Reviewer's Comment

Although this study identified hybridisation between oil seed rape and *Sinapis arvensis*, such a finding needs to be interpreted with caution. The frequency of such an event in the field is likely to be very low, as highlighted by the fact it has never been detected in numerous previous assessments. Furthermore, the conditions where the hybrid was found appear to be quite unusual, restricted as it was to a case where Sinapis was sufficiently abundant in a crop to act as a significant conspecific pollen donor. The consequences of the transfer of the herbicide tolerance trait on the fitness and persistence of *Sinapis arvensis* were not assessed in this study but are presumed to be negligible (Hails & Morley[1]). Nevertheless, this unusual occurrence merits further study in order to adequately assess any potential risk of gene transfer.

[1] Hails, R.S. & Morley, K. (2005) Genes invading new populations: a risk assessment perspective. Trends in Ecology and Evolution 20(5):245-252

THE POTENTIAL FOR DISPERSAL OF HERBICIDE TOLERANCE GENES FROM GENETICALLY-MODIFIED, HERBICIDE-TOLERANT OILSEED RAPE CROPS TO WILD RELATIVES

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SUMMARY

1. The commercial growing of genetically modified, herbicide-tolerant (GMHT) oilseed rape is seen to result in the potential for the inserted gene to escape from the crop and become incorporated in the genomes of one or more related wild crucifer species, potentially giving a competitive advantage to the recipients.
2. The possibility of such gene transfer may be greatest where the wild relative is closely related to the crop, where the two grow in close proximity and where they flower at the same time.
3. Because oilseed rape is grown as either a winter-sown or a spring-sown crop, there are two flowering periods; winter rape in late March to mid-May and spring rape in June. There may be a short overlap period when some individuals in both crops are in flower at the same time. The existence of these two flowering periods extends the period over which both crop and wild relatives may be in flower together.
4. The most closely related species to oilseed rape (*Brassica napus*), and those which have been considered as potential recipients of herbicide tolerance are other members of the genus *Brassica: B. oleracea, B. rapa, B, nigra*, together with *Raphanus raphanistrum* and *R. sativus*, and two species of *Sinapis*, *S. arvensis* and *S. alba*.
5. As an adjunct to the Farm Scale Evaluations of GMHT oilseed rape, DEFRA funded a three-year project to examine the extent to which transfer of herbicide tolerance from the oilseed rape crops to wild relatives in the vicinity did occur. This report details the result of that study.
6. Transfer of herbicide tolerance was assessed in the field and in the laboratory. In the field, plants were tested by the application of a small quantity of glufosinate ammonium (*Liberty*™) to individual leaves and observing whether any necrosis resulted. Seed collected from plants growing in or near oilseed rape fields were germinated and the resulting seedlings were sprayed with *Liberty*™ to assess tolerance. Any plants showing signs of tolerance to the herbicide were subjected to PCR to identify whether the gene was present.
7. The most common wild relative found in fields in the trial was *Sinapis arvensis***.** In contrast, *Brassica rapa* was only found adjacent to a single field in the winter oilseed rape trial.
8. A total of 95459 seedlings of wild relatives were grown and tested. Of these, only 2 plants, of *Brassica rapa* showed resistance to the treatment.
9. In the year after the trial, a sub-set of fields was revisited and wild relatives growing in or around the subsequent crop were tested by herbicide application. A single plant of *Sinapis arvensis* showed no reaction to the application and a leaf of this plant was taken for PCR analysis. The gene construct was found to be present.
10. Because weed control is generally very efficient in cereal fields, few volunteer oilseed rape plants survive in wheat or barley fields sown following the harvest of an oilseed rape crop.
11. We examined some fields in the first and second years following the oilseed rape trials and found that volunteer populations did occur and that a proportion of the plants were tolerant of glufosinate ammonium.

12. Transfer of herbicide tolerance to wild relatives is not seen as a major problem, especially as it would not be expected to confer any selective advantage in the absence of the appropriate herbicide application.
13. The persistence of herbicide-tolerant volunteer populations of oilseed rape in subsequent crops may pose agronomic problems, especially if the same gene construct is introduced into other crop species.

1. INTRODUCTION

One of the concerns raised by the introduction of genetically modified crop plants is that the gene construct may move from the modified crop into wild relatives of that crop (Crawley et al, 1993; Champolivier et al, 1999; Snow et al, 1999). Once within wild populations, it is suggested that a selective advantage could be conferred on the recipients, so altering their biology and influencing their ecological relationship with native genotypes or other species (Klinger & Ellstrand, 1994; Lefol et al, 1997; Linder, 1998). It is considered by these and other authors that this could constitute a threat to biodiversity.

The possibilities for transfer of any trait from crop to weed will depend on the two occurring together, their synchronous flowering, successful pollen transfer and compatibility of the pollen which would allow successful fertilisation and embryo development. Any seed produced would then need to germinate and the trait would need to be exhibited in the resulting plant. To maintain the trait, success as a pollen donor, as a seed producer, or both, would be needed. Where a trait carrier is self fertile or where more than one individual has been produced, the F_2 generation may be produced by hybrid mating, though the more likely scenario (given that the population of such hybrids may be very small) is for introgression into the recipient species' genome as a result of backcrossing.

Crop plants and some weeds are derived from the same ancestors and retain a number of common characteristics. They may also still grow in close association within the geographical area in which both originated and give rise to crop-weed complexes (van Raamsdonk and van der Maesen, 1996) in which introgression of weed characters into the crops and crop characters into the weeds can occur, and may have done so over an extended period of time.

Among the crops closest to clearance for commercial planting in Britain are herbicide-tolerant maize, beet and oilseed rape. Of these, maize has no close relatives in Britain so that, although there may be problems of introgression within its native range, in Central America, it would not be subject to gene exchange with wild relatives in Europe. Of the other two crops, beet does have a close wild relative, sea-beet (*Beta maritima*), which is the progenitor of the crop. This is a plant of cliffs and the upper parts of salt marshes. Hybridisation between the two is known to occur (Desplanque et al, 2002). Under normal cultivation conditions where beet (a biennial) is grown either as a source of sucrose or as a fodder crop, the objective is to harvest the plant after the first growing season and, hence, before flowering. Occasionally, bolting plants will flower in their first year. In areas where seed production is the objective, e.g. the Crimea and parts of southern France, the crop does flower in the vicinity of sea beet.

Oilseed rape is an allotetraploid (AACC, 2n = 38) derived from *B. rapa* (AA, 2n = 20) and *B. oleracea* (CC, 2n=18). The wild relatives are defined in this context as members of the tribe *Brassicaceae* of the family *Cruciferae* (see Clapham, Tutin and Warburg, 1962) and in Europe, oilseed rape has a number of wild relatives either within the same genus or in closely related genera. In Britain, these include its parents, *B. rapa* and *B. oleracea*, *B. nigra*, *Sinapis arvensis* and *S. alba*, and *Raphanus raphanistrum*. The extent to which hybrid formation can occur differs

widely among these species. Spontaneous hybrids between *B. napus* and *B. rapa* are known to occur with either species as the pollen donor (Hauser et al, 1998). Where *B rapa* is the pollen receptor, the chances of hybridisation occurring will depend on the relative amounts of crop and conspecific pollen: where there is a large excess of crop pollen, such as when a single individual is present in a field of rape, chances of hybridisation are enhanced considerably (Jørgensen et al,1996; Scott and Wilkinson, 1998). Crosses between *B. oleracea* and *B. napus* are much less easily produced (Scheffler and Dale, 1994) and, although they have been found in experimental plots (Chèvre et al, 1998), they have not been recorded in natural populations (Raybould and Gray, 1993). Crosses with *Raphanus raphanistrum* have also been reported from experimental plots in which the *Raphanus* was grown at a very low density: no hybrids were found when *Raphanus* was present at high density (Darmency et al, 1998). The cross is more likely to occur when oilseed rape is the maternal parent and where pollen competition is absent (Chèvre et al, 1996). Spontaneous hybrids have also been found when *Hirschfeldia incana* was grown at low density in experimental plots (Lefol et al, 1996b). Hybrids with *B. nigra* have only been produced in the laboratory following embryo rescue. This technique has also been used to produce hybrids with *Sinapis arvensis* and *S. alba*. No spontaneous hybrids with *S. arvensis* as the maternal parent have been found in experimental plots or fields (Lefol et al, 1996a; Moyes et al, 2002), although Moyes et al (2002) obtained hybrids with both *B. napus* and *S. arvensis* as the maternal parent in trials using hand pollination techniques; these hybridisation rates were very low however and the single hybrid plant developed from *S. arvensis* as the maternal parent did not produce viable pollen or seed.

Although laboratory and glasshouse tests of compatibility and small scale trials can provide data on compatibility, experimental approaches cannot always be scaled up to provide a realistic assessment of the situation in the field, under agricultural conditions. The Farm Scale Evaluations of genetically modified herbicide-tolerant (GMHT) crops provided the opportunity to test for hybridisation between oilseed rape and these related species at a large number of sites throughout England and Scotland.

The two main objectives of this study were:

1) to assess at the field scale, the extent of transfer of herbicide tolerance from GMHT oilseed rape crops to adjacent wild relatives where these were present within or around the FSE sites, and to determine the persistence within the environment of any herbicide-tolerant hybrids that may have occurred.
2) to assess the persistence of GMHT oilseed rape volunteers within the crops grown following the FSE trials.

2. DISTRIBUTION AND ECOLOGY OF WILD RELATIVES

Brassica rapa is a species which appears in two forms in Britain. "Weedy" B. rapa appears to be a relic of past cultivation of turnip rape (*B. rapa* ssp. *oleifera*) and occurs as an occasional weed in a limited number of fields in the Humberside area. It may also be found in fields in Scotland. During the cereal stages of a normal crop rotation, this weed is controlled by herbicides acting on broadleaf weeds, but in break crops (typically of oilseed rape) it may re-appear as a weed, forming patches within the crop. The "wild" form of *B. rapa* (wild turnip, bargeman's cabbage), referred to as ssp. *sylvestris* (Rich, 1991) is a plant of riversides and canal banks and does not appear to spread beyond the limits of flooding from these systems. Where it is present, it may occur as scattered individuals or as larger colonies of plants. The plant is essentially a winter annual, requiring cold stratification before coming into flower in spring/early summer. The distribution of this species has been subject to doubt largely because of the difficulty of distinguishing it from feral oilseed rape. However, it is the subject of detailed studies at present (Wilkinson et al, 2003) and it appears to be more widespread in central and southern England than was previously thought. It has been found within the Severn, Trent and Thames catchments and their inter-connecting canal systems.

Brassica oleracea (wild cabbage) is a coastal plant of calcareous soils. It is found predominantly in South and South-West England and in South Wales, although scattered populations are present elsewhere in England and in Scotland. This is the plant from which cultivated cabbage is descended and inland, occasional escapes are found in suitable open habitats such as waste ground or disturbed roadsides.

Brassica nigra (black mustard) is most widespread and abundant in southern and south-eastern Britain, though its range does extend to southern Scotland. It is found mainly along riverbanks but also grows on coastal cliffs, shingle or embankments, or as a casual on waste ground.

Sinapis arvensis (charlock) is a widespread and abundant arable weed found in fields, along roadsides and riverbanks and on waste ground throughout Britain, except for the upland and montane areas. This is the most common of the oilseed rape relatives found in and around arable fields.

Sinapis alba (white mustard) is more limited in distribution than S. arvensis. It is most common in southern England, where it is restricted mostly to calcareous soils. However, at sites where it is present it can form large populations.

Raphanus raphanistrum (wild radish, runch) may have yellow or white flowers or, more rarely, purple ones. It is a common plant of fields and waste ground, though may avoid the heavier, wetter, soils favoured by species such as *Brassica nigra*. Seeds tend to have strong inherent dormancy and may require higher temperatures for germination than other oilseed rape relatives. This means that flowering may not begin until June. Where plants develop rapidly, flowering can coincide with that of winter oilseed rape as well as with spring oilseed rape.

3. METHODS

3.1 Field sampling and testing, and glasshouse trials

The FSE fields used a 'split-field' design with one half of the field sown with the GM crop and the other half of the field sown with the conventional equivalent crop. At each winter and spring oilseed rape field in the FSE trials an initial assessment of wild relative presence was made by local field teams and this information used to select fields for sampling.

Shortly before the FSE trial was harvested, seeds of wild relatives were collected from a representative sample of the populations present. Seeds were sampled from plants growing amongst the oilseed rape crop and within a 10m strip next to the crop edge, with a population being defined as a group of individuals at least 10m from its nearest neighbour of the same species. Population size was estimated in broad categories (abundant, limited, patchy, scattered and individuals). Sampling included fields with a range of these densities, from extensive populations within the crop (and hence a greater number of plants to intercept the wind-blown rape pollen but also increased competition from relative pollen which may then germinate preferentially on a stigma of the same species), to scattered or individual plants (with reduced competition from same-species pollen and so potentially greater opportunities for germination of the crop pollen, if inherent incompatibility mechanisms can be overcome).

Samples were taken by stripping all fruits from a number of individuals within each population and taking the harvested fruits to CEH Dorset, where seeds were separated from husks by hand. Representative sub-samples (comprising approximately 200 seeds) from each plant were sown in John Innes seed compost in half seed trays placed in an unheated glasshouse.

Following germination, the number of seedlings present in each tray was counted when plants were between the expanded cotyledon stage and the 3-4 leaf stage. Plants were then sprayed with a 1% solution of *Liberty*™ (supplied by Bayer CropScience). After the seedlings had collapsed following herbicide damage, a second dose of *Liberty*™ was applied so as to treat any seedlings that had been shielded previously and, after at least a further week, the number of survivors was counted. Leaf samples were taken from surviving seedlings and tested by DNA analysis for presence of the herbicide-tolerant (*bar*) gene. Where the *bar* gene was found to be present, another leaf sample was taken and tested by flow cytometry to determine the chromosome number and so assess whether the surviving seedling was a hybrid. Hybrids formed between tetraploid oilseed rape plants and wild, diploid relatives will be triploid, so that the amount of DNA expected would be halfway between that of the two parents.

During the first year of the trial, seeds were sown without any pre-treatment and it was noted that germination occurred over an extended period of several months. In subsequent years, seeds were treated by soaking overnight in a 0.1% solution of gibberellic acid. They were washed, to remove any excess and prevent continued effect of the treatment, before being sown. This procedure resulted in quicker and more even germination, and allowed more rapid turnover of samples.

In the year following the trial, a sub-set of fields was revisited to record presence of wild relatives and of volunteer rape plants, and to test these for herbicide tolerance. Plants *in situ* were tested by applying 1% *Liberty*™ to parts of single leaves using a paintbrush. Glufosinate ammonium is a contact herbicide with only limited translocation, so that effects are limited largely to the area of application. A brown stripe across the leaf was indicative of susceptibility. After approximately one week the fields were revisited and plants were scored for the effect of the herbicide. Herbicide spotting in the field was limited to fewer fields than may have been desirable both as a result of limitation of resources and the restricted number of sites available to test. In the case of the wild relatives this was dependent on the possibility of hybrids being present in the follow-on crop, from the first year study. For the volunteers, effective weed control often meant that there were no volunteers present to sample in the follow-on crops.

Leaves were also taken from a sample of volunteer plants at selected fields and DNA extracted from them was tested for the presence of the gene construct.

3.2 DNA extraction and PCR

To detect presence of the herbicide tolerance (*bar*) gene, a novel DNA extraction method was followed which used a 96-well microtitre plate system (Mogg, 2003). Leaf samples from surviving plants were put into 1.2ml racked microtubes arranged in a 96 well format. Samples were labelled with a code, therefore the PCR testing was carried out "blind". To each collection microtube one tungsten carbide bead and 400µl of extraction buffer (containing 100mM Tris, 50mM EDTA, 500mM NaCl, 0.7% SDS, 50µg/ml Proteinase K and 50µg/ml RNase) was added. The plant material was disrupted using a MM300 mixer mill (Qiagen) and the samples incubated overnight at 37^{o}C. After overnight incubation, 260µl of 5M NaCl was added to each tube, the samples mixed thoroughly and then centrifuged for five minutes at 4000 rpm to pellet the plant debris. The DNA containing supernatant was transferred to a new 1.2ml microtube containing 800µl of 85% isopropanol. The samples were mixed by inverting, placed in the fridge for 3 hours (although 1 hour in the freezer was found to be equally efficient) and then centrifuged at 5000 rpm to pellet the DNA. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 500µl of 1xTE.

PCR reactions were set up using the Qiagen Multiplex PCR Kit. The kit contains a master mix specially designed for multiplex PCR applications. The master mix contains pre-optimized concentrations of HotStarTaq DNA Polymerase, $MgCl_2$, dNTPs and a PCR buffer newly developed for multiplex PCR reactions. Each 10µl PCR reaction contained 1µl of template DNA, 5µl of the Qiagen master mix, and 10 pmoles/µl of primers MDB246 (Pssuara primer), MDB151 (bar primer), CVZ7 (endogenous primer) and CVZ8 (endogenous primer) respectively. Primer sequences were supplied by Bayer CropScience. After an initial 10 minute hot start at 94°C, PCR amplification was carried out for 35 cycles consisting of 1 minute at 94°C, 1 minute at 57°C and 1 minute at 72°C, followed by a final extension of 10 minutes at 72°C. The PCR products were resolved on a 1.5% agarose gel, with Hyperladder (Bioline) to size the amplified fragments. Two positive controls, (genomic DNA samples known to contain the transgenic sequences, supplied by Bayer CropScience), two wildtype controls, (genomic DNA samples not containing the transgenic

sequences, *B. napus* cultivar Maskot grown at Scottish Crop Research Institute) and a negative control (no DNA) were run out on each gel.

3.3 Flow cytometry

Whole leaves were removed from putative hybrids between *B. napus* and *B. rapa* (those seedlings showing tolerance to *Liberty*™), and from seedlings of *B. napus* and *B. rapa* grown alongside the test plants. These were packed in polythene bags, to retain moisture and condition of the leaves, and posted to Prof A.V. Roberts, University of East London for testing by flow cytometry. Testing was carried out within 24 hours of detaching leaves in order to use material in which degradation of DNA was minimal.

Initial measurements were based on 4' 6' diamidino-2-phenylindole (DAPI) as the fluorochrome. DAPI is perfectly reliable for ploidy measurements but is less reliable than the more complicated propidium iodide (PI) staining method for measurements of absolute DNA amounts. Previous experience with *Brassica* material indicated that DAPI and PI give very similar estimates of DNA amounts. Subsequently, the PI method was used. The 2C DNA amounts of young leaves (ten samples per plant) of *B. rapa*, *B. napus* and one of the putative hybrids were measured by flow cytometry using propidium iodide as the fluorochrome and *Petroselinum crispum* 'Champion Moss Curled' (2C DNA amount 4.46 pg) as an internal calibration standard, as described by Yokoya et al (2000).

3.4 Statistical analysis

Statistical analysis was performed using the program StatXact® (Cytel Software, 1998) to calculate the exact 95% confidence for the proportion of wild relatives that were tolerant, based on the method of Clopper and Pearson (1934). Exact statistics are used in cases where the estimated proportions are zero or very small and so standard methods of estimating (symmetrical) confidence limits would not be valid. Chi-squared was used to assess differences within and between fields of volunteers.

4. RESULTS

4.1 Prevalence of relatives

A number of FSE sites had no close relatives within 10m of the crop (i.e. within the crop, any marginal cultivated area or adjacent hedges or ditches).
Among the FSE sites where relatives were present, over the three years of the trial we sampled wild relative seed from 18 spring oilseed rape fields and 10 winter oilseed rape fields, covering a total of 12 counties in the UK (Table 1). Eight winter and fifteen spring oilseed rape fields had populations of *Sinapis arvensis* (seed from two winter rape sites failed to germinate in the glasshouse trials). One winter and five spring fields had *S. alba. B nigra* was in one spring and two winter oilseed rape fields. *Raphanus raphanistrum* was sampled from two winter rape and two spring rape fields and *R. sativus* was only found in a single spring rape field. *B. rapa* was not found until the final year of the winter oilseed rape trial, when it was present at the edge of a single field. No fields had adjacent *B. oleracea* populations. Other crucifers growing in or around rape fields were *Sisymbrium officinale* (frequently), *Alliaria petiolata* (recorded in one field) and *Barbarea vulgaris* (recorded in one field), though population sizes of these were not recorded and samples were not collected. In the final year, we concentrated on trying to find populations of *Raphanus* and *Brassica rapa,* as the two most likely species (on the basis of published work elsewhere) to form hybrids.

The wild relatives present varied in their population structures, with seed samples taken from a range of densities where possible.
Sinapis arvensis, the most frequent wild relative found in the study ranged from extensive populations within the crop, to small populations both within the crop and margin, scattered individuals, and a solitary individual from one field (seeds of which failed to germinate).
Sinapis alba population structures varied from a block of plants approx 10m away from the GM crop, populations along both the GM and conventional crop edges, and individuals.
Brassica nigra seed was also sampled from a range of population sizes including a dense population in the conventional half of the field but near to the split, and a patch of plants, scattered plants and individuals in the GM half.
Raphanus raphanistrum population structures in the GM half of the field included a more or less continuous population in the crop margin, dispersed plants within the crop and individual plants.
Raphanus sativus was found in a strip of game cover at the end of the FSE field and *Brassica rapa* was found in three discrete clumps within 10m of the conventional unit of the oilseed rape crop.

Location in the UK	Number of FSE fields with seed collected					
	Sinapis arvensis	*Sinapis alba*	*Brassica nigra*	*Raphanus raphanistrum*	*Raphanus sativus*	*Brassica rapa*
Aberdeenshire	1			1		
Dorset	1	2				
East Riding of Yorkshire	2					
Gloucestershire	3		1			
Hertfordshire	3	2				
Lincolnshire	4					
Norfolk	4	1		1	1	
Nottinghamshire	1			1		
Oxfordshire				1		
Shropshire	1	1				1
Warwickshire	2		1			
Worcestershire	1		1			
TOTAL	**23**	**6**	**3**	**4**	**1**	**1**

Table 1. Number of FSE oilseed rape fields from which wild relative seed was collected in years 2000 – 2002. The number of fields for each species (with some sites containing more than one species) and within each county is shown.

4.2 Herbicide tolerance in wild relatives

4.2.1 Herbicide tolerance in seedlings grown in the glasshouse trials

Data from different fields within each year have been bulked. Tables 2 and 3 show the number of seedlings of each species tested for herbicide tolerance and the number of parent plants from which the seeds originated. As all the plants from all species produced either no resistant offspring, or only one resistant offspring for 2 of the 97 *Brassica rapa* plants, there is obviously no evidence for differences in resistance rates of offspring between parent plants. Therefore confidence limits for the proportion of offspring in the whole study population of each species, is based on the individual seeds regardless of the number of parent plants.

A total of 95459 seedlings were tested and of these only 2 seedlings, of *Brassica rapa,* were herbicide-tolerant, giving an estimated resistance rate of 0.00021. Herbicide resistance was not evident in the other species of wild relative tested.

Species	2000	2001	2002	Total (all years)	Tolerant	U_1 (all years)
Sinapis arvensis	19606	30533	10629	60768	0	0.0001
Sinapis alba	1288	661	18694	20643	0	0.0002
Brassica nigra	147	2444	0	2591	0	0.0014
Raphanus raphanistrum	47	556	1190	1793	0	0.0021
Raphanus sativus	117	0	0	117	0	0.031
Brassica rapa	0	0	9547	9547	2	0.0008
TOTAL				**95459**		

Table 2. Number of seedlings of each species tested for herbicide tolerance by *Liberty*™ spraying in the glasshouse trials. The number of seedlings grown, from seed collected at the FSE fields in years 2000 – 2002, and the number of survivors are shown.
U_1 = upper 95% confidence limit for the proportion of offspring which are tolerant (treats seeds as the basic sampling unit and totals for all three years have been used in the analysis).
L_1 (lower 95% confidence limit) for B. rapa = 0.0000.

Species	2000	2001	2002
Sinapis arvensis	488	255	75
Sinapis alba	>7	9	79
Brassica nigra	6	37	0
Raphanus raphanistrum	4	*	88
Raphanus sativus	*	0	0
Brassica rapa	0	0	97

Table 3. Number of parent plants from which each of the species tested for herbicide tolerance originated. Plants were sampled from the FSE fields in years 2000 – 2002.
** indicates where the total number of parent plants is not known.*

Brassica rapa

Brassica rapa seeds were collected from the single site at which it was found. This site was adjacent to the end of the conventional crop unit furthest from the GM oilseed rape. The seedlings were germinated and tested for *Liberty*™ resistance and three seedlings were initially found to show no reaction to the application of herbicide. DNA was extracted from each of the three seedlings and tested for the presence of the gene construct and a positive result was found in two of the three samples (Figure 1). A further application of *Liberty*™ resulted in herbicide damage to the sample that returned a negative PCR result, suggesting initial shielding from the herbicide.

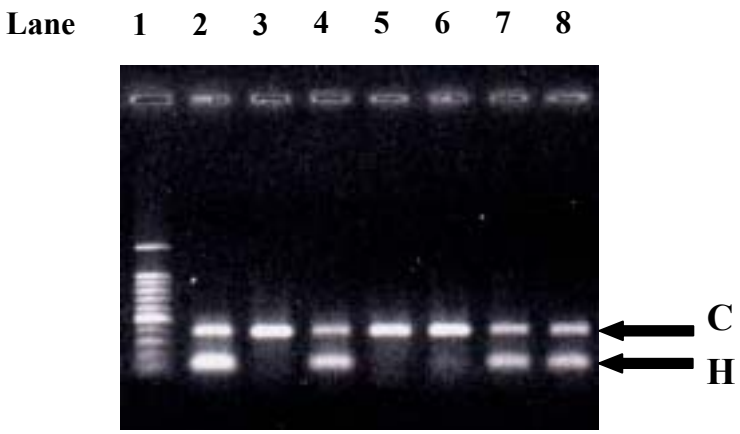

Figure 1. PCR amplification of the *B. rapa* survivors.
Lane 1 contains 5µl of 100bp ladder (Bioline). Lanes 2-5 contain the control samples which alternate between positive control (containing the GM gene) and wildtype control (conventional oilseed rape). Lanes 6-8 contain the three surviving seedlings. The seedling in lane 6 subsequently died after the third round of herbicide application. The arrow labelled C shows the position of the control PCR product, the arrow labelled H shows the position of the bar gene PCR product.

Results from the PCR amplification show that plants containing the herbicide tolerance gene amplify two bands, the 394 bp control band and the 142 bp bar gene band, whereas plants not containing the herbicide tolerance gene amplify only the 394 bp control band. Therefore it was shown that in two seedlings the herbicide tolerance gene had been both incorporated into the *B. rapa* genome and expressed in the seedlings as susceptibility to the herbicide.

Flow cytometry was used to determine the hybrid status of the herbicide-tolerant seedlings. One of the two suspected hybrids had subsequently died, but the remaining *B. rapa* sample was tested by flow cytometry to determine DNA quantities (Table 4).

Species	**DAPI DNA amount**	**PI 2C DNA amount**
B.napus	2.377 pg (mean of 3 measurements)	2.421 pg (± 0.015 pg)
B.rapa	1.049 pg (mean of 4 measurements)	1.059 pg (s.d. ± 0.004 pg)
Expected amount for *B.rapa X B.napus* hybrid	1.713 pg	1.740 pg
Putative *B.rapa X B.napus* hybrid	1.716 pg (mean of 4 measurements)	1.756 (s.d. ± 0.014 pg)

Table 4. Flow cytometry results of *B. napus*, *B. rapa* and the putative hybrid.

Using the DAPI method, where only readings with cvs below 5% were accepted, the results were clearly consistent with the expected hybrid origin of plants showing herbicide tolerance. Also, using the PI method, the 2C DNA amounts of the putative hybrid of 1.756 pg (s.d. ± 0.014 pg) did not differ significantly from the expected value for a *B. rapa* × *napus* hybrid of 1.740 pg in a Student's t-test ($t=1.717$; d.f.=27; $p>0.05$).

4.2.2 Persistence of herbicide tolerance in the field

Sinapis arvensis

At two sites, *Sinapis arvensis* plants present around the margins of follow-on crops were tested in the field. At one site a single individual showed no apparent reaction to the application of *Liberty*™ to a single leaf. DNA was extracted from a leaf sample collected from this plant and tested for the presence of the gene construct. A positive result was found. We are satisfied that the plant treated in the field was correctly identified as *Sinapis arvensis* and not *Brassica napus* as there is little ambiguity between mature specimens of these two species. We are also satisfied that our method of DNA extraction does not lead to cross-contamination of samples (Mogg, 2003). No leaves were present for assay of the small number of plants growing at the same location in the following year (by the time the presence of the gene construct had been definitively confirmed, *S. arvensis* plants at the site had mature seeds, but leaves had died back). However, seeds were collected from these plants and the seedlings grown up and tested for *Liberty*™ resistance. No evidence of herbicide tolerance was found in any of the seedlings.

4.3 Herbicide tolerance in volunteers

Samples of volunteer oilseed rape plants found in follow-on crops were tested in the field (by leaf spotting) and the laboratory (by PCR).

The oilseed rape line used in the FSEs is Ms8xRf3, an F1 hybrid between Ms8 and Rf3 both of which contain the HT gene. The gene (in single copy) is dominant and the two versions of the HT gene are unlinked so segregate independently. On the basis of normal Mendelian inheritance if the F1 selfs, the F2 seed would contain the following proportions of the three gene combinations: nine with both HT genes, six with one or other of the HT genes and one with no HT genes. Herbicide tolerance thus segregates 15:1 (i.e. 94% are herbicide tolerant). Where non-GM parents were involved in the production of the F2 generation (e.g. via pollen flow between the field halves), the proportion of susceptible plants would be expected to be greater, with one containing no HT gene to every three with at least one copy of the gene. Results carried out on volunteers in follow-on crops are shown in Tables 5 & 6.

Follow on year	Field	Crop	Crop unit	No. plants	Volunteer density	Eaten	Susceptible	Resistant	% resistant
1st									
	A	beans	GM	19	$2.5m^{-2}$	1	6	12	66.7
2nd									
	B	beet	GM	34	$2.0m^{-2}$	1	6	27	81.8
	C	wheat	GM	88	$1.0m^{-2}$	7	23	58	71.6
			Con	142	$2.9m^{-2}$	4	138	0	0
	D	maize	GM (SW)	48	$>10m^{-2}$	4	19	25	56.8
			GM (NW)	50	$>10m^{-2}$	4	34	12	26.1
			Con	47	$>10m^{-2}$	1	46	0	0

Table 5. Results of field spot-testing of oilseed rape volunteers in the 1st and 2nd year follow-on crops after the FSE trial.
Calculations for volunteer densities have been given where known or otherwise estimates made. 'Eaten' denotes where the tested leaves had been eaten by herbivores before herbicide damage had been recorded.

Follow on year	Field	Crop	Crop unit	No. plants	Volunteer density	Null	+ *bar*	- *bar*	% with *bar*
1st									
	A	beans	GM	25	2.0m^{-2}	0	23	2	92.0
			Con	25	2.0m^{-2}	0	18	7	72.0
	E	wheat	GM	3	-	0	2	1	66.7
	F	setaside	GM	61	-	0	51	10	84.0
	G	wheat	GM	60	-	1	10	49	16.9
2nd									
	B	beet	GM (1)	32	2.0m^{-2}	1	28	3	90.3
			GM (2)	33	2.0m^{-2}	1	27	5	84.4
			GM (3)	33	2.0m^{-2}	0	29	4	87.9
			GM (4)	42	2.0m^{-2}	0	37	5	88.1
			Con (1)	44	2.0m^{-2}	0	7	37	15.9
			Con (2)	43	2.0m^{-2}	0	1	42	2.3
			Con (3)	43	2.0m^{-2}	0	1	42	2.3
			Con (4)	38	2.0m^{-2}	0	15	23	39.5
	D	maize	GM (SW)	104	>10m^{-2}	0	54	50	52.0
			GM (NW)	103	>10m^{-2}	0	43	60	42.0
			Con	53	>10m^{-2}	0	1	52	1.9
	H	potatoes	GM	50	>0.02m^{-2}*	0	37	13	74.0

Table 6. Results of PCR testing of oilseed rape volunteers in the 1st and 2nd year follow-on crops after the FSE trial, showing the proportion of volunteers containing the bar gene.
*Calculations for volunteer densities have been given where known or estimates made. *= samples were collected from an uncultivated strip, approx 5m wide, around the edge of the field. A 'null' result was recorded where there was no PCR amplification in the DNA analysis.*

First year follow-on

In the first year following spring oilseed rape trials, volunteers were found and tested in two fields. In one, the follow-on crop was beans and in the other winter wheat. In the first year following winter oilseed rape trials, volunteers from two fields were also tested. One of these fields was in winter wheat and the other in setaside.

In the wheat fields, the tramlines were searched and only volunteers growing in what had been the GM half of the field were collected and tested, using PCR on leaf samples. In what had been the FSE winter oilseed rape field only three volunteers were found and, although two of them possessed the gene construct, the sample size is too small to draw any conclusions. In the field that had been spring oilseed rape, the proportion of plants with the gene construct was surprisingly low (16.9%). This could

be due to the presence of a seedbank, and subsequent volunteer population that included seed from a previous non-GM crop of oilseed rape.

In the setaside field 84% of plants tested contained the gene construct. This result compares with the results obtained from the field of beans in which both leaf spotting and PCR methods were used.

Second year follow-on

Volunteers were only found in second year follow-on fields that had been in spring rape in the FSE trial. One was in beet, one in potatoes, one in wheat and one was in the FSE maize trial. Spot testing of plants was carried out in three of the fields and PCR on leaf samples from three fields. Results are shown in Tables 5 & 6.

Plants used in the wheat field were located along two lines parallel with what had been the division between GM and conventional crop units in the trial. Volunteers tested (by leaf spotting in the field) were all growing in a strip approximately 1m wide across the whole width of the field and centred on a tramline. One transect was approximately 60m from the split in the "ex-GM" half of the field and the other was the equivalent position in the "ex-conventional" half. There were more volunteers in the ex-conventional half of the field (2.9 plants m^{-2}) than in the ex-GM (1 plant m^{-2}). Whilst the proportion of resistant plants in the ex-GM half of the field corresponded approximately to expected values, all plants were susceptible to *Liberty*™ on the ex-conventional half of the field. This suggests that there was little gene flow at this distance from the GM half of the field.

Spot tests and PCR assays were carried out on volunteers in the beet field. Plant density was much the same in both halves of the field (2 plants m^{-2}) but field testing was only carried out on a few plants on the ex-conventional side of which all five plants tested were susceptible. Of the 34 plants on the ex-GM side tested, 27 were resistant. More structured sampling for PCR involved collecting from four locations in each half of the field. In each half-field two sets of leaves were taken from within 10m of the division between GM and conventional (samples 1 & 4) and two sets close to the boundaries furthest from where the division had been (samples 2 & 3). Results within the ex-GM half of the field are consistent (X^2= 0.53; 3df; $p>0.05$), but in the ex-conventional half, a significantly higher proportion of volunteers nearer the division carried the *bar* gene than at the furthest points away from the GM side (X^2= 20.05; 1df; $p<0.001$).

A similar, structured approach to sampling was carried out in the maize field. Over the greater part of the conventional half of the field, there were no volunteer rape plants, so sampling was restricted to a marginal area, where such plants were present. Two samples were taken from close to the corners of the GM half of the field furthest from the conventional (designated NW and SW).

Leaf spotting indicated that no plants among the population in the conventional half of the field were resistant, whilst in the GM half the proportion of resistant plants was lower than expected if all pollination had been among GM plants. Of 53 plants sampled and subjected to PCR in the conventional half of the field, only one had the

bar gene. On the GM half of the field only around half of the plants tested had the bar gene with similar proportions at the two different locations within the field (X^2= 2.15; 1df; p>0.05). This suggests that either there had been other rape seed within the seedbank or that there had been movement of pollen from the conventional half of the trial.

Differences in proportions of herbicide-tolerant volunteers were found when comparing the second year follow-on fields of beet (B), maize (D), potatoes (H) and wheat (C) (X^2= 65.03; 3df; p<0.001). The beet crop contained a significantly higher proportion of GMHT volunteers than each of the other crops respectively (X^2_{1df} {field B v field D}= 44.65; p<0.001, {BvH}= 5.12; p<0.05, {BvC}= 8.84; p<0.01). The wheat and potato crop contained similar proportions to each other (X^2= 0.09; 1df; p>0.05) and the maize field contained a significantly lower proportion than each of the other crops (X^2_{1df} {DvB}= 44.65; p<0.001, {DvH}= 11.89; p<0.001 and {DvC}= 14.34; p<0.001). The wheat crop was included in analysis although testing was by herbicide spotting, as when comparing the two methods of detecting herbicide tolerance (herbicide spotting and PCR) for the maize field, there was found to be no significant difference between the two (X^2= 3.35; 1df; p>0.05).

Potential third year follow on

No measurements were made of volunteers in 2003 fields that had been in the trial in 2000. However some seeds collected from plants present in two fields in 2002 were screened (Table 7). All 29 seedlings developing from seed of one individual from the GM half of one field contained the *bar* gene. In the other field seed was collected from plants on both ex-GM and ex-conventional sides, germinated and tested by PCR. Of 60 seedlings from seed of five plants on the ex-GM side 73% carried the bar gene: of 30 seedlings from a single plant on the ex-conventional side, only one had the gene.

Follow on year	**Field**	**Crop**	**Parent plant**	**Seedlings**	**Null**	**+ *bar***	**-*bar***	**% with *bar***
3rd	A	wheat	GM 1	29	0	29	0	100
	B	beet	GM 1	3	0	2	1	66.7
			GM 2	11	0	9	2	81.8
			GM 3	6	0	6	0	100
			GM 4	4	0	4	0	100
			GM 5	36	0	23	13	63.9
			Total GM	***60***	***0***	***44***	***16***	***73.3***
			Con 1	2	0	0	2	0
			Con 2	30	0	1	29	3.3
			Total Con	***32***	***0***	***1***	***31***	***3.1***

Table 7. Results of PCR testing of potential oilseed rape volunteers in the 3rd year follow-on crops after the FSE trial, showing the proportion of volunteers containing the bar gene.
A 'null' result was recorded where there was no PCR amplification in the DNA analysis.

5. DISCUSSION & CONCLUSIONS

5.1 Wild relatives

The risks of transfer of herbicide tolerance to wild relatives of oilseed rape appear to be minimal. *Brassica rapa*, the most likely wild relative to hybridise with oilseed rape, was found to have the herbicide tolerance gene in two of the seedlings grown from seed collected from one FSE site. *B.rapa* has a limited distribution, however, which will to some extent limit the potential for cross-pollination and gene flow to occur. Although we have found evidence of possible hybridisation between *Sinapis arvensis* and *Brassica napus*, this is the first time this has been reported, at least with *S. arvensis* as the maternal parent. The incidence of such crosses must be very low, even though this is the most common wild relative found associated with arable fields in Britain: no other examples were found in our study nor in the those undertaken elsewhere, in particular France, where over 2 million seeds have been tested.

The most likely scenario for the production of a hybrid as a result of pollen flow from oilseed rape to *S. arvensis* would be expected to be where a single plant of the latter was present within, or close to, a field of oilseed rape. In such a case, the self-incompatible *Sinapis* would be subjected to a massive overload of rape pollen. One such situation was found in a large field in Lincolnshire. A single plant of *S. arvensis* was present in the middle of the conventional crop unit. Although this was a large plant, overtopping the oilseed rape crop, with a much-branched inflorescence, it carried only three pods and yielded only eight seeds. None of these was viable. The location in which the putative hybrid was found was in a field where there was a very large population of *S. arvensis* within and around the spring oilseed rape crop – so much so that the crop could not be harvested for oil extraction but was taken for game food. Under these conditions, there should have been ample opportunity for the transfer of conspecific pollen to the maternal plant producing the individual in question.

Hybridisation between different members of the *Brassicaceae*, with the exception of those forming U's triangle (containing the parent species: *B. rapa, B. oleracea and B. nigra,* and the hybrids that have arisen from them: *B. napus*, *B. juncea* and *B. carinata*) is not reported in the literature, which suggests that, even if occasional episodes of gene flow occur from oilseed rape to either *Brassica rapa* or *Raphanus raphanistrum* (or even *Sinapis arvensis*), this is not likely to result in a general spread of the gene through a range of species.

The consequences of any transfer of herbicide tolerance needs to be seen in the context of possible competitive advantage. At present we do not know whether there is a fitness cost to the possession of herbicide tolerance or whether any competitive advantage is conferred in the absence of herbicide application. Snow et al (2001) found that fitness was reduced in wild-crop hybrids (between wild and cultivated radishes) because of delayed flowering, reduced pollen viability and reduced seed output. The consequences of such gene transfer, rather than the fact of its occurrence, need to be examined more closely.

5.2 Volunteers

Volunteer populations of oilseed rape in follow-on crops show a variable pattern of continued resistance to *Liberty*™. Whilst, in general, there is only low occurrence of resistance in what had been the conventional halves of fields in the trial (indicative of generally low rates of gene flow via pollen), the results in the ex-GM halves of the fields show a wide range in the proportion of tolerant individuals. This variation may be indicative of patchy dispersal of pollen, such that some maternal parents have a relatively high proportion of GM offspring whilst others have received no pollen as a result of long distance (in excess of a few metres) transfer. Where populations of volunteers consist of groups of plants derived from the same parent, and where that parent is herbicide tolerant, then a relatively high proportion of any seed produced might be expected to be tolerant. Where that seed is shed there is potential for local populations of herbicide tolerant volunteers to persist over many years, especially where weed control measures are not highly effective. Such populations may appear sporadically over time, as in the case of one of the fields observed over two years of winter wheat cropping: in the first year there were no volunteers, but in the second year a relatively high density (with a high proportion of tolerant individuals where the GM crop unit had been grown) was found. Whilst other weed control measures may be taken to minimise the problems caused by these volunteer populations, extension of the use of GM glufosinate ammonium-tolerance to other crops could cause problems (as suggested in the field where a maize trial was planted as a second year follow up to a spring oilseed rape trial).

The large-scale study of the Farm Scale Evaluations enabled us to sample wild relative seed from across a wide range of locations in Great Britain. From these, there were only three recordings of herbicide resistance; 2 individuals, of *Brassica rapa*, were found to be herbicide resistant in glasshouse trials of collected seed, and one plant of *Sinapis arvensis* in a follow-on crop when tested by herbicide spotting. As there was no evidence for persistence of herbicide tolerance of *S. arvensis* at that location and *B. rapa* has a limited distribution in the UK, this leads us to suggest that there is low risk of extensive gene flow of the herbicide resistance gene through the environment via introgression into populations of wild relatives.
Evidence, however, from the study of herbicide-resistant volunteers present in the following two and potential third year crop following the FSE leads us to suggest that volunteer oilseed rape may pose a greater risk for gene flow of the *bar* gene into the environment. This highlights implications for the EU threshold limits of GM content in oilseed rape crops set at 0.1%, 0.3% and 0.9% for organic seed, certified seed and food & feed, respectively.

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