Rapeseed cytoplasm gives advantage in wild relatives and complicates genetically modified crop biocontainment

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Summary

• Biocontainment methods for genetically modified crops closest to commercial reality (chloroplast transformation, male sterility) would be compromised (in absolute terms) by seed-mediated gene flow leading to chloroplast capture. Even in these circumstances, however, it can be argued that biocontainment still represses transgene movement, with the efficacy depending on the relative frequency of seed- and pollen-mediated gene flow.

• In this study, we screened for crop-specific chloroplast markers from rapeseed (*Brassica napus*) amongst sympatric and allopatric populations of wild *B. oleracea* in natural cliff-top populations and *B. rapa* in riverside and weedy populations.

• We found only modest crop chloroplast presence in wild *B. oleracea* and in weedy *B. rapa*, but a surprisingly high incidence in sympatric (but not in allopatric) riverside *B. rapa* populations. Chloroplast inheritance models indicate that elevated crop chloroplast acquisition is best explained if crop cytoplasm confers selective advantage in riverside *B. rapa* populations.

• Our results therefore imply that chloroplast transformation may slow transgene recruitment in two settings, but actually accelerate transgene spread in a third. This finding suggests that the appropriateness of chloroplast transformation for biocontainment policy depends on both context and geographical location.

Introduction

The prevention of gene flow from genetically modified (GM) crops would circumvent the need to assess many ecological risks. The bewildering array of strategies and protocols that have been proposed for this purpose are collectively known as biocontainment methods. They include physical separation (Tackaberry *et al.*, 2003), natural genetic containment (Anon, 2004), chloroplast transformation (Daniell *et al.*, 1998), conditional lethality (Kriete *et al.*, 1996), engineered male sterility (Denis *et al.*, 1993), terminator concept and seed lethality (Daniell, 2002), apomixis (Anon, 2004), cleistogamy (Lu 2003), transgene mitigation (Al-Ahmad & Gressel, 2005), recoverable block function (Kuvshinov *et al.*, 2001) and transgene excision (Hare & Chua, 2002). Despite many recent advances, the prospect of effective biocontainment remains elusive over the near future, largely because the vast majority of methodologies have been demonstrated only in principle. Indeed, male sterility and chloroplast transformation remain the only genetic options that have progressed to field trials or commercial release (Dunwell & Ford, 2005; Supporting Information Notes S1). These two protocols thus provide the best immediate hope for the biocontainment of most GM crops.

Most interest in biocontainment has focused on crops such as rapeseed, whose cultivated range coincides with several cross-compatible relatives (Ellstrand *et al.*, 1999). The key limitation of the two currently available biocontainment

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methods lies in the fact that they only restrict pollen-mediated gene flow (Daniell *et al.*, 2005). Their efficacy therefore depends partly on whether seed-mediated gene flow occurs from the crop, on the fitness of any resultant transgenic introgressants (Chapman & Burke, 2006) and on the extent to which chloroplast inheritance can be effected through the male germline (paternal transmission of chloroplasts). Regulators thereby require baseline information (preferably at the national scale) on whether crop chloroplasts are routinely captured by wild relatives, the underlying processes leading to chloroplast capture (seed dispersal and/or paternal inheritance) and whether the crop cytoplasm influences the fitness of the relative within-recipient habitats.

For the vast majority of higher plant species, chloroplasts are reported to be exclusively maternally inherited, although low-level paternal chloroplast leakage has been seen in species previously believed to inherit their chloroplasts maternally (for example, Azhagiri & Maliga, 2007; Ruf *et al.*, 2007; Svab & Maliga, 2007; Chandler & Dunwell, 2008), and occasional biparental inheritance in others (Mogensen & Rusche, 2000; James *et al.*, 2001; Bogdanova, 2007; Hu *et al.*, 2008; Matsushima *et al.*, 2008). Such 'leakiness' in chloroplast inheritance would complicate predictive modelling of chloroplast exchange between crops and their relatives if it occurred at significant rates. Therefore, it is important that paternal transmission rates are evaluated separately for the specific species combination of concern.

Even in situations in which chloroplasts are predominantly maternally inherited, capture can nevertheless occur at significant frequencies in natural ecosystems, presumably via seed dispersal (for example, Rieseberg & Soltis, 1991; Lanner, 1998; Yuan & Olmstead, 2008). In these instances, a uniparental inheritance pattern of transmission greatly simplifies the prospects of modelling the transmission and spread of chloroplasts in the event that these organelles confer fitness advantage, cost or play no role in determining fitness.

In England, rapeseed (*Brassica napus*, AC genomes) hybridizes spontaneously with A genome-containing *B. rapa* (Scheffler & Dale, 1994) and the C genome species *B. oleracea* (Ford *et al.*, 2006). *Brassica rapa* is a common riverside species and an infrequent agricultural weed (Wilkinson *et al.*, 2003), whereas *B. oleracea* inhabits scattered sea-cliff communities (Mitchell & Richards, 1979). In this study, we examine the extent to which crop chloroplast capture has occurred historically between conventional rapeseed crop and both relatives across England. Wild and weedy *B. rapa* populations are considered separately because they differ in exposure to rapeseed and occupy divergent habitats.

Moreover, the current study uses a combination of species-specific chloroplast markers, population modelling and *in situ* competition experiments to explore the extent to which currently available biocontainment methodologies would be effective in preventing or repressing transgene spread from a hypothetical GM line of rapeseed in the UK.

Materials and Methods

Plant material

***Brassica* reference panels** Genetically diverse reference panels were established from material collected across Eurasia. These panels comprised 81 *B. napus*, 72 *B. oleracea* and 84 *B. rapa* accessions and were provided by Warwick HRI (Wellesbourne, Warwickshire, UK) (Tables S1a–c, see Supporting Information).

Wild and weedy populations Leaf material of *B. oleracea* was collected from 716 individuals from 28 populations periodically sympatric with rapeseed and from 454 plants from seven allopatric populations, as described by Ford *et al.* (2006) (Table S3a,b, see Supporting Information). Leaf material of weedy *B. rapa* was collected from 407 individuals from five arable fields in Humberside, as described in Table S4a–c (see Supporting Information). In 2002 and 2003, leaf material was collected from 65 riverside populations of *B. rapa* (Rivers Thames and Nene, UK). Samples comprised 1014 individuals from 27 populations periodically sympatric with cultivated rapeseed, and 1020 plants from 38 allopatric populations (as described in Table S5a–c, see Supporting Information). Further field work was performed in 2002 and 2003, and leaf material was collected from a further eight rivers comprising an additional 837 *B. rapa* individuals originating from 27 sites on the banks of the rivers Avon1, Avon2, Derwent, Dove, Parret, Ouse, Stour, Trent, Welland and Brue (Table S6a,b, see Supporting Information).

Interspecific crosses Reciprocal interspecific crosses were performed between both relatives (*B. rapa* and *B. oleracea*) and rapeseed. Details of all crosses are provided in Table S2a–d (see Supporting Information).

DNA extraction

All DNA extractions were performed from fresh leaves using QIAgen DNeasy plant extraction kits (Qiagen, St Albans, Hertfordshire, UK) according to the manufacturer's instructions.

Molecular markers

Crop-specific chloroplast markers We used four chloroplast simple sequence repeat (cpSSR) markers [Chloro 39, Chloro P and Chloro O from Allender *et al.* (2007) and Chloro H from *YCF3* (forward primer, 5′-gcttcttcccctgtgcctcc-3′; reverse primer, 5′-agtgcagccttagatgcttc-3′)]. These markers were amplified and fractionated according to Allender *et al.* (2007). We also used one cleaved amplified polymorphic sequence (CAPS1) marker (part of *YCF3*) and two high-resolution melt (HRM) analysis single nucleotide polymorphism markers (SNP1 targeting *YCF2*, SNP2 targeting *cytochrome F*). Collectively, these markers were deployed to characterize the chloroplast haplotypes of the three *Brassica* species. Details of the assay

design and conditions used are provided in Supporting Information Notes S2. The species specificity of all haplotypes was tested using the reference panels described above.

Crop-specific nuclear markers The A genome-specific SSR marker BRMS043 (Suwabe *et al.*, 2006) was used to confirm the F_1 hybrid status of triploids secured from rapeseed × *B. oleracea* interspecific crosses, as described by Ford *et al.* (2006). Five additional SSRs [BN83b1, AP1C 5r, Na10A08 (Allainguillaume *et al.*, 2006) and BRMS005 (allele 140 bp) and BRMS098 (allele 178 bp) (Suwabe *et al.*, 2006)] were screened across the *B. napus* and *B. rapa* reference panels using the conditions described by Allainguillaume *et al.* (2006).

Maternal chloroplast inheritance in interspecific crosses

F_1 hybrid identity was inferred by flow cytometry (Wilkinson *et al.*, 2000) and the presence of crop-specific nuclear SSRs (Allainguillaume *et al.*, 2006; Ford *et al.*, 2006). Chloroplast inheritance was determined using the chloroplast-specific markers described above. Details of all crosses and the markers used to confirm hybrid status are provided in Table S2a–d.

Survey of wild populations for crop-specific chloroplast markers

B. oleracea We screened all *B. oleracea* individuals for the presence of rapeseed chloroplasts using marker CAPS1 or SNP2. Ploidy levels and the presence of hybrids have been determined in a previous study (Ford *et al.*, 2006). Details of all material screened are presented in Table S3a,b.

Weedy *B. rapa* We screened weedy *B. rapa* individuals for the rapeseed chloroplast and for triploid hybrids. All plants were flow sorted (Wilkinson *et al.*, 2000) to identify triploids, and then hybrids were confirmed using C genome-specific SSR markers. SSR markers were established as C genome specific by their presence in all rapeseed individuals and their absence from all reference *B. rapa* (Table S1a–c).

Riverside *B. rapa* Populations originating from the primary surveys of the Thames and Nene (see above) were screened for rapeseed chloroplast capture using all the species-specific chloroplast markers described above. (Samples described in Table S5a–c.) Populations from the second, broader survey (Avon1, Avon2, Derwent, Dove, Parret, Ouse, Stour, Trent, Welland and Brue) were screened using the chloroplast marker CAPS1 (Table S6a,b).

Minimum spanning network between *Brassica* species haplotypes

The minimum spanning network between the nine *Brassica* haplotypes was computed using software ARLEQUIN version 3.1 (Excoffier *et al.*, 2005). The distance matrix between haplotypes was calculated assuming unidirectional stepwise mutation for the chloroplast SSR loci.

Frequency-based Wright–Fisher simulation of chloroplast assimilation in *B. rapa*

We studied the theoretical dynamics of chloroplast capture in model populations using modified frequency-based Wright–Fisher simulations to explain the incidence of chloroplast capture in riverside *B. rapa* as described below. The model was implemented using Borland C++ Builder v5. The Windows executable can be downloaded.

Model description The population is assumed to be of constant size N, represented by N individual chloroplasts. These can be either of *B. rapa* (N_r) or rapeseed (N_n) origin. Random mating is assumed. The initial model state is 100% *B. rapa* chloroplasts ($N_r = N$). F_1 hybrids possessing rapeseed chloroplasts are formed at a rate m in generations 1 yr after sympatry (based on casual observations, we assume feral rapeseed persists in *B. rapa* populations for 1 yr following sympatry). These are expected to contribute $Nm\lambda_{F1}$ rapeseed chloroplasts to the following generation, where λ_{F1} is the fitness coefficient of F_1 hybrids relative to *B. rapa* ($\lambda_r = 1$). Where rapeseed chloroplasts are already present, the effective contribution of F_1 hybrids will be $N_r m\lambda_{F1}$. Similarly, the relative fitness of *B. rapa* with rapeseed chloroplasts (λ_n) will modify the contribution from captured chloroplasts. Thus, the proportion of the population with rapeseed chloroplasts (p_r) in successive generations is calculated as:

$$p_r^{t+1} = \frac{N_n^t}{N^t}\lambda_n + \frac{N_r^t}{N^t} m\lambda_{Fl} \qquad \text{Eqn 1}$$

where the right-hand side of the equation is applied only in the years following sympatry. In the deterministic model, the number of individuals with the rapeseed chloroplast is then taken as Np_r, and N_n is calculated as $N - N_r$. For the stochastic simulations, a sample is taken from a binomial distribution, taking p_r as the expected value.

Seed bank and variable population size Detailed surveys of the River Thames and Nene over 4 yr (J. Allainguillaume, unpublished data) indicate that the location and size of *B. rapa* populations are dominated by disturbance. Populations vary in size considerably from year to year, and even large populations may not always be present. The seed bank is apparently a key feature of *B. rapa* dynamics (Allainguillaume *et al.*, 2006). As such, we felt that it was necessary to explore variations on the basic model in order to summarize the implications of a seed bank and variable population size.

Populations are assumed to vary in size log-normally, with a defined mean population size (N_{mean}). The range of this

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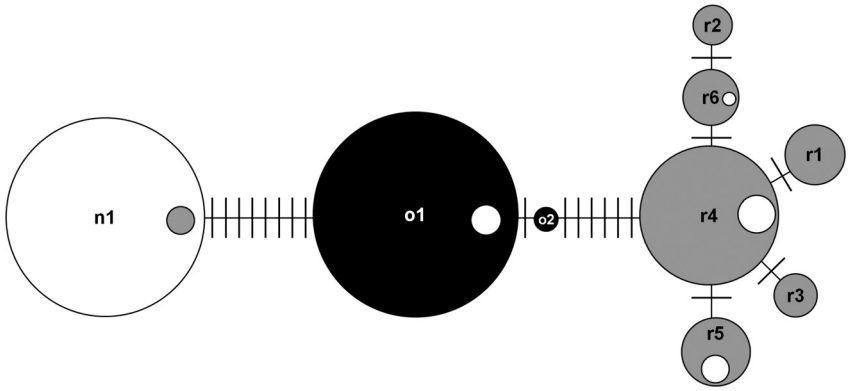

Fig. 1 Minimum spanning network of nine chloroplast DNA haplotypes (n1, o1, o2, r1–r6) in 81 *Brassica napus* (white), 72 *B. oleracea* (black) and 84 *B. rapa* (grey) accessions (Table S1a–c). Each circle represents a single haplotype in which the circle size is proportional to the frequency of occurrence within a species (inside circle corresponding to the less frequent species displaying that haplotype). Each haplotype is separated by a number of mutations which are represented by lines.

distribution is generated as a random variate about 2 log N_{mean} for each model run within limits of 0 and 7. The new population size N_{t+1} is generated annually from the log-normal distribution independently of N_t.

Buried *B. rapa* seeds have been successfully germinated after 23 yr (Madsen, 1962), > 40 yr (Chippindale & Milton, 1934) and > 660 yr (Odum, 1965). A 20-yr seed bank is modelled, assuming 80% germination in the first season, followed by a standard 50% annual reduction in contribution (Harper, 1977). The contribution from each year of the seed bank (C_{year}) is thus 0.8, 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.0015625, 0.00078125, 0.000390625, 0.000195313, 9.76563E-05, 4.88281E-05, 2.44141E-05, 1.2207E-05, 6.10352E-06, 3.05176E-06, 1.52588E-06, 7.62939E-07, 3.8147E-07. The size of the population (N^{year}) and the relative numbers of chloroplasts present in the seed bank are recorded for each year. The starting state is for the seed bank to be fixed *B. rapa* and of the same size N_{mean}. Equation 1 is modified to take the contribution of each generation in the seed bank into account:

$$P_r^{t+1} = \sum_{year=1}^{year=20} C_{year}\left(\frac{N_n^{year}}{N^{year}}\lambda_n + \frac{N_r^{year}}{N^{year}} m\lambda_{F1}\right) \qquad \text{Eqn 2}$$

where year 1 is time t and year x is time $(t + 1 - x)$.

Parameters for weedy and wild *B. rapa* are available in Supporting Information Notes S3.

***In situ* competition experiment** We empirically tested for the possibility of selective advantage being conferred by the crop chloroplast in riverside *B. rapa* populations by planting 1 : 1 mixes of *B. rapa* seeds collected from 20 divergent parental *B. rapa* plants containing two chloroplast haplotypes (those characteristic of rapeseed and *B. rapa*) collected from two mixed populations (Table S7a, see Supporting Information). Six thousand seeds were treated with 100 ppm GA_3 to remove dormancy (Allainguillaume *et al.*, 2006), and 500 were planted (November 2004) in each of 12 freshly disturbed sites known to contain *B. rapa*. The use of locations with resident *B. rapa* known to lack crop chloroplasts (Table S7a) ensured that the results had maximal ecological relevance, although difficulty lay in the presence of a *B. rapa* seed bank. Moreover, emergent seedlings with the *B. rapa* chloroplast type will include both planted and resident genotypes. Wide genetic diversity amongst the residents, the parents used and the uncertainty of paternal parentage precluded the use of molecular markers to identify (and discount) resident recruits. We therefore estimated the relative contribution of resident seeds using seedling recruitment data collected from 16 undisturbed stationary quadrats in three nearby and equivalent sites (Table S7b). The retention of competing vegetation in these reference quadrats was intended to reduce seed bank recruitment and so provide a conservative underestimate of resident seed contribution among experimental quadrats.

Results

All chloroplast markers generated polymorphisms between species in the reference panels and revealed nine haplotypes (Table S1a–c). Considering all markers, samples were divided into distinct, species-specific clusters, with a marked absence of intermediate haplotypes (Fig. 1). Occasional atypical samples clustering with another species were invariably cultivars/volunteers whose position could be readily explained by chloroplast capture during breeding (Table S1a,c). Overall, there were no *B. oleracea* plants and only three *B. rapa* plants (all Italian accessions, two of which were cultivated Broccoletto varieties) that possessed the rapeseed haplotype. Accordingly, we elected to use these markers (Table S1a) to survey for crop chloroplast capture.

We next verified maternal chloroplast inheritance in interspecific crosses between rapeseed and both wild relatives. We screened 192 offspring from reciprocal crosses between two *B. rapa* accessions and two rapeseed lines; all were triploid F_1 hybrids, as revealed by flow cytometry and the presence of all C genome crop-specific SSRs. All hybrids bore the maternal chloroplast (Table S2b,c). By contrast, 109 crosses between rapeseed and *B. oleracea* yielded only seven offspring. All seven were triploid F_1 hybrids and inherited the crop chloroplast maternally (Table S2d).

Wild populations were subsequently screened for chloroplast capture from rapeseed. Just four of 716 (i.e. 0.6%) wild *B. oleracea* plants from 28 sympatric populations possessed rapeseed chloroplasts. These individuals originated from four sites. They included one triploid F_1 hybrid and three presumably introgressed diploids exhibiting the *B. oleracea* phenotype (Table S3a,b). All other sympatric and allopatric plants contained *B. oleracea* chloroplasts.

Rapeseed chloroplast capture was similarly uncommon among weedy *B. rapa*, with only six of 407 (1.5%) weeds containing rapeseed chloroplasts. From the 407 weedy plants, 26 were triploid F_1 hybrids, 77 were confirmed nuclear introgressants [diploid *B. rapa* containing at least one (of five) crop-specific SSR alleles] and the remaining 304 lacked any nuclear rapeseed markers (unconfirmed introgressants). The six individuals bearing the crop chloroplast haplotype represented 15% (4/26) of triploid F_1 hybrids (Table S4a), 1.3% (1/77) of confirmed nuclear introgressants, but just 0.3% (1/304) of plants showing no evidence of introgression (Table S4a–c).

Weedy *B. rapa* is constantly exposed to the slow influx of crop chloroplasts via hybridization on rapeseed. Crop chloroplasts should consequently accumulate over an unknown time frame unless their presence influences fitness. We constructed a frequency-based Wright–Fisher simulation to predict the rate of chloroplast assimilation into weedy *B. rapa* assuming no advantage (see Supporting Information Notes S3). Without advantage, the model can consistently explain the observed low abundance of crop chloroplast types within the three decades, as the crop was widely cultivated in England (Inglis *et al.*, 1989). Indeed, passive fixation of crop chloroplasts into *B. rapa* was not predicted to occur even within a 200-yr timeframe ($P < 0.00001$). The introduction of a fitness weighting relative to *B. rapa* of up to 1.3 similarly fails to lead to crop chloroplast fixation among the weedy *B. rapa* population within 30 yr ($P < 0.001$), although values above 1.4 did so (data not shown).

By contrast, the 65 riverside *B. rapa* populations (Rivers Thames and Nene) screened in 2002–03 exhibited extreme bias favouring the presence of rapeseed crop chloroplasts in sympatric populations ($P < 0.0001$, $\chi^2 = 99.1$) (Table S5a,b). Moreover, 123 of 1014 (12.1%) of *B. rapa* contained rapeseed chloroplasts in sympatric sites, but only nine of 1020 (0.9%) did so in allopatry. The vast majority (49/50) of triploid F_1 hybrids from sympatric sites (Table S5c) contained rapeseed chloroplasts, although the hyperabundance of rapeseed chloroplasts in surrounding *B. rapa* means we cannot infer how many were formed maternally on (presumably feral) rapeseed rather than on introgressed *B. rapa* that had already captured the rapeseed chloroplast. Nevertheless, these results indicate extensive local chloroplast capture among riverside *B. rapa*.

We constructed a second model to simulate recent chloroplast movement into riverside *B. rapa* (Supporting Information Notes S3) over the *c.* 30 yr since modern rapeseed has been extensively grown. Without advantage, this model predicts a negligible probability of generating the observed frequencies of chloroplast capture after 30 yr (Fig. 2), or even after 200 yr ($P < 0.0001$). One possibility is that excessive variation in population size may have enhanced crop chloroplast abundance through stochastic, local bottleneck effects. Although the presence of a large seed bank in *B. rapa* (Hails & Morley, 2005) buffers against such influences, we elected to incorporate wide stochastic variation in plant number between years and modelled without a seed bank to exaggerate the influence of population size fluctuation. Despite this measure, the probability of reaching the observed rapeseed chloroplast frequencies within 30 yr remained negligible ($P < 0.0001$). We next considered the possibility that raised hybridization frequency causes the high incidence of crop chloroplast capture. Certainly, in one population that approached fixation (Bath, R79), F_1 hybrid abundance was a remarkable 17.5% (36/206). Even when this level of hybrid formation was unrealistically assumed to represent the constant F_1 hybrid abundance during the year following sympatry, the probability of reaching the observed crop chloroplast abundances within 30 yr remained negligible ($P < 0.0001$).

We thereafter considered the possibility that the crop cytoplasm confers advantage. Here, we restored the seed bank (to buffer against accumulation), assumed that the mean hybrid abundance matches previous observations (i.e. 1.46%; Wilkinson *et al.*, 2003) applied across all sympatric populations, and imposed relative fitness coefficient values between 1 and 1.8 to determine the optimal value required to explain various crop chloroplast frequencies. When all hybrids were unrealistically assumed to be formed on rapeseed, mean fitness coefficients of 1.1–1.2 could comfortably explain all observed frequencies of crop chloroplasts in riverside *B. rapa* (Fig. 3).

The reduction of maternal hybridization rates to a more reasonable 10% of F_1 hybrids increased the required range of fitness coefficients slightly to 1.2–1.3 to explain the observed chloroplast frequencies (Fig. 3). Thus, the relatively high abundance of crop chloroplasts in sympatric sites is easily explained only if their presence (or that of associated mitochondria) also confers advantage.

We later surveyed riverside *B. rapa* more broadly to assess the extent to which our observations on chloroplast capture apply generally across England (Table S6a). In total, we sampled 2871 individuals from 92 populations throughout the area of greatest *B. rapa* abundance (central and southern England). Most populations lacked crop chloroplasts (65/92, 70.6%), although the crop chloroplast approached fixation (> 70%) in six (6.5%) of dispersed sites and was apparently fixed in one (Table S6b).

Finally, we sowed *B. rapa* seed mixtures consisting of 50% carrying the crop chloroplast and 50% from the same populations with *B. rapa*-type chloroplasts into populations that lacked crop chloroplasts to provide preliminary '*in situ*' evidence that

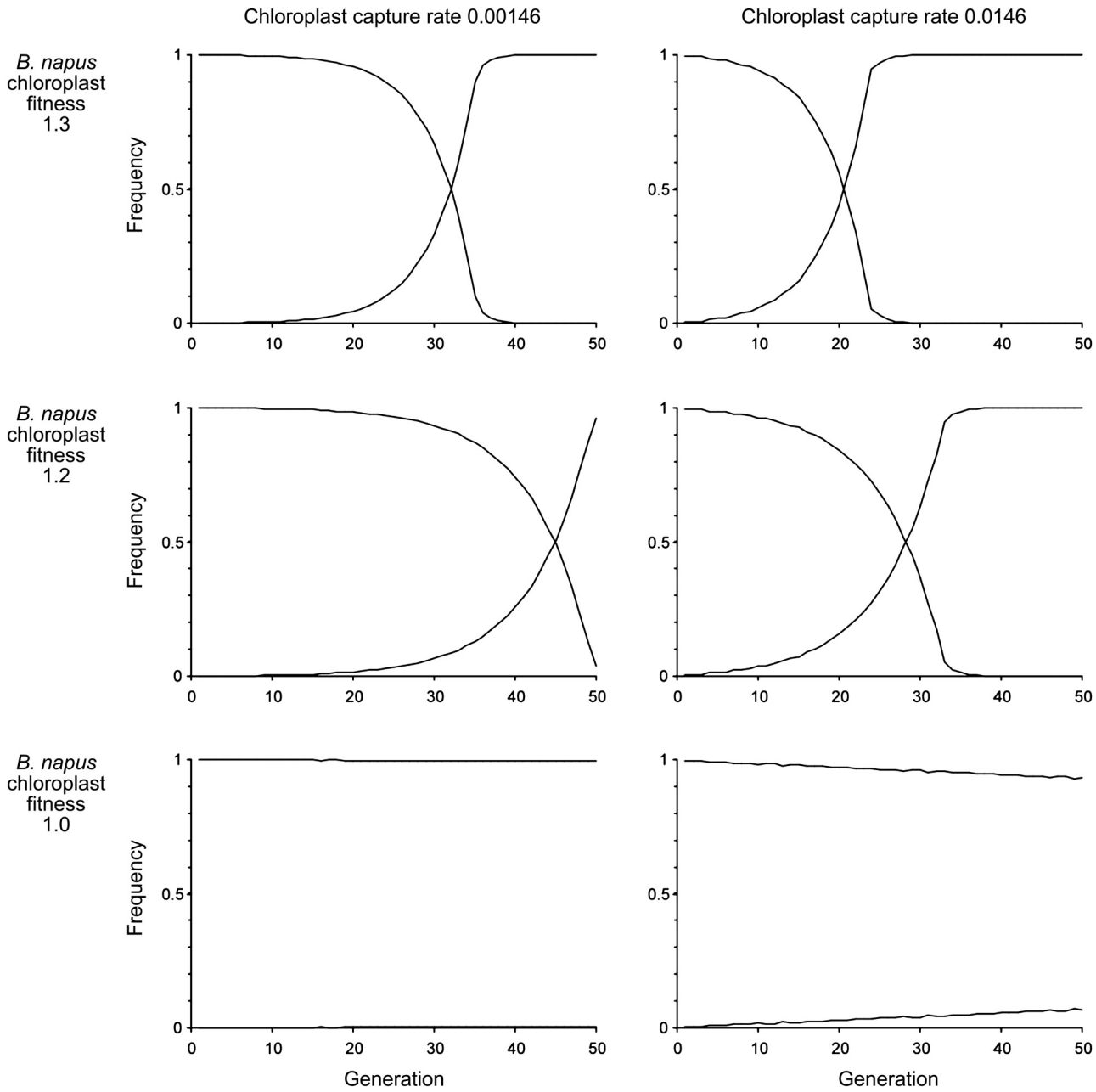

Fig. 2 The effect of varying fitness in *Brassica rapa* plants containing rapeseed chloroplasts on the accumulation of crop chloroplasts in a population of fixed size (5000) starting as pure *B. rapa*. Chloroplast capture is shown for two rates, 0.00146 and 0.0146, representing 10% and 100% of the total F_1 hybridization rate, respectively, in years of sympatry (every 3 yr). Full lines represent rapeseed and broken lines represent *B. rapa* chloroplasts. When the fitness is unity, a ratio of 0.5 : 0.5 is achieved in 436 generations for a capture rate of 0.0146, but is not achieved in 100 000 generations for a capture rate of 0.00146.

possession of the crop plasmid influences performance. We first generated a 'best case' scenario estimate of *B. rapa* seedling recruitment from the existing seed bank in unsown quadrats. Here, mean seedling germination per unsown quadrat was 5.75 ± 2.22 [two standard errors of the mean (SEM)], providing an expected minimal resident seed bank recruitment to first leaf stage across all experimental (i.e. sown) quadrats of 69 ± 26.64 seedlings (5.75 ± 2.22 seedlings/quadrat × 12 quadrats). After discounting for local recruitment, seedlings with rapeseed chloroplasts were invariably significantly more abundant amongst the emergent seedlings (Table S7a,b), thereby indicating a divergence of performance associated with chloroplast type and supporting the prediction of crop cytoplasm advantage.

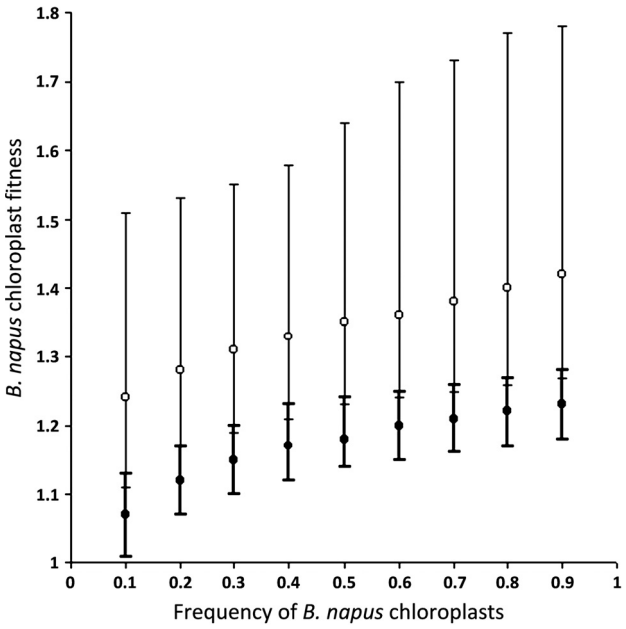

Fig. 3 Expected fitness of rapeseed chloroplasts relative to *Brassica rapa* chloroplasts (fitness 1.0) to attain different crop chloroplast frequencies within a population of fixed size (5000) within 30 generations, with sympatry every 3 yr. Estimates obtained from 10 000 stochastic simulations, taking the rate of maternal hybridization events leading to chloroplast capture as 0.00146 (open circles) and 0.0146 (filled circles), representing 10 and 100% of total F_1 hybridization rates, respectively. Circles show the mean fitness, and the error bars the range of fitness values, which were the lowest fitness advantages to achieve each frequency.

Discussion

The ability to distinguish between characteristic chloroplast haplotypes of cross-compatible species is a requirement of any study of chloroplast capture. Although the seven chloroplast markers used in the present work revealed some level of infraspecific variation for all three species, this was minor compared with the interspecific variation. Clear distinction between rapeseed and relatives was noted in all markers across the reference panel, and this allowed us to screen for chloroplast capture with confidence provided that the presumption of maternal inheritance holds for interspecific crosses.

Numerous studies illustrate that, for the vast majority of plant species, chloroplasts are invariably inherited maternally in infraspecific crosses (see Daniell, 2007). This leads to the expectation of maternal chloroplast inheritance in interspecific crosses, although it is important to test this premise. For crosses between rapeseed and *B. rapa*, there are at least two independent studies confirming that chloroplasts are maternally inherited amongst modest numbers of offspring (Scott & Wilkinson, 1999; Johannessen *et al.*, 2005). The results presented here included larger numbers of offspring (from four cross combinations) and included crosses in both directions. In all cases, chloroplasts were maternally inherited, further supporting the presumption of obligate (or near-obligate) maternal inheritance. Crosses involving *B. oleracea* were far more difficult and produced only seven offspring, all of which nevertheless inherited their chloroplast maternally. Thus, our results are only consistent with obligate maternal inheritance for this cross.

It may be reasonably argued that, for most crop–wild relative combinations, at least some seed-mediated gene flow can be expected whenever hybrid frequency is relatively high. There are nonetheless several features of these *Brassica* species that could act to repress the incidence of chloroplast capture, and so may throw this presumption into question. For instance, the strong sporophytic self-incompatibility of *B. rapa* and *B. oleracea* will impede introgression through the maternal line of the wild recipient via selfing, and so lower the frequency of chloroplast capture. There is likewise some evidence that rapeseed carries 'cryptic' self-incompatibility alleles that may then become functional in *B. rapa* when captured (Okamoto *et al.*, 2007). If this effect were to apply generally, it would also favour introgression through the crop maternal line over that through the *B. rapa* maternal line, thereby reducing chloroplast capture. It follows that the detection here of chloroplast capture in sympatric sites, where hybrids are relatively abundant, although not entirely surprising, does nevertheless serve to indicate that these features have not prevented spontaneous chloroplast capture in either recipient species.

Modern rapeseed has only been widely grown in England since the mid-1970s, with the area assigned to the crop increasing from 9530 ha in 1970 to over 556 000 ha in 2006 (Supporting Information Notes S1). The large abundance of crop chloroplasts in riverside *B. rapa* consequently requires explanation. However, in the late 19th century, *B. napus* was widely grown (primarily swede, but also rapeseed for steam engine lubrication). For example, strikingly, the total area recorded during the period 1880–96 for turnips (*B. rapa*) and swedes (*B. napus*) was 890 000 ha (2.2 million acres) per annum. During the latter part of this period, these staple and fodder vegetable crops were grown on a similar acreage as wheat. Thus, chloroplast capture by wild *B. rapa* is either a recent phenomenon or is relatively ancient and dates back over 100 yr.

The close spatial association between the positioning of contemporary rapeseed fields and the appearance of rapeseed chloroplasts in riverside *B. rapa* populations implies that capture occurred in modern times. This inference is further supported by the notable lack of spread from these sites despite high local frequencies, and by the presence of rapeseed chloroplasts in *B. rapa* individuals positioned throughout the entire shared rapeseed–*B. rapa* boundaries within the contemporary field layouts (data not shown). This being so, the fact that sympatric riverside populations adjacent to rapeseed fields exhibited more capture than weedy *B. rapa* actually growing within the crop is strikingly counter-intuitive and

requires explanation. Perhaps the most plausible hypothesis stems from the occasional presence of feral rapeseed in riverside *B. rapa* populations in the year following sympatry (for example, Allainguillaume *et al.*, 2006). Feral rapeseed is typically infrequent and, as such plants are greatly outnumbered by *B. rapa* in riverside populations, hybrid seed set on these plants should be significantly elevated. Further study is required to characterize the importance of this route of F_1 hybrid formation, although the high incidence of chloroplast capture noted here implies that the contribution to the total could be significant.

The transmission of rapeseed chloroplasts into sympatric *B. oleracea* populations is perhaps also surprising given that the hybrid abundance for this species probably numbers in hundreds rather than thousands (see Ford *et al.*, 2006; unpublished). Conversely, the fact that we frequently noted occasional feral rapeseed plants within *B. oleracea* populations (data not shown) provides a similar scenario to that outlined above for riverside *B. rapa*, and requires that greater account should be taken of feral crops when modelling transgene spread on a landscape scale. Observations for weedy *B. rapa* are in keeping with reports of infrequent chloroplast capture by volunteer rapeseed plants from sympatric weedy *B. rapa* in Denmark (Hansen *et al.*, 2003), and no capture in two UK weedy *B. rapa* populations (Haider *et al.*, 2009), and are more easily explained. Moreover, weedy *B. rapa* plants are typically greatly outnumbered by surrounding crop plants, and the consequential over-abundance of rapeseed pollen renders hybridization relatively less likely on rapeseed and more likely on *B. rapa*.

Irrespective of the mechanisms leading to rapeseed chloroplast capture by all relatives studied here, its occurrence means that, at best, chloroplast transformation will merely reduce transgene recruitment (a point made previously by Hansen *et al.*, 2003). Thus, chloroplast transformation cannot be used as a basis for circumventing exposure analysis for hazards associated with gene flow, although it could reduce hybrid abundance provided that the crop chloroplast confers no advantage. It is here that careful consideration is needed of the large abundance of rapeseed chloroplasts in sympatric riverside *B. rapa* populations. The large abundance of crop chloroplasts among F_1 hybrids occupying sympatric sites characterized in this study implies that either more F_1 hybrids are returned from seed of feral rapeseed and/or that their offspring have a fitness advantage. Examination of the surrounding *B. rapa* plants from these populations also revealed a hyperabundance of rapeseed chloroplasts when compared with allopatric populations.

The maternal inheritance of chloroplasts in *Brassica* means that modelling approaches are particularly well suited to investigating the possible causes of these observations. The consistent failure of 'nonadvantage' models parameterized on existing population size and hybrid abundance to explain the observed rapeseed chloroplast frequencies, even when these models are adjusted to exaggerate the effects of stochasticity, bottlenecks or elevated hybridization frequency, implies that the crop cytoplasm may confer advantage. This tenet was supported when the model was adjusted to more realistic parameters, but various levels of advantage were attached to the possession of rapeseed cytoplasm, with the model explaining the current abundance of rapeseed chloroplasts in sympatric riverside *B. rapa* with only modest levels of advantage. Preferential survival of seedlings carrying rapeseed chloroplasts during the *in situ* seedling competition experiments adds further support to the theory. We therefore infer that the advantage given by the possession of rapeseed cytoplasm in riverside habitats probably led to their widespread local accumulation in periodically sympatric *B. rapa* populations across England. This observation compromises the utility of chloroplast transformation as a biocontainment strategy within this setting, as it means that the recruitment of neutral and advantageous transgenes could actually be accelerated rather than repressed by deployment of the technology. Furthermore, such advantage would persist until all of these sites became fixed for the crop chloroplast type (our model predicts that this is still several decades away). The same is not true for *B. rapa* in a weedy setting in the UK or in wild *B. oleracea*, where recruitment would be initially repressed by the technology and spread would depend on any advantage accruing from the transgene.

We are unaware of similar reports of context-specific advantage being predicted in a wild relative attributable to capture of a crop chloroplast, and so it is difficult to assess the extent to which our findings apply more generally. Such data may emerge as the number of quantitative trait locus-based studies identifying genomic regions associated with adaptive differences between ecotypes (for example, Gardner & Latta, 2006) or crop–wild hybrids (Baack *et al.*, 2008) proliferates. Studies of this nature will also uncover the extent to which genes and gene regions within the nuclear genome are also likely to confer advantage in a wild or weedy context. Nevertheless, there are several contemporary studies showing that context can profoundly affect the fitness of crop–relative hybrids and introgressed populations. For instance, Campbell *et al.* (2006) found that location and context produced a pronounced effect on the fitness of introgressed populations of *Raphanus raphanistrum* × *R. sativus* hybrids. A fitness of between 1 and 2.7 was observed in advanced generations of *R. raphanistrum* × *R. sativus* hybrids relative to wild *R. raphanistrum*. Mercer *et al.* (2007) found that crop–wild hybrids of sunflower (*Helianthus annuus*) were markedly less fit than wild *H. annuus* when grown under benign conditions, but that this difference was strongly attenuated or even negated when the populations were exposed to stressful conditions. Vacher *et al.* (2004) similarly found that the direct advantage conferred by the Bt transgene in *B. napus* × *B. rapa* F_1 hybrids was only maintained in the face of active herbivore pressure, and that release from selection resulted in the transgenic experiencing a significant fitness cost.

Table 1 The range of currently available containment strategies (+, strategies compromised by seed-mediated gene flow; –, techniques unaffected) (Daniel, 2002; Anon, 2004; Gleba *et al*., 2004)

Technique	Impact of seed-mediated gene flow
Chloroplast transformation	+
Engineered male sterility	+
Apomixis	+
Cleistogamy	+
Physical separation	–
Natural genetic containment	–
Inducible promoters	–
Terminator concept/seed lethality	–
Transgene mitigation	–
Inteins	–
Auxotrophy	–
Transgene excision	–

These findings have variable relevance for different biocontainment strategies. In the case of male sterility, advantage and subsequent accumulation of the rapeseed chloroplast will not influence the rate of subsequent transgene spread, except in those instances in which sterility is at least partly conferred by the chloroplast itself. Nevertheless, the fact that maternal introgression does occur in all three scenarios means that the approach would not be completely effective on a landscape scale and could only delay transgene spread. Although we conclude that the value of chloroplast transformation is limited for biocontainment in these contexts, we strongly emphasize that these findings do not compromise the utility of chloroplast transformation as a biotechnological tool *per se*. Moreover, chloroplast transformation also facilitates high levels of transgene expression, multigene engineering and lacks some of the drawbacks of nuclear insertion, such as gene silencing, position and pleiotropic effects (Daniell *et al*., 2005). It is also entirely possible that there may be a few contexts in which chloroplast transformation will provide a more effective biocontainment measure.

In conclusion, we feel that the search for truly effective biocontainment strategies should continue. There are numerous alternative containment technologies that are either marginally affected or unaffected by seed-mediated gene flow (Table 1). Currently, these technologies are only in the concept or developmental stage, and we argue that there is a pressing need for further support to develop and evaluate the efficacy of emerging biocontainment technologies.

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References

Al-Ahmad H, Gressel J. 2006. Mitigation using a tandem construct containing a selectively unfit gene precludes establishment of *Brassica napus* transgenes in hybrids and backcrosses with weedy *Brassica rapa*. *Plant Biotechnology Journal* **4**: 23–33.

Allainguillaume J, Alexander M, Bullock JM, Saunders M, Allender CJ, King G, Ford CS, Wilkinson MJ. 2006. Fitness of hybrids between rapeseed (*Brassica napus*) and wild *Brassica rapa* in natural habitats. *Molecular Ecology* **15**: 1175–1184.

Allender CJ, Allainguillaume J, Lynn J, King GJ. 2007. Simple sequence repeats reveal uneven distribution of genetic diversity in chloroplast genomes of *Brassica oleracea* L. and ($n = 9$) wild relatives. *Theoretical and Applied Genetics* **114**: 609–618.

Anon. 2004. Bioconfinement of plants. In: *Biological confinement of genetically engineered organism*. Washington, WA, USA: The National Academies Press, 65–129.

Azhagiri AK, Maliga P. 2007. Exceptional paternal inheritance of plastids in Arabidopsis suggests that low-frequency leakage of plastids via pollen may be universal in plants. *Plant Journal* **52**: 817–823.

Baack EJ, Sapir Y, Chapman MA, Burke JM, Rieseberg LH. 2008. Selection on domestication traits and QTLs in crop–wild sunflower hybrids. *Molecular Ecology* **17**: 666–677.

Bogdanova VS. 2007. Inheritance of organelle DNA markers in a pea cross associated with nuclear-cytoplasmic incompatibility. *Theoretical and Applied Genetics* **114**: 333–339.

Campbell LG, Snow AA, Ridley CE. 2006. Weed evolution after crop gene introgression: greater survival and fecundity of hybrids in a new environment. *Ecology Letters* **11**: 1198–1209.

Chandler S, Dunwell JM. 2008. Gene flow, risk assessment and the environmental release of transgenic plants. *Critical Reviews in Plant Sciences* **27**: 25–49.

Chapman MA, Burke JM. 2006. Letting the gene out of the bottle: the population genetics of genetically modified crops. *New Phytologist* **170**: 429–443.

Chippindale HG, Milton WEJ. 1934. On the viable seeds present in the soil beneath pastures. *Journal of Ecology* **22**: 508–531.

Daniell H. 2002. Molecular strategies for gene containment in transgenic crops. *Nature Biotechnology* **20**: 581–586.

Daniell H. 2007. Transgene containment by maternal inheritance: effective or elusive? *Proceedings of the National Academy of Sciences, USA* **104**: 6879–6880.

Daniell H, Datta R, Varma S, Gray S, Lee SB. 1998. Containment of herbicide resistance though genetic engineering of the chloroplast genome. *Nature Biotechnology*, **16**: 345–348.

Daniell H, Kumar S, Dufourmantel N. 2005. Breakthrough in chloroplast genetic engineering of agronomically important crops. *Trends in Biotechnology* **23**: 238–245.

Denis M, Delourme R, Gourret JP, Mariani C, Renard M. 1993. Expression of engineered nuclear male sterility in *Brassica napus* (genetics, morphology, cytology, and sensitivity to temperature). *Plant Physiology* **101**: 1295–1304.

Dunwell JM, Ford CS. 2005. Technologies for biological containment of GM and non GM crops. DEFRA Contract CPEC 47, http://www.defra.gov.uk/science/Project_Data/DocumentLibrary/CB02036/CB02036_3629_FRP.doc.

Ellstrand NC, Prentice HC, Hancock JF. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Sysematics* **30**: 539–563.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47–50.

Ford CS, Allainguillaume J, Grilli-Chantler P, Cuccato G, Allender CJ, Wilkinson MJ. 2006. Spontaneous gene flow from rapeseed (*Brassica*

napus) to wild *Brassica oleracea*. *Proceedings of the Royal Society B* **273**: 3111–3115.

Gardner KM, Latta RG. 2006. Identifying loci under selection across contrasting environments in *Avena barbata* using quantitative trait locus mapping. *Molecular Ecology* **15**: 1321–1333.

Gleba Y, Marillonet S, Klimyuk V. 2004. Design of safe and biologically contained transgenic plants: tools and technologies for controlled transgene flow and expression. *Biotechnology and Genetic Engineering* **21**: 325–367.

Haider N, Allainguillaume J, Wilkinson MJ. 2009. The inefficiency of chloroplast transformation in preventing transgene flow: a case study of chloroplast capture. *Current Genetics* **55**: 139–150.

Hails RS, Morley K. 2005. Genes invading new populations: a risk assessment perspective. *Trends in Ecology and Evolution* **20**: 245–252.

Hansen LB, Siegismund HR, Jørgensen RB. 2003. Progressive introgression between *Brassica napus* (oilseed rape) and *B. rapa. Heredity* **91**: 276–283.

Hare PD, Chua NH. 2002. Excision of selectable marker genes from transgenic plants. *Nature Biotechnology* **20**: 575–580.

Harper JL. 1977. *Population biology of plants*. London, UK: Academic Press.

Hu Y, Zhang Q, Rao G, Sodmergen. 2008. Occurrence of plastids in the sperm cells of *Caprifoliaceae*: biparental plastid inheritance in angiosperms is unilaterally derived from maternal inheritance. *Plant Cell Physiology* **49**: 958–968.

Inglis R, Thearle RJP, Issacson AJ. 1989. Wood pigeon (*Columba palumbus*) damage to oilseed rape. *Crop Protection* **8**: 299–309.

James CM, Barret JA, Russell SJ, Gibby M. 2001. A rapid PCR based method to establish the potential for paternal inheritance of chloroplasts in *Pelargonium*. *Plant Molecular Biology Reports* **19**: 162–167.

Johannessen MM, Andersen BA, Damgaard C, Jørgensen RB. 2005. Maternal inheritance of chloroplasts between *Brassica rapa* and F1-hybrids demonstrated by cpDNA markers specific to oilseed rape and *B. rapa*. *Molecular Breeding* **16**: 271–278.

Kriete G, Niehaus K, Perlick AM, Pühler A, Broer I. 1996. Male sterility in transgenic tobacco plants induced by tapetum-specific deacetylation of the externally applied nontoxic compound *N*-acetyl-L-phosphinothricin. *Plant Journal* **9**: 809–818.

Kuvshinov V, Koivu K, Kanerva A, Pehu E. 2001. Transgenic crops expressing synthetic *cry9Aa* gene are protected against insect damage. *Plant Science* **160**: 341–353.

Lanner C. 1998. Relationships of wild *Brassica* species with chromosome number $2n = 18$, based on comparison of the DNA sequence of the chloroplast intergenic region between trnL (UAA) and trnF (GAA). *Canadian Journal of Botany* **76**: 228–237.

Lu BR. 2003. Transgene containment by molecular means – is it possible and cost effective? *Environmental Biosafety Research* **2**: 3–8.

Madsen SB. 1962. Germination of buried and dry stored seeds. *Proceedings of the International Seed Testing Association* **27**: 920–928.

Matsushima R, Hu Y, Toyoda K, Sodmergen, Sakamoto W. 2008. The model plant *Medicago truncatula* exhibits biparental plastid inheritance. *Plant Cell Physiology* **49**: 81–91.

Mercer KL, Andow DA, Wyse DL, Shaw RG. 2007. Stress and domestication traits increase the relative fitness of crop–wild hybrids in sunflower. *Ecology Letters* **10**: 383–393.

Mitchell ND, Richards AJ. 1979. Biological flora of the British Isles no.145 *Brassica oleracea* L. ssp. *Oleracea*. *Journal of Ecology* **67**: 1087–1096.

Mogensen H, Rusche M. 2000. Occurrence of plastids in rye (*Poaceae*) sperm cells. *American Journal of Botany* **87**: 1189–1192.

Odum S. 1965. Germination of ancient seeds. *Dansk Botanisk Arkiv* **24**: 1–70.

Okamoto S, Odashima M, Fujimoto R, Sato Y, Kitashiba H, Nishio T. 2007. Self-compatibility in *Brassica napus* is caused by independent mutations in the S-locus genes. *Plant Journal* **50**: 391–400.

Rieseberg LH, Soltis DE. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* **5**: 65–84.

Ruf S, Karcher D, Bock R. 2007. Determining the transgene containment level provided by chloroplast transformation. *Proceedings of the National Academy of Sciences, USA* **104**: 6998–7002.

Scheffler JA, Dale PJ. 1994. Opportunities for gene transfer from transgenic oilseed rape (*Brassica napus*) to related species. *Transgenic Research* **3**: 263–278.

Scott SE, Wilkinson MJ. 1999. Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. *Nature Biotechnology* **17**: 390–392.

Suwabe K, Tsukazaki H, Iketani H, Hatakeyama K, Kondo M, Fujimura M, Nunome T, Fukuoka H, Hirai M, Matsumoto S. 2006. Simple sequence repeat-based comparative genomics between *Brassica rapa* and *Arabidopsis thaliana*: the genetic origin of clubroot resistance. *Genetics* **173**: 309–319.

Svab Z, Maliga P. 2007. Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proceedings of the National Academy of Sciences, USA* **104**: 7003–7008.

Tackaberry ES, Prior F, Bell M, Tocchi M, Porter S, Mehic J, Ganz PR, Sardana R, Altosaar I, Dudani A. 2003. Increased yield of heterologous viral glycoprotein in the seeds of homozygous transgenic tobacco plants cultivated underground. *Genome* **46**: 521–526.

Vacher C, Weis AE, Hermann D, Kossler T, Young C, Hochberg ME. 2004. Impact of ecological factors on the initial invasion of Bt transgenes into wild populations of birdseed rape (*Brassica rapa*). *Theoretical and Applied Genetics* **109**: 806–814.

Wilkinson MJ, Davenport IJ, Charters YM, Jones AE, Allainguillaume J, Butler HT, Mason DC, Raybould AJ. 2000. A direct regional scale estimate of transgene movement from genetically modified oilseed rape to its wild progenitors. *Molecular Ecology* **9**: 983–991.

Wilkinson MJ, Elliott LJ, Allainguillaume J, Shaw MW, Norris C, Welters R, Alexander M, Sweet JB, Mason DC. 2003. Hybridization between *Brassica napus* and *B. rapa* on a National Scale in the United Kingdom. *Science* **302**: 457–459.

Yuan YW, Olmstead RG. 2008. A species-level phylogenetic study of the *Verbena* complex (*Verbenaceae*) indicates two independent intergeneric chloroplast transfers. *Molecular Phylogenetics and Evolution* **48**: 23–33.

Supporting Information

Additional supporting information may be found in the online version of this article.

Notes S1 (1) GM field trial applications: online databases; and (2) agricultural statistics.

Notes S2 Crop-specific chloroplast markers: (1) chloroplast CAPS1 assay; (2) chloroplast high-resolution melt SNPs.

Notes S3 Frequency-based Wright–Fisher simulation to assess the time frame of chloroplast assimilation into *B. rapa*: (1) parameter description for weedy and wild *B. rapa*; (2) seed bank; (3) variable population size; (4) calculation of required fitness advantage.

Table S1 (a–c) Chloroplast haplotype distribution in *B. napus*, *B. oleracea* and *B. rapa* reference panels

Table S2 (a–d) Maternal inheritance of chloroplasts in inter-specific crosses between *B. napus* and *B. rapa* or *B. oleracea*

Table S3 (a–b) Crop-specific chloroplast screening in wild *B. oleracea* populations

Table S4 (a–c) Crop-specific chloroplast screening in weedy *B. rapa* populations

Table S5 (a–c) Crop-specific chloroplast screening in wild *B. rapa* populations from the River Thames and the River Nene

Table S6 (a–b) Chloroplast haplotype screening of wild *B. rapa* in central and southern England

Table S7 *In situ* competition experiment using mixed seed (rapeseed + *B. rapa*) plantings in stationary quadrats within natural riverside populations of *B. rapa*.

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