



# CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAcability

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# 1 Summary

A study performed by Agro-PM will explore the challenges, gaps, and opportunities linked to the production of CMS crops on the basis of evidence from printed publications and internet sites, addressing problem fields as (i) impact of the different resistance management strategies (separate refuges vs. random mixtures) on the development of Bt-resistance in maize, (ii) stability of the CMS trait, properties of the various sterile cytoplasm (T, C, and S) that exist in maize, (iii) suboptimum seed set brought about by insufficient supply of exogenous pollen to the CMS plants, (iv) prospects for enhancing the quality (oil content, composition of carbohydrates) and boosting the grain yield via xenia for different crops, (v) experiences thus far with CMS crops (maize, rapeseed, rye etc.).

This review on biological gene containment strategies with special focus on CMS maize, cleistogamic rapeseed and chloroplast transformation is provisional, because field experiments within WP1 will provide the material to explore the challenges, gaps, and opportunities linked to the production of CMS crops for submission of a research paper to a scientific international journal.

## 2 REVIEW OF CMS, CLEISTOGAMY, AND CHLOROPLAST TRANSFORMATION AS BIOLOGICAL CONTAINMENT STRATEGIES

### 2.1 Transgene containment in maize by growing mixtures of CMS GM plants and male-fertile non-GM plants

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#### Dispersal of maize pollen

Maize (*Zea mays* L.) is a wind-pollinated grass. Like other wind-pollinated species, it produces huge amounts of pollen. Even though the pollen grains are voluminous (about 100 µm diameter), they can be transported over long distances in a viable state (reviewed by Feil and Schmid, 2002). Among other things, the frequency of outcrossing depends on the longevity of the foreign pollen (maize pollen has been reported to function for a few minutes to 24 hours), the pollination type of the crop (self-pollination prevents outcrossing), and the extent of pollen competition (ratio of foreign to own pollen) in the pollen receptor crop (Feil and Schmid, 2002). If maize pollen is viable for one day (Purseglove, 1972) and if the wind velocity is high, then successful pollination may be possible at a distance of almost 1000 km from the pollen source (Emberlin et al., 1999). Maize is predominantly cross-pollinated, whereby the rate of cross-fertilization varies from 85 to 99% (Purseglove, 1972). Despite the potentially long lifespan of maize pollen, the fact that it can remain airborne over relatively long periods, as well as the reproduction biology of maize, outcrossing seldom occurs at long distances from the pollen emitter due to strong pollen competition. Most outcrossing events take place in the immediate vicinity of the pollen donor (reviewed by Treu and Emberlin, 2000; Eastham and Sweet, 2002; Feil and Schmid, 2002), but outcrossing has been observed to occur 2 km from the pollen source (Fleischmann, 1942).

Cultivated maize is derived from or is closely related to the diploid species teosinte (*Zea mexicana* [Schrad.] Reeves & Mangel.). Maize and teosinte are sexually compatible and produce fertile hybrids. Thus, it cannot be ruled out that transgenes migrate into the wild relative teosinte and enhance its weediness. This potential threat is geographically restricted, because teosinte grows only in Central America. In the ancient maize production areas of Mexico and Guatemala, pollen-mediated gene transfer from GM maize (as well as from modern non-GM maize cultivars) to landraces can be a problem (Luna V. et al., 2001).

#### Male sterility as an option for controlling the flow of GM pollen

The concerns put forward by the opponents of gene technology should be taken seriously and measures for preventing the escape of transgenes from GM fields via pollen flow sought. Spatial isolation and border rows may help reduce the frequency of outcrossing, but it is evident that these measures cannot reliably prevent unwanted pollination. Therefore, several other strategies for controlling the dispersal of transgenes have been suggested, including apomixis, cleistogamy, and plastid transformation (Daniell, 2002; Eastham and Sweet, 2002). Some authors stated that male sterility can also be used to eliminate the risks associated with the release of GM pollen, but this option has received little attention, for the most part, because GM pollen control through male sterility is considered to be applicable only to crops, the seeds or fruits of which are not the harvested end

However, it is by all means possible to produce grains on male-sterile GM plants. Feil and Stamp (2001) proposed that the dispersal of GM maize pollen be controlled by growing 80:20% or similar mixtures of cytoplasmic male-sterile (CMS) GM maize and male-fertile conventionally bred maize, whereby the latter component acts as pollen donor for the entire stand. The pollen required for fertilization may either come from the male-fertile counterpart of the CMS hybrid (= isogenic pollination) or from a phenologically compatible foreign pollen donor (= non-isogenic pollination). As will be explained below, the latter approach offers the opportunity of exploiting xenia effects on grain yield.

Since the fertility of the GM hybrid plants is not restored, they release no pollen or no functional pollen and, thus, the transgenes cannot escape from the GM maize field. One major advantage of this GM maize production system over alternative approaches to transgene containment is evident: it can be put into practice immediately, because it is unnecessary to genetically engineer maize for male sterility and inexpensive seed of unrestored CMS hybrids can be produced in large quantities using existing standard technology.

### **What is CMS?**

Cytoplasmic male sterility (CMS) in plants is defined as the maternally inherited inability to produce functional pollen (male gametes) in individuals from an otherwise hermaphroditic species. Specific mutations in mitochondrial DNA (mtDNA) are responsible for a dysfunction in the respiratory metabolism occurring in anther-tapetum cells during sporogenesis (Schnable and Wise, 1998; Budar et al., 2003; Cilier et al., 2004). This disturbance inhibits the production of viable pollen. The CMS trait has been observed in more than 150 plant species (Laser and Lersten, 1972) and has been characterized in many crop species, including beet, carrot, maize, onion, petunia, rice, rye, sorghum, sunflower and wheat.

### **Cytoplasmic male sterility in maize**

Rhoades (1933) was the first to describe cytoplasmic male sterility (CMS). Breeders were quick to realize that the cytoplasmic male-sterile trait could be used to enable crosses without manual detasseling, thereby saving time and money.

### **The CMS types**

Hundreds of CMS sources were rapidly identified in the following years. There are three CMS types in which the sporogenesis stage is affected (Duvick, 1965, Beckett, 1971):

CMS-T {Texas} (Rogers and Edwardson, 1952),

CMS-C {Charrua} (Beckett, 1971),

CMS-S {USDA} (Jenkins in Jones et al., 1957).

Today, the above CMS types still represent the main classes of male-sterile cytoplasm, although many new sources have been found. CMS-T affects the earliest stages of sporogenesis and therefore leads to sporophytic sterility. This results in a complete and very stable sterility, in contrast to CMS-S, which occurs later (gametophytic sterility) and is thus prone to spontaneous reversal to fertility.

### **Restoration of fertility**

The three main CMS types are defined according to the specific restorer genes (Rf) that counteract the CMS trait and restore fertility. The diversity in restoration systems extends to the number of restorer genes. In some systems, one or two major restorer loci confer complete restoration. In others, full restoration depends on the concerted action of a number of genes, many of which have only small incremental effects (Schnable and Wise, 1998).

In CMS-S maize, male sterility and fertility are determined by mitochondrial nuclear interactions in the haploid male gametophyte (Buchert, 1961; Kamps et al., 1996). The nuclear allele that restores fertility in CMS-S plants is referred to Rf3 (mapped on chromosome 2) (Duvick, 1965). Several Rf alleles are found at the rf3 locus, whereas several other Rf alleles are at other loci, which are also necessary for restoration.

In CMS-T maize, fertility restoration is sporophytic. Rf1 (on chromosome 3) and Rf2 (on chromosome 9) are unlinked restorers of fertility genes required to suppress or compensate for the CMS effects of the T-urf13 mitochondrial gene in CMS-T (Duvick, 1965). Nevertheless, the rare Rf8 and Rf\* alleles can each substitute for the Rf1 allele to partially restore fertility in the presence of Rf2 (Dill et al., 1997; Wise et al., 1999). Rf2 is present in almost all maize lines. This suggests that the Rf2 gene has been maintained by selection during evolution and must, therefore, have a significant function other than restoration (Schnable and Wise, 1994). In CMS-C maize, fertility restoration is sporophytic too. Several nuclear restorer-of-fertility genes (Rf4, Rf5, Rf6, etc.) seem to be involved in the restoration mechanism in this type of CMS (Kheyr-Pour et al., 1981; Josephson et al., 1978; Gabay-Laughnan and Laughnan, 1994). The exact interaction between nuclear and cytoplasmic genes is still unclear.

Male fertility results from a nucleo-mitochondrial conflict between nuclear restorer-of-fertility alleles and mitochondrial CMS genes (Budar et al., 2003). Specific nuclear restorer-of-fertility genes (Rf genes), carried by pollen, counteract the mitochondrial dysfunction caused by the altered mtDNA and therefore reverse the progeny to fertility (Gabay-Laughnan and Laughnan, 1994). For example, in the presence of the Rf1 restorer, the accumulation of 1.6-kb and 0.6 kb T-urf13 transcripts in T-CMS maize is greatly enhanced and the accumulation of the 13-kDa URF13 protein is reduced (Wise et al., 1996). Alternatively, loss of a nuclear function essential for the expression of CMS may also condition male fertility (Wen et al., 2003).

Only one restorer gene has been cloned to date. The Rf2 gene, required for fertility restoration in CMS-T maize, encodes a predicted protein with a high degree of sequence similarity to mammalian mitochondrial aldehyde dehydrogenases (Cui et al., 1996). Based on this finding, several hypotheses have been put forward to explain the mechanism, by which this gene functions. According to the metabolic hypothesis, URF13 alters mitochondrial function so that additional aldehydes are produced. The Rf2 protein would detoxify these aldehydes or catalyze their oxidation to compounds that are essential in plants harboring T-cytoplasm mitochondria.

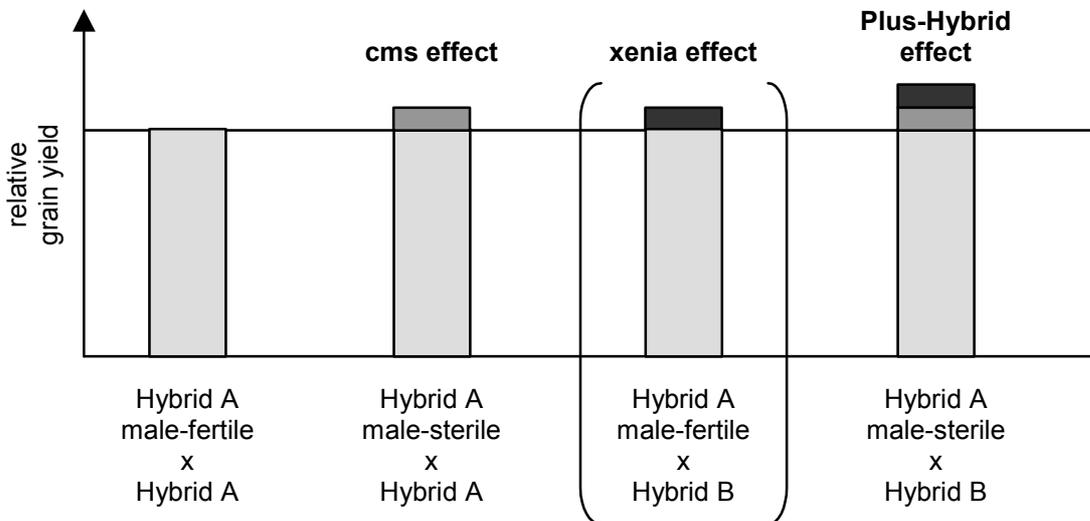
### ***Current and future uses of CMS in maize production***

Cytoplasmic male sterility has been widely used in the commercial production of hybrid seeds, according to a method that had been developed to restore the fertility with nuclear restorer-of-fertility genes. In 1970, 85% of US hybrid maize was produced with CMS-T (Dewey and Korth, 1994). The CMS-T type was the most extensively used because of the ease of finding suitable restorer genotypes and because it is a reliable and stable source of CMS for seed production (stable absence of pollen). However, in the early 1970s, a new race of *Bipolaris maydis* caused an epidemic of Southern Corn Leaf Blight in maize produced with CMS-T genotypes. Both the male sterility and the disease susceptibility traits appear to be closely linked and are associated with a mitochondrial gene, T-urf13, which encodes a 13-kilo Dalton polypeptide (URF13) (Levings, 1990). An interaction between fungal toxins and URF13, which results in permeabilization of the inner mitochondrial membrane, accounts for the specific susceptibility to the fungal pathogens. This epidemic involved a loss of yield of about 50% due to this susceptibility of CMS-T (Braun et al., 1992) and an immediate reversal to the use of normal male-fertile cytoplasm. However, because of the high costs of producing hybrid seed on manually detasseled plants, some of today's breeders are relying on CMS to produce hybrid seed.

A potential future application of CMS is the Plus-Hybrid system (Weingartner et al., 2002). Figure 1 illustrates that the Plus-Hybrid effect on grain yield results from the combined effects of male sterility and xenia. In the Plus Hybrid system, the male-sterile plants may be transgenic and the male-fertile plants conventionally bred (Feil et al., 2003). If this concept is applied, the Plus-Hybrid system offers these advantages:

- containment of the transgenes integrated in the male-sterile plants
- increased grain yield

Potential problems are:  
 spontaneous reversal to fertility  
 existence of restorer-of-fertility genes (Rf)



**Figure 1: The Plus-Hybrid effect on grain yield. The third column (in parentheses) refers to a system that is impracticable in the field due to uncontrolled pollination.**

**Effect of male sterility on grain yield**

As early as in the 19<sup>th</sup> century, long before the discovery of CMS and the implementation of hybrid seeds in commodity maize production, Watson (1893) presented results of comparative trials with manually detasseled and untreated maize, indicating that detasseling may increase the grain yield by 20 and 50%. Watson concluded that the over-production of pollen in a maize field is an exhaustive process. With the production of hybrid seed in the 1930s, interest in the grain yield of male-sterile breeding material after detasseling grew rapidly (Leonard and Kiesselbach, 1932; Dungan and Woodworth, 1939; Kiesselbach, 1945; Grogan, 1956). Not long after the discovery of CMS, breeders began to compare the effects of detasseling and CMS on grain yield and other agronomic attributes (Rogers and Edwardson, 1952; Duvick, 1958; Duvick, 1959; Everett, 1960; Chinwuba et al., 1961; Sanford et al., 1965). At first, single-cross hybrids were the subject of interest, because they were preferred as female parents in the production of hybrids. However, as soon as improved high-yielding inbred lines were available, researchers started to concentrate on these (Grogan et al., 1971). After the epidemic of *Helminthosporium maydis* in the USA, when hybrid seed production relying on CMS-T was discontinued, the number of published studies on this topic decreased dramatically. In recent years, there has been a renewed interest in the use of CMS in commercial maize production (Stamp et al., 2000; Weingartner et al., 2002a; Weingartner et al., 2002b).

The effects of detasseling and CMS on grain yield were inconsistent, but usually increases in yield were observed. Most researchers found a highly significant dependence on the investigated genotype and/or the environmental conditions (Duvick, 1965). The physiological background of the positive grain yield response to CMS is not yet fully understood. In the experiments of Weingartner et al. (2002a), CMS increased the number of kernels per unit area, suggesting that CMS favors the survival of kernel primordia, possibly because male sterility promotes the allocation of nutrients such as nitrogen and other growth factors to the female organs.

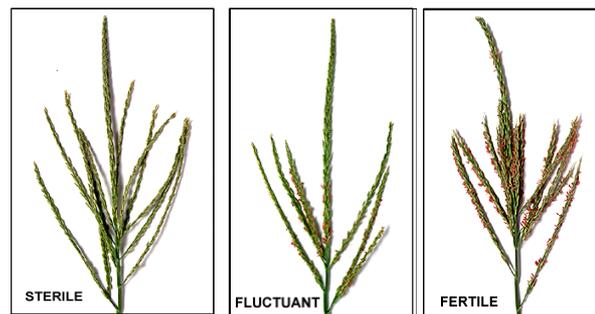
**Effect of xenia on grain yield**

The maize kernel can be divided into three main parts: the embryo (about 10% of the kernel moisture-free weight), the endosperm (about 85%), and the pericarp together with remnants of the nucellus and seed coats and the pedicel (remaining 5%) (Kiesselbach, 1949). In the

embryo, 50% of the genome originates from the pollen, and in the endosperm 33% of the genome originates from the pollinator. In conventional hybrid seed production, one can take advantage of the heterosis by crossing two distantly related inbred lines. The offspring represents the F1 generation. In conventional commodity maize production systems only one variety is planted. The harvested kernels represent the F2 generation, in which the extent of homozygosity, i.e. the extent inbred depression, is higher than in the F1 generation due to isogenic pollination. In non-isogenic pollination, paternal effects, encoded in pollen genes, can influence the size (and quality) of the seeds and, thus, increase grain yield. One of the first studies on this topic was carried out by Kiesselbach and Cook (1924), who observed xenia effects in various maize varieties. In 1998, Bulant and Gallais reported similar findings for inbred lines and single-cross hybrids. These authors pointed out that yield increases are more likely to occur when the pollen parent is not closely related to the seed parent.

### **Stability of the CMS trait**

To the best of our knowledge, no studies have been published that focus on the stability of male sterility in maize. Publications dealing with CMS in other crops (rice, cotton, tomato) mention temperature, photoperiod, and water availability as factors that can influence the stability of male sterility. It is generally agreed that sterility increases with increasing temperature and day length (Marshall et al., 1974; Latha et al., 2004; Sawhney, 2004). Several breeders also reported that spontaneous reversal to fertility can occur late in the growing season when the days become shorter. The reversal can be total or partial; the mechanisms involved are still unclear. The figures below show that the reversal may occur to varying extents.



The tassels of completely sterile plants have no anthers and, therefore, are unable to produce pollen. Unstable CMS plants may have either a so-called “fluctuant tassel” with anthers only on the secondary branches or anthers on the whole tassel. In both cases, the anthers can produce more or less viable pollen.

### **Implementation of the system**

Is the proposed system for controlling GM pollen really feasible? From the farmers’ point of view, the grain yield is the crucial factor. Mixtures of CMS hybrids and male-fertile pollinator plants have already been grown on a large area (> 400,000 ha in 1999) in the USA for the production of high-oil maize. As in the system proposed for GM pollen control, a blend of two types of maize is planted in the so-called TopCross system. One type, the grain parent, is the CMS version of an elite hybrid. It represents 92% of the seed in the blend, while the second type, the pollinator, represents only 8% of the seed. The pollinator contributes little to grain yield, but its pollen contains genes that produce kernels with a higher content of oil. Experiments and on-farm studies indicate that the blends yield less than the check hybrids, but there is little evidence that this is due to a reduced kernel set caused by insufficient pollen supply (Thomison et al., 2002).

Weingartner et al. (2002a) tested several combinations of European CMS hybrids and pollinator hybrids and found that the best combination increased grain yield by more than 20% compared with the male-fertile version of the cms hybrid. Large-strip mixture trials, conducted with local germplasm in the USA and Switzerland, confirmed the observations

made in small-scale experiments, but the European maize seemed to be more responsive to the Plus-Hybrid system than the US maize (Weingartner et al., 2002b). The authors attributed this to differences in the genetic distance between the components of the mixtures and to the fact that the male-sterile cytoplasm used was not the same in Europe and the USA.

In summary, there is experimental evidence that controlling the spread of transgenes by cultivating associations of cms GM hybrids and non-GM male-fertile pollinator hybrids does not necessarily lead to lower grain yields. Quite the contrary, marked increases in grain yield can be achieved if the male-sterile GM hybrids are mixed with carefully selected non-GM pollen donors. The Plus-Hybrid systems used by Weingartner et al. (2002b) contained 15 and 20% male-fertile plants, respectively. Since maize produces a large surplus of pollen, a portion of only about 10% male-fertile non-GM maize may suffice to ensure maximum grain set in the whole stand (Poehlman and Sleper, 1995). However, a higher portion of pollen-producing plants and the blending of two or more pollinator hybrids may be advisable to reduce the risk of crop failure.

### Outlook

In principle, the proposed system is applicable to all cross-pollinating crops that produce a sufficient surplus of pollen, such as rape seed (*Brassica napus* L.) and rye (*Cereale secale* L.). In the 1999/2000 cropping season, 17, 8, and 5% of the total rape seed hectareage in France, the UK and, Denmark, respectively, was planted with 70:30% or 80:20% mixtures of un-restored male-sterile hybrids and male-fertile pollinator plants (Pinochet and Bertrand, 2000). Thus, it may be feasible to produce GM rape seed in varietal associations that comprise a GM hybrid without fertility restoration and one or two male-fertile non-GM pollen donors in order to control the spread of transgenes via pollen flow. It is still unclear, however, whether the yield of rape seed is similarly responsive to CMS and xenia as that of maize.

Needless to say, growing blends of genetically dissimilar hybrids rather than genetically uniform hybrids will not be met with enthusiasm by breeders and farmers, because it complicates the breeding and production of GM crops. It is, however, assumed that breeders and farmers will be less skeptical if the yield advantages over male-fertile non-GM hybrids outweigh the additional expenditures.

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## 2.2 Cleistogamy in oilseed rape

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Conventional oilseed rape is a hermaphrodite plant with a highly variable autogamy rate. The autogamy rate seems to mainly depend on the genotype but also on the environmental conditions. Intraspecific and interspecific cross pollination can occur and pollen transfer is partly due, in various proportions, to wind or pollinating insects according to the situation.

The a priori interest of the cleistogamy (closed flowers) lies in two ways:

to favour self pollination by limiting allopollen deposition on the stigma

to reduce gene flow by limiting pollen dispersal.

The cleistogamous trait has been selected from induced mutagenesis in oilseed rape and patented (Renard and Tanguy, 1997). This trait is controlled, in major part, by one gene (Clg1).

There is only restricted literature on cleistogamic rapeseed lines, because INRA Rennes's team is the only one in the world having such kind of biological tool to reduce gene flow. This material has never been given outside the INRA group before the COEXTRA Project

Since 1998, several field experiments have been carried on cleistogamous lines of oilseed rape to study their impact on pollination. In particular the evaluation of the benefits brought by cleistogamy to increase harvest purity in several cropping systems has been simulated using a spatio-temporal model: the GENESYS-RAPE (Colbach et al, 2001; Fargue, 2003; Colbach et al, 2005). Nevertheless, some results were impaired by the lack of stability of cleistogamy in the newly created lines (Fargue et al., 2006). Therefore, a positive effect of the cleistogamy on the autogamy rate was not clearly demonstrated.

Presently, lines with a good stability exist and the aim is to verify the impact of cleistogamy both on autogamy and pollen dispersal limitation under several climatic and agricultural conditions or cultivation techniques. Preliminary results from 2004-05 have shown that the main effect is a reduction of pollen dispersal. Nevertheless, autogamy seems not to be increased by cleistogamy, in such experiments conducted with only 2% of cleistogamous plants among classical oilseed rape canopy, that is when cleistogamous flowers are under a very high pressure of external pollen. Such results have to be improved in future field experimentations.

Another aim is to verify the stability of the cleistogamous trait under several environmental conditions in order to be certain of its interest in various situations. Multilocalized field experiments are of prime importance to test stability. From the integration of these results into the GENESYS-Rape model, the benefits of cleistogamy could be considered. During the present experimental year 2 cleistogamous genotypes are being tested in the field at 5 locations (2 in UK, 2 in Germany and 1 in France in WP1 of Co Extra), and under 2 treatments (with or without application of a growth regulator when the growth is restarting at the end of winter).

On the other hand, studies have also been undertaken on the impact of the closing of the flowers on the pollinating insects behaviour. It has been shown that honeybees and bumblebees visit closed flowers (Pierre and Renard, 1999) but do not open them to collect pollen. They visit cleistogamous flowers only to probe nectar (giving honey) by gaps existing at the base of the corolla. Doing so, they have no contacts with the reproductive organs of the closed flower. This typical behaviour is observed when they are foraging in a homogeneously cleistogamous field. In that case, they do not imperil self-pollination and have not a high impact on the pollen dispersal (Pierre et al., 2002). Nevertheless, their behaviour is still unknown when very few cleistogamous plants are placed in a conventional field and reciprocally, i.e. when volunteers have a floral morphology unlike the major plants

present in the field. Thus the relations between honeybees and both cleistogamous and conventional flowers have to be taken in account to reliably evaluate the efficiency of cleistogamy on plant containment and coexistence. Presently, behavioural studies of foraging honeybees are performed by INRA Rennes in fields sown with 98% of classical oilseed rape and 2% of cleistogamous one.

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## 2.3 Evaluation of plastid transformation as containment strategy

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Plants with transgenic plastid genomes (“transplastomic” plants) offer an attractive alternative to conventional transgenic plants (Bock, 2001, 2004; Ruf et al., 2001). Besides several technological attractions (e. g., high-level transgene expression, transgene stacking in operons, absence of epigenetic transgene instability), the increased biosafety provided by transplastomic plants is particularly important to future applications of genetic engineering in agriculture and biotechnology: In most crops, plastids are maternally inherited excluding plastid-localized transgenes from pollen transmission and thus greatly reducing the risk of unwanted transgene transfer from fields with GM plants to neighboring fields with non-GM plants. How absolute maternal inheritance can be is a highly controversial issue (Huang et al., 2003; Wang et al., 2004; Maliga, 2004; Bock 2005) and reliable quantitative data on maternal inheritance versus possible occasional paternal transmission (“paternal leakage”) are largely lacking. As such data are of outstanding importance to the critical evaluation of the biosafety of transplastomic technologies as well as to the mathematic modeling of possible outcrossing scenarios, we have begun to develop an experimental system suitable to determine the frequency of occasional paternal transmission of plastid transgenes. The plant species used in these studies is tobacco for various reasons: (i) it is currently the only species in which plastid transformation is a routine technique, (ii) it produces enormous numbers of seeds per plant as required to be able to detect even very low frequencies of paternal transmission, (iii) tobacco seeds can be easily harvested in very large quantities (from dried pods) and conveniently assayed (by germination on filter paper wetted with the selecting antibiotic), (iv) unlike many other crop species, tobacco displays high sensitivity to prokaryotic translational inhibitors providing a simple and clear visual assay system for paternal transmission, and (v) tobacco is one of the prime candidate species in agricultural biotechnology with numerous pharmaceutical-producing lines being already grown in large-scale field trials in the US.

The experimental strategies are summarized in Figure 1. The system involves large-scale genetic crosses between a wild-type plant as female parent and a transplastomic plant as a paternal parent. To facilitate highly sensitive detection of occasional paternal chloroplast transmission, the transplastomic parent contains an antibiotic resistance gene (conferring spectinomycin and streptomycin resistances) and a visual reporter of gene expression as marker (GFP). The wild-type parent should be male sterile to facilitate directed crosses at large-scale. Thus, the design of the system involves: (i) selection of male sterile recipient lines, (ii) generation of transplastomic lines as pollen donors which carry plastid-localized antibiotic resistance genes, (iii) large-scale genetic crosses between the two lines followed by selection of the progeny for antibiotic resistance and (iv) genetic and molecular analyses to distinguish pollen transfer from spontaneous antibiotic resistance as acquired through specific point mutations in the target molecules of the antibiotics. The antibiotics used for selection are spectinomycin and streptomycin, two inhibitors of prokaryotic translation whose specific target in plant cells is the chloroplast ribosome.

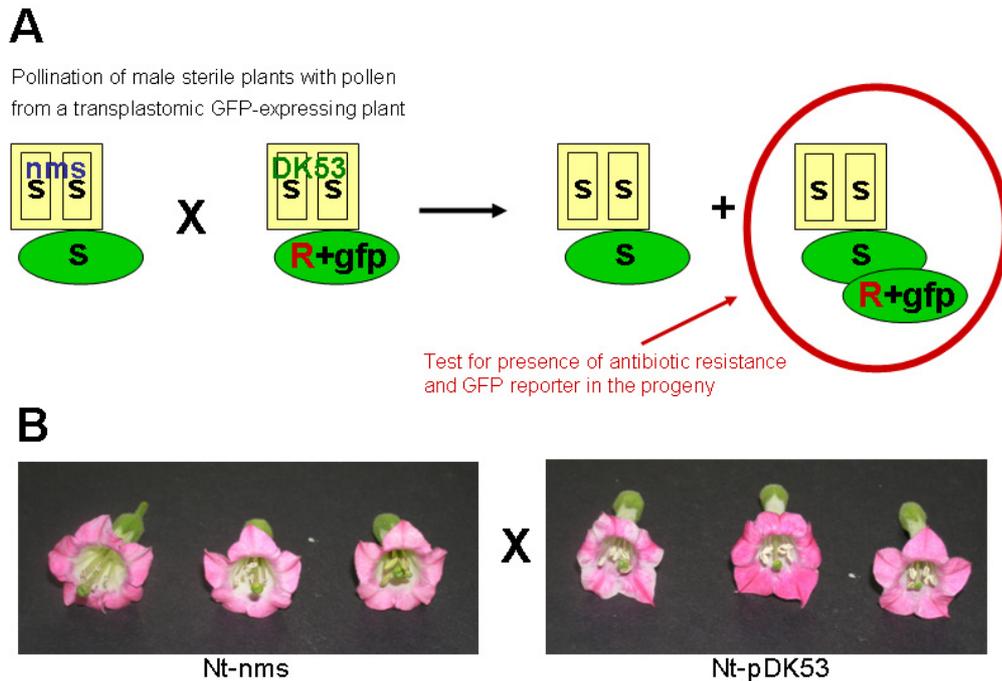


Figure 1: Experimental strategy and current status of the development of an experimental system for evaluating the biosafety of transplastomic plants. **(A)** Selection strategy for occasional parental chloroplast transmission (see text for details). **(B)** Flowers from isolated male sterile plants (left; *nms* for nuclear male sterility) and from transplastomic male fertile plants harbouring both an antibiotic resistance gene and the *gfp* reporter gene in their plastid genome.

Using somaclonal variation and tissue culture procedures, we successfully isolated several male sterile tobacco lines (Fig. 1B) which produced no viable pollen and whose sexual reproduction thus was dependent on cross-pollination. To obtain a suitable paternal parent, we constructed a chloroplast transformation vector containing both a spectinomycin/streptomycin resistance gene and a reporter gene for *in vivo* visualization of gene expression (*gfp*; Fig. 1A). Chloroplast transformation experiments were conducted and transplastomic lines were successfully isolated and brought to homoplasmy (Bock, 2001). Importantly, transplastomic lines proved male fertile (Fig. 1B) and thus are now used to conduct large-scale crosses to the male sterile maternal parent line (Fig. 1A). Seedlings from the crosses will be subjected to selection for spectinomycin and streptomycin resistance to identify events of occasional paternal transmission. Seedlings resulting from normal maternal chloroplast inheritance will bleach out on antibiotic-containing medium, whereas only those that have received paternal plastids will be green or at least contain green sectors.

In sum, the system proposed to be developed is well underway and the essential components have already been successfully generated. First results from the screens for paternal inheritance will be available in one to two years. The system can not only be used to assess the level of containment provided by chloroplast transformation (by determining the frequency of occasional parental chloroplast inheritance), but it will also be suitable to quantitatively determine the influence of environmental factors (e. g. abiotic and biotic stresses) on plastid transgene inheritance.

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