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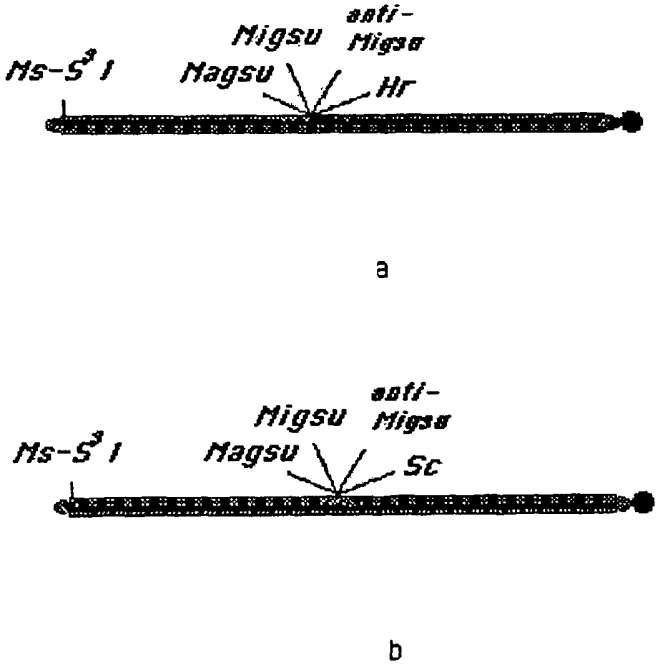
(54) Title: A METHOD TO MAINTAIN A GENIC MALE-STERILE FEMALE PARENTAL LINE OF WHEAT THROUGH SELFING OF THE MAINTAINER LINE



(57) Abstract: The present invention provides a method for stably maintaining a genic male-sterile female parental line of bread wheat, durum wheat and triticale through selfing of the maintainer line. The male-sterile female line is used for the production of low-cost hybrid wheat or hybrid triticale seeds. It also provides a male-sterile female line homozygous for a recessive male-sterility allele, and a maintainer line which is readily and stably propagated. The maintainer line is isogenic to the female line but has an alien chromosomal arm, added to the wheat or triticale complement, carrying a dominant male-fertility allele that restores fertility to the maintainer line, heterologous microgametophytic- and macrogametophytic-suicide genes that kill pollen grains and embryo sacs carrying them thereby preventing the transmission of this chromosome arm to the progeny, a heterologous inducible anti macrogametophytic-suicide gene or anti microgametophytic-suicide gene and a heterologous selectable marker. Because of the activity of the microgametophyticand macrogametophytic-suicide genes all the progeny of the selfed maintainer are male-sterile female plants. When the activity of the anti macrogametophytic- or anti microgametophytic-suicide gene is induced, also female gametes or male gametes, respectively, carrying the alien chromosome arm are viable and since the frequency of these gametes is about 20%, the progeny of the selfed maintainer contains a mixture of female plants (80%) and maintainer plants (20%). The selectable marker facilitates the selection of maintainer plants among the progeny of the selfed maintainer line and thus, maintenance of the maintainer itself. The female lines and the hybrid lines do not contain heterologous genes and therefore, are not genetically modified organisms (GMO).

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A METHOD TO MAINTAIN A GENIC MALE-STERILE FEMALE PARENTAL LINE OF WHEAT THROUGH SELFING OF THE MAINTAINER LINE

FIELD OF THE INVENTION

The present invention is a stand-alone improvement to our previous original patent application (U.S. Provisional Patent Application No. 60/259,725 filed on 04.01.2001), entitled "Method to Maintain a Genic Male-Sterility Female Parental Line for the Production of Hybrid Wheat". It concerns the production of low-cost hybrid seeds of common (bread) wheat, durum wheat and triticale. More specifically, the present invention concerns a new improved method for maintaining a male-sterile female parental line for use in the production of hybrid wheat and hybrid triticale seeds, which female line is homozygous for a recessive male-sterility mutant allele (*ms*), and a new maintainer line for maintaining the female parental line which is isogenic to the female line but has an alien chromosomal arm, as a monotelosomic addition, carrying a dominant male-fertility allele (*Ms*) homoeoallelic to the recessive male-sterility allele, a heterologous microgametophytic-suicide (*Migsu*) gene capable of killing pollen grains carrying it, a heterologous macrogametophytic-suicide (*Magsu*) gene capable of killing embryo sacs carrying it, a heterologous inducible anti microgametophytic- or macrogametophytic-suicide (*Anti Migsu* or *Anti Magsu*) gene, and a heterologous selectable marker such as a herbicide resistance (*Hr*) gene or a seed color (*Sc*) gene. These four heterologous genes and the male-fertility native gene on the alien chromosomal arm are permanently linked due to lack of pairing and recombination between the alien chromosomal arm and the wheat or triticale chromosomes. The presence of the macrogametophytic- and the microgametophytic-suicide genes on the alien chromosomal arm ensures that all the maintainer viable gametes lack this chromosomal arm and consequently, self pollination of the maintainer yields uniform seeds, all of which are homozygous for said male-sterility allele, and lack the alien chromosome arm, and therefore are not GMO, said seeds, when grown, developing into male-sterile female plants. On the other hand, when the inducible promoter of the anti microgametophytic- or macrogametophytic-suicide gene is switched on by application of a suitable chemical, all male gametes or female gametes, respectively, are viable resulting, upon selfing, in a mixture of seeds, 80% of which lacking the

alien chromosome arm and 20% having a single alien chromosome arm. When the *Migsu* or *Magsu* gene encodes a protein that binds a metabolic essential factor and this leads to degeneration of the microgametophytes or macrogametophytes carrying this gene, respectively, exogenous supply of this essential factor prevents the degeneration of male or female gametes carrying the alien chromosome arm resulting, upon selfing, in a mixture of seeds, 80% of which lacking the alien chromosome arm and 20% have it in a single dose. When the selectable marker is a gene conferring resistance to a specific herbicide, it is possible by way of spraying with this herbicide, to kill all plants lacking the alien chromosomal arm thus allowing the growth of only the plants that contain the alien chromosomal arm and therefore, are resistant to said herbicide. When the selectable marker is seed color, such as blue grains, it is possible, by way of seed sorting, to separate the seeds of the genotype with a single alien chromosome arm (the blue seeds) from the female seeds (the red or white seeds).

Alternatively, the alien chromosome arm of the maintainer can carry, in addition to the male-fertility gene, a heterologous gametophytic-suicide gene (*Gsu*) that kills both pollen grains and embryo sacs carrying it, a heterologous anti gametophytic-suicide gene, a heterologous herbicide resistance gene, and heterologous discriminating gene that facilitates the discrimination of plants carrying it in two doses. The presence of the gametophytic-suicide gene ensures that all the maintainer viable gametes lack the alien chromosome arm and consequently, self pollination of the maintainer yields uniform seeds, all of which are homozygous for said male-sterility allele and lacking the alien chromosome arm and therefore, are not GMO, said seeds, when grown, developing into male-sterile female plants. On the other hand, when the inducible promoter of the anti gametophytic-suicide gene is switched on by application of a suitable chemical, all gametes are viable, or when the *Gsu* gene encodes a protein that binds a metabolic essential factor and this leads to degeneration of the gametophytes carrying this gene, exogenous supply of this essential factor prevents the degeneration of gametes carrying the alien chromosome arm and consequently, all gametes are viable, resulting, upon selfing, in a mixture of seeds, 64% of which lacking the alien chromosome arm, 32% and 4% having it in a single dose and two doses, respectively. When the selectable marker is a gene conferring resistance to a specific herbicide, it is possible by way of spraying this herbicide, to kill all plants lacking the alien chromosomal arm thus

allowing the growth of only the plants that contain this arm and therefore, are resistant to said herbicide. Of the latter, about 11% have a pair of the alien chromosome arm and will be discriminated by the action of the discriminating gene and 89% are the maintainer seeds. When the discriminating gene is seed color, such as blue grain, it is possible, by way of seed sorting, to separate the seeds of the genotype with a single alien chromosome arm (the light blue seeds) from those with a pair of the alien arm (the dark blue seeds) and from the female seeds (the red or white seeds). The invention further provides the maintainer line.

The resulting hybrid plants do not contain the heterologous genes and are all heterozygous for the dominant male-fertility allele and for the recessive male-sterility allele and are, therefore, male-fertile.

BACKGROUND OF THE INVENTION

Hybrid wheat lines and hybrid triticale lines yield higher than pure, true breeding lines, and exhibit increased yield stability, improved quality and greater tolerance to environmental and biotic stresses (Wilson and Driscoll, 1983; Pickett, 1993; Bruns and Peterson, 1998; Jordaan et al., 1999; Oettler et al., 2001). Common (bread) wheat, *Triticum aestivum* L. ssp. *aestivum* MacKey, durum (macaroni) wheat, *T. turgidum* L. ssp. *durum* (Desf.) Husn., and hexaploid triticale, *Triticosecale* Wittmack, are predominantly self-pollinating species and every flower contains both female and male organs. To produce hybrid seeds, it is therefore necessary to male-sterilize the female parent. Since hand emasculation is impractical in wheat, male-sterility may be brought about by application of chemical hybridizing agents (CHAs) or by genetic means. Utilization of a CHA to male-sterilize wheat plants is expensive, inefficient and pollutant.

The following conditions are required for the production of hybrid wheat seeds by genetic means: 1) complete and stable male-sterility of the female parent; 2) complete and stable fertility restoration by the male parent; and 3) easy propagation of the male-sterile female parent by the male-fertile maintainer line. Although these conditions are well known to wheat geneticists there has, however, not been a

breakthrough in hybrid wheat production during the 50 years since the first male-sterile wheat was described (Kihara, 1951).

There are two main types of genetic male-sterility that can be exploited for hybrid seed production: cytoplasmic male-sterility in nuclear substitution or alloplasmic lines, caused by the incompatible interaction of an alien cytoplasm with the common wheat nuclear genome, and genic male-sterility in euplasmic lines, caused by a recessive mutation or a deletion of a nuclear gene(s) that is essential to male-fertility in common wheat.

Whereas in many commercial crops it is the genic male-sterility that prevails, this type has not yet been fully exploited in common or durum wheat. Most previous attempts in common wheat have been directed to producing hybrid seeds on the basis of cytoplasmic male-sterility. However, the use of an alien cytoplasm as a sterilizing factor in common wheat has a major drawback since various important traits including grain yield are negatively affected by the interaction between the wheat nuclear genome and the alien cytoplasm. In addition, it has been difficult to find stable fertility restoration genes for the alloplasmic male-sterile lines, which are highly effective in a wide range of genotypes. Moreover, the system requires breeding of the male parent too (e.g. introduction of genes that can restore male-fertility to the alien cytoplasm), thus rendering hybrid seed production more expensive and limiting the number of male parents that can be tested for combining ability (contribution to a significant heterosis).

Genic male sterility, on the other hand, is expressed in a normal wheat cytoplasm and does not involve deleterious effects on plant performance. Further, using a female parent homozygous for a recessive male-sterility allele, any wheat cultivar which is by its nature homozygous for the dominant allele conferring male-fertility, can be used as a male parent that will restore complete fertility to the F_1 hybrids. There is no need to breed for male lines and no limitation exists for the number of males which can be crossed with the male-sterile females and evaluated for their combining ability.

Several chromosome arms have been described in common wheat which carry genes affecting male-fertility, e.g. chromosome arms of group 4 : the long arm of chromosome 4A (4AL), the short arm of chromosome 4B (4BS) and the short arm of chromosome 4D (4DS), carrying the normal male-fertility *Ms-A1*, *Ms-B1* and *Ms-D1* genes, respectively, and the long arms of the group 5 chromosomes : 5A, 5B and 5D (5AL, 5BL and 5DL, respectively), carrying the *Ms-A2*, *Ms-B2* and *Ms-D2* genes, respectively. However, until now, only in the *Ms-B1* locus, on the distal region of chromosome arm 4BS, three recessive alleles that cause male sterility were found or induced. These alleles, namely, *ms-B1-a*, *ms-B1-b* and *ms-B1-c* (often also called *ms1a*, *ms1b* and *ms1c*, respectively), were reported not to cause any effect, beyond male-sterility, on plant performance (reviewed by Wilson & Driscoll, 1983).

Maintenance of the male-sterile female lines remains the major obstacle for a successful hybrid production system based on genic male-sterility. One way is to equip the maintainer line with an alien male-fertility allele homoeoallelic to the recessive mutant male-sterility allele, which is not transmitted into the female line. An example of this approach of maintaining the male-sterile female is the XYZ system of Driscoll (1972). Three decades ago he suggested to add into the male-sterile female Z line (homozygous for the recessive mutant allele *ms-B1-c*) an extra single (in Y line) or a pair (in X line) of an alien chromosome carrying the dominant *Ms* homoeoallele which, in turn, confers fertility to X and Y lines. The alien chromosome does not pair with its wheat homoeologous chromosomes and in the Y line (maintainer) is transmitted through the pollen in a very low frequency and thus the pollinated male-sterile female line produces seeds, most of which will germinate into male-sterile plants. Since the maintainer (Y) is not a true-breeding line, it is produced by pollinating the male-sterile female (Z) by the disomic alien addition line (X). This system was characterized by two major drawbacks: some transmission of the alien chromosome occurred through the pollen of the maintainer line which introduced male fertility to the new generation of the male-sterile female line; and addition decay occurred in the X line impairing its purity. These are possibly the

reasons why this system has never come into practical (commercial) use.

More recently, Driscoll (1985) proposed a modification of the above XYZ system of producing hybrid wheat. In this system, a selfed Y-line replaces the Y-line to maintain and propagate the male-sterile Z-line. This modification eliminates the need for the X-line that was originally needed to generate a large quantity of Y-line plants. Moreover, the newly proposed Y-line carries an alien isochromosome so that the compensating male-fertility homoeoallele is in two doses. While the modified XYZ system requires fewer crosses between the various parental plants in order to maintain and propagate the male-sterile female plants, than the original XYZ system, the drawbacks characterizing the original XYZ system as noted above, do however, also exist in the modified XYZ system and limit its use in commercial production of hybrid seeds.

In view of the above, it therefore seems that traditional methods of hybrid production are not efficient enough and new approaches are needed. One such new approach, based on an improvement of the above XYZ system of Driscoll (1972), has been described in the International PCT Patent Application Nos. PCT/AU91/00319 (WO 92/01366) and PCT/AU93/00017 (WO93/13649), which concerns the production of hybrid cereal crops such as common wheat. In these publications there are described plant lines used for the production of hybrids which have an alien chromosome or chromosome segment bearing a dominant male-fertility allele homoeoallelic to the male-sterility mutant allele and a color marker gene conferring coloration on the progeny seed. The maintenance of the male-sterile (female) parental line is accomplished by physically separating the progeny seeds by color sorting. Such genetically altered common wheat plants contain a modified chromosome with a dominant normal male-fertility allele from the diploid wheat *Triticum monococcum* as an addition or substitution for one of the wheat 4B chromosomes. The modified chromosome carries the short arm of chromosome $4A^m$ of *T. monococcum* ($4A^mS$) carrying the *Ms-A^{m}1* allele and a second arm with a proximal segment from the long arm of either chromosome $4A^m$

of *T. monococcum* ($4A^mL$) or chromosome 4E of *Agropyron elongatum* (4EL) with the coloration allele (C) and a distal segment of wheat chromosome arm 4BL. Part of this modified chromosome is homologous and part of it is homoeologous to the wheat chromosome 4B bearing the recessive male-sterility allele. The homologous part, i.e. the distal region of 4BL can pair with the normal wheat 4BL, thus ensuring regular segregation at meiosis. Another possibility to mark this chromosome carrying the normal dominant male-fertility allele, *Ms-A^{m}1*, is by the use of a gene conferring increased plant height on progeny plants.

However, the above hybrid-production system has too a number of drawbacks as regards the efficient maintenance of the parental lines. First, pollination of female plants by the maintainer will yield a larger number of seeds with the recombinant alien/4BL chromosome, which will develop into male-fertile plants. Secondly, the maintenance of the male-sterile female parent involves a complex procedure of progeny selection based on marker genes. Thirdly, the maintainer line for the female (male-sterile) parental line is also a genetically unstable line in that it carries 20 pairs of normal common wheat chromosomes, one 4B chromosome carrying the male-sterility (*ms-B1-b*) mutant allele (known as 'Probus') (Wilson and Driscoll, 1983) and one recombinant alien group 4/4BL chromosome having the normal, male-fertility *Ms-A^{m}1* allele and the seed coloration allele. Thus, the maintainer line is male-fertile, and upon selfing will yield fertile plants homozygous or heterozygous for the modified chromosome. It will thus be impossible to distinguish between the two genotypes on the basis of the coloration gene and very difficult on the basis of the height gene. Hence, the propagation of the maintainer and its use to provide the male-sterile female line is laborious and not practical for large-scale commercial applications.

To overcome the difficulties of mechanical or other indirect means of selection against the alien chromosome carrying the male fertility *Ms* allele, T.R. Endo, Kyoto University, Kyoto, Japan, suggested (as cited by Tsujimoto and Tsunewaki 1983) to use the gametocidal gene *Gc1* and link it to the male sterility allele *ms*. The

gametocidal allele, originated from *Ae. speltoides*, brings about abortion of gametes not carrying it (but rather carrying the native recessive *gc1* allele). According to Endo's proposal, the male-sterile female line is homozygous for both *ms* and *Gc1*, which are tightly linked, while a male-fertile line (the maintainer) isogenic to the female line but having *Ms* and *gc1* alleles, is used to pollinate the female line to yield a double heterozygote *msMsGc1gc1.* Due to abortion of gametes carrying *gc1,* all the progeny of such selfed line will be homozygote *msmsGc1Gc1* and identical to the female line. However, according to their proposal the male line (R line) in the hybrid production system should also be bred to contain the *Gc1* allele otherwise the fertility of the F_1 hybrid will be reduced. Moreover, *Gc1* causes the abortion of female as well as male gametes and therefore, a cross (between the female and the maintainer) and a self (of the double heterozygote) are required each year to renew the female seed stock. This is a drawback in time and cost. Another disadvantage of Endo's proposal stems from the fact that the male-sterile female parent contains an alien chromosome segment carrying the *Gc1* allele that was derived from *Ae. speltoides*. This segment may carry also alleles with negative effect on the performance of the female, increasing the cost of hybrid seed production, or even affecting the yield of the hybrid.

In our previous patent [application No. PCT/IL98/00220 (WO 98/51142)] a method was provided for the production of hybrid wheat based on the ability to stably maintaining a genic male-sterility female parental line of common and durum wheat. The method was based on the production of a male-sterile female line homozygous for a recessive male-sterility allele and for a dominant pollen-killing allele, and a maintainer line which is readily and stably propagated. The maintainer line is isogenic to the female line but has an alien engineered chromosome carrying a dominant male-fertility allele that restores fertility to the maintainer line, a recessive pollen-killing allele that is susceptible to the killing effect of the native pollen-killer thus preventing the transmission of this chromosome to the female line, and one or more selectable markers that facilitate the maintenance of the maintainer itself.

However, the engineered chromosome, due to some frequency of transversal division of the centromere, was found to be unstable resulting in male gametes that contain the chromosome arm with the male-fertility allele. These male gametes were transmitted upon pollination of the female plants resulting in a certain degree of male-fertile female plants, which led to reduced hybrid purity. Consequently, this method was not used in commercial practice. An improved invention (U.S. Provisional Patent Application No. 60/259,735 filed on 04.01.2001) arranged the three genes on one alien arm thereby overcoming the above mentioned obstacle.

Another method to obtain male-sterile plants based on the use of foreign DNA causing male-sterility has been developed recently in several crop plants such as oilseed rape or maize, aiming to use it also in wheat. The male-sterility gene is a heterologous gene comprising foreign DNA that codes for a cytotoxic protein such as RNase (European Patent Application No. EP 0,412,911) or for a protein that binds an essential cellular factor such as biotin (International PCT Patent Application No. WO 96/40949), and a plant promoter that enables the expression of the foreign DNA only in stamen cells. This method requires the transformation of the male parent too with another heterologous gene comprising antisense DNA that is capable to inhibit the cytotoxic effect by suppressing the transcription activity of the male-sterility DNA or coding for a protein that inhibits the cytotoxic effect, with a plant constitutive promoter. To maintain the male-sterile female line there is a need for additional heterologous gene with an inducible promoter or to treat the male-sterile female plants with an exogenous compound such as biotin, to supply the essential factor. Hence, the method requires breeding of the male parent too rendering the hybrid seed production expensive. In addition, the hybrid plants contain at least two heterologous genes rendering their grains unsuitable for human consumption in the present time.

In a previous patent (U.S. Provisional Patent Application No. 60/259,725 filed on 04.01.2001) a method was provided for stably maintaining a genic male-sterile female parental line of wheat for the production of hybrid wheat. The female is

homozygous for a mutant male-sterility allele and the maintainer line is isogenic to the female line but has an alien chromosomal arm, added to the wheat complement carrying a dominant male-fertility allele that restores fertility to the maintainer line, a heterologous microspore- or microgametophytic-suicide gene that kills microspores or pollen grains carrying it thereby preventing the transmission of this chromosome arm through the male gametes, a heterologous seedling-killer gene that kills seedlings carrying the alien chromosome arm, thus allowing the development of only male-sterile female plants in the progeny of the selfed maintainer, and a heterologous selectable marker that facilitates the selection of the maintainer line and thus, maintenance of the maintainer. In this method the seeds that are planted to obtain male-sterile female plants contain also about 20% maintainer seeds incapable of germination (or die at an early developmental stage). This requires planting of the female population at a higher seed rate. Moreover, the maintainer seeds contain heterologous genes (i.e., they are GMO) and, even though they are incapable of germination or their plants will not survive, the planting of GMO seeds is more complicated due to the requirement of a special permission.

To overcome these two drawbacks we developed an improved method assuring that all the progeny of the selfed maintainer are male-sterile female plants.

As regards the importance of common wheat hybrid production, it should be noted that different reports on experimental hybrid performance indicate a yield increase of the best wheat hybrids of up to 30% above the leading best cultivars (Wilson and Driscoll, 1983). Further, it is well known that many hybrids exhibit an increased yield stability, improved quality and greater tolerance to environmental and biotic stresses than the conventional cultivars. Since the yield increase in successful hybrid wheat is commonly around 15% and the net profit hardly covers the high cost of hybrid seeds, it is essential to produce low cost hybrid seeds. Producing the seeds in plots of mixed female and male population, as described in our previous patent (U.S. Provisional Patent Application No. 60/259,764 filed on 04.01.2001), addresses this target.

DEFINITIONS

Throughout the description and the claims, the following terms and abbreviations will be used:

Common wheat = bread wheat, *Triticum aestivum* L. ssp. *aestivum* MacKey, being an allohexaploid species (2n=42) having the three genomes ABD.

Durum wheat = macaroni wheat, *Triticum turgidum* L. ssp. *durum* (Defs.) Husn., being an allotetraploid species (2n=28) having the two genomes AB.

Triticale = hexaploid (2n=42) triticale (*Triticosecale* Wittmack), a man made crop, derived from hybridization, followed by chromosome doubling, between durum wheat and rye, having the three genomes ABR.

Aegilops searsii = a diploid species (2n=14), closely related to the donor of the B genome of durum and common wheat having genomes S^S, whose chromosomes are homoeologous (partial homologous) to those of wheat.

4BS = the short arm of chromosome 4B of common and durum wheat.

$4S^SS$ = the short arm of chromosome $4S^S$ of *Aegilops searsii.*

AA= an alien chromosomal arm $4S^sS$ of *Aegilops searsii,* added to the complement of common wheat as a monotelosomic addition.

Ms = a dominant allele responsible for male-fertility in wheat.

Ms-B1 = a dominant allele for male-fertility in durum and common wheat located on 4BS.

ms = a recessive mutant allele of *Ms* that confers male-sterility.

ms-B1 = a recessive mutant allele of *Ms-B1*, that confers male-sterility in durum and common wheat, when present in homozygous state.

ms-B1-a = *ms1a* which is the 'Pugsley' mutant *ms-B1* allele.

ms-B1-b = *ms1b* which is the 'Probus' mutant *ms-B1* allele.

ms-B1-c = *ms1c* which is the 'Cornerstone' mutant *ms-B1* allele.

Ms-S^S*1* = a dominant allele for male-fertility, homoeoallelic to *Ms-B1*, on $4S^SS$.

Gsu = a gametophytic-suicide gene; a heterologous gene capable of killing

gametophytes (pollen grains and embryo sacs) carrying it, controlled by a gametophytic-specific promoter.

Magsu = a macrogametophytic-suicide gene; a heterologous gene capable of killing macrogametophytes (embryo sacs) carrying it, controlled by a macrogametophytic-specific promoter.

Migsu = a microgametophytic-suicide gene; a heterologous gene capable of killing microgametophytes (pollen grains) carrying it, controlled by a microgametophytic-specific promoter.

Anti Gsu = a heterologous gene that counteracts the action of *Gsu* by either suppressing *Gsu* activity or inactivating the protein which is coded by *Gsu.*

Anti Magsu = a heterologous anti macrogametophytic-suicide gene that counteracts the action of *Magsu*.

Anti Migsu = a heterologous anti microgametophytic-suicide gene that counteracts the action of *Migsu*.

Rip = a ribosome-inhibitor protein that destroys ribosomes.

BaRNase = a gene from *Bacillus amyloliquefaciens* coding for RNase.

Barstar = a gene from *Bacillus amyloliquefaciens* coding for a protein that inhibits RNase.

Hr = a heterologous specific herbicide-resistance gene by which plants having this gene can be selected, controlled by a constitutive promoter.

Bar = a dominant glufosinate (Basta)–resistance gene.

Sc = a heterologous seed color gene by which plants having this gene can be selected, controlled by an endosperm-specific promoter.

Sd = a heterologous seed dormancy gene by which seeds having this gene in two doses are dormant and do not germinate, controlled by a seed-specific promoter.

Sg = a heterologous seed germination gene by which seeds having this gene in two doses do not germinate, controlled by a seed-specific promoter.

Ss = a heterologous seed size gene by which seeds having this gene in two doses

are smaller than seeds having it in one dose, controlled by a seed-specific promoter.

Rht = a heterologous plant reduced height gene by which plants having this gene in two doses are shorter than plants having it in one dose, controlled by a constitutive promoter.

Vrn = a heterologous vernalization requirement gene by which plants having this gene in two doses require vernalization, controlled by a constitutive promoter.

Le = a heterologous gene causing an early death or slow development of plants carrying it in two doses, controlled by a constitutive promoter.

cv. = cultivar.

Female line = a male-sterile line of common and durum wheat homozygous for one of the recessive male-sterility alleles of *ms-B1.*

Male line = a male-fertile line of common and durum wheat homozygous for the dominant male-fertility allele *Ms-B1.*

Maintainer line = a male-fertile line isogenic to the male-sterile female line but contains the alien chromosomal arm AA, carrying a dominant male-fertility allele (*Ms*) linked to several heterologous genes.

SUMMARY OF THE INVENTION

In order to overcome the above mentioned drawbacks of the prior art, it is an object of the present invention to provide an efficient and inexpensive method for maintaining a genic male-sterile female parental line of a common wheat, durum wheat or triticale cultivar, which method provides for a simple means for stably maintaining the male-sterile female parental line.

Yet another object of the present invention is to provide a maintainer line for use in the above method, which maintainer line is easily, rapidly and stably propagated.

The present invention makes possible the commercial production of low-cost

hybrid seeds of common wheat, durum wheat and triticale. In one aspect, the invention provides a novel method for the maintenance of a male-sterile female parental line that is homozygous for a recessive male-sterility mutant (*ms*) allele. The maintainer is isogenic to the female line and has further the alien chromosomal arm $4S^sS$ of *Aegilops searsii* as a monotelosomic addition, herein referred to as AA, carrying a dominant male-fertility allele (*Ms*) linked to a heterologous macrogametophytic- and microgametophytic-suicide gene (*Magsu or Migsu*) capable of killing embryo sacs and pollen grains carrying them, controlled by a macrogametophytic- and microgametophytic-specific promoters, respectively, a heterologous anti macrogametophytic-suicide or anti microgametophytic-suicide gene capable of counteracting the action of *Magsu* or *Migsu*, respectively, controlled by an inducible promoter and a heterologous selectable marker gene, such as a specific herbicide-resistance gene (*Hr*), controlled by a constitutive promoter, or a seed color gene (*Sc*) such as blue aleurone (*Ba*), controlled by an endosperm-specific promoter (Figs. 1-2). Male or female gametes of the maintainer containing the AA are not functional.

Alternatively, the AA of the maintainer carries, in addition to the dominant male-fertility allele, a heterologous gametophytic-suicide (*Gsu*) gene capable of killing pollen grains and embryo sacs carrying it, controlled by a gametophytic-specific promoter, a heterologous anti gametophytic-suicide (*Anti Gsu*) gene capable of counteracting the action of the *Gsu* gene, controlled by an inducible promoter, and either a heterologous selectable marker gene, such as specific herbicide resistance gene (*Hr*), controlled by a constitutive promoter, and a dosage-dependent discriminating gene, such as reduced height, controlled by a constitutive promoter, or a seed color gene, such as blue aleurone (*Ba*), controlled by an endosperm-specific promoter (Fig. 3). Male and female gametes of the maintainer containing the AA are not functional.

Thus, a simple system has been developed in accordance with the present invention, by which the male-sterile female parental line is maintained by self-

pollinating of maintainer line, and all of the resulting progeny are male-sterile female plants (Figs. 4 and 5). Based on the percentage of the male and female gametes lacking the AA, 80% seed set is expected in the selfed maintainer, all of which, when germinated, will grow into male-sterile female plants. The maintainer line is itself easily maintained by the application of a specific chemical that induces the expression of the anti gametophytic-suicide gene and self-pollination, resulting in a mixture of progeny seeds. In one case, when the anti gametophytic-suicide gene is *Anti Magsu* or *Anti Migsu*, the progeny seeds of the selfed maintainer consists of about 20% carrying the AA, when grown, developing into male-fertile plants, and 80% lacking the AA, when grown, developing into male-sterile plants. Spraying said progeny plants with the specific herbicide kills the male-sterile plants (Fig. 4a). When the selectable marker is seed color, such as blue aleurone (*Ba*), the red or white seeds, when grown, developing into male-sterile female plants, can be separated by a seed sorter from the blue seeds, when grow, developing into male-fertile maintainer plants (Fig. 4b). In the second case, when the anti gametophytic-suicide gene is *Anti Gsu*, the mixture of the progeny seeds contain about 36% that carry the AA (32% carry it in one dose and 4% in two doses), when grown, developing into male-fertile plants, and about 64% lack this alien chromosomal arm, when grown, developing into male-sterile plants. Spraying said progeny plants with the specific herbicide kills the male–sterile plants while plants with a pair of the AA are selected against on the basis of the phenotype of the plants having two doses of the discriminating gene, thereby allowing only the male-fertile maintainer plants to grow (Fig. 5a). When the selectable marker is seed color, the red or white seeds, when grown, developing into male-sterile female plants, can be separated by a seed sorter from the light blue seeds, when grown, developing into male-fertile maintainer plants and from the dark blue ditelosomic addition AA seeds (Fig. 5b).

The AA was derived from *Ae. searsii*. It is the short arm of chromosome $4S^s$ ($4S^sS$). Being an alien single telocentric chromosome that was added to the complement of common wheat, it does not pair during meiosis and only enters to

about 20% of the gametes, either male or female. This AA is normally not transmitted through the male and female gametes because of the presence of the gametophytic-suicide gene (s).

The *Ms-S^{s}1* gene is homoeoallelic to the *Ms-B1* gene and dominant over the *ms-B1* alleles of common and durum wheat and is expressed in all tested genetic backgrounds. The heterologous gametophytic-suicide gene *(Gsu)*, or the microgametophytic- or macrogametophytic-suicide gene (*Migsu* or *Magsu*, respectively) is any gene that codes for heterologous protein which is toxic to microgametophytes (pollen grains) and macrogametophytes (embryo sacs) possessing it or disturbs their metabolism. It consists of a foreign DNA sequence coding for a toxic protein, which is toxic to gametes, or codes for a protein that significantly disturbs the metabolism and thereby the viability of the gametes, inserted into an expression cassette under control of a gametophytic-specific promoter or by a microgametophytic- or macrogametophytic-specific promoter. The heterologous anti gametophytic gene is any gene that counteracts the action of the *Gsu* gene or the *Migsu* or *Magsu* genes and its promoter can be induced by a chemical. It consists of a foreign DNA sequence coding for a molecule that binds and inactivates the toxic protein encoded by the *Gsu* gene or the *Migsu* or *Magsu* genes and thereby allows for gametes containing the AA to be viable, inserted into an expression cassette under control of an inducible promoter. The heterologous specific herbicide-resistance gene (*Hr*) codes for a heterologous protein that confers resistance to a specific herbicide under control of a constitutive promoter. The heterologous seed color gene (*Sc*) codes for an anthocyanin and thus conferring coloration of the grains containing it, inserted into an expression cassette under control of an endosperm-specific promoter. The discriminating gene is a gene that results in a different phenotype when present in two doses, thereby allowing the selection against this genotype or is a lethal gene that causes early death when present in double but not in a single dose. It is inserted into an expression cassette under the control of a constitutive promoter.

The above mentioned heterologous genes are introduced to the AA by standard wheat transformation procedures.

In still another aspect, the invention relates to a method for producing a hybrid plant line of common or durum wheat, wherein the male-sterile female parental line is crossed with any cultivar of the same species, which by its nature is male-fertile homozygous for the *Ms-B1* allele, to yield F_1 hybrid progeny that are all fertile and heterozygous (*Ms-B1ms-B1*).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts schematic drawings of the alien chromosomal arm $4S^sS$ of *Aegilops searsii* (AA) carrying the dominant male-fertility allele *Ms-S^{s}1*, a heterologous microgametophytic-suicide gene (*Migsu*) with a microgametophytic-specific promoter, a heterologous macrogametophytic-suicide gene (*Magsu*) with a macrogametophytic-specific promoter, a heterologous *Anti Migsu* gene controlled by an inducible promoter, and a heterologous selectable marker gene, either specific herbicide resistance (*Hr*) controlled by a constitutive promoter (1a); or seed color gene (*Sc*) controlled by an endosperm-specific promoter (1b).

Fig. 2 depicts schematic drawings of the alien chromosomal arm $4S^sS$ of *Aegilops searsii* (AA) carrying the dominant male-fertility allele *Ms-S^{s}1*, a heterologous microgametophytic-suicide gene (*Migsu*) with a microgametophytic-specific promoter, a heterologous macrogametophytic-suicide gene (*Magsu*) with a macrogametophytic-specific promoter, a heterologous *Anti Magsu* gene controlled by an inducible promoter, and a heterologous selectable marker gene, either specific herbicide resistance (*Hr*) controlled by a constitutive promoter (2a); or seed color gene (*Sc*) controlled by an endosperm-specific promoter (2b).

Fig. 3 depicts schematic drawings of the alien chromosomal arm $4S^sS$ of *Aegilops searsii* (AA) carrying the dominant male-fertility allele *Ms-S^{s}1*, a

heterologous gametophytic-suicide gene (*Gsu*) with a gametophytic-specific promoter, a heterologous inducible *Anti Gsu* gene controlled by an inducible promoter, and a heterologous selectable marker gene, either specific herbicide resistance (*Hr*) controlled by a constitutive promoter and a heterologous plant reduced height (*Rht*) gene which, due to its dosage effect, plants having it in two doses are much shorter than plants having it in one dose, controlled by a constitutive promoter (3a); or lacking the selectable marker gene and the reduced height gene and having the seed color (*Sc*) gene, controlled by an endosperm-specific promoter (3b).

Fig. 4 depicts a general scheme for maintaining a male-sterile female parental line by selfing the maintainer line and obtaining progeny seeds all of which, when grown; developing into the male-sterile female line, and maintaining the maintainer line by applying a specific chemical to the maintainer plants that activates the *Anti Migsu* (or *Anti Magsu*) gene and thus obtaining progeny seeds of which 80%, when grown, developing into the female line, and 20%, when grown, developing into the maintainer line. When the selectable marker is Basta herbicide resistance, the maintainer line is selected by spraying with Basta (4a); when the selectable marker is the seed color gene, such as the blue aleurone (*Ba*) gene, the maintainer is selected by a seed sorter (4b).

Fig. 5a depicts a general scheme for maintaining a male-sterile female parental line by selfing the maintainer line and obtaining progeny seeds all of which, when grown, developing into the male-sterile female line, and maintaining the maintainer line by applying a specific chemical to the maintainer plants that activates the *Anti Gsu* gene and thus obtaining progeny seeds of which 64%, when grown, developing into the female line and are susceptible to Basta, 4% having a pair of the AA, when grown, developing into short plants and 32% having a single AA, when grown, developing into taller plants. The latter are the maintainer line and are selectively harvested due to height differences.

Fig. 5b depicts a general scheme for maintaining a male-sterile female

parental line by selfing the maintainer line and obtaining progeny seeds all of which, when grown, developing into the male-sterile female line, and maintaining the maintainer line by applying a specific chemical to the maintainer plants that activates the *Anti Gsu* gene and thus obtaining progeny seeds of which 64% are red or white, when grown, developing into female line plants, 4% are dark blue, when grown, developing into plants with a pair of the AA, and 32% are light blue, when grown, developing into the maintainer line plants. The latter are selected by separating the light blue seeds (the maintainer seeds) from the red or white seeds (the female seeds) and from the dark blue seeds (having a pair of the AA) by a seed sorter.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention a simple system has been developed for common wheat, durum wheat and triticale as depicted in Figs. 4 and 5 by which the male-sterile female parental line is maintained by self-pollinating of the maintainer line, and all of the resulting progeny are male-sterile female plants. Similarly, the maintainer line is itself easily maintained by specific chemical application to the maintainer plants and self-pollination. In the case of activation of *Anti Migsu* or *Anti Magsu* the mixture of the progeny seeds of the selfed maintainer contains 20% seeds, when grown, developing into male-fertile plants identical to the maintainer line and carrying the AA, and 80% seeds, when grown, developing into male-sterile plants due to the absence of the AA and consequently, the *Ms-S^{s}1* allele (Fig. 4). Spraying the progeny plants of the selfed maintainer line with a specific herbicide (Fig. 4a), kills the male-sterile plants while the male-fertile plants carrying the AA with the specific herbicide resistance gene survive. In the case of activation of *Anti Gsu*, the mixture of the progeny seeds of the selfed maintainer contains about 32% seeds, when grown, developing into male-fertile plants identical to the maintainer line and carrying a single AA, 4% carrying a pair of AA, and about 64%, when grown, developing into male-sterile plants due to the absence of the AA and consequently, the *Ms-S^{s}1* allele (Fig. 5). Spraying the progeny plants of the

selfed maintainer line with the specific herbicide (Fig. 5a), kills the male-sterile plants while the male-fertile plants carrying the AA with the specific herbicide resistance gene survive. However, those with a pair of the AA will be short plants due to the *Rht* gene and the maintainer plants, which will be taller, are selectively harvested. Alternatively, separating the seeds on the basis of their different colors with a seed sorter (Fig. 5b) allows the selection of maintainer seeds.

For hybrid seed production, the male-sterile female parental line is crossed with any common or durum wheat cultivar, which by its nature is male-fertile homozygous for the *Ms-B1* allele, to yield F_1 hybrid offspring that are all heterozygous *Ms-B1ms-B1* and therefore, male-fertile.

Accordingly, in one aspect, the present invention provides a method for the maintenance of a male-sterile female parental line of common wheat, durum wheat or triticale (Fig. 4) for use in the production of hybrid wheat, said method comprising:

(a) selfing a maintainer line isogenic to the female line and homozygous for the same *ms-B1* allele of the female line, and having AA, as a monotelosomic addition, carrying the dominant male-fertility allele *Ms-S^s1,* linked to four heterologous genes, a microgametophytic-suicide gene (*Migsu*) that disturbs the metabolism of pollen grains carrying it, thus rendering them inviable, controlled by a microgametophytic-specific promoter, a macrogametophytic-suicide gene (*Magsu*) that disturbs the metabolism of embryo sacs carrying it, thus rendering them inviable, controlled by a macrogametophytic-specific promoter, a heterologous anti microgametophytic- or macrogametophytic-suicide gene (*Anti Migsu* or *Anti Magsu*) that counteracts the action of *Migsu* or *Magsu*, respectively, by coding for a protein that remedies the damage caused by the suicide gene, controlled by an inducible promoter, and heterologous selectable marker gene by which plants having AA are selected. Alternatively, AA contains in addition to the *Ms-S^s1*, four or three heterologous genes, a gametophytic-suicide gene (*Gsu*) that disturbs the metabolism of those male and female

gametophytes carrying it thus rendering them inviable, controlled by a gametophytic-specific promoter, an *Anti Gsu* gene that counteracts the action of *Gsu* by coding for a protein that remedies the damage caused by it, controlled by an inducible promoter, and either a heterologous selectable marker gene by which plants having this chromosomal arm can be selected, such as a specific herbicide-resistance gene (*Hr*), controlled by a constitutive promoter, and a heterologous reduced height gene controlled by a constitutive promoter or, alternatively, a seed-color gene (*Sc*), controlled by an endosperm-specific promoter. Gametes of the maintainer containing the AA are not functional; and

(b) harvesting the seeds from the selfed maintainer of (a), all of which are homozygous for said male-sterility allele and lack the AA, said seeds, when grown, developing into said male-sterile female line.

Any male-sterility *ms-B1* allele may be used according to the invention such as, for example, the *ms-B1-a, ms-B1-b* and *ms-B1-c* alleles or any other allele of this locus or another locus of common wheat, durum wheat or triticale inducing male-sterility.

The heterologous microgametophytic-suicide (*Migsu*) gene, macrogametophytic-suicide (*Magsu*) gene and gametophytic-suicide gene (*Gsu*) are any gene that codes for heterologous protein which is toxic to microgametophytes (pollen grains) and macrogametophytes (embryo sacs) possessing it, or codes for a protein that disturbs the metabolism of male and female gametes possessing it. It consists of a foreign DNA sequence coding for a toxic protein such as *BaRNase* that destroys RNA (Mariani et al., 1992), or ribosome-inhibitor protein (RIP) that destroys ribosomes (Logemann et al., 1992; Jach et al., 1995), or codes for a protein that specifically binds an essential factor such as streptavidin which specifically binds the metabolically-essential factor biotin, inserted into an expression cassette under control of a microgametophytic-, macrogametophytic- or gametophytic-specific

promoter.

The heterologous inducible anti microgametophytic-suicide (*Anti Migsu*) gene, anti macrogametophytic-suicide (*Anti Magsu*) gene and anti gametophytic-suicide gene (*Anti Gsu*) is any gene that codes for heterologous protein which inactivates the toxin encoded by *Migsu, Magsu* or *Gsu* and allows for viable gametes. It consists of a foreign DNA sequence coding for a protein which binds and inactivates the toxic protein, inserted into an expression cassette under control of an inducible promoter.

The heterologous selectable marker by which maintainer plants or seeds are selected among the progeny of the selfed maintainer, can be a specific herbicide-resistance gene (*Hr*) or a gene conferring seed color (*Sc*). The heterologous specific herbicide-resistance gene (*Hr*) codes for a heterologous protein that confers resistance to a specific herbicide such as glufosinate (Basta) (De Block et al., 1987; Weeks et al., 1993), under control of a constitutive promoter. The heterologous seed-color gene (*Sc*) controlled by an endosperm-specific promoter, codes for an anthocyanin that is accumulated in the aleurone layer or in the endosperm resulting in dosage dependent intensity of blue coloring of the grain (Dooner et al., 1991).

The discriminating gene can be any gene affecting morphological, physiological or biochemical trait in two doses and enables the selection against this genotype, such as reduced height gene that reduces plant height when present in two doses but not in one dose.

In another aspect, the present invention provides a male-fertile maintainer line of common or durum wheat which is itself maintained by selfing of maintainer plants that were treated by a specific chemical that induces the expression of the *Anti Migsu*, *Anti Magsu* or *Anti Gsu* gene (Figs. 4 - 5), said maintainer line being isogenic to the female parent and homozygous for any one of the *ms-B1* male-sterility alleles of the female parent, and having an AA as a monotelosomic addition, carrying the *Ms-S^{S}1* male-fertility allele, a heterologous microgametophytic-suicide gene (*Migsu*), under control of a microgametophytic-specific promoter, a heterologous macrogametophytic-suicide gene (*Magsu*), under control of a macrogametophytic-

specific promoter, a heterologous *Anti Migsu* or *Anti Magsu* gene that counteracts the action of *Migsu* or *Magsu* by coding for a protein that remedies the damage caused by them, controlled by an inducible promoter, and a heterologous selectable marker gene by which plants having the AA can be selected, such as a specific herbicide resistance gene (*Hr*), controlled by a constitutive promoter, or a seed color gene (*Sc*), controlled by an endosperm-specific promoter. Being an alien monotelosomic addition, the AA does not pair during meiosis and consequently, enters only to about 20% of the gametes, either male or female. Hence, since under the control of the *Anti Migsu* gene or the *Anti Magsu* gene the AA is transmitted through the male or female gametes, respectively, the amount of seeds containing a single AA among the progeny of the selfed maintainer is about 20%. These seeds, when grown, developing into plants resistant to the specific herbicide and are selected by it. When the *Migsu* or *Magsu* gene encodes a protein that binds a metabolic essential factor and this leads to degeneration of the microgametophytes or macrogametophytes carrying this gene, respectively, exogenous supply of this essential factor prevents the degeneration of male or female gametes carrying the AA resulting, upon selfing, in a mixture of seeds, 80% of which lacking the alien chromosome arm, when grown, developing into male-sterile plants and 20% have it in a single dose, when grown, developing into male-fertile plants which are resistant to the specific herbicide and are selected by it. Alternatively, when the selectable marker is a heterologous seed-color gene (*Sc*), the blue seeds containing the AA can be separated from the red or white seeds lacking the AA by a seed sorter. The blue seeds, when grown, developing into male-fertile maintainer plants, and the red or white seeds, when grown, developing into male-sterile female plants.

An alternative AA carries, in addition to the *Ms-S^{s}1* gene, a heterologous gametophytic-suicide gene (*Gsu*), under control of a gametophytic-specific promoter, a heterologous *Anti Gsu* gene that counteracts the action of *Gsu* by coding for a protein that remedies the damage caused by it, controlled by an inducible promoter and either a heterologous selectable marker gene by which plants having the AA

can be selected, such as a specific herbicide resistance gene (*Hr*), controlled by a constitutive promoter, and a heterologous discriminating gene by which plants having a pair of AA can be selected against, such as reduced height (*Rht*) which plants having it in two doses are short or, alternatively, a seed color gene (*Sc*), controlled by an endosperm-specific promoter. Being an alien monotelosomic addition, AA does not pair during meiosis and consequently, enters only to about 20% of the gametes, either male or female. Hence, since under the control of the *Anti-Gsu* gene the alien chromosomal arm is transmitted through the gametes, the amount of seeds containing a single or a pair of the AA chromosomal arm among the progeny of the selfed maintainer is about 32% and 4%, respectively. These seeds, when grown, developing into plants resistant to the specific herbicide and can be selected by it. Plants with a pair of AA are undesirable and are short because of the presence of two doses of the *Rht* gene. Alternatively, when the selectable marker is a heterologous seed color gene (*Sc*), the light blue seeds containing a single AA can be separated from the red or white seeds lacking AA and from the undesirable dark blue seeds having a pair of AA by a seed sorter. The light blue seeds, when grown, developing into male-fertile maintainer plants, and the red or white seeds, when grown, developing into male-sterile female plants.

Yet another aspect of the present invention is a method for producing hybrid plants of common or durum wheat, comprising:

(a) crossing a male parent with a male-sterile female parent of the same species, wherein said male parent is selected from any desired common or durum wheat cultivar, which, by its nature, is homozygous for the dominant wild-type male-fertility (*Ms-B1*) allele, and said male-sterile female parent is a line of said wheat species being homozygous for any one of the recessive mutant male-sterility (*ms-B1*) alleles, said male-sterile female parent being maintained by a maintainer line of the invention as noted above; and

(b) collecting the progeny seed of the cross of (a), which seeds, when grown,

develop into progeny hybrid plants all of which are male-fertile and are heterozygous for the said mutant male-sterility allele, i.e., *ms-B1Ms-B1.*

The present invention will now be described in more detail in the following non-limiting examples and their accompanying drawings.

Example 1 :

The hybrid system

The conditions required for a successful production of hybrid seeds from a male-sterile female parent and a male parent by genetic means are as follows : 1) complete and stable male-sterility of the female parent, called the 'Female line'; 2) complete and stable male-fertility restoration by the male parent, called the 'Male line'; and 3) easy propagation of the female line by a male-fertile maintainer line, called the 'Maintainer line'. The F_1 hybrid seeds produced in this way are all male-fertile. The female line is propagated by planting the progeny of the selfed maintainer line, and the maintainer line is itself maintained by specific chemical application and selfing, and the desired male-fertile plants among the progeny of the selfed maintainer are selected each generation by the use of a selectable marker characterizing the maintainer.

An example of production of hybrid common wheat, durum wheat or triticale using this method is described below. A cultivar, herein designated cv. 'One', equipped with genes for male-sterility, i.e. homozygous for one of the male-sterility recessive mutant alleles (*ms-B1ms-B1*) on the short arm of chromosome 4B, is used as the male-sterile female line. Three such male-sterility alleles have been described (review in Wilson and Driscoll, 1983). These alleles are 'Pugsley' (Pugsley & Oram, 1959 - spontaneous appearance; *ms-B1-a*), 'Probus' (Fossati & Ingold, 1970 - induced by X rays; *ms-B1-b*) and 'Cornerstone' (Driscoll, 1977 - induced by gamma-irradiation; *ms-B1-c*). The alleles *ms-B1-b* and *ms-B1-c* are terminal deletions. Several additional mutant alleles were previously induced by us either by gamma-irradiation or by treatment with ethyl methanesulfonate (EMS) (our unpublished data). The EMS-treated mutants can be distinguished from the various

deletions of the *Ms-B1* locus by the presence in these mutants of a terminal C-band on 4BS. The DNA marker *Xpsr921* which is located on the distal region of 4BS, is absent in *ms-B1-c* and in several of our gamma-irradiated mutants (and possibly also in *ms-B1-b*) (our unpublished data), i.e., it is located in the deleted segment and its absence can mark homozygosity for the deletion.

The maintainer line is of the same cultivar as the female line, i.e., cv. 'One', is homozygous for the same *ms-B1* allele present in the female line but has the alien chromosomal arm as a monotelosomic addition (Figs. 4 and 5), consisting of $4S^{Ss}S$ of *Aegilops searsii*, that carries the dominant male-fertility allele *Ms-*S^{Ss}*1*, and the heterologous *RIP* gene coding for a protein that inhibits ribosome formation and consequently, causes cell death, controlled by a microgametophytic-specific promoter, a heterologous *BaRNase* gene coding for RNase that degrades RNA and consequently, causes cell death, controlled by a macrogametophytic-specific promoter, a heterologous *Barstar* gene that codes for a protein that inhibits RNase and therefore counteracts the action of *BaRNase*, controlled by an inducible promoter, and a heterologous *Bar* gene conferring resistance to the herbicide Basta. Because of the presence of the *RIP* and the *BaRNase* genes, all male gametes carrying the *RIP* gene and the female gametes carrying the *BaRNase* gene die and thus, the AA is not transmitted to the progeny. Self pollination of the maintainer line yield therefore, only seeds, when grown, developing into male-sterile plants. On the other hand, activation of the *Barstar* gene, by treatment with specific chemical that induces the activity of the *Barstar* promoter, counteracts the RNase and consequently, female gametes carrying the AA are also viable and transmitted to the progeny. Thus, self pollination of the maintainer yields a mixture of seeds, of which 80%, when grown, developing into male-sterile plants, and 20%, when grown, developing into male-fertile plants. When the selectable marker is herbicide resistance gene such as *Bar* (Fig. 4a), the male-sterile plants, lacking this gene, are killed by Basta. When the selectable marker is seed color gene, such as blue aleurone (*Ba*) (Fig. 4b) the red or white seeds, when grown, developing into male-

sterile plants are seperated by a seed sorter from the blue seeds, when grown, developing into male-fertile plants. An alternative maintainer contains on the AA, in addition to the *Ms-S^{s}1* gene, a heterologous *BaRNase* gene, controlled by a gametophytic-specific promoter, a heterologous *Barstar* gene controlled by an inducible promoter and either a heterologous *Bar* gene controlled by a constitutive promoter and a heterologous reduced height gene which plants having it in two doses are short, controlled by a constitutive promoter (Fig. 5a), or a seed color gene, such as blue aleurone (*Ba*) with an endosperm-specific promoter (Fig. 5b). Because of the presence of the *BaRNase* gene, all the male and female gametes carrying it die and thus, the alien chromosomal arm is not transmitted through the pollen grains and the embryo sacs. Self pollination of the maintainer line yields only seeds when grown, developing into male-sterile plants. On the other hand, activation of the *Barstar* gene allows also for the transmission of gametes with the AA. Thus self pollination of the maintainer line yields a mixture of seeds, of which about 64%, when grown, developing into male-sterile plants, and about 36%, when grown, developing into male-fertile plants which, in turn, have a single (32%) or a pair (4%) of AA (Figs. 5a and 5b). Due to the presence of the selectable marker which is a heterologous gene either conferring glufosinate (Basta) resistance (Fig. 5a) or coding for anthocyanin in the aleurone layer (Fig. 5b), on the AA, the male-fertile offspring of the maintainer are either resistant to glufosinate while the male-sterile offspring are susceptible (Fig. 5a), or the color of the seeds which, when grown, developing into male-fertile plants, is blue while that of the other seeds, when grown, developing into male-sterile plants, is red or white. This differential reaction to glufosinate facilitates the selection of the male-fertile offspring. Undesirable plants which are resistant to glufosinate but have a pair of the alien arm are eliminated by selection against short plants and harvesting only the taller ones. In case of difference in seed color it is possible to select male-fertile maintainer seeds from the female and from the undesirable plants containing the AA in two doses, thus growing only the maintainer line.

The male parent (cv. 'Two') is any normal common wheat, durum wheat or triticale cultivar, which by its nature is homozygous for the male-fertility *Ms-B1* allele.

Thus, by the described method, hybrid seeds of common wheat, durum wheat or triticale are readily and efficiently produced as the F_1 progeny, all of which are heterozygous for the male-sterility alleles (*Ms-B1ms-B1*) and therefore, are male-fertile. So far, all cultivars that were used as male parents were able to fully restore the male fertility of the F_1 hybrid.

Example 2

Testing the expression of male-fertility, brought about by one dose of the male-fertility allele *Ms-S^{s}1* of *Aegilops searsii*, in *ms-B1ms-B1* genotype of common wheat

In order to test the feasibility of the above hybrid production system and maintenance of the male-sterile female parental line, it was first necessary to produce an alien monotelosomic addition line in which chromosomal arm 4S^{s}S of *Aegilops searsii,* carrying the dominant male-fertility allele *Ms-S^{s}1*, was added to the full complement of common wheat homozygous for the male-sterility allele *ms-B1-c.* This was carried out by crossing the ditelosomic 4S^{s}S addition line (derived from crosses of *Aegilops searsii* acc. TE-10 with the common tall wheat cultivar Chinese Spring and kindly provided by Prof. G. Hart, Soil and Crop Science, Texas A & M University, College Station, Texas) which is homozygous for *Ms-B1* and carries two doses of *Ms-S^{s}1*, with the *ms-B1-cms-B1-c* male-sterile genotype of the semi-dwarf common wheat cv. Gamenia (kindly provided by Dr. M. MacKay, curator of the Australian Winter Cereal Collection, RRM 944, Calala Lane, Tamworth, NSW 2340, Australia). The resultant F_1 plants were all heterozygous *Ms-B1ms-B1-c* and carried one additional chromosomal arm of 4S^{s}S with the *Ms-S^{s}1* allele. These plants were selfed and F_2 plants homozygous for *ms-B1-c* (as was indicated by the absence of the DNA marker *Xpsr921*) were selected. All euploid homozygous *ms-B1-cms-B1-c*

plants were, as expected, male-sterile, but those homozygous that had an additional 4S^{s}S chromosomal arm carrying *Ms-S^{s}1,* were all male-fertile, indicating the complete dominance of *Ms-S^{s}1* over two doses of *ms-B1-c*.

Example 3:

Construction of the alien chromosomal arm 4S^{s}S carrying the *Ms-S^{s}1* allele, the heterologous *RIP* gene controlled by a microgametophytic-specific promoter, the heterologous *BaRNase* gene controlled by a macrogametophytic-specific promoter, the heterologous *Barstar* gene controlled by an inducible promoter, and the heterologous *Bar* gene controlled by a constitutive promoter

Plants homozygous for the male-sterility recessive allele *ms-B1-c* and ditelosomic addition for the alien chromosomal arm 4S^{s}S carrying the dominant male-fertility allele *Ms-S^{s}1,* are transfected with the four heterologous genes, *RIP*, controlled by a microgametophytic-specific promoter, *BaRNase*, controlled by a macrogametophytic-specific promoter, *Barstar*, controlled by an inducible promoter, and *Bar* gene, controlled by a plant constitutive promoter. The transgenic plants thus obtained are screened and those carrying the four heterologous genes on the alien chromosomal arm are selected and analyzed for gene expression. Those plants with the desirable heterologous gene expression are selected.

Example 4:

Construction of the alien chromosomal arm 4S^{s}S carrying the *Ms-S^{s}1* allele, the heterologous *BaRNase* gene controlled by a gametophytic-specific promoter, the heterologous *Barstar* gene controlled by an inducible promoter, the heterologous *Bar* gene controlled by a constitutive promoter and the reduced height gene

Plants homozygous for the male-sterility recessive allele *ms-B1-c* and ditelosomic addition for the alien chromosomal arm $4S^sS$ carrying the dominant male-fertility allele *Ms-S^{s}1,* are transfected with the four heterologous genes, *BaRNase*, controlled by a gametophytic-specific promoter, *Barstar*, controlled by an inducible promoter, *Bar*, controlled by a plant constitutive promoter, and a reduced height gene controlled by a constitutive promoter. The transgenic plants thus obtained are screened and those carrying the four heterologous genes on the alien chromosomal arm are selected and analyzed for gene expression. Those plants with the desirable heterologous gene expression are selected.

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[For Pugsley and Oram, 1959; Fossatti and Ingold, 1970; and Driscoll, 1977 see above review by Wilson and Driscoll, 1983].

CLAIMS

1. A plant homozygous for a recessive male-sterility allele and having in the nuclear genome of its cells an alien chromosomal arm carrying:

a male-fertility dominant allele (*Ms*);

a heterologous microgametophytic-suicide (*Migsu*) gene comprising:

i. a microgametophytic-suicide DNA encoding a heterologous protein that is either cytotoxic to or disturbs the metabolism of those pollen grains carrying it thus rendering them inviable; and

ii. a microgametophytic-specific promoter directing the expression of said microgametophytic-suicide DNA selectively in the pollen grains that possess it;

a heterologous macrogametophytic-suicide (*Magsu*) gene comprising:

i. a macrogametophytic-suicide DNA encoding a heterologous protein that is either cytotoxic to or disturbs the metabolism of those embryo sacs carrying it thus rendering them inviable; and

ii. a macrogametophytic-specific promoter directing the expression of said macrogametophytic-suicide DNA selectively in the embryo sacs that possess it;

a heterologous inducible *Anti Migsu* gene that counteracts the *Migsu* gene comprising:

i. a foreign DNA encoding a heterologous protein that binds and inactivates the product of the *Migsu* in the pollen grains;

ii. a chemically inducible constitutive promoter directing the expression of said *Anti Migsu* DNA;

a heterologous selectable marker comprising:

i. a selectable-marker DNA encoding for a heterologous protein that facilitates the selection of plants carrying it; and

ii. a promoter, either constitutive or tissue specific, directing the

expression of said selectable marker DNA in all plant tissues or in a specific tissue, respectively.

2. The plant of claim 1, having on its alien chromosomal arm a heterologous inducible *Anti Magsu* gene instead of *Anti Migsu* gene, that counteracts the *Magsu* gene.

3. A plant homozygous for a recessive male-sterility allele and having in the nuclear genome of its cells an alien chromosomal arm carrying:

a male-fertility dominant allele (*Ms*);

a heterologous gametophytic-suicide gene comprising:

i. a gametophytic-suicide DNA encoding a heterologous protein that is either cytotoxic to or disturbs the metabolism of those gametes carrying it thus rendering them inviable; and

ii. a gametophytic-specific promoter directing the expression of said gametophytic-suicide DNA selectively in the gametes that possess it;

a heterologous inducible *Anti Gsu* gene that counteracts the *Gsu* comprising:

i. a foreign DNA encoding a heterologous protein that binds and inactivates the action of the *Gsu* in the gamete;

ii. a chemically inducible constitutive promoter directing the expression of said *Anti Gsu* DNA;

a heterologous selectable marker comprising:

i. a selectable-marker DNA encoding for a heterologous protein that facilitates the selection of plants carrying it; and

ii. a promoter, either constitutive or tissue specific, directing the expression of said selectable marker DNA in all plant tissues or in a specific tissue, respectively.

4. The plant of claim 3, having also on its alien chromosomal arm a discriminating

gene, by which, plants carrying it in two doses can be discriminated.

5. The plants of claims 1-4, wherein said male-sterility allele is a heterologous gene.

6. The plants of claims 1-4, wherein said male-sterility allele is one of the recessive male-sterility alleles of common wheat, durum wheat, triticale or another species of the Triticeae.

7. The plants of claims 1-4, wherein said male-sterility allele is one of the male-sterility alleles of the *Ms-B1* locus either *ms-B1-a, ms-B1-b*, *ms-B1-c* or any other allele of *MS-B1*.

8. The plants of claims 1-4, wherein said dominant male-fertility allele (*Ms*) is a heterologous gene.

9. The plants of claims 1-4, wherein said dominant male-fertility allele (*Ms*) is one of the male-fertility alleles of the Triticeae.

10. The plants of claims 1-4, wherein said dominant male-fertility allele (*Ms*) is *Ms-S^s1* of chromosome arm 4S^sS of *Aegilops searsii*.

11. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene is any of the genes affecting the viability of male gametes, female gametes or male and female gametes, respectively, that carry it in an active state.

12. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene is any of the genes encoding a protein that is capable of binding a plant essential factor thus causing depletion

of said essential factor and consequently, resulting in reduced plant viability and degeneration of plants tissues.

13. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene encodes avidin or sterptavidin of variant thereof.

14. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene encodes RNase of variant thereof.

15. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene encodes ribosomal-inhibitor protein (RIP) of variant thereof.

16. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-specific promoter is any promoter that is expressed in plants directing gene expression in the microspore, microgametophyte and pollen grain, or in the megaspore, macrogametophyte or embryo sac, or in pollen grain and embryo sac, respectively.

17. The plants of claims 1-4, wherein the said microgametophytic-, macrogametophytic-, or gametophytic-suicide gene encoding for a plant essential factor is counteracted by an exogenous supply of this essential factor.

18. The plant of claim 1-4, wherein the *Anti Migsu*, *Anti Magsu* or *Anti Gsu* genes counteracting the effect of *Migsu*, *Magsu* or *Gsu*, respectively, encode biotin of variant thereof.

19. The plant of claim 1-4, wherein the *Anti Migsu*, *Anti Magsu* or *Anti Gsu* genes counteracting the effect of *Migsu*, *Magsu* or *Gsu*, respectively, encode RNase antisense of variant thereof.

20. The plant of claim 1-4, wherein the *Anti Migsu*, *Anti Magsu* or *Anti Gsu* genes counteracting the effect of *Migsu*, *Magsu* or *Gsu*, respectively, encode RIP antisense of variant thereof.

21. The plant of claims 1-4, wherein the said inducible promoter is any promoter that is expressed in plants directing gene expression in the plants.

22. The plant of claims 1-4, wherein the said inducible promoter is induced by a chemical.

23. The plant of claims 1-4, wherein the said inducible promoter is induced by tetracyclin.

24. The plants of claims 1-4, wherein the heterologous selectable marker is any gene encoding for a heterologous protein that facilitates the selection of plants carrying it.

25. The plants of claims 1-4, wherein the heterologous selectable marker is a specific herbicide resistance gene.

26. The plants of claims 1-4, wherein the specific herbicide resistance gene is *Bar* conferring resistance to glufosinate (Basta).

27. The plants of claims 1-4, wherein the constitutive promoter of the specific herbicide resistance gene is expressed in plants, directing gene expression in the

plants.

28. The plants of claims 1-4, wherein the heterologous selectable marker is a plant reduced height gene reducing the height of plants having it.

29. The plants of claims 1-4, wherein the promoter of the reduced height gene is a constitutive promoter.

30. The plants of claims 1-4, wherein the heterologous selectable marker is a seed-color gene coloring the endosperm.

31. The plants of claims 1-4, wherein the heterologous selectable marker is a seed color gene coloring the aleurone layer of the endosperm.

32. The plants of claims 1-4, wherein the promoter of the seed color gene is an endosperm-specific promoter.

33. The plants of claims 1-4, wherein the promoter of the seed color gene is an aleurone-specific promoter.

34. The plants of claim 4 wherein said heterologous discriminating gene is any gene encoding for a heterologous protein affecting morphological, physiological or biochemical traits by which plants carrying it in two doses can be selected against.

35. The plants of claim 4 wherein said heterologous discriminating gene is a plant reduced height gene causing plants having it in two doses to be short.

36. The plants of claim 4 wherein said heterologous discriminating gene is a

vernalization requirement gene causing plants having it in two doses to develop slower in non vernalizing conditions.

37. The plants of claim 4 wherein said heterologous discriminating gene is a lethal gene causing plants having it in two doses to be albino or to exhibit any other chlorophyll defect from which they either die or develop considerably slower.

38. The plants of claim 4 wherein said heterologous discriminating gene is a lethal gene, when present in two doses, encodes for a compound that kills the cells containing it or slow their development.

39. The plants of claim 4 wherein the discriminating gene has a constitutive promoter.

40. The plants of claim 4 wherein the discriminating gene is a seed trait gene, affecting the morphology or the physiology of seeds having it in two doses.

41. The plants of claim 40 wherein the seed trait gene is a seed color (*Sc*) gene.

42. The plants of claim 40 wherein the seed trait gene is a seed size (*Ss*) gene.

43. The plants of claim 40 wherein the seed trait gene is a seed germination (*Sg*) gene.

44. The plants of claim 40 wherein the seed trait gene is a seed dormancy (*Sd*) gene.

45. The plants of claim 40 wherein the seed trait gene has a seed specific promoter.

46. The plants of claims 1-4, wherein the alien chromosomal arm is any arm of a Triticeae species carrying a dominant male-fertility allele.

47. The plants of claims 1-4, wherein the alien chromosome arm is chromosomal arm $4S^sS$ of *Aegilops searsii*.

48. The plants of claims 1-4, which is selected from any of the Gramineae species.

49. The plants of claims 1-4, which is selected from wheat, triticale, barely, rye, oat, rice, and maize.

50. A method for the maintenance of a male-sterile female parental line of common wheat, durum wheat or triticale for use in the production of hybrid wheat, said method comprising:

(a) selfing a maintainer line as defined in claims 1-4; and

(b) harvesting from the selfing of (a) the progeny seed, all of which are homozygous for said male-sterility allele, and lacking the alien chromosomal arm, said seeds, when grown, developing into said male-sterile female plants.

51. A method for the maintenance of a male-fertile maintainer line of common wheat, durum wheat or triticale for use in the maintenance of male-sterile female line, said method comprising:

(a) treating with a specific chemical that induces the expression of the *Anti Migsu* gene or *Anti Magsu* gene, a maintainer line isogenic to the female line and homozygous for the same *ms-B1* allele of the female line, and having the alien chromosomal arm $4S^sS$ that derived from *Aegilops searsii*, as a monotelosomic addition, herein referred to as AA, carrying the dominant male-fertility allele *Ms-S^s1*, a heterologous

microgametophytic-suicide gene (*Migsu*) a heterologous macrogametophytic-suicide gene (*Magsu*), both heterologous genes are capable of coding for a protein that binds an essential factor thus causing a depletion in this essential factor and eventually death of the cell containing them, or destroys an essential compound and thus causes cell death, controlled by a microgametophytic- or macrogametophytic-specific promoter, respectively, a heterologous inducible *Anti Migsu* or *Anti Magsu* gene that counteracts the action of *Migsu* or *Magsu,* respectively, controlled by an inducible promoter, a heterologous selectable marker controlled either by a constitutive promoter or by a tissue–specific promoter, by which plants having this chromosome arm can be selected, and selfing them;

(b) harvesting from the maintainer of (a) its progeny seed, all of which are homozygous for said male-sterility allele, of which 80% lack the alien chromosome arm, said seeds, when grown, developing into said male-sterile female plants, and 20% have a single alien chromosome arm, said seeds, when grown, developing into male-fertile maintainer plants; and

(c) growing the seeds of (b) and selecting the maintainer plants by the selectable marker.

52. A method for the maintenance of a male-fertile maintainer line of common wheat, durum wheat or triticale for use in the maintenance of male-sterile female line, said method comprising:

(a) treating with a specific chemical that induces the expression of the *Anti Gsu* gene, a maintainer line isogenic to the female line and homozygous for the same *ms-B1* allele of the female line, and having the alien chromosomal arm $4S^{s}S$ that derived from *Aegilops searsii*, as a monotelosomic addition, herein referred to as AA, carrying the dominant

male-fertility allele *Ms-S^{s}1,* a heterologous gametophytic-suicide gene (*Gsu*) capable of coding for a protein that binds an essential factor thus causing a depletion in this essential factor and eventually death of the cell containing it, or destroys an essential compound and thus causes cell death, controlled by a gametophytic-specific promoter, a heterologous inducible *Anti Gsu* gene that counteracts the action of *Gsu,* controlled by an inducible promoter, a heterologous selectable marker controlled either by a constitutive promoter or by a tissue–specific promoter, by which plants having this chromosome arm can be selected, and a lethal gene which is active in two doses but not in a single dose and selfing them;

(b) harvesting from the maintainer of (a) its progeny seed, all of which are homozygous for said male-sterility allele, of which 64% lack the alien chromosome arm, said seeds, when grown, developing into said male-sterile female plants, 32% have a single alien chromosome arm, said seeds, when grown, developing into male-fertile maintainer plants, and 4% have a pair of the alien arm; and

(c) growing the seeds of (b) and selecting the maintainer plants by the selectable marker.

53. A method according to claims 51, 52, wherein the selectable marker gene is a specific herbicide resistance gene (*Hr*), controlled by a constitutive promoter.

54. A method according to claims 51, 52, wherein the selectable marker gene is a seed color gene (*Sc*), coding by an endosperm-specific promoter.

Figure 1a

Figure 1b

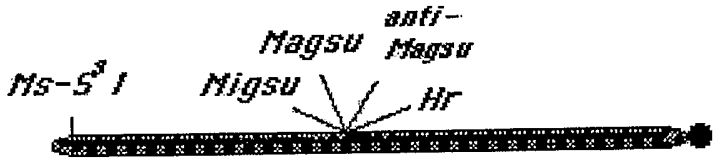

Figure 2a

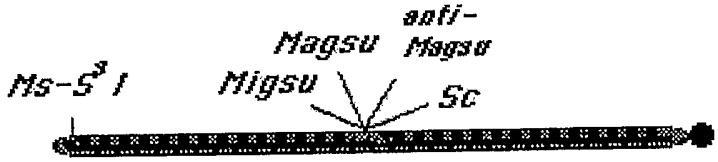

Figure 2b

Figure 3a

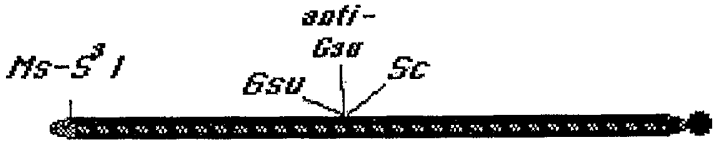

Figure 3b

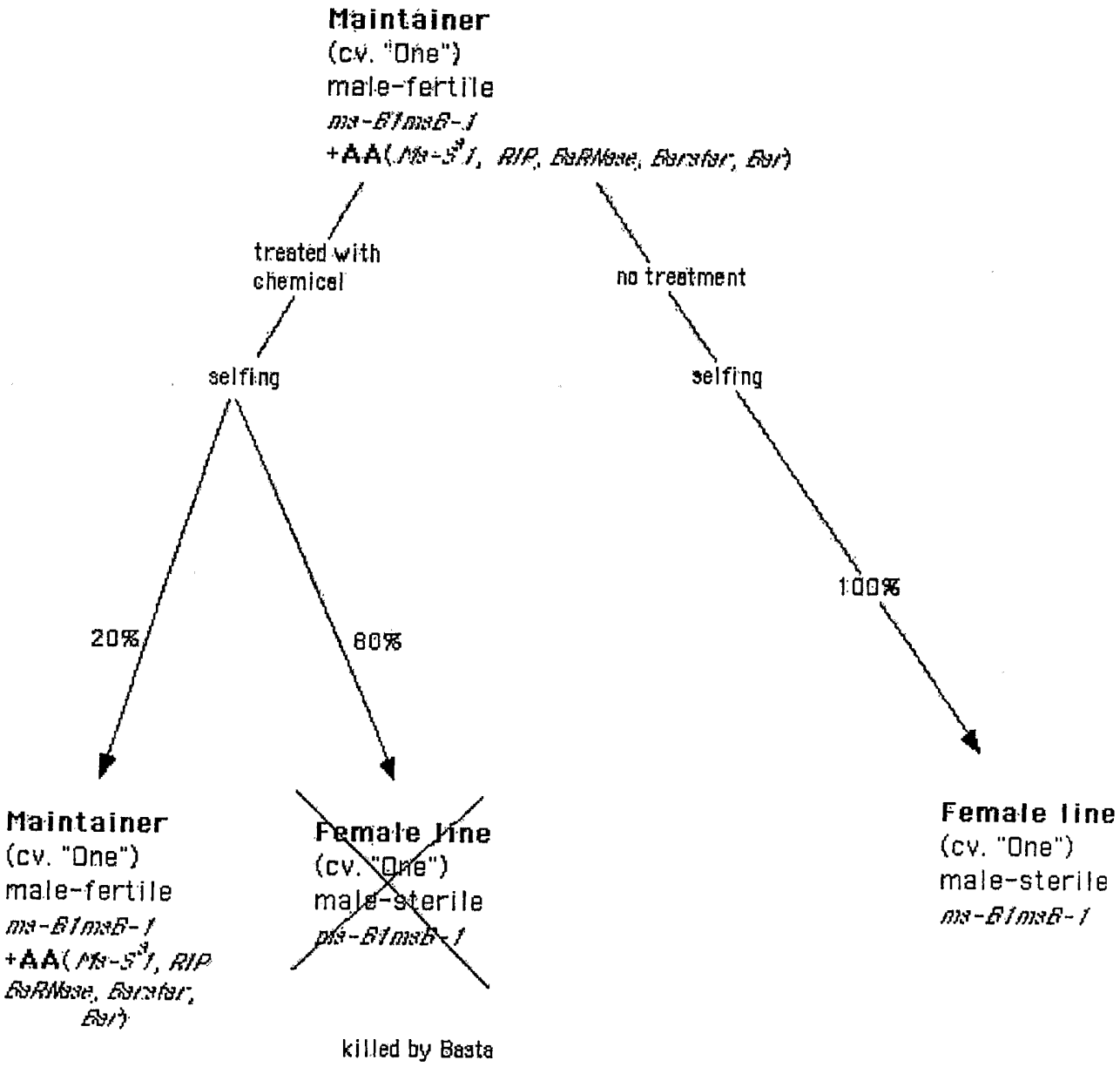
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+AA(Ms-3'1, RIP, BsRNase, Barstar, Bar)
treated with chemical
no treatment
selfing
selfing
20%
80%
100%
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+AA(Ms-3'1, RIP BsRNase, Barstar, Bar)
Female line
(cv. "One")
male-sterile
ms-B1msB-1
killed by Basta
Female line
(cv. "One")
male-sterile
ms-B1msB-1

Figure 4a

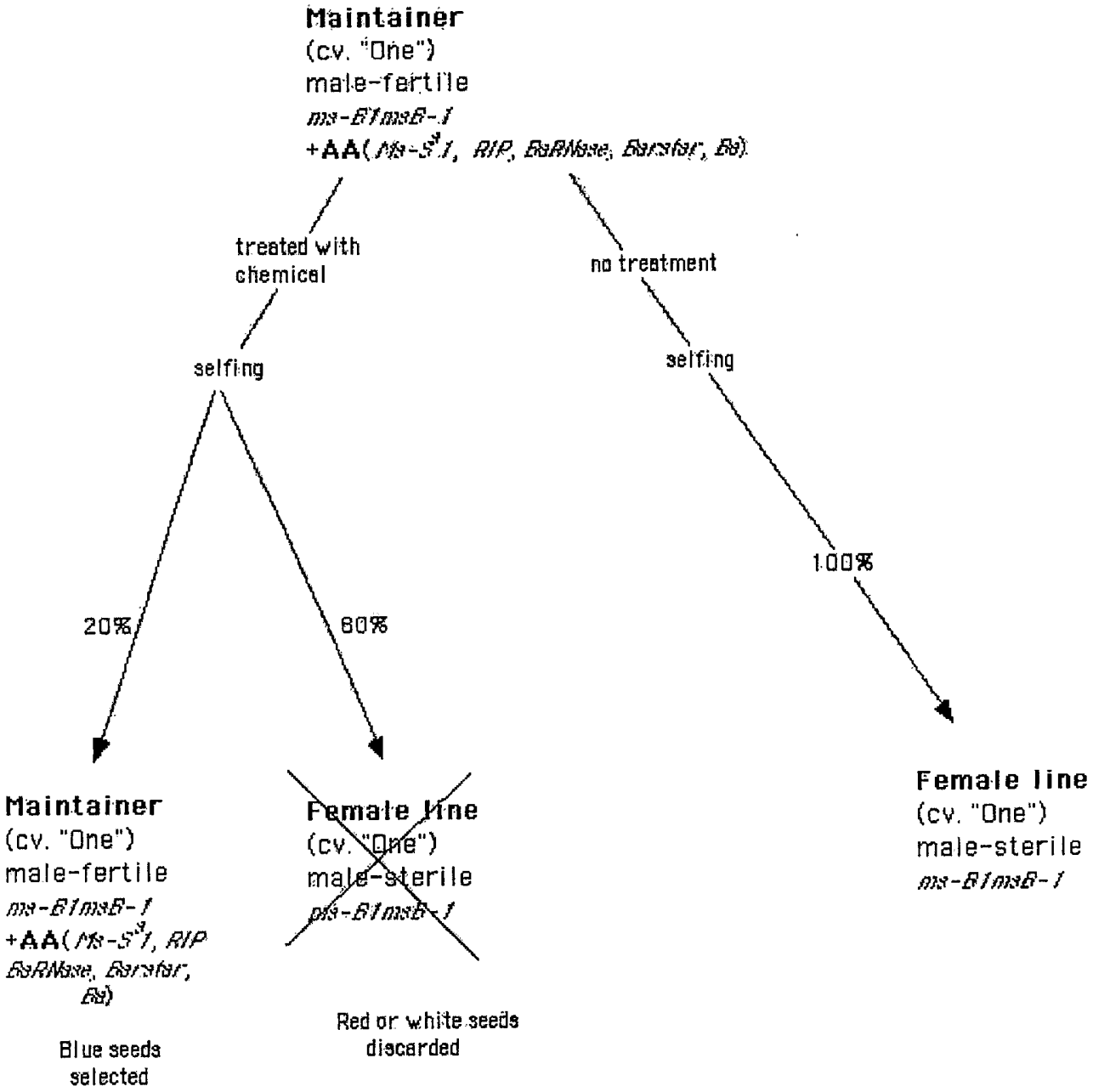
Maintainer
(cv. "One")
male-fertile
ms-E1msE-1
+AA(Ms-S^3.1, RIP, BsRNase, Barstar, Bs)
treated with chemical
no treatment
selfing
selfing
20%
80%
100%
Maintainer
(cv. "One")
male-fertile
ms-E1msE-1
+AA(Ms-S^3.1, RIP BsRNase, Barstar, Bs)
Blue seeds selected
Female line
(cv. "One")
male-sterile
ms-E1msE-1
Red or white seeds discarded
Female line
(cv. "One")
male-sterile
ms-E1msE-1

Figure 4b

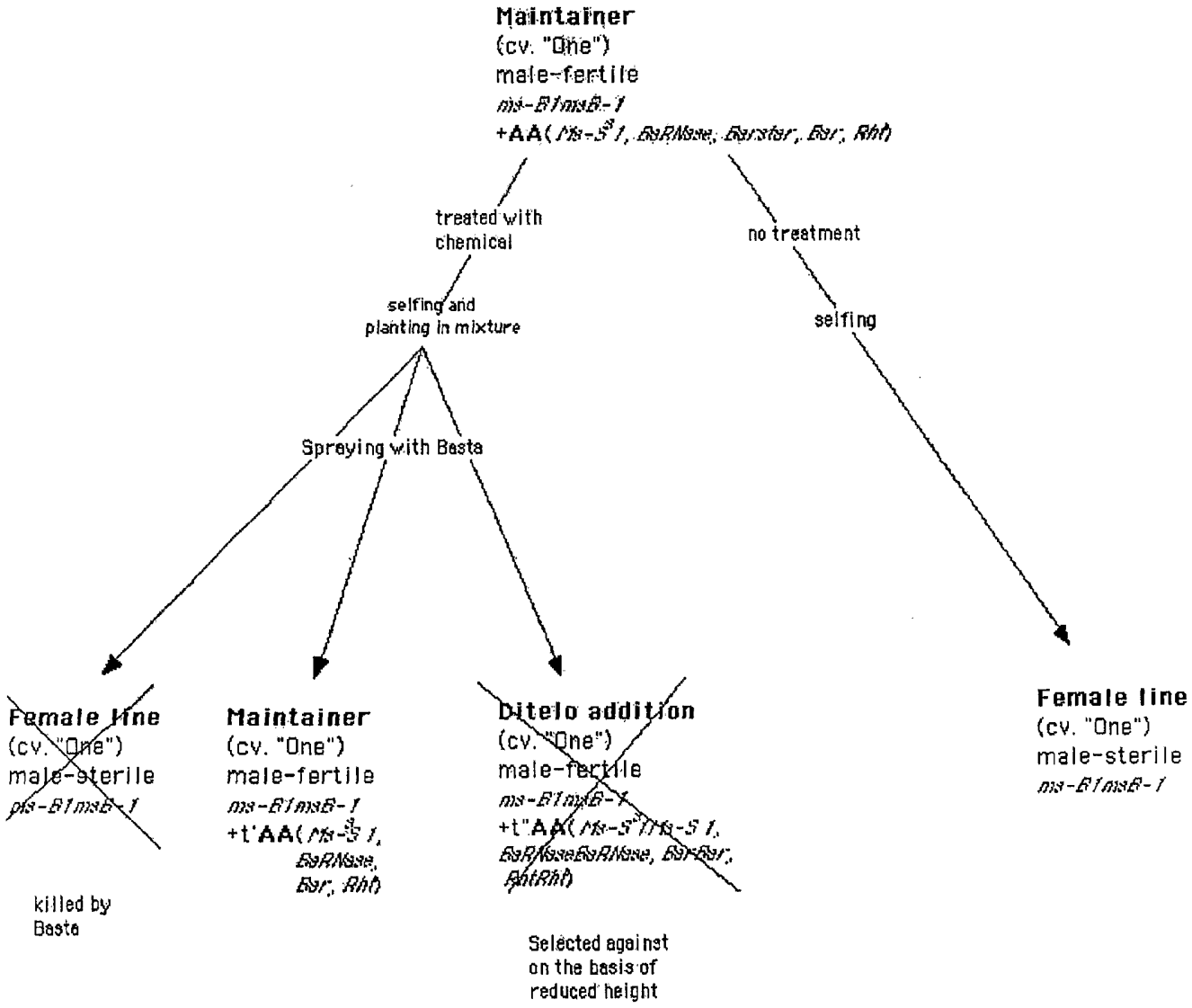
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+AA(Ms-S1, BaRNase, Barstar, Bar, Rht)
treated with chemical
no treatment
selfing and planting in mixture
selfing
Spreying with Basta
Female line
(cv. "One")
male-sterile
ms-B1msB-1
killed by Basta
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+t'AA(Ms-S1, BaRNase, Bar, Rht)
Ditelo addition
(cv. "One")
male-fertile
ms-B1msB-1
+t"AA(Ms-S1Ms-S1, BaRNaseBaRNase, BarBar, RhtRht)
Selected against on the basis of reduced height
Female line
(cv. "One")
male-sterile
ms-B1msB-1

Figure 5a

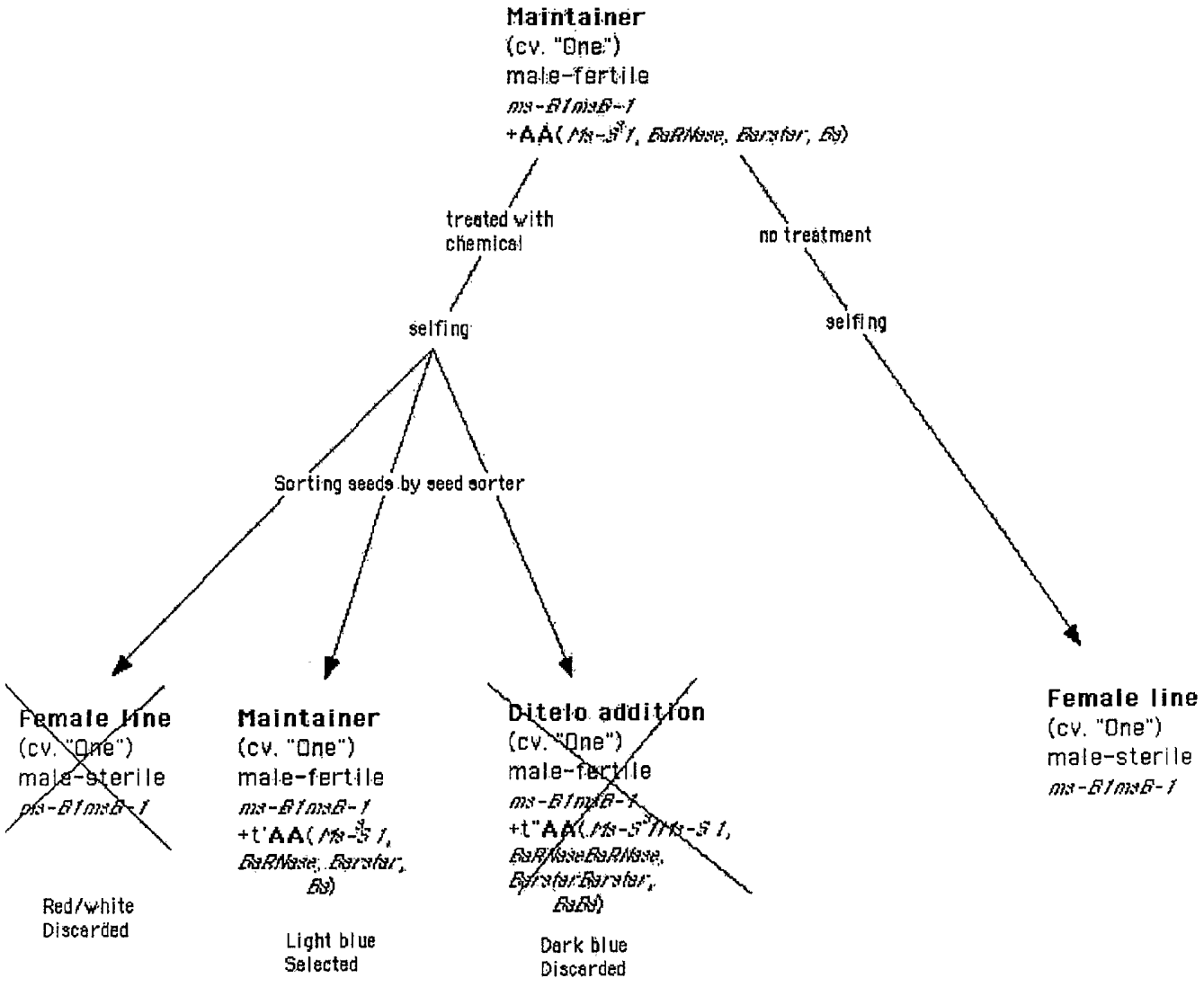
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+AA(ms-3 1, BaRNase, Barstar, Bs)
treated with chemical
no treatment
selfing
selfing
Sorting seeds by seed sorter
Female line
(cv. "One")
male-sterile
ms-B1msB-1
Red/white
Discarded
Maintainer
(cv. "One")
male-fertile
ms-B1msB-1
+t'AA(ms-3 1, BaRNase, Barstar, Bs)
Light blue
Selected
Ditelo addition
(cv. "One")
male-fertile
ms-B1msB-1
+t"AA(ms-3 1/ms-3 1, BaRNaseBaRNase, Barstar/Barstar, BsBs)
Dark blue
Discarded
Female line
(cv. "One")
male-sterile
ms-B1msB-1

Figure 5b