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Low frequency transmission of a plastid-encoded trait in *Setaria italica*

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Abstract It has been claimed that engineering traits into the chloroplast will prevent transgene transmission by pollen, precluding transgene flow from crops. A *Setaria italica* (foxtail or birdseed millet) with chloroplast-inherited atrazine resistance (bearing a nuclear dominant red-leaf base marker) was crossed with five male-sterile yellow- or green-leafed herbicide susceptible lines. Chloroplast-inherited resistance was consistently pollen transmitted at a 3×10^{-4} frequency in >780,000 hybrid offspring. The nuclear marker segregated in the F₂, but resistance did not segregate, as expected. Pollen transmission of plastome traits can only be detected using both large samples and selectable genetic markers. The risk of pollen transmission at this frequency would be several orders of magnitude greater than spontaneous nuclear-genome mutation-rates. Chloroplast transformation may be an unacceptable means of preventing transgene outflow, unless stacked with additional mechanisms such as mitigating genes and/or male sterility.

Introduction

There have been many recent claims that inserting transgenes into the plastome (chloroplast genome) of crops would preclude gene flow from such crops (Bogo-

rad 2000; Bock 2001; Daniell 2002; Maliga 2002), on the presumption that chloroplast traits are completely maternally inherited in many species. This view that gene flow would be prevented may be flawed on three accounts: (1) not all species have maternal inheritance, and one does not know whether an untested species has maternal inheritance until there is an adequate marker; (2) the possibility for non-transgenic species to hybridize, with the crop as the female parent; and (3) the inability to detect low frequencies of pollen transmission.

These can be explained as follows:

- (1) there is a variation among species in the frequency of paternal inheritance of chloroplast genome-inherited traits, which precludes generalizations. Considerable biparental inheritance was found in crops such as rye, sweet potato, chickpea, vetch, alfalfa, petunia, common beans and geranium, and other angiosperm plant species (Tilney-Bassett and Abdel-Wahab 1979; Corriveau and Coleman 1988; Derepas and Dulieu 1992; Hagemann 1992; Reboud and Zeyl 1994; Mogensen and Rusche 2000). Chloroplast inherited traits are inherited paternally in all gymnosperm species assayed (e.g. Shiraishi et al. 2001) and even in the dicotyledonous *Actinidia* (kiwifruit) (Chat et al. 1999). Thus, it will be necessary to ascertain frequencies of paternal inheritance with each crop situation where chloroplast inheritance occurs. The utility of maternal inheritance for transgene containment is even less guaranteed in far crosses, where it was deemed most useful. A breakdown in the control mechanisms of maternal inheritance has been reported when plants are forced into wide-crosses, as could occur between crops and distantly related weed or wild species. The organelle inheritance in F₁ of crosses between *Festuca pratensis* and *Lolium perenne* appeared to be maternal when probed with plastid and mitochondrial DNA. Still, a high frequency of pollen transmission was found when the F₁ progeny were backcrossed with *Lolium* (Kiang et al. 1994), which also demonstrates the limitations of DNA probing;

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- (2) an intercrossing species can still form hybrids between such a crop and the related species, with the crop as the female parent and the related species as the male (pollen) parent in the initial cross that forms a hybrid. Continued backcrosses with the crop-relative, serving as the pollen parent, would fix the plastome trait in the relative, allowing complete flow of a plastome-inherited gene or transgene from the crop to the other species. Plastome-inherited, newly evolved triazine herbicide resistance was thus successfully transferred by breeders from weeds to crops using this strategy in reverse (Souza-Machado 1982; Darmency and Pernes 1985; Wang et al. 2000);
- (3) laboratory evidence has indicated that maternal uniparental inheritance is leaky at a low frequency, but hard to detect in species where maternal inheritance was thought to be absolute. Earlier evidence of occasional rare biparental transmission was shown in *Antirrhinum majus* (Diers 1971) and discussed in Corriveau and Coleman (1988) and Hagemann (1992). Large numbers of hybrids must be generated and assessed to ascertain a possible low frequency of pollen transfer of plastome-encoded traits to demonstrate that pollen transfer is below an acceptable-risk threshold. Such experiments are hard to perform without the ability to generate large numbers of hybrids using either self-incompatibility to crossing, or at least partial male sterility, together with nuclear markers that allow distinguishing between spurious self-pollinated progeny from the hybrid progeny when crosses are made. A lack of such male-sterile lines in a self compatible species requires the emasculation of large numbers of flowers to obtain sufficient seed, and bagging flowers to guard against promiscuous pollination. The plastome trait must allow rapid verification of the low frequency of pollen transfer, such as plastome-inherited herbicide resistance. In the few cases where such markers and systems were available for laboratory studies, plastome-inherited tentoxin resistance in tobacco (Avni and Edelman 1991) and atrazine resistance in various weeds (Darmency 1994; Gawronski 1985) was evidence of a frequency of more than 10^{-4} transmission of the trait by pollen. Reboud and Zeyl (1994) even suggested that "leaky" transfer by pollen would indeed be the general rule.

In the study reported here, we used such easy to detect nuclear markers to detect intraspecific hybrids, together with male sterility to assess the frequency of pollen transfer of a chloroplast trait in the field, in the crop *Setaria italica* (foxtail or birdseed millet). This self-pollinating cereal is cultivated in parts of China and India as a high quality food that is also drought-tolerant and can produce a crop even on infertile land. It is still grown for birdseed in Europe and as a fodder plant in South America. *S. italica* is related to and capable of intercrossing with many members of a group of globally distributed pernicious weedy *Setaria* species (Holm et al. 1977). Because of weed problems limiting cultivation of

this crop, we carried out a breeding program that included transferring a maternally inherited herbicide resistance from the weed *Setaria viridis* to this crop (Darmency and Pernes 1985; Wang et al. 2000). Herbicide resistance in the crop would allow control of weedy *Setaria* as well as other species. Both spontaneous mutations to resistance in wild populations and the possible transfer of herbicide resistance through intercrossing could lead to the proliferation of this trait to wild relatives, similar to that feared from transgenic crops. Pollen flow of nuclear markers has been described in this autogamous crop (Wang et al. 1997). The level of gene-introgression was quantified to predict the spread of plastid-inherited herbicide resistance genes into the wild species. The *psbA* chloroplast gene inherited triazine herbicide-resistance selectable marker transferred at a low but consistent frequency in the field, suggesting that the use of maternal inheritance can substantially delay such gene transfer, but not preclude it.

Materials and methods

Breeding stocks

The possible inter-dependence between chloroplast- and mitochondria-inherited organelles in recipient male-sterile lines could result in biased chloroplast paternal transmission. Five different male-sterile lines were chosen to test the consistency of chloroplast pollen transfer in several distinct genetic backgrounds. The five stable male-sterile *S. italica* lines were selected from different breeding material and used as females for making large quantities of hybrid seed with different female genetic backgrounds (Fig. 1). (1) Line 1066A (Wang and Du 1994) is a commercial, highly recessive male sterile line with yellow seedlings made from the cross of a yellow male-sterile mutant and cv Nihuang. (2) Ba-ai 2 (kindly provided by Prof. Z. Zhao of Zhangjiakou Agricultural Institute, Zhangjiakou, China) is also a highly recessive male-sterile line, with green seedlings. (3) Sda-1 is a progeny of Ms^{ch} (kindly provided by Prof. H. Hu of Chifeng Agricultural Institute, Chifeng, China), and is a dominant male-sterile line derived from an Australian *S. italica* × cv Tulufan (Hu et al. 1986). (4) Line 350A (Wang and Du 1994) is a highly recessive male-sterile line with green seedlings, selected from a summer *S. italica*. (5) Line Ba951 (kindly provided by Prof. Z. Zhao) has photoperiodically conditional male sterility, with male sterility appearing at day lengths above 14 h. This line was derived from a cross between an Australian *S. italica* × cv Zhongweizuyueqing (Zhao et al. 1996).

The pollen parent AR608 is nuclearly homozygous for red-collar of the seedling with cytoplasmically inherited atrazine resistance. It was selected from an atrazine-resistant line D90-23 produced by INRA in France by crossing the plastome of a French triazine-resistant *S. viridis* into *S. italica* as the pollen parent and as the recurrent pollen-backcross parent (Darmency and Pernes 1985). One of those lines, D90-23, was then pollinated by Hui 468 (red-leaf collar pigmentation), then twice backcrossed using male parents for improving its agronomy traits. The selected elite progeny were bred to stable lines, including AR 608. It was homozygous for red pigmentation. All the female (male-sterile) plants were homozygous for green or yellow pigmentation, which is recessive to the red-pigmentation of the pollen parent.

Crossing

All the material was sown in experimental fields in Beijing as well as 180-km north at Zhangjiakou (China) in May 1999. Two rows of the pollen parent line AR608 were arranged between every two

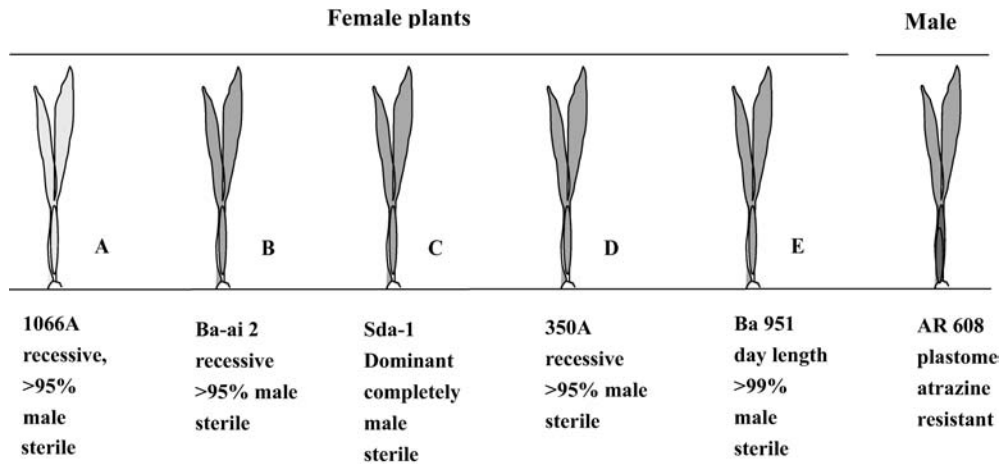


Fig. 1 Parental lines: the different types of yellow or green male-sterile lines crossed with the (nuclear) homozygous red-leaf collared and plastomic atrazine resistant pollen parent. As described in more detail in Material and methods: *A* Line 1066A is a highly recessive male-sterile line with yellow seedlings; *B* Line

Bai-ai 2 is a recessive male-sterile line, with green seedlings; *C* Line *Sda-1* is a dominant male-sterile line; *D* Line 350A is a highly recessive male-sterile line with green seedlings. *E* Line Ba951 is a day-length dependent male-sterile with green seedlings. All true hybrid offspring had red collars

rows of the seed-parent plants. One of two rows AR608 was sown 7–10 days later to ensure simultaneous flowering with the male-sterile parents. The experimental field was surrounded by a weed-free buffer zone of 1 km to preclude any pollen transfer to wild *Setaria* spp. Hybrids were made by enclosing 1–3 panicles of the resistant male plants with 3–5 panicles of female parent plants in paper bags before flowering. The bags were tapped daily at 10–12 a.m. during the flowering period to ensure pollination. This is the standard procedure used by Chinese breeders to make commercial hybrid seed of this species, which does not produce copious amounts of pollen. After pollination, the paper bags were only put on the female plants to prevent pollination of later-opening florets and protect against bird damage. All samples were harvested in late September, air-dried and hand-threshed to prevent cross-contamination.

As expected, not all seeds derived from the crosses with the red atrazine-resistant AR608 line were true hybrids due to incomplete male sterility of some of the female lines. Non-hybrid plants without the red collar were removed by hand. The true hybrid seeds from different crosses and control seeds (see below) of a certified commercial hybrid variety (1066A×Hui 445), as well as its parents, were sown in an irrigated field near Beijing. The whole field was treated with commercially formulated atrazine (Xuanhua Agricultural Chemical Factory, China) at the 1.8-kg active ingredient per hectare, double the highest recommended dose, both at the 5-leaf stage and again at the 7-leaf stage, to eliminate any possible susceptible escapes. A rate of 0.3 kg/ha was previously shown to kill the susceptible type. Here, the atrazine-susceptible commercial hybrids and parents were used as controls to ascertain that there was no low-frequency of resistance within susceptible populations. Three weeks after the second treatment, surviving and dead plants were counted.

As a further test of maternal inheritance of the resistance trait once paternally transferred, flowers of several-resistant hybrid plants from two of the crosses were separately bagged for self-pollination and the F_2 seeds harvested, and sown the following season. The segregation of color and fertility were noted and the plants treated with atrazine, as above. The viability of the stand was noted.

Results

Pollen transmission verified in the field

Using the appropriate genetic material prepared for this study, we could perform a large-scale field study to assess the frequency of pollen transfer of a plastome-inherited trait utilizing the crop *S. italica* (foxtail or birdseed) millet. Crosses were made in the field using the homozygous nuclear dominant red-colored, plastome-inherited triazine resistant line of *S. italica* as the pollen parent, and variously colored different types of male-sterile lines as the female parent (Fig. 1) to attempt to transfer the maternally inherited trait via pollen. The seeds of these crosses were planted the following season to ascertain whether indeed there had been pollen transmission of this trait. Additionally, atrazine-susceptible hybrid seed and seed of the parents was planted and treated with atrazine to easily assay for spontaneous mutations to triazine resistance. The field was twice-treated with a very high rate of atrazine. The first treatment was to kill susceptible individuals and the second to ensure that there were no escapes or late-germinating individuals.

There were no surviving individuals among the 300,000 control plants, demonstrating that: (1) spontaneous evolution of resistance would be an unlikely cause of appearance of resistance among the crosses (Table 1); and (2) the use of two treatments provides a satisfactory screening tool. Such a mutation might occur if there was a nuclear-inherited plastome mutator gene (Gressel 2002) in the populations of all the male-sterile strains. In the past, we have treated more than 1,600,000 susceptible individuals of *S. italica* and *S. viridis*, from more than 1,000 germplasm resources, and have never found an atrazine-resistant mutant. Atrazine has been used to continuously control *Setaria* species on millions of hectares of croplands, with only a few known locations

Table 1 Low frequency of paternal inheritance of chloroplast-encoded triazine resistance. See Fig. 1 for an explanation of the lines used

Material	Total seed produced	No. of seeds germinating	No. of hybrid seedlings	No. of resistant seedlings	Frequency of resistance
Controls—cross and parents					
A×Hui	123,904	116,428	107,142	0	0
A (selfed)		93,750		0	0
Hui (selfed)		103,448		0	0
Plastome donor crosses					
A×AR	228,318	204,587	186,732	67	3.6×10^{-4}
B×AR	165,913	143,749	127,581	32	2.5×10^{-4}
C×AR	211,468	192,514	192,437	58	2.8×10^{-4}
D×AR	161,192	133,871	123,514	30	2.4×10^{-4}
E×AR	184,166	163,046	157,065	54	3.4×10^{-4}
Total	951,057	837,767	787,329	241	3.1×10^{-4}

Table 2 F₂ segregation of atrazine-resistant F₁ hybrids from two representative crosses. See Fig. 1 for an explanation of the lines used

F ₂	No. red collared	No. yellow or green	χ^2 (3:1) value	χ^2 probability	% atrazine susceptible	% atrazine resistant
A×AR	9,235	2,962	3.29	0.070	0	100
C×AR	4,421	1,378	4.66	0.031	0	100

of atrazine resistance appearing in the USA, Canada, France, Spain and the former Yugoslavia (Heap 2003), suggesting an exceedingly low yet finite frequency of mutation to this phenotype. The estimated frequency is well below 10^{-12} (Gressel 2002); how much lower, remains an open question that cannot be easily tested experimentally due to the vast numbers required.

We generated more than an unprecedented 750,000 true hybrids using the atrazine-resistant strain as the pollen parent and the five male-sterile varieties. The red marker was very important, allowing easy culling of as many as 14% of the non-hybrids in some crosses, due to incomplete male sterility allowing some self fertilization (Table 1). Only about 0.03% of the true hybrids later survived two sequential herbicide treatments; a pollen transmission frequency of this plastome inherited a trait of 3×10^{-4} .

Maternal inheritance after pollen transmission

We further verified that indeed this limited number of hybrids were true hybrids, and that the atrazine resistance was predominantly maternally inherited, by bagging the flowers of randomly chosen F₁ plants of two of the crosses. F₂ progeny were obtained on all of the selfed individuals from one cross, with the expected 3:1 ratio for the nuclear-inherited trait (Table 2). Only half of the F₂ individuals of self pollination of the other cross, set seed because the parent had been heterozygous dominant for male sterility, and the F₁ was thus basically a backcross vis a vis male sterility, meaning that half the F₂ would be male-sterile and incapable of setting seed by self pollination. The segregation was close to 3:1 ($P=0.07$), possibly due to a slight linkage in that cross with male sterility. All the F₂ individuals were atrazine resistant, which follows from the maternal inheritance of this trait.

Discussion

This pollen transmission of the plastome trait should not be confused with the recently reported (Huang et al. 2003) putative transfer of chloroplast-encoded transgenes to the nuclear genome; as after pollen transmission to the host in the cases described above, triazine resistance remained predominantly maternally inherited (Table 2). The frequency of 3×10^{-4} pollen transmission of a plastome-inherited trait in the field is about an order of magnitude lower than had been found for other species in the laboratory (Avni and Edelman 1991; Darmency 1994). The difference could also be due to species variability, rather than the experimental conditions. Still, this frequency of transmission is very high when compared to the $<10^{-6}$ frequency typical for ubiquitous nuclear mutations for herbicide resistance, and even more so than expected for plastome mutations. Despite the widely different genetic backgrounds of the male-sterile lines, pollen transmission of the plastome trait was very similar. Nuclear inherited resistance to other herbicides is widespread in *Setaria* species, despite fewer years of use of alternative herbicides over a smaller area (Heap 2003).

The results should not be wholly unexpected, either from the laboratory results or from the considerations of how maternal-inheritance is controlled. Many known and possible mechanisms of maternal plastome gene inheritance have been described, (e.g. Hagemann and Schroeder 1989; Hagemann 1992; Birky 1995; Mogensen 1996). Plastome transmission can be blocked by a variety of mechanisms and at different stages of reproduction in different species. None of the mechanisms described as the basis for maternal inheritance of chloroplast traits would alone be absolute. The plastome can even be heteroplasmic for traits such as triazine resistance (Frey et al. 1999). In gymnosperms where the reverse situation of paternal inheritance of chloroplast traits is the rule, the

transmission is also leaky, with some biparental inheritance (Shiraishi et al. 2001).

Of course, the biosafety containment implications of this frequency of gene flow must be dealt with case by case, considering the nature of the transgene and its products, as well as the nature of the related species. For example, if the measured frequency of nuclear gene flow at that isolation distance used is less than 10^{-5} , and the frequency of plastid gene flow is 3×10^{-4} that of the nuclear gene flow, then the expected risk of plastid gene flow to that distance is $<10^{-5} \times 3 \times 10^{-4} = <3 \times 10^{-9}$. Conversely, the risk of consequential plastome gene flow would be much too high for a herbicide resistance gene in a crop such as rice having red-rice (also *Oryza sativa*, a highly weedy, feral form of rice) growing in its midst.

The risks of negative consequences of gene flow from the transgenic crop could be substantially lowered by also using mitigation technologies. This can be done by tandemly attaching a 'mitigator' gene to the gene of choice to be transformed into the chloroplast (Gressel 1999). A mitigator gene is one that is neutral (or beneficial) to the crop but would be deleterious to a crop-weed hybrid and to backcrosses into the weed, e.g. a dwarfing gene or any other important character such as the low tillering, non-shattering of seed, etc. Such a mitigator gene has been shown to be effective in conveying unfitness to a recipient, at least at the greenhouse simulation level (Al-Ahmad et al. 2003).

Thus, while not absolute, plastome insertion of transgenes can be effective in lessening transgene movement, which may represent a sufficient degree of biosafety. The results illustrate the requirement for large numbers of crosses to assess the lessening of risk by any single technology. To show that the risk of gene flow is less than 10^{-8} would have required approximately 10^9 plants. Combining containment and mitigating systems that compound the reduction of the risk is probably the best-management strategy. Such stacking of containment and mitigation barriers will be most efficient if practiced from the onset of cultivation of 'at risk' transgenic crops, and not as a recourse once genes have flowed, at which time stacking would be ineffectual.

A novel additional combination that considerably lowers the risk of gene outflow within a field (but not gene influx from related strains or species) can come from utilizing the male-sterile, atrazine-resistant lines that appeared in the F_2 generation, after they are further stabilized. In cases where there is incomplete male sterility, the (predominantly) plastome-inherited herbicide resistant male-sterile variety can be cultivated as such (without "maintenance"), as it sets seed by self-pollination with the limited amount of pollen it produces (about 1% the normal amount). In situations where there is no intense field sympatry with fertile crop/weed hybrids, such a system would further reduce the risk of gene outflow by two orders of magnitude. Such a double failsafe containment method might be considered sufficient where there are highly stringent requirements for preventing gene outflow to other varieties (e.g. to

organically cultivated ones), or where pharmaceutical or industrial traits are engineered into a species. Introducing the plastome-inherited traits into varieties with complete male sterility would vastly reduce the risk of transgene flow, except in the small isolated areas required for line maintenance. Plastome-encoded transgenes for non-selectable traits (e.g. for pharmaceutical production) could be transformed into the chloroplasts together with a trait such as atrazine resistance as a selectable plastome marker, e.g. a tobacco variety with tentoxin resistance or a crop already bearing atrazine resistance. With such mechanisms to further reduce out-crossing risk, plastome transformation can possibly meet the initial expectations.

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