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# Hybridization between oilseed rape (*Brassica napus*) and different populations and species of *Raphanus*

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**When cultivating genetically modified varieties, the spontaneous gene flow between crop and wild relatives could be of concern. We analyzed spontaneous hybridization between a transgenic male-sterile line of oilseed rape (*Brassica napus*, 2*n*=38, AACC) and, as pollen donors, three European populations of wild radish (*Raphanus raphanistrum*, 2*n*=18, Rr,Rr) and a variety of cultivated radish (*Raphanus sativus*, 2*n*=18, RR). Seeds showed size and shape dimorphism that correlated to the frequency of hybrids. The offspring were scored morphologically and analyzed using DNA markers (inter-simple sequence repeats) to quantify hybrid frequencies. Seed set ranged from 0.4–1.2 seeds per pod, and 0.02–0.6 seeds per pod were confirmed as hybrids. The frequency of confirmed hybrids differed significantly among populations of *R. raphanistrum*. In the cross with a French population, all offspring were hybrids; in the cross with a Swiss population, 53% of the offspring were hybrids; and in the cross with a Danish population, only 2% of the offspring were found to be hybrids. The remaining offspring apparently belonged to two groups: the majority was *B. napus*-like plants, possibly of matromorphic origin, and a minority from the Danish cross seemed to carry fragments of the *Raphanus* genome. In the cross with a cultivated *R. sativus*, all offspring were found to be hybrids. This is the first report on spontaneous hybridization between *B. napus* and *R. sativus.* Hybrids from all cross-combinations had low pollen fertility (0–15%). If *R. raphanistrum* occurs where male-sterile *B. napus* is cultivated, large regional differences in hybridization frequencies between the species could complicate environmental risk assessment of transgenic oilseed rape.**

**Keywords:** hybridization frequencies / population differences / ISSR / seed dimorphism

## INTRODUCTION

One concern of growing transgenic oilseed rape is the risk of transgene flow from the genetically modified crop to wild or weedy relatives, and the consequences gene dispersal might have for wild populations and for their ability to create weed problems (Scheffler and Dale, 1994). For the overall estimation of the potential risks of cultivating genetically modified (GM) crops, the European Union has common risk-assessment guidelines (Directive 2001/18/EC). The risk assessment procedure demands evaluation of gene flow from a genetically modified crop to wild recipients, and in this respect it is important that the variation in gene flow is known and not just assumed to be identical in all regions and for all genotypes. Therefore, we compared hybridization frequencies between a male-sterile line of *B. napus* and three *R. raphanistrum* populations from different regions in Europe. Hybrids between *B. napus* and *R. raphanistrum* have been found to occur spontaneously (Baranger et al., 1995; Darmency et al., 1998; Eber et al., 1994; Rieger et al., 2001; Warwick et al., 2003), but spontaneous hybridization between *B. napus* and *R. sativus* has not been reported previously, and we therefore included *R. sativus* in our crossing experiment. A male-sterile line of *B. napus* was used as maternal line in the hybridizations, which is relevant as varietal associations with male-sterile *B. napus* are cultivated in Europe (Booth, 1998). Male-sterile plants could potentially have increased outcrossing, for instance with GM fields in the neighborhood. Also, hybrid varieties of oilseed rape may retain some male sterility that also enhances outcrossing (Eastham and Sweet, 2002).

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*R. raphanistrum* is self-incompatible (Sampson, 1964), and occurs as a weed in agricultural fields and as a ruderal plant on sandy soils (Chèvre et al., 2004; Tutin et al., 1980). Its seeds can remain viable in the soil for many years (Roberts and Boddrell, 1983), and it is an economically damaging weed worldwide (Holm et al., 1997). *R. sativus* is also self-incompatible (Karron et al., 1990), and cultivated in large areas of the world, where it can escape from cultivation and colonize disturbed sites such as roadsides, fields and coastal sand dunes (Snow et al., 2001). Overlap in flowering time between crop and weeds makes interspecific pollination events possible in Europe, since *R. raphanistrum* and *R. sativus* flower in early to late summer and *B. napus* in spring to early summer (winter oilseed rape) and early to mid summer (spring oilseed rape).

In the field, the frequency of spontaneous hybridization between *R. raphanistrum* and fertile lines of oilseed rape was found to be extremely low (Chèvre et al., 2000; Rieger et al., 2001; Thalmann et al., 2001; Warwick et al., 2003), as were frequencies of backcrosses to *R. raphanistrum* in field experiments (Chèvre et al., 1998). When male-sterile *B. napus* is pollinated by *R. raphanistrum*, hybridization frequencies increase (Baranger et al., 1995). Hybrids between *B. napus* and *R. raphanistrum* are vigorous and almost sterile (Lefol et al., 1997), but fertility increases in the following backcross generations (Chèvre et al., 1998). Some of the backcross plants with *R. raphanus* had chromosome numbers close to that of the wild parent, and retained *B. napus* markers and the transgene from the crop (Chèvre et al., 1998). However, these *B. napus* markers were apparently not stably recombined into the *R. raphanistrum* genome. As the transgene is retained, the hybrids – $F_1$ and wider hybrid generations – may potentially affect the environment in unwanted ways.

In the present study we set off to analyze if hybridization frequencies between oilseed rape and *R. raphanistrum* was influenced by populational differences. Our results are discussed in relation to environmental risk assessments of GM plants.

## RESULTS

Table 1 shows the data obtained on seed set, seed size, seed weight, pollen fertility, the morphological categories, genetic marker analysis, and ploidy. For each cross-combination the results in Table 1 were obtained from the same sets of plants, although sample sizes vary among traits.

### Variation in seed size and seed set

Within the four cross combinations, the 20 *B. napus* plants seemed to contribute almost equally to pod and seed set, and therefore seeds were harvested on a population basis. Seeds from the *R. sativus* cross and the cross with the French *R. raphanistrum* population had similar sizes, and both these crosses had 2-3 times fewer seed per pod than plants from the Danish cross and the Swiss cross. Seed morphology results from all crosses are shown in Figure 1. The seeds from the *R. sativus* and French crosses were all quite small (0.8–1.5 mm), whereas seeds from the Swiss cross fell into two groups, the larger group (68%) was similar to the small-sized seeds from the *R. sativus* cross and the French cross (0.8–1.5 mm). The other group (32%) consisted of larger seeds (1.5–2.1 mm). Seeds from the Danish cross also fell into these two size-groups; very few (9%) were small (0.8–1.5 mm) whereas the majority (91%) was larger (1.5–2.1 mm). The large seeds did not have the characteristic round and smooth morphology of normal *B. napus* seeds. Instead, these seeds were asymmetric, rough and each seed had its own characteristic shape (Fig. 1). The weight of 100 seeds was very similar in the French *R. raphanistrum* cross and the *R. sativus* cross ($p = 0.944$), and significantly less than in the other two crosses ($p < 0.001$), which also differed significantly from each other ($p < 0.001$).

### Morphology of the offspring plants

There did not seem to be substantial differences in seed germination among crosses after the dormancy breaking treatment. Seeds of each seed size were grown in a separate experiment to evaluate the correlation between seed size and morphology of the offspring plants. This experiment showed that plants reared from small seeds had hybrid morphology and plants from large seeds had *B. napus*-like morphology.

The morphology of approximately 100 offspring plants per cross-combination is given in Table 1. The offspring plants produced from the cross with the French population of *R. raphanistrum*, and those produced with *R. sativus* all looked like interspecific hybrids. Except for a few hybrid-looking plants, morphology of the offspring fathered by the Danish population of *R. raphanistrum* was *B. napus*-like. However, some of these had a few prickly hairs on the leaves and could not be morphologically categorized as either *B. napus* or as hybrids (19.6% of the offspring from this cross, NC = non-categorized, see Tab. 1). Such deviating morphology was also found among the offspring plants from the cross with the Swiss

**Table 1.** Spontaneous crosses between male sterile *B. napus* (female) and accessions of *Raphanus raphanistrum* or *Raphanus sativus*: seed set, seed sizes, pollen fertility, morphological and genetic identification of offspring.

| $F_1$ offspring between *B. napus* and *Raphanus* | | | | |
|---|---|---|---|---|
| Male parent | Swiss population | Danish population | French population | *sativus* population |
| **Seed data** | | | | |
| Pods (20 pl.) / per pl. | 1726 / 86.3 | 662 / 33.1 | 1548 / 77.4 | 1739 / 87.0 |
| Seeds (20 pl.) / per pl. | 2021 / 101.1 | 755 / 37.8 | 541 / 27.1 | 1065 / 53.3 |
| Seeds per pod | 1.17 | 1.14 | 0.35 | 0.61 |
| Seed size, mm [a] | 0.8 < 68% <1.5<br>1.5 < 32% < 2.1 | 0.8 < 9% < 1.5<br>1.5 < 91% < 2.1 | 0.8 < 100% < 1.5 | 0.8 < 100% < 1.5 |
| Weight of 100 seed, g | 0.169 ± 0.007 | 0.346 ± 0.010 | 0.061 ± 0.000 | 0.064 ± 0.003 |
| **Morphological evaluation** | | | | |
| Hybrids, % (# plants) | 57.4 (54) | 4.3 (4) | 100 (100) | 100 (100) |
| *B. napus*-like, % (# pl.) | 31.9 (30) | 76.1 (70) | 0 (0) | 0 (0) |
| NC, % (# pl.) | 10.6 (10) | 19.6 (18) | 0 (0) | 0 (0) |
| Total, % (# pl.) | 100 (94) | 100 (92) | 100 (100) | 100 (100) |
| **Genetic analysis** | | | | |
| Hybrids, % (# pl.) | 53.2 (50) | 1.7 (2) | 100.0 (20[c]) | 100.0 (20[c]) |
| *B. napus*-like, % (# pl.) | 44.7 (42) | 75.0 (90) | 0.0 (0) | 0.0 (0) |
| FRB, % (# pl.) | 0.0 (0) | 23.3 (28) | 0.0 (0) | 0.0 (0) |
| Total, % (# pl.) | 100.0 (92) | 100.0 (120) | 100.0 (20) | 100.0 (20) |
| Seeds per pod that were hybrids [b] | 0.62 | 0.02 | 0.35 | 0.61 |
| **Pollen fertility, %** | | | | |
| Parental plants | 87 (7) ± 27 | 79 (7) ± 27 | 74 (6) ± 30 | 86 (7) ± 20 |
| Hybrids | 3 (9) ± 5 | nd[d], (FRB > 90% (4)) | 1 (10) ± 1 | 6 (9) ± 5 |
| **Chromosome number or ploidy level** | | | | |
| Chromosome number, (# pl.) | | 2*n*=28 (1), 2*n*=38 (4) | 2*n*=28 (3), 2*n*=56 (2) | |
| Flow cytometry, (# pl.) | triploid (1) | tetraploid (28) | | triploid (1) |

The table shows the percentages (the number of plants given in brackets) of plants analysed and classified as hybrids, as *B. napus*, as non-categorized (NC), or as containing a Few *Raphanus* Bands (FRB).
[a] Distribution of the total amount of seeds within size categories defined by the seed diameter.
[b] Based on the results of the ISSR marker analysis.
[c] As all 100 plants analysed morphologically were clearly hybrids, only 20 of them underwent ISSR analyses.
[d] The two $F_1$ hybrids from the cross-combination with the Danish population inherited the male sterility trait, which made estimation of pollen fertility impossible.

population (10.6% of the offspring from this cross), whereas the remaining offspring from this cross was found to be either hybrid-like or *B. napus*-like plants (Tab. 1).

## Genetic marker analysis

In the ISSR analysis, there was a clear distinction between the parental species *B. napus* and *Raphanus* (Fig. 2), as several markers were specific for one or the other species. No variation was found within *B. napus*, as all markers were monomorphic in the 80 plants analyzed (randomly selected from the same seed sample as the maternal plants). Quite opposite, ISSR analysis of the three *R. raphanistrum* accessions displayed much intrapopulational variation in accordance with the outcrossing nature of this species; *R. sativus* was also quite variable. *Raphanus*-specific markers were easily found, but none of them were monomorphic over all four populations. As a consequence

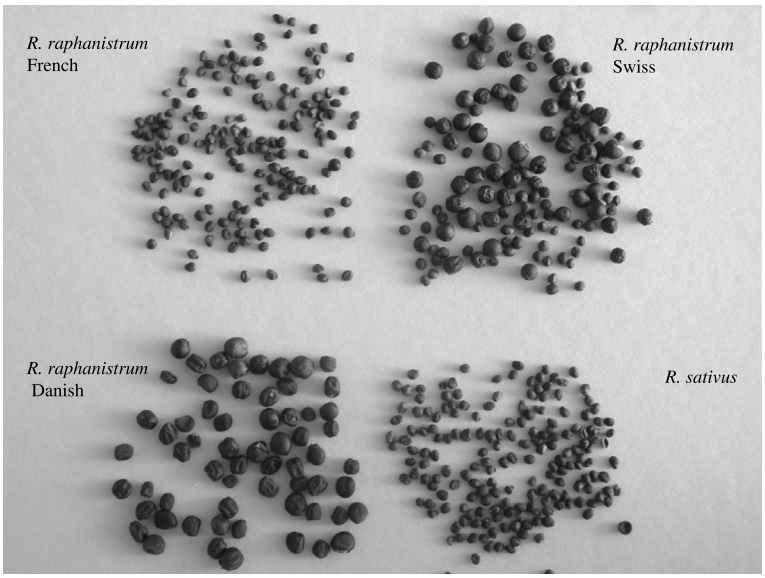


**Figure 1.** Size and shape dimorphism of seeds from crosses between male-sterile *Brassica napus* and one cultivar of *Raphanus sativus* and three different populations of *Raphanus raphanistrum* – a French, Swiss and Danish.

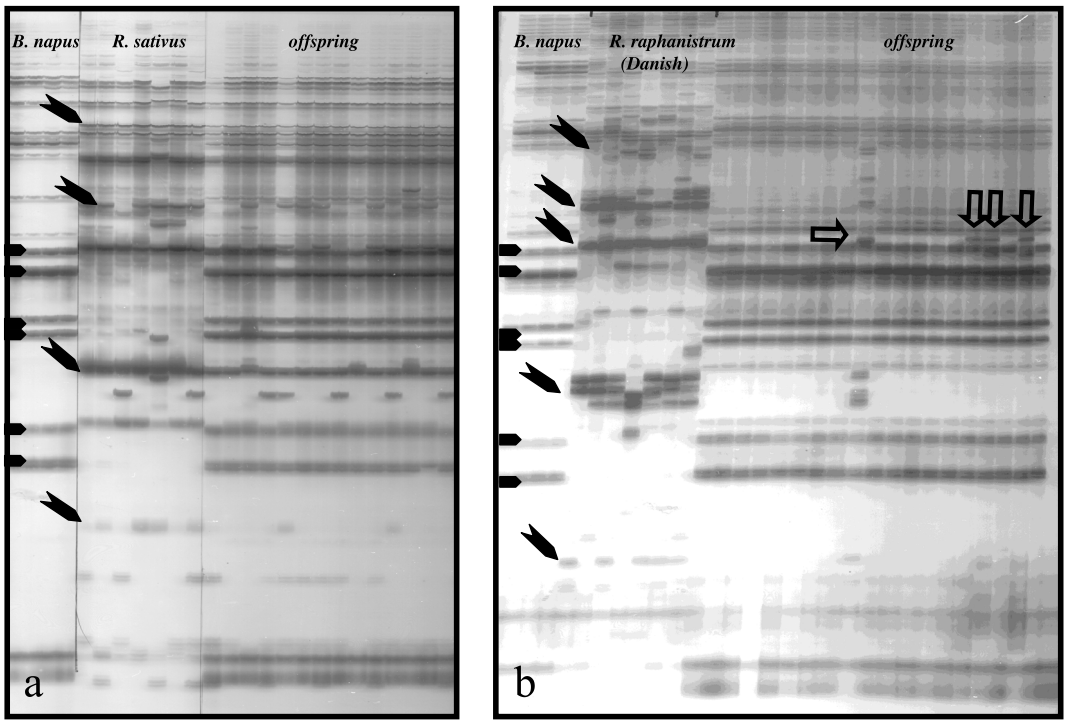


**Figure 2.** ISSR profiles of parents and offspring from the *Brassica napus* and *Raphanus* crosses; (a) cross with *R. sativus*; (b) cross with the Danish population of *R. raphanistrum*. Some of the gel zones with diagnostic *Raphanus* bands are given (long filled arrows), as are six *B. napus* bands, never observed in *Raphanus* (short filled arrows). All offspring in panel a are hybrids; the hybrid plant in panel b is indicated by the unfilled horizontal arrow, and three offspring plants revealing only some of the *Raphanus* bands (FRB plants) are shown by unfilled vertical arrows.

of the genetic variability in *Raphanus*, different sets of population-specific markers were used for identification of the hybrids in the four crosses. All *Raphanus* individuals had 8–27 specific markers differentiating them from oilseed rape, and *B. napus* had a minimum of 9–12 specific markers depending on which of the *Raphanus* accessions that were used as pollinator. Some of these crop-specific bands were never seen in any of the four *Raphanus* populations (Fig. 2).

Within the different cross-combinations, the hybrids showed variation in the bands inherited from the *Raphanus* parent, suggesting that their paternal DNA originated from different individuals. As seen in Figure 2, the interspecific hybrids could easily be detected by the ISSR-markers, as all hybrids had many *Raphanus* markers. However, some offspring with *B. napus*-morphology from the combination with the Danish *R. raphanistrum* were difficult to place as being either hybrids or *B. napus*. These plants had only 1–3 recurrent *Raphanus* markers (Fig. 2: plants designated as FRB = few *Raphanus* bands), whereas two hybrids from this cross and hybrids from the other crosses always inherited 7–15 of the *Raphanus* markers. The estimated numbers of hybrid seeds per pod based on the marker analysis are shown in Table 1.

### Ploidy levels

Chromosome numbers were counted in five offspring from each of the French and the Danish cross-combinations with *R. raphanistrum*, as these two crosses represented high-frequency hybridization and low-frequency hybridization, respectively. In five hybrid-looking offspring from the French cross, three plants had 28 chromosomes, corresponding to what would be expected for an $F_1$ hybrid (genome ACRr), and two plants had 56 chromosomes (genome AACCRrRr?). All five plants from the Danish population resembled *B. napus*; however, one plant produced no pods when selfed (individual plants shaken to promote pollination), while the others had a pod and seed production equivalent to *B. napus*. Four of these plants had 38 chromosomes, like *B. napus,* whereas the one plant not producing pods had 28 chromosomes.

Flow-cytometric analysis was performed on 30 plants that were also evaluated by ISSR markers and morphology. They were four FRB plants and 24 *B. napus*-like offspring from the Danish cross and two hybrid plants, one each from the *R. sativus* and the Swiss *R. raphanistrum* cross. All 28 offspring from the Danish cross were categorized as tetraploid, and the hybrids from the *R. sativus* and the Swiss *R. raphanistrum* cross were triploids.

### Pollen fertility

As expected from the hemizygous nature of the male-sterile *B. napus* parent, 48% of the offspring identified as hybrids (from genetic marker analyses) were male-sterile. The male-fertile offspring had a low pollen fertility: between 0% and 15% for the 28 hybrids plants tested, and 1–6% for the four offspring populations (Tab. 1). Pollen fertility of the FRB offspring plants from the cross with the Danish population was more than 90% (Tab. 1), and at flowering, these plants were morphologically like oilseed rape. For comparison the paternal pollen fertility is given in Table 1.

Female fertility of the offspring was not accessed.

## DISCUSSION

### Size dimorphism of seeds

In this study, first generation hybrids between male-sterile *B. napus* and three populations of *R. raphanistrum* and one cultivar of *R. sativus* were produced spontaneously. The resulting seeds showed size dimorphism. In the French *R. raphanistrum* cross and the *R. sativus* cross, where all offspring were found to be hybrids (based on genetic markers and morphological analyses), all the seeds produced were small (< 1.5 mm). In the crosses with the Danish and Swiss *R. raphanistrum* populations, where only part of the offspring were hybrids and the remaining *B. napus*-like plants, the seeds grouped into small seeds (< 1.5 mm) and large seeds (> 1.5 mm). Such relationship between small seed size and hybrid identity has previously been described for crosses between male-sterile *B. napus* and *R. raphanistrum* (Baranger et al., 1995), and is also known from other *Brassica* crosses (Eenink, 1974 a, b; 1975). In contrast to the experiment by Baranger et al. (1995), our crosses were made in pollen-tight chambers with no fertile *B. napus* grown in the vicinity, and we therefore believe that the seedlings resembling *B. napus* could not derive from unintended pollination by *B. napus* pollen. This is supported by the fact that no *B. napus*-like plants were obtained from two of the crosses. Observations on size and shape dimorphism of seeds containing matromorphic plants (matromorphy ~ development of unfertilized ovules) have previously been described in crosses between *B. oleracea* and related species (Eenink, 1975). Hence, the seeds producing *B. napus*-like plants might be matromorphs, produced

asexually by the *B. napus* mother, possibly stimulated by the foreign pollen of *R. raphanistrum*. However, as the marker analysis revealed (see discussion of *B. napus* × *R. raphanistrum* crosses), these seeds could also represent plants that were derived from hybrids that had lost most of the *Raphanus* genome.

## Fertility of the hybrids

Pollen fertility of the hybrids was quite low (0–15%), and in agreement with previous results from hybrids of *B. napus* × *R. raphanistrum* crosses (Baranger et al., 1995; Chèvre et al., 1998; Chèvre et al., 2000). This implies that fertilization by the $F_1$ hybrids will be limited. The low male fertility is likely to slow down further introgression of transgenes, as is also suggested by the low frequency of spontaneous production of backcross plants; when $F_1$ hybrids were pollinated by *R. raphanistrum* in the field, 0.78 seeds/plant were produced (Chèvre et al., 1998). However, the $F_1$ hybrids – the first step in the introgression process – could themselves be problematic to the environment.

## Crosses

### *The* B. napus x R. sativus *cross*

Hybrids between *B. napus* and *R. sativus* have previously been reported using embryo rescue (Lelivelt et al., 1993; Paulmann and Röbbelen, 1988; Takeshita et al., 1980). However, the present study demonstrates that hybridization between these species is indeed possible without hand pollination or embryo rescue, using male-sterile *B. napus*.

The reason we obtained so many hybrids was likely due to the lack of competition from conspecific pollen on the *B. napus* stigma. Experiments on pollen competition in crosses between *B. napus* and *B. rapa* have shown that *B. rapa* pollen in *B. napus* styles had a significantly lower fitness than the conspecific pollen (Hauser et al., 1997). As fertile lines of *B. napus* are highly selfing, the likelihood of heterospecific pollen fertilizing a *B. napus* plant under field conditions is limited. However, varietal associations with a mix of male-sterile and -fertile *B. napus* plants are cultivated (Booth, 1998), and also hybrid varieties may have some male sterility (Eastham and Sweet, 2002). The chance of outcrossing in these situations is substantially increased, and hence also the chance of interspecific hybridization (Chèvre et al., 2000; Eastham and Sweet, 2002).

Spontaneous hybridization between *B. napus* and *R. sativus* has not been considered in the environmental risk assessment prior to release of transgenic *B. napus*, since it has not been known to occur. If it does take place in nature, this route of gene escape should also be considered when releasing GM oilseed rape, as *R. sativus* may occur as a feral or volunteer and hybridize readily with *R. raphanistrum* (Klinger et al., 1992; Snow et al., 2001; Warwick and Francis, 2005). Post-release monitoring might detect the occurrence of such hybrids in the future, but investigations of the frequency of interspecific hybridization under natural conditions seem necessary in order to include the likelihood of the event in pre-release risk assessment of a specific transgenic line (directive 2001/18/EC). Any possible environmental consequences of this hybridization then have to be considered.

### *The* B. napus x R. raphanistrum *crosses*

The DNA marker analysis revealed that the French and Swiss crosses produced 18–30 times more hybrid seeds per pod than the Danish cross. This suggests a difference in hybridisation potential relating to the genotype of the *R. raphanistrum* population. Although chamber effects are possible, genetic differences in pollen source are the most likely explanation for the observed differences, as chambers were identical as to size, orientation etc., and only had natural light.

The ability of *R. raphanistrum* pollen to penetrate the micropyle and fertilize the ovule was analyzed for two oilseed rape varieties by Gueritaine et al. (2003). They observed variations between seven *R. raphanistrum* plants from a single population and suggested that the variation in fertilization frequency depended on the genotype of the wild *R. raphanistrum* parent (Gueritaine et al., 2003). Genotypic differences between plants within the populations could be the reason for our observed differences in hybridization frequencies, but in contrast to the experiment by Gueritaine et al. (2003), we used 20 plants from each of four different populations of *Raphanus* and studied the seed production and offspring generation. Within the four cross-combinations, the variation in the bands inherited from the *Raphanus* parent suggests that the paternal DNA originated from different individuals. Hence, our results on the ability to produce interspecific hybrids appear to relate to population differences rather than individual variations among plants.

Several explanations for the difference in hybridisation frequencies among *R. raphanistrum* populations are possible. One of them could be that the hybridization potential differs due to different levels of already existing

introgression of *R. sativus* genes into the *R. raphanistrum* local population. Gene flow from *R. sativus* to *R. raphanistrum* is known to occur (Klinger et al., 1992; Snow et al., 2001). Since our results have shown a high frequency of hybridization with *R. sativus*, the presence of introgressed *R. sativus* genes in a population of *R. raphanistrum* might result in a higher ability to hybridize with *B. napus*, such as we found for the French population and the Swiss population in our experiment.

For the Swiss and Danish cross-combinations, the frequency of hybrids identified by morphology *versus* marker analysis was not quite identical. This discrepancy is probably due to that morphological scoring of hybrids at the 4-leaf stage is more uncertain than at later developmental stages, where pod production could reveal plant fertility/sterility. In conclusion, identifying $F_1$ plants between *B. napus* and *R. raphanistrum* cannot be done reliably using morphology alone.

### *Odd cytotypes and offspring with few Raphanus bands*

In the Danish cross, 23.3% of the offspring plants could not be classified as either hybrids or matromorphs, due to the occurrence of a few *Raphanus* specific bands in an otherwise *B. napus* genetic fingerprint (FRB = few *Raphanus* bands, Fig. 2). The bands could be linked, as it was always the same 1–3 bands that were inherited. Repetition of the ISSR analysis confirmed the presence and high frequency of FRB in the offspring from this cross. Our results from ISSR analysis of the *B. napus* variety showed that the bands did not originate from the genetically homogeneous *B. napus* variety.

In a field experiment, Warwick et al. (2003) found a spontaneous hybrid between *R. raphanistrum* and *B. napus.* This hybrid probably had the genome constitution RrRrAC (2*n*=37), and originated from fertilization of an unreduced *Raphanus* female gamete. This plant had morphology similar to *R. raphanistrum,* and its pollen fertility was very low (0.12%). The four FRB plants analysed by flow-cytometry were apparently tetraploid (or had a chromosome number close to the tetraploid number), their pollen fertility was normal (> 90%) and their morphology *B. napus*-like. Therefore, in contrast to the hybrid found by Warwick et al. (2003), the FRB plants most likely contained only a small amount of DNA from *R. raphanistrum*.

One explanation of the presence of FRB is that the bands are pollen-borne cytoplasmic DNA. Plant genera displaying some degree of biparental cytoplasmic DNA inheritance has been found in some taxa (*e.g.*, Mogensen, 1996). Reciprocal crosses between *B. napus* and *R. raphanistrum* could reveal a possible paternal cytoplasmic inheritance between these species. Johannessen et al. (2005) found the chance of paternal inheritance of cytoplasmic DNA in reciprocal crosses between *B. napus* and *B. rapa* – another weedy relative of *B. napus* – to be less than 0.015. Another possibility is that the FRB plants developed from interspecific hybrids that lost most of the *Raphanus* genome by chromosome elimination early in their embryo or seedling stage, combined with a doubling of the genome. Genome doubling and chromosome elimination has been suggested to occur in interspecific hybridizations between *B. napus* and *Orychophragmus violaceus* (Cheng et al., 2002). If the FRB plants developed from a process involving elimination and genome doubling, then the *B. napus*-like plants assumed to be matromorphic might also have developed from a hybridization event, but here elimination of the *Raphanus* genome was more complete or at least not evident with the present set of ISSR markers. If the FRB plants carry just a small fragment of the genome from the father, hybridization may be difficult to detect. Investigations of the possible chromosome elimination involved in the production of the *B. napus*-like plants will be the aim of a separate study. If the majority of *B. napus*-like plants developed as true matromorphs without preceding hybridization and chromosome elimination, then the differences between the Danish and the Swiss populations may relate to the ability to produce such matromorphs.

### *Conclusions*

To summarize, using morphological and ISSR analysis we observed large differences in the frequencies of hybrids from crosses between oilseed rape and three populations of the weedy relative *R. raphanistrum*. As a consequence of this population-dependent hybridization frequency, introgression of oilseed rape genes to wild recipients is likely to take place with different speed in different regions. In the future, more studies with free pollen competition under natural conditions are needed to confirm the existence of the regional differences in hybridization found in the greenhouse. If large population differences exist in hybridization frequency, this should be considered when making predictions about the level of risk when growing transgenic *B. napus*. However, it is important to remember that even if spontaneous hybridization with oilseed rape is frequent for some *Raphanus* populations, further introgression of the

transgene will probably be slowed, due to reduced hybrid fertility (Chèvre et al., 1998). Also, the fact that *B. napus* hybridized readily with *R. sativus* in the present experiment ought to be investigated in more detail by evaluating frequencies of hybridization and spontaneous gene transfer in different combinations between *B. napus* and a range of *R. sativus* cultivars.

Hybridization followed by chromosome elimination and genome doubling are the most likely events that resulted in the offspring plants with few *Raphanus* bands (FRB). The finding of FRB plants suggests that hybridization events between *B. napus* and *R. raphanistrum* may occur more often than previously expected. If so, new considerations on the likelihood of transgene spread will have to be included in the risk assessment of growing transgenic oilseed rape.

## MATERIALS AND METHODS

### Plant material

A transgenic nuclear male-sterile *B. napus* ssp. *oleifera* (DC.) (2*n*=38, AACC) of the variety Drakkar was used as maternal parent in all crosses (provided by Plant Genetic Systems, Belgium). The plants express a transgenic tapetum specific ribonuclease (*barnase*) that completely prevents pollen development (Mariani et al., 1990). The trait is hemizygous and inherited in a Mendelian manner. This line was one of the first transgenic oilseed rape lines to be approved by EU (GMOaut, 2004), and it seems to be stable both as to inheritance and gene expression (Ammitzbøll et al., 2005).

Three different populations of *R. raphanistrum* (L.) (2*n*=18, RrRr) and one cultivar of *R. sativus* (L.) (2*n* = 18, RR) were used as pollinators. The seeds of the *R. raphanistrum* populations were from a Danish population collected at northwest Zealand, a French population from Bretagne (kindly provided by A. Chèvre, Institut National de la Recherche Agronomique, Rennes), and a Swiss population (from near Zurich; kindly provided by M. Meier, Swiss Federal Institute of Technology, Zurich). The *R. raphanistrum* populations seemed morphologically very alike (but morphometric analysis was not performed); the most obvious difference being flower color: the plants germinated from the Danish and French populations had yellow flowers, while individuals from the Swiss population had white flowers. The *R. sativus* seeds were produced by the seed company Hammenhögs (Weibull Trädgård AB, 276 50 Hammenhög, Sweden). The variety was Flamboyant 3.

### Spontaneous crosses

The crosses took place during May in four separate pollen-tight chambers in a greenhouse with a constant temperature at 20 °C and no artificial light. In each chamber, 20 male-sterile *B. napus* plants were placed randomly between 20 plants of the specific *R. raphanistrum* or *R. sativus* population. Captive bumblebees, reared on artificial feed in mini-bee-hives, pollinated the plants for four weeks, while both *B. napus* and the *Raphanus* species were flowering. Bee activity seemed to be similar in the four chambers. Bees were replaced with new hives after two weeks, to ensure high pollination activity and no bees were allowed to move between chambers.

### Seed germination and seed size

The pods formed on *B. napus* were harvested and their number determined per population (see Tab. 1). Offspring seeds were weighed in groups of 100 seeds, and the seed number (weight of all seeds/average weight 100 seeds) and seeds per pod were determined. Seed sizes were quantified by sorting the seeds through 11 sieves with circular holes of different diameters ranging from 0.5–2.3 mm (0.5, 0.75, 0.8, 1, 1.25, 1.5, 1.75, 1.85, 2, 2.1 and 2.3 mm). To visualize any connection between seed size and hybrid morphology of the emerging seedlings, an extra batch of seeds was sown: 14–30 small seeds (< 1.5 mm) and 30 (where possible) large seeds (> 1.5 mm) from each cross. Plants were grown to maturity.

From each cross-combination, randomly selected offspring seeds were sown together with 50 parental *Raphanus* seeds. For morphological comparison, 50 seeds from the mother line of *B. napus* (male-sterile var. Drakkar) were also sown. All seeds germinated in 40 × 60 cm soil-trays. The trays were placed in a growth chamber, and a dormancy breaking temperature cycling was applied (30 °C / light 9 h, 20 °C / light 7 h and 17 °C / darkness 8 h). The temperature cycle was repeated for 96 h.

### Morphological analysis

At the 4-leaf stage ~100 emerging offspring plants from each cross (see Tab. 1), and from the seed batches separated according to size, were classified as being hybrid-like or *B. napus*-like. Two types of offspring plants were identified – hybrid-like with intermediate traits between *R. raphanistrum* and *R. sativus*, with more lobed

leaves, with prickled hairs and leaves without a visible wax layer, and *B. napus*-like plants that had less lobed leaves without prickled hairs but with a grayish wax layer. A third group of offspring that were not easily assigned to one of these two categories were designated as "non-categorized". Two people independently scored the morphology of the seedlings and obtained identical results.

### *Genetic marker analysis*

Genetic marker analysis was applied on offspring from crosses, to analyze to what extent morphological classifications of the offspring as hybrids/non-hybrids were correct. At the 4-leaf stage, a 2-$cm^2$ piece of each leaf was collected from individual plants and frozen. DNA was extracted by taking the samples directly from liquid nitrogen, and macerating them in a mixer mill together with two steel beads. Thereafter the DNA was extracted using the CTAB-method modified by Steward and Via (1993) from Doyle and Doyle (1990). We used dominant ISSR markers (Inter Simple Sequence Repeats) according to the method described by Charters et al. (1996). Markers were scored as present or absent. The primers used were BDB-$(CA)_7$.

For identification of species-specific markers, individuals from the parental accessions were screened: 80 individuals from the oilseed rape parent and 60 *Raphanus* individuals, 15 plants from each of the four *Raphanus* types. Subsequently, offspring plants were analyzed for the presence of ISSR markers. As all offspring from the French cross and the *R. sativus* cross had clear hybrid morphology and were very uniform, only 20 plants from each of these crosses were analyzed with molecular markers. As the offspring from the Danish and Swiss crosses had both hybrid-like and *B. napus*-like morphotypes, approximately 100 offspring from each of these morphologically characterized offspring populations were analyzed, plus 28 additional plants from the Danish cross that were also subjected to flow-cytometry. Offspring from the cross with the Danish populations were run twice to confirm reproducibility.

### Chromosome counts and flow-cytometry

Chromosome numbers were counted in five offspring plants from each of the Danish and the French population crosses. Root tips were pre-treated in a 0.05% colchicine solution for two hours. The root tips were stained with Feulgen and squashed in 45% acetic acid. Chromosomes in three cells from each plant were counted.

Using flow-cytometry, ploidy level was determined for 30 offspring plants that were characterized by genetic markers and morphology (for plant identity, see Results): 28 plants from the Danish cross, one from each of the Swiss and the *R. sativus* crosses. As standards, the ploidy levels in three parental *B. napus* (tetraploid) and four parental Danish *R. raphanistrum* plants (diploid) were used. Three leaves were collected from each plant and sent to DLF Trifolium, Research Division, Højerupvej 31, DK-4660 Store Heddinge that performed the flow-cytometry analysis. Fresh leaf material (0.5 $cm^2$) was extracted and stained using the CyStain UV Presice P Kit (Partec, Münster). Samples were filtered though 50 µm pore-size nylon filters (Partec CellTrics, Partec, Münster) and incubated at room temperature for 5 min. A Partec PA Flow cytometer was used for the analysis.

### Pollen viability

To determine the pollen viability, anthers from two flowers of each plant were collected and stained with cotton blue. The percentage of viable pollen grains was determined by finding the ratio of viable to non-viable pollen from 200 pollen grains per flower. Pollen viability was analyzed in flowers from 9-10 hybrids (confirmed by ISSR analysis) of each of the Swiss, French and *R. sativus* crossing combinations; in total 28 hybrids. From the Danish cross, the two hybrids found and the four FRB plants (few *Raphanus* bands, see results for a definition) exposed to flow-cytometry were analyzed. For comparison, flowers from 6-7 plants of each of the four paternal types were collected and pollen viability was determined.

### Statistical analysis

Statistical analyses were made using the statistical software package SPSS for Windows (version 11.5; SPSS Inc., Chicago, USA). The significant level used was $\alpha = 0.05$. Differences in seed weights were analyzed using Univariate Analysis of Variance, including a Post Hoc Tukey B test. Differences in the number of hybrid offspring based on the results from the genetic marker analysis were analyzed by a Chi-Square test.

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