

Evaluating biological containment strategies for pollen-mediated gene flow

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Several biological containment methods have been developed to reduce pollen dispersal; many of them only have a proof of concept in a model plant species. This review focuses on biological containment measures which were tested for their long-term efficiency at the greenhouse or field scale level, *i.e.* plastid transformation, transgene excision, cleistogamy and cytoplasmic male sterility (CMS). Pollen-mediated gene transfer in transplastomic tobacco could occur at very low frequencies if the predominant mode of inheritance is maternal. Transgene excision from tobacco pollen can be made highly efficient by coexpression of two recombinases. For cleistogamous oilseed rape it was shown that some flowers were partially open depending on genotypes, environment and recording dates. Reports on the stability of CMS in maize and sunflower indicated that there is a high variability for different genotypes under different environmental conditions and over successive years. But for both crop types some stable lines could be selected. These data demonstrate that the biological containment methods discussed are very promising for reducing gene flow but that no single containment strategy provides 100% reduction. However, the necessary efficiency of biological containment methods depends on the level of containment required. The containment level may need to be higher for safety purposes (*e.g.* production of special plant-made pharmaceuticals), while much lower containment levels may already be sufficient to reach coexistence goals. It is concluded that where pollen-mediated gene flow must be prevented altogether, combinations of complementary containment systems will be required.

Keywords: biological containment / biosafety / cleistogamy / cytoplasmic male sterility, plastid transformation / transgene excision

INTRODUCTION

The cultivation of genetically modified (GM) plants has been increasing over the past few years. In the context of biosafety of GM crops, gene flow is considered to be the most important factor. Gene flow indicates the movement of genes or genetic materials from one plant into another. Three possibilities for gene flow occur: pollen-mediated, seed-mediated and vegetative-propagule-mediated gene flow. Pollen-mediated gene flow will be primarily determined by the pollination biology of the plant species, outcrossing rates and pollen competition between donors and recipients. Physical or environmental conditions, such as distances between pollen donors and recipients, the strength and direction of wind, temperature and air humidity will also influence pollen-mediated gene flow to a great extent. Since pollination will cause hybridisation or further introgression into the environment, it could be a first, but important, step in the persistence and spread of

a transgene in wild populations and the environment (Lu, 2008). A range of possible environmental consequences (*e.g.*, creation of new weeds and loss of genetic diversity in crop landraces and wild relatives) are conceivable and have been reviewed elsewhere (Conner et al., 2003; den Nijs et al., 2004; Peterson et al., 2000).

In its Scientific Opinion on Guidance for the risk assessment of GM plants used for non-food or non-feed purposes (EFSA, 2009) the EFSA Panel on Genetically Modified Organisms describes the importance of risk management systems, such as post-market environmental monitoring, standard production protocols/stewardship, or confinement strategies to reduce exposure to plants used for non-food or non-feed purposes. The risk assessment for these plants has to take into account the confinement measures when applied. In cases where gene flow from GM plants may result in adverse effects on human and animal health and the environment, it is essential to find efficient and reliable biological containment systems to prevent pollen-mediated gene flow during cultivation. The necessary efficiency of biological containment

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methods depends on the level of containment required. The containment level may need to be higher for safety purposes, while much lower containment levels may be sufficient to reach coexistence goals. A wide variety of biological containment strategies for pollen-mediated gene flow have been proposed (Bock and Timmis, 2008; Daniell, 2002; Gressel and Al-Ahmad, 2005; Murphy, 2007), either naturally occurring or engineered, such as plastid transformation, cleistogamy, male sterility, complete sterility by non-flowering, transgene excision or incompatible genomes. It should be noted here that the European Commission funded a Specific Targeted Research or Innovation Project in the Sixth Framework Programme to develop efficient and stable biological containment systems for GM plants (de Maagd and Boutilier, 2009). Most of the proposed strategies did not move beyond a proof of principle in model plants and were not studied for their use as a biological containment strategy in practice. However, there is a need to ensure that instabilities do not limit or even prevent such strategies and that their long-term effectiveness under various environmental and agricultural conditions has been tested. Here, we review the most promising containment methods for pollen-mediated gene flow, which were assessed for their stability and reliability at a greenhouse (transplastomic tobacco and transgene excision in tobacco and *Arabidopsis*) and field scale level (cleistogamous oilseed rape and cytoplasmic male sterile maize and sunflower) published in peer-reviewed literature.

BIOLOGICAL CONTAINMENT STRATEGIES FOR RESTRICTING POLLEN-MEDIATED GENE FLOW

Plastid transformation

An acknowledged strategy for the prevention of pollen-mediated gene flow is the targeting of the transgene to the chloroplast genome in species in which chloroplasts are generally maternally inherited (Birky, 1995; Bock and Khan, 2004; Dong and Wagner, 1994; Testolin and Cipriani, 1997). In addition to maternal inheritance, the transplastomic approach offers a number of advantages including high-level transgene expression (DeCosa et al., 2001); multi-gene engineering in a single transformation event (Quesada-Vargas et al., 2005; Ruiz and Daniell, 2005); lack of gene silencing, position effect and pleiotropic effects (Daniell et al., 2005; Grevich and Daniell, 2005).

Plastid transformation has been achieved in many important crops, including tobacco, soybean, potato, tomato, cotton, as well in minor species, such as lettuce, petunia and *Lesquerella fendleri* (Brassicaceae, cultivated for oil production) and in non-crop systems, such as the alga *Clamydomonas reinhardtii* (Murphy, 2007). The

cultivation of transplastomic GM cultivars revealed some disadvantages in respect to containment: Although maternal inheritance is widely assumed to be the general rule in most angiosperms, rare pollen-mediated gene transfer has been detected in a number of cases (Murphy, 2007). Rare, but possible, transmission of plastid DNA *via* pollen (Cummins, 1998; Lu, 2003; Wang et al., 2004), even with very low levels of paternal gene transfer, may be sufficient for the escape and spread of a transgene (Haygood et al., 2004). It is also conceivable that transplastomic GM crops could be fertilized by pollen from other plants resulting in hybridisation (Dunwell and Ford, 2005; Lu, 2003, 2008). The resulting hybrid seeds could disperse in the environment. Furthermore, chloroplast DNA may be transferred to the nucleus at low frequencies and be integrated into the nuclear genome (Haygood et al., 2004; Medgyesy et al., 1980). In this case, the transgene is inherited biparentally resulting in a failure of the containment strategy (Sheppard et al., 2008; Stegemann and Bock, 2006).

Several studies assessed the rate of pollen-mediated plastomic or chloroplast gene transfer. Wang et al. (2004) found in *Setaria italica* (foxtail bristlegrass, often cultivated in Asia) a paternal inheritance of 0.03% of all seedlings. The rate of pollen-mediated gene transfer in the ornamental *Petunia hybrida* was even 2% of all progenies (Derepas and Dulieu, 1992). Azhagiri and Maliga (2007) reported a low frequency of 3.9×10^{-5} of transmission of paternal plastids in *Arabidopsis thaliana*. In a field study, Allanguillaume et al. (2009) found only modest crop chloroplast presence in wild *Brassica oleracea* and in weedy *B. rapa*, but a surprisingly high incidence in sympatric (but not in allopatric) riverside *B. rapa* populations. Several studies focussed on the pollen-mediated gene transfer in tobacco (Avni and Edelman, 1991; Medgyesy et al., 1986; Svab and Maliga, 2007). Ruf et al. (2007) assessed the extent to which plastid transformation technology can be used as a biological mitigation technique in tobacco. They described an experimental system facilitating stringent selection for occasional paternal plastid transmission. In a large screen, they detected low-level paternal inheritance of transgenic plastids in tobacco. Whereas the frequency of transmission into the cotyledons of F1 seedlings was 1.58×10^{-5} (on 100% cross fertilization), transmission into shoot apical meristems was significantly lower (2.86×10^{-6}). The authors concluded that plastid transformation provides an effective tool to increase the biosafety of transgenic plants. Moreover, the frequency of pollen-mediated gene transfer under lab conditions (stringent selection) is probably much higher than under field conditions. It can be summarized that more field scale research is needed in this promising area to perfect this approach for biological containment of transgenes.

Transgene excision

Pollen-mediated gene flow can be effectively eliminated by incorporation of a transgene excision system into the process of pollen formation (Mlynárová et al., 2006). Transgene excision resembles the removal of a transgene construct or parts of it from the plant genome by means of recombinase enzymes acting on two target sites flanking the DNA to be excised. The best-known recombination systems are CRE/*lox* from bacteriophage P1, FLP/*frt* from *Saccharomyces cerevisiae* and R/*RS* from *Zygosaccharomyces rouxii* (Gidoni et al., 2008). Most plant applications use the CRE/*lox* system. Transgene excision has been demonstrated in many crops, including tobacco, *Arabidopsis*, tomato, potato, soybean, wheat and maize. Recombinase activity can be induced by heat or chemical treatments or by the use of a developmentally regulated promoter (Gidoni et al., 2008).

Promoter-driven removal of transgenic DNA during pollen development results in male gametes that do not carry the transgene. Experimental data are limited to tobacco (*Nicotiana tabacum*) and *Arabidopsis* focusing on the removal of a selectable marker from the construct in a process known as auto-excision (Luo et al., 2007; Mlynárová et al., 2006). However, these results can be extrapolated to approaches intended for biological containment of the whole construct before or during pollen development. Excision of transgenes regulated in the pollen from *Arabidopsis* and tobacco can be highly efficient (Mlynárová et al., 2006) and was achieved by using the tobacco NTM19 microspore-specific promoter. Activation of the promoter during pollen formation resulted in recombinase-mediated auto-excision of all genetic material flanked by recombination sites in pollen only. As a result, all pollen of the GM plant should be wild type, except for the presence of a single recombination site. Small scale seed germination assays ($n = 100$ per line) of both tobacco and *Arabidopsis* seeds from backcrosses of single-event transgenic plants to wild type plants did not yield any kanamycin-resistant plants out of a total of 1800, indicating 100% transgene excision. Large scale assays ($n = 3000$ per line) on back-crossed tobacco lines yielded two non-excised plants out of a total of 15 000 analyzed, corresponding to a failure rate of only 0.027% (Mlynárová et al., 2006). Luo et al. (2007) demonstrated an increased efficiency of transgene removal in tobacco when they have combined *lox-frt* recognition sites with pollen-specific expression of either Cre or FLP recombinases, compared to non-fused recognition sites of Cre/*lox* or FLP/*frt* recombination systems. The combination of Cre/*lox* and FLP/*frt* in a single construct resulted in 100% excision in approximately 25 000 progeny of backcrosses to wild-type for six lines tested, which corresponds to less than 0.008% failure rate in tobacco (de Maagd and Boutilier, 2009). In addition, Luo et al. (2007) have

demonstrated that the high excision efficiency remains stable in vegetatively propagated progeny plants.

In summary, the results presented show the feasibility of generating transgene-free pollen from transgenic plants under greenhouse conditions. However, if a recombinase is used to excise a transgene, the system must be working at an unexpectedly high efficiency to be an effective biological containment strategy at a field scale level. A calculation of Haygood et al. (2004) suggested that a failure rate of less than 10^{-3} is needed for large scale crop cultivation. In addition, it should be noted that the recombination systems used so far leave a single recombination recognition site footprint (32 bp for *lox*). The resulting plants will still be considered transgenic and could be identified as such (Mlynárová et al., 2006).

Cleistogamy

Cleistogamy (CL), the trait of non-opening flowers, could be used to reduce pollen flow. Self-pollination and fertilisation take place within the closed bud and there is no release of pollen or exposure of the gynoecium to out-crossed pollen. CL is wide-spread among plants, but rare among crop species. Examples of crop species that exhibit some degree of cleistogamy are barley (Kurauchi et al., 1994; Turuspekov et al., 2004), soybean (Benitez et al., 2010; Miyashita et al., 1999) and rice (Connor, 1979; Hoshikawa, 1993).

Transgene containment by the use of CL appears to be easy to apply for some crop species, but it is not practical for all crops (Lu, 2003). It is extremely difficult to create cleistogamous plants for allogamous crops such as maize (Dunwell and Ford, 2005). There is considerable evidence in the literature that suggests that cleistogamy in allogamous species is unlikely to be an evolutionary stable strategy (Diaz and MacNair, 1998). Only few examples exist of biocontainment research on cleistogamy in crops. To date cleistogamy is available for biological containment of pollen in rice (Yoshida et al., 2007) and oilseed rape (Leflon et al., 2009b). There is only limited information about the genetic control and regulatory mechanism of CL occurring in autogamous plant species. In rice, Nagao and Takahashi (1963) identified a CL rice mutant. They reported that the single recessive gene *d7* determined this mutant phenotype, and that the gene was possibly linked to the gene *d6* on chromosome 4. Yoshida et al. (2007) identified another CL rice mutant harbouring a missense mutation. In this case, mutation of the class-B MADS-box gene, SUPERWOMEN-1 with altered lodicule identity, prevents flower opening. The reliability of this CL rice mutant was tested under field conditions in the central region of Japan. The CL trait was permanently stable and completely expressed; data about pollen dispersal were not collected. On the other

side it was shown that in rice lines exhibiting cleistogamy, pollen-mediated gene flow occurs between feral and cultivated forms (Gealy, 2005; Gressel and Valverde, 2009). In oilseed rape CL plants do not occur in the genetic resources. A cleistogamous oilseed rape line was obtained by chemically induced mutagenesis of the cultivar 'Primor' at the National Institute for Agronomic Research in France (INRA Rennes; Patent FR 97 15768). Analysis of segregating phenotypic oilseed rape lines suggested that the cleistogamous trait is controlled by at least two genes (*Clg1* and *Clg2*) with an epistatic dominant effect of *Clg1* over *Clg2* (Lu et al., 2008). Leflon et al. (2009b) examined the reliability of the cleistogamous trait for two inbred oilseed rape lines in field trials. The flower opening level was measured at different dates during the flowering period in six field experiments (three sites \times two years). The results showed a line with promising stability and a line with highly unstable behaviour. Some flowers were partially opened with rates varying from 0.5 to 33% depending on genotypes, trials (site and year) and recording dates. Leflon et al. (2009a, 2009b) considered that, even when partially unstable, CL could be beneficially used in combination with other means in a containment strategy. Despite the fact that cleistogamous lines are stably expressed under various environmental and agricultural conditions and show high heritability, it is also important to investigate the autogamy rates of cleistogamous lines in order to ensure the purity of any "specialty" crop. In another study the rate of allo-pollination in cleistogamous lines under several environmental conditions was assessed (Leflon et al., 2009a). Although differences among sites and years were observed, average allogamy rates of cleistogamous lines grown under a high pressure of allo-pollen varied in three locations between 4.4 and 16.2%. Conventional oilseed rape also shows a considerable degree of receptiveness to pollen from other varieties. These estimates range from 12 to and 55%, averaging 30% (Hüsken and Dietz-Pfeilstetter, 2007). In samples collected from open pollinated cleistogamous plants at 0 m from the pollen source the percentage allogamy rate was 1.64% and it was 0.26% at 6 m distance. Allo-pollination may occur at larger distances to the pollen source, although at levels $< 0.2\%$. These data suggest that cleistogamy could be a promising strategy for transgene containment in autogamous crops, however in the case of instabilities, data about the actual amount of pollen dispersal or the frequency of transgene transmission are still lacking.

Cytoplasmic male sterility

Cytoplasmic male sterility (CMS) is a naturally occurring and maternally transmitted trait, whereby a plant is unable to produce viable pollen. CMS results from

gain-of-function mutations in the plant mitochondrial genome (Chase and Gabay-Laughnan, 2004), responsible for a dysfunction in the respiratory metabolism which can lead to deleterious phenotypes (Chase, 2007; Cilier et al., 2004), resulting in the abnormal production of male gametes (Budar et al., 2003). Therefore, plants either produce no pollen or pollen that is not viable. However, female fertility is not affected by CMS. Kriete et al. (1996) reported naturally occurring CMS for maize, oilseed rape, rice, and *Beta* beets. CMS inbred lines have been widely used for hybrid seed production of many crops (Chase, 2006; Havey, 2004; Pelletier and Budar, 2007; Perez-Prat and van Lockeren Campagne, 2002). Reports on the stability of CMS indicated that there is a high variability for different genotypes under different environmental conditions and over successive years. This was observed in cotton (Marshall et al., 1974; Sarvella, 1966), oilseed rape (Fan and Stefansson, 1986) and onion (Peterson and Foskett, 1953). Temperature, photoperiod and water availability are described as factors that can influence the stability of male sterility. It is generally agreed that sterility increases with increasing temperature and day length (Latha et al., 2004; Marshall et al., 1974; Sawhney, 2004). Stamp et al. (2000) suggested that this trait could be an important tool for enabling efficient and reliable coexistence between GM and non-GM cultivation by biocontainment of GM maize pollen. In this context, Weider et al. (2009) investigated the stability of the cytoplasmic male sterility trait of 22 CMS versions of modern European maize hybrids in 17 different environments in Europe over two years. Of the three types of male-sterile cytoplasm (T-, S- and C-type), the sterility of the T- and C-cytoplasm hybrids was most stable. S-cytoplasm hybrids often showed partial restoration of fertility. Of the eight C-cytoplasm hybrids tested in the study, five were fully sterile. Of the eight T-cytoplasm hybrids also five were fully sterile across all environments. The genetic instability of S-cytoplasm, coupled with its sensitivity to environmental conditions makes the S system generally unreliable for hybrid seed production and biocontainment. Climatic factors, especially air temperature, humidity and evapotranspiration in the ten days before as well as during anthesis, were correlated positively or negatively with the partial reversion of male fertility. T- and C-cytoplasm sterile maize hybrids show viable prospects for containing transgenic pollen (Weider et al., 2009). CMS T was identified as the most stable type of male sterile cytoplasm; nevertheless, due to its susceptibility to the fungi *Bipolaris maydis*, its use may be limited to the growth of small scale transgenic fields, e.g. molecular farming. To achieve a useful agricultural biocontainment system sowing a mixture of GM CMS-maize hybrid (80%) and an unrelated conventional fertile hybrid (20%) is recommended, whereby the latter component acts as

pollen donor for the entire stand. In this Plus-Hybrid System pollination of the crop is ensured while the release of GM pollen is minimised (Munsch et al., 2008). In addition, appropriate combinations of CMS hybrids and fertile pollinators could lead to a significant gain in yield (Munsch et al., 2010; Stamp et al., 2000; Weingartner et al., 2002), due to combining potential benefits of male sterility (CMS effect) and allopollination (xenia effect).

In sunflower, the use of CMS could also serve as an alternative route to prevent transgene escape. In a 3-years study in different locations in Bulgaria, six CMS sunflower lines were evaluated concerning the stability of the CMS trait (Hvarleva et al., 2009). Four lines were fully sterile across all environmental conditions, while two lines showed unstable sterility. The data suggest that the climatic conditions may affect the stability of sterility depending on their genotype.

In summary, we expect that CMS can provide a powerful tool to manage gene flow issues by eliminating or reducing the release of pollen. However there is instability in these systems and data about the actual amount of pollen dispersal or the frequency of transgene transmission, at field scales of production, are still lacking.

CONCLUSION

Despite the great progress that has been made in the development of approaches to reduce or prevent transgene flow through pollen, the biological containment strategies discussed here all show the tendency of not being completely effective. In addition, data about the actual amount of transgene pollen dispersal or the frequency of transgene transmission at the field level are still lacking. However, there might be tolerance of incomplete biocontainment, depending on the trait/plant combination under consideration. Biological containment methods can be used in combination and also combined with physical containment methods, such as wide distances between donor and recipient fields as barriers for pollen flow, in order to further reduce frequencies of pollen transmission. In cases where pollen transmission must be prevented altogether, more research is required to determine effective combinations of the different containment methods required to achieve this.

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