines

The propagation of nuclear male-sterile plants can also be achieved through cross-breeding with a maintainer plant that is male-fertile but produces 100% male-sterile progeny when used to pollinate male-sterile plants. Perez-Prat and van Lookeren Campagne (2002) developed pollen lethality and colour maintainer lines that are useful for propagating both dominant and recessive male-sterile lines. The maintainer plants are genetically identical to the nuclear male-sterile plants with the exception of a transgenic maintainer locus that renders it male-fertile. This system does not require chemical application but a fertility restorer gene and, in the case of colour maintainers, seed sorting might also be needed.

14.5 Commercial Use of Male Sterility

A number of CMS systems have been and are being used in traditional plant breeding in order to generate hybrid varieties. From the many potential procedures for obtaining transgenic male sterility, only a few have been developed so far for commercially available crops. A compilation of these crops is given in Table 14.1. The events include several in canola, one in chicory, and three in maize. In almost all cases, the Barnase/Barstar system is being employed, with the notable exception of a DNA adenine methylase from *E. coli* that causes male sterility if expressed in certain plant tissues. Recently, the Barnase/Barstar system was adopted to Indian oilseed mustard (*Brassica juncea*; Ray et al. 2007), but this has not yet been developed for commercial use.

14.6 Conclusions and Future Perspectives

The use of hybrid crops has been a very important agricultural advance in recent years, because hybrids have an increased yield and a wider environmental adaptability and are more insect- and disease-resistant. One strategy that has been utilized for hybrid crop production is male sterility. Biotechnology has enabled new methods for obtaining male-sterile plants and developing several new

Table 14.1 Commercially used male-sterile plants. Data are from AGBIOS GM database (http://www.agbios.com). Barnase and barstar genes are from *Bacillus amyloliquefaciens*. *fr* Fertility restoring line, *ms* male sterile line, *PPT* phosphinothricin-N-acetyltransferase gene from *Streptomyces hygroscopius* or *S. viridochromogenes*

Event	Company	Description	Marker gene
Brassica napus (Canola)			
MS1, RF1	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNAse inhibitor gene	ms + fr: PTT
MS1, RF2	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNAse inhibitor gene	ms + fr: PPT
MS8xRF3	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene; fr: barstar RNAse inhibitor gene	ms + fr: PPT
PHY14, PHY35	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNAse inhibitor gene	ms + fr: PPT
PHY36	Aventis Crop Science (formerly Plant Genetic Systems)	ms: barnase ribonuclease gene; fr: barstar RNAse inhibitor gene	ms + fr: PPT
Cichorium intybus (Chicory)			
RM3-3, RM3-4, RM3-6	Bejo Zaden BV	ms: barnase ribonuclease gene	PPT
Zea mays (Maize)			
676, 678, 680	Pioneer Hi-Bred International Inc.	ms: DNA adenine methylase from *E. coli*	PPT
MS3	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene	PPT
MS6	Bayer Crop Science (Aventis Crop Science; AgrEvo)	ms: barnase ribonuclease gene	PPT

pollination control systems that could be useful for hybrid seed production. However, the inability to propagate the male-sterile female parent line in a cost-effective and efficient way limits the potential application of commercial hybrid production. Future research should take into account the importance of developing solutions for propagation because, for many crops, it is the limiting factor in the large-scale production of hybrids. Male sterility systems are also being developed for tree species which in the future may be used in other tree species (Höfig et al. 2006).

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