Theor Appl Genet (2007) 114:209–221
DOI 10.1007/s00122-006-0424-x

ORIGINAL PAPER

Modelling gene flow between oilseed rape and wild radish. I. Evolution of chromosome structure

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Received: 3 May 2006 / Accepted: 23 September 2006 / Published online: 8 November 2006

Abstract The assessment of gene flow from crop species to weeds has found a new emphasis over the last years because of the marketing of transgenic crops and the possible selective advantage that crop (trans)gene may confer to the weeds. Several studies focused on the F1 interspecific hybrid production but few data are available on the factors affecting the genetic structure of advanced generations. It depends on the genomic structure of the species concerned as well as on the degree of their genome homology that affect the occurrence of intergenomic recombination. Oilseed rape (*Brassica napus*, AACC, $2n = 38$)-wild radish (*Raphanus raphanistrum*, RrRr, $2n = 18$), a distantly related weed, is a good model to address such questions. From seven male sterile oilseed rape lines carrying an herbicide tolerance transgene, F1 interspecific hybrids and four advanced generations were produced under field conditions with wild radish as pollinator. Observation of hybrid chromosome numbers across four generations revealed a high variability, especially in the ''BC1'' generation. A regression model was fitted in order to describe the relationship between parent and offspring chromosome numbers. The effects of generation, transgenic line and selection pressure on the mean relationship were investigated. The first two factors had an influence on the rate of decrease of chromosome numbers, whereas selection pressure resulted in the presence of an additional chromosome in the herbicide treated plants. The model provided a convenient framework for analysing how chromosome numbers evolve over successive hybridization events and it may prove useful as a basis for simulation-based approaches.

Communicated by G. Wenzel.

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Introduction

Gene exchange has always been a major determinant of the evolution in crop–weed-wild complexes (Small 1984; Harlan 1992). During the last decade, the prospect of commercial release of genetically engineered plants has put new emphases on this issue. Many studies have now demonstrated that hybridization occurs spontaneously under natural conditions among crop plants and their wild relatives (Jenczewski et al. 2003). However, very few studies have considered all the subsequent steps that must be completed for efficient gene flow to occur: (1) production of fertile and fit offspring from these hybrids, (2) gene transmission during the successive generations, (3) effective gene introgression through recombination between genomes, and (4) maintenance of the transgene in the natural populations.

When it comes to environmental concern, crop-to-wild gene flow has usually to be considered in the context of wide interspecific hybridization. It is then important to take into account the fact that crop species and their relatives may differ in their numbers of chromosomes, as well as in the degree of relatedness between these chromosomes. These two biological attributes have a profound impact on both gene dispersal and gene establishment because they can contribute to genetic isolation through various biological processes: post-zygotic selection, unfair segregation leading to modified transmission of chromosomes during successive generations of hybridization, and reduced intergenomic recombination. Complex genome structures are commonplace among crop–wild hybrids because many crops were taken into cultivation only after genome duplication (polyploidy), which often accompanied interspecific hybridization (e.g. *Triticum aestivum*, *Gossypium hirsutum*, *Coffea arabica*, *Brassica napus*); these crops consequently lack wild relatives at the same level of ploidy. In a few cases, hybridization between diploid crops and their polyploid relatives has also been documented (e.g. *Sorghum bicolor* and *S. halepense*, Arriola and Ellstrand 1996). Studying the role of hybrid genome structure in the promotion or prevention of crop-to-wild gene flow is therefore important, but has not been extensively explored. This is the main concern of this paper.

Oilseed rape (*Brassica napus*, AACC, $2n = 38$) and its wild relatives represent a very good model to address this question. This crop species is a natural allopolyploid species that originated from wide hybridization(s) between *B. rapa* (AA, $2n = 20$) and *B. oleracea* (CC, $2n = 18$). Interspecific hybridization is possible between oilseed rape and a wide range of related species (Chèvre et al. 2004, for a review). For example, spontaneous F1 hybrids between *B. napus* and wild *B. rapa* have been observed under field conditions in Europe and Canada (Hansen et al. 2001; Wilkinson et al. 2003; Warwick et al. 2003). The two species share a common genome and extensive transfer of nuclear as well as plastid DNA from oilseed rape into a self-maintained weedy population of *B. rapa* has been documented in Denmark (Hansen et al. 2001). These *B. rapa*-like plants have a proportional decrease in oilseed rape-specific markers, suggesting that the markers were introgressed beyond the stage of backcross hybrids. Other studies have shown that gene introgression occurs more easily when the transgene is originally carried by the A genome of oilseed rape (Mikkelsen et al. 1996) rather than by the C genome (Metz et al. 1997). Backcrossing the AAC F1 hybrids ($2n = 29$) to *B. rapa* allows segregation of the C chromosomes that can be modelled (Tomiuk et al. 2000; Lu et al. 2002).

Intergeneric hybridization has also been documented between oilseed rape and wild radish (*Raphanus raphanistrum*, RrRr, $2n = 18$), a more distantly related weed that frequently occurs in oilseed rape fields. The rate of spontaneous intergeneric hybridization between these two species is low under field conditions (Chèvre et al. 2000; Rieger et al. 2001; Warwick et al. 2003). Chèvre et al. (1997a, 1998) have produced five generations of advanced hybrids by backcrossing F1 oilseed rape × wild radish ACRr hybrids to wild radish. This experiment has been conducted in field test plots where an equal number of herbicide resistant hybrids and wild radish had been planted. These authors observed a wide range of hybrid genomic structures and showed that the percentage of herbicide resistant plants as well as their fertility varied extensively. While some herbicide resistant plants demonstrated a level of female fertility equivalent to that of *R. raphanistrum*, none possessed 18 chromosomes as *R. raphanistrum*. This suggested that the transgene had not been introgressed into the *R. raphanistrum* genome through recombination. Herbicide resistant hybrids with the *R. raphanistrum* cytoplasm always carried at least one additional oilseed rape chromosome and had a growth pattern similar to that of *R. raphanistrum* plants, but with male and female fitness values twice as low as those of *R. raphanistrum* (Guéritaine et al. 2002).

The aim of this paper is to study the evolution of hybrid genome structures during the successive generations that follow the formation of intergeneric *B. napus* × *R. raphanistrum* hybrids, in order to set up a framework for modelling gene flow between these two species. We have considered here seven GM oilseed rape lines that have the same genetic background but different insertion sites of the same transgene. We have modelled the evolution of the number of chromosomes in interspecific hybrids produced under field conditions for these seven GM lines and a wild population of *R. raphanistrum*. More precisely, we have addressed the following questions: (1) what is the relationship between the number of chromosome of a female parent and the mean number of chromosomes of its offspring? (2) Does this relationship vary among the successive generations? (3) Does it vary among the different GM oilseed rape lines? (4) Is this relationship modified by selection for the presence of the transgene?

Materials and methods

Plant material

Seven transgenic lines were provided by Bayer Crop Science. They were produced independently from Canadian spring-type variety *B. napus* cv Westar, and were homozygous for one insertion of the *brnx* gene conferring tolerance to Oxynil herbicide. The transgene is under control either of the 35S promotor (lines 235.1, 235.2, 235.3, 235.4, 235.5) or of the promotor of the small unit of the RuBisCo gene (lines 237.1, 237.2). These lines were crossed as male with a female European spring-type *B. napus* cv FuBrutor which carries an Ogu-INRA cytoplasm inducing a male sterility (Pelletier et al. 1983). The male sterile F1 hybrids produced were hemizygous for the transgene. The *R. raphanistrum* populations used were locally collected at Rennes and Dijon, France. The genealogy of the material produced by open pollination is presented in Fig. 1. From the ''BC1'' generation, half of the hybrids were herbicide treated and at the following generations only the seedlings obtained from herbicide tolerant plants were treated. The presence of *R. raphanistrum* chromosomes was checked on the progeny of ''BC1'' plants, except for hybrids obtained from 235.3 line for which ''BC2'' progeny was used.

Field design

Seed germination was carried out in Petri dishes and seedlings were transplanted in the greenhouse. At the 4–5 leaf stage, plants were vernalized at 4°C for one month and then transplanted out into the field in Rennes. All field trials were surrounded by a 6 m wide band of *R. raphanistrum* to prevent pollen dispersal outside the plots and all were spatially isolated from any other oilseed rape field and flowering crucifer species by at least 500 m. All the experiments were performed with the agreement of the French Committee for Molecular Engineering (CGB).

There were three replications arranged in a randomized block design. Each plot consisted in three rows of one oilseed rape F1 hybrid for the production of F1 intergeneric hybrid (first generation) or of intergeneric hybrids from one original transgenic line (advanced generations) and of three rows of wild radish.

Oxynils treatment

In the greenhouse, 3–4 leaf stage seedlings were treated by spraying a solution of 3 g/l of oxynil. The number of herbicide tolerant plants was observed 10 days later.

Cytogenetic studies

Flow cytometry

From previous studies (Eber et al. 1997), a linear regression was established between the chromosome number observed from root tips and flow cytometry data. Cytometry analyses were performed from leaves of the hybrids to assess their chromosome number as described by Eber et al. (1997).

Meiotic behaviour

For meiotic analyses, floral buds were prepared as described by Eber et al. (1994).

Genomic in situ hybridization (GISH)

Cytological preparations were produced from root tips of seedlings as previously described by Snowdon et al. (1997). For the GISH procedure, total genomic DNA from *R. raphanistrum* was used as a probe. DNA was isolated from young leaves according to the method described by Doyle and Doyle (1990) and then labelled with biotin-16-dUTP by nick translation (Invitrogen life technology). Total genomic DNA from *B. napus* leaves was isolated using the same method and then autoclaved to give fragments of 100–500 bp and used as blocking DNA at a ratio 1:50 (probe:blocking). Hybridization was performed as described by Snowdon et al. (1997). Fluorescence images were captured using CoolSnap HQ camera (Photometrics) on Axioplan 2 microscope (Zeiss) and analysed using MetaVue™ (Universal Imaging Corporation™).

Statistical analyses

Model definition

We apply a mixed-effects linear model for describing the relationship between the chromosome number of the female hybrid k in the generation n, denoted by $Y_{n,k}$, and the chromosome number of the *lth* individual in its progeny, denoted by $Y_{n+1,kl}$. The basic model is:

$$Y_{n+1,kl} = a(Y_{n,k} - y_0) + b + \varepsilon_{n+1,kl} \quad (1)$$

where a and b are model parameters, y_0 is given constant and $Y_{n,k}$ is treated as a known covariate. The regression function passes through the point (y_0, b), so

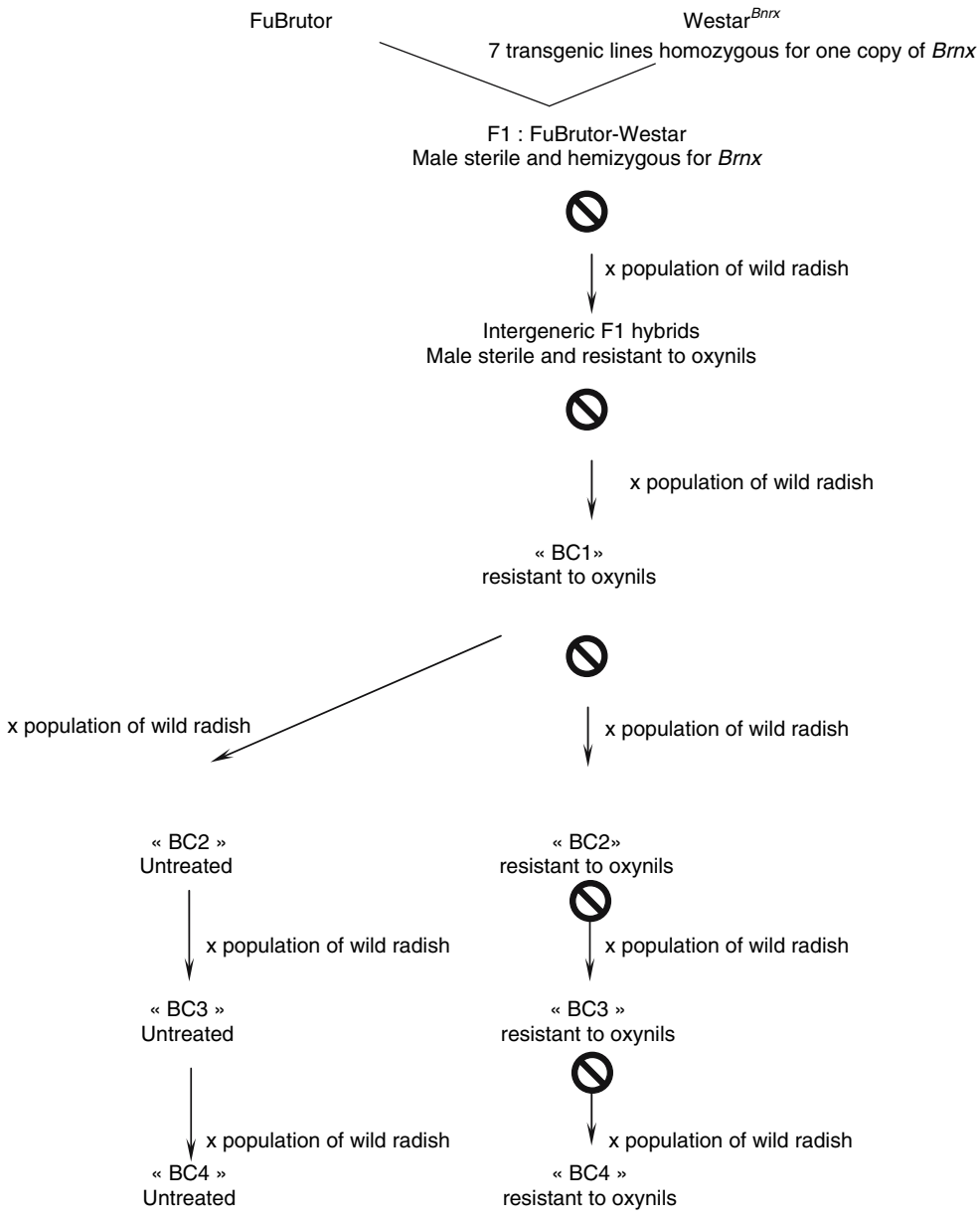

Fig. 1 Genealogy of the hybrids produced under field conditions from seven transgenic lines. ⊘ means that hybrids were treated with oxynils before they were transplanted in the field

the parameter b represents the mean chromosome number in the progeny of an hybrid with y_0 chromosomes. The value of the constant y_0 may be fixed arbitrarily, without loss of generality for the model. Here we have chosen $y_0 = 19$ to check in a convenient way whether the 19-chromosome hybrids retain an additional *B. napus* chromosome or not. The parameter a controls the rate of decrease in chromosome number from one generation to the next one: the value of a is expected to be positive and smaller than 1, indicating that the chromosome number decreases with n, at a rate inversely proportional to a.

Two components of residual variability were taken into account in the model. The first component was the measurement error when counting chromosomes and the second one was the natural variability of chromosome numbers, which was found to increase with the number of chromosomes. Consequently, the residual term was assumed to follow a centered Gaussian distribution with variance $\mathrm{var}(\varepsilon_{n+1,kl}) = \sigma^2(1 + y_+)^p$, where $y_+ = Y_{n,k} - 18$ is the number of additional *B. napus* chromosomes and p, σ are variance parameters.

Introduction of factorial effects

In order to check whether the basic chromosome-number model in Eq. 1 was (1) constant along the generations, (2) dependent or not on the transgenic

lines, (3) dependent or not on selection pressure, these factors were introduced into the model by expressing the parameters of Eq. 1 as the sum of their main effects:

$$a = a_{n,ijk} = \alpha_0 + \alpha_n^{(G)} + \alpha_i^{(T)} + \alpha_j^{(L)} + \mathrm{A}_{n,k}^{(G)}$$
$$b = b_{n,i} = \beta_0 + \beta_n^{(G)} + \beta_i^{(T)} \quad (2)$$

where α_0 denotes the mean slope, $\alpha_n^{(G)}$ denotes the effect of parent generation n, with n in {$BC1$, $BC2$, $BC3$}, $\alpha_j^{(L)}$ denotes the effect of transgenic line j, with j in {235.1, 235.2, 235.3, 235.4, 235.5, 237.1, 237.2} and $\alpha_i^{(T)}$ denotes the effect of oxynil treatment with i in {T, NT} (T represents hybrids treated by oxynil). The decomposition of intercept b was slightly different, excluding the line effect term. The hypothesis that the mean chromosome number $b_{n,i}$ of 19-hybrid progeny does not depend on a transgenic line is addressed in the Results section. The factorial effects appearing in Eq. 2 satisfied standard identifiability constraints:

$$\sum_n \alpha_n^{(G)} = \sum_i \alpha_i^{(T)} = \sum_j \alpha_j^{(L)} = 0$$
$$\sum_n \beta_n^{(G)} = \sum_i \beta_i^{(T)} = 0$$

In order to assess a possible correlation between offspring chromosome numbers from the same female parent, the model includes the random effect of female parent k, denoted by $A_{n,k}^{(G)}$. The parental effect $A_{n,k}^{(G)}$, is assumed to follow a Gaussian distribution with variance depending on the generation: var $(A_{n,k}^{(G)}) = \tau^2_n$.

Statistical inference

The parameter estimates and their asymptotic standard errors have been computed using maximum likelihood method (Lindstrom and Bates 1990). Likelihood ratio tests have been performed to test for the absence of factorial effects. The model fit and related inference are assessed using the lmepackage of the R software (Pinheiro and Bates 2000).

Results

F1 hybrids between oilseed rape and wild radish

More than 500 F1 intergeneric hybrids were obtained from each of the seven male sterile *B. napus* hybrids that were hemizygous for the *brnx* gene. All these hybrids had the expected ACRr genomic structure with $2n = 28$ chromosomes. As expected, the percentage of resistant plants among these hybrids ranged from 43.3 to 52.7% and their female fertility was low, ranging from 1.14 to 3.76 seeds/plant.

"BC1" hybrids between oilseed rape and wild radish

The "BC1" hybrids were obtained from the seeds harvested on F1 intergeneric hybrids. Their germination rate and the percentage of herbicide tolerant plants are reported in Table 1.

Contrary to expectations, a majority of "BC1" plants did not have 37 chromosomes; this genomic structure would have been expected if 28-chromosome unreduced gametes from the hybrid had been fertilized by reduced 9-chromosome gametes of *R. raphanistrum*. In the current study, less than 18% of the BC1 plants displayed this expected structure (a maximum of 30% of 37-chromosome BC1 plants has been observed in the progenies of lines 235.2, 237.1 and 237.2).

A wide range of chromosome numbers was observed (Fig. 2) varying from 24 to 80 (flow cytometry estimation). Distributions of chromosome numbers were clearly different between families (i.e. offspring from each single GM line). For example, up to 25 and 60% of the BC1 plants originating from lines 235.1 and 235.3, respectively, had 28 chromosomes whereas less than 15% of the offspring produced from the other GM lines had less than 37 chromosomes. In order to check that such plants contained the *R. raphanistrum* genome, we analyzed their "BC2"or "BC3" progeny by genomic in situ hybridization (GISH). We observed that each plant carried at least 18 chromosomes of wild radish. The results are reported in Table 2 and two examples are presented in Fig. 3.

Table 1 Characterization of the "BC1" plants obtained from F1 herbicide tolerant interspecific hybrids

Original transgenic lines	Percentage of germination	Percentage of resistant plants	Number of plants transplanted
235.1	48.8	69.5	49
235.2	33.2	67.9	32
235.3	17.2	97.6	59
235.4	35.3	73.2	33
235.5	49.2	77.8	40
237.1	53.6	80.5	77
237.2	45.6	82.9	56

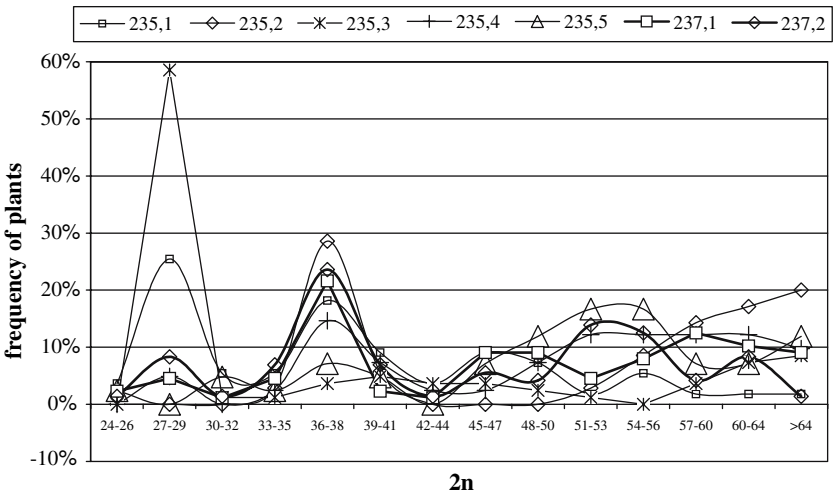

Fig. 2 Chromosome numbers of the ''BC1''plants

Evolution of chromosome number in the following generations

The chromosome numbers of the ''BC2'', ''BC3'' and ''BC4'' hybrids were assessed by flow cytometry and confirmed by meiotic analyses on a sample of plants at each generation (data not shown). As expected, the chromosome numbers decreased progressively during three backcross generations for the majority of ''BC1'' hybrids (Fig. 4). Nevertheless, some hybrids, especially the plants that had a ''BC1'' mother-plant with a high chromosome number, displayed a constant or even a higher number of chromosomes in the following generations. At the same time, the hybrids with high chromosome number in ''BC1'' were in general less fertile: most of them had no progeny in ''BC4'', with a majority having no progeny even in ''BC2''. The differences between transgenic lines observed in ''BC1'' were less pronounced across the following generations, apart from the line ''235.3''. This line displayed a very particular chromosome number distribution in ''BC1'' with a large proportion of plants with $2n = 28$ chromosomes. It continued to evolve in a different way; more frequently than with the other lines, the ''BC2'' hybrids of the line ''235.3'' had more chromosomes than their mother-plant and in the following generations, some plants kept the same chromosome number as the mother-plant whereas the other showed a decrease of the chromosome number (Fig. 4). In the sequel, we have focussed on describing a mean trend in chromosome number evolution rather than explaining the exceptions from the rule, thus the hybrids of the

Table 2 Identification of the wild radish chromosome number by GISH in ''BC2'' plants

Original transgenic lines	Chromosome number of the ''BC1'' mother-plants	Chromosome number of the ''BC2'' plants	Number wild radish chromosomes
235.1	36	26	18
235.2	64	50	22
	36	30	18
		28	18
	36	26	18
235.3[a]	28	26	18
		26	18
235.4	36	27	18
		26	18
235.5	56	50	18
		58	20
237.1	35	36	18
		36	18
237.2	33	26	18
		24	18
		27	18

[a] For this line, seeds from the ''BC2'' plants were used and the number of wild radish chromosomes was assessed in ''BC3''plants

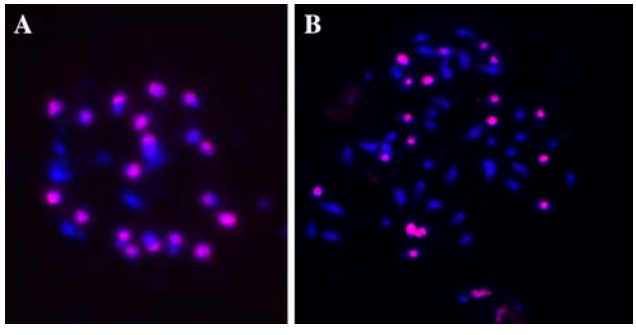

Fig. 3 GISH on metaphase chromosomes of two hybrids taken from the progenies of line 235.3 (**a** $2n = 26$) and line 235.5 (**b** $2n = 58$), and that show 18 and 20 wild radish chromosomes, respectively

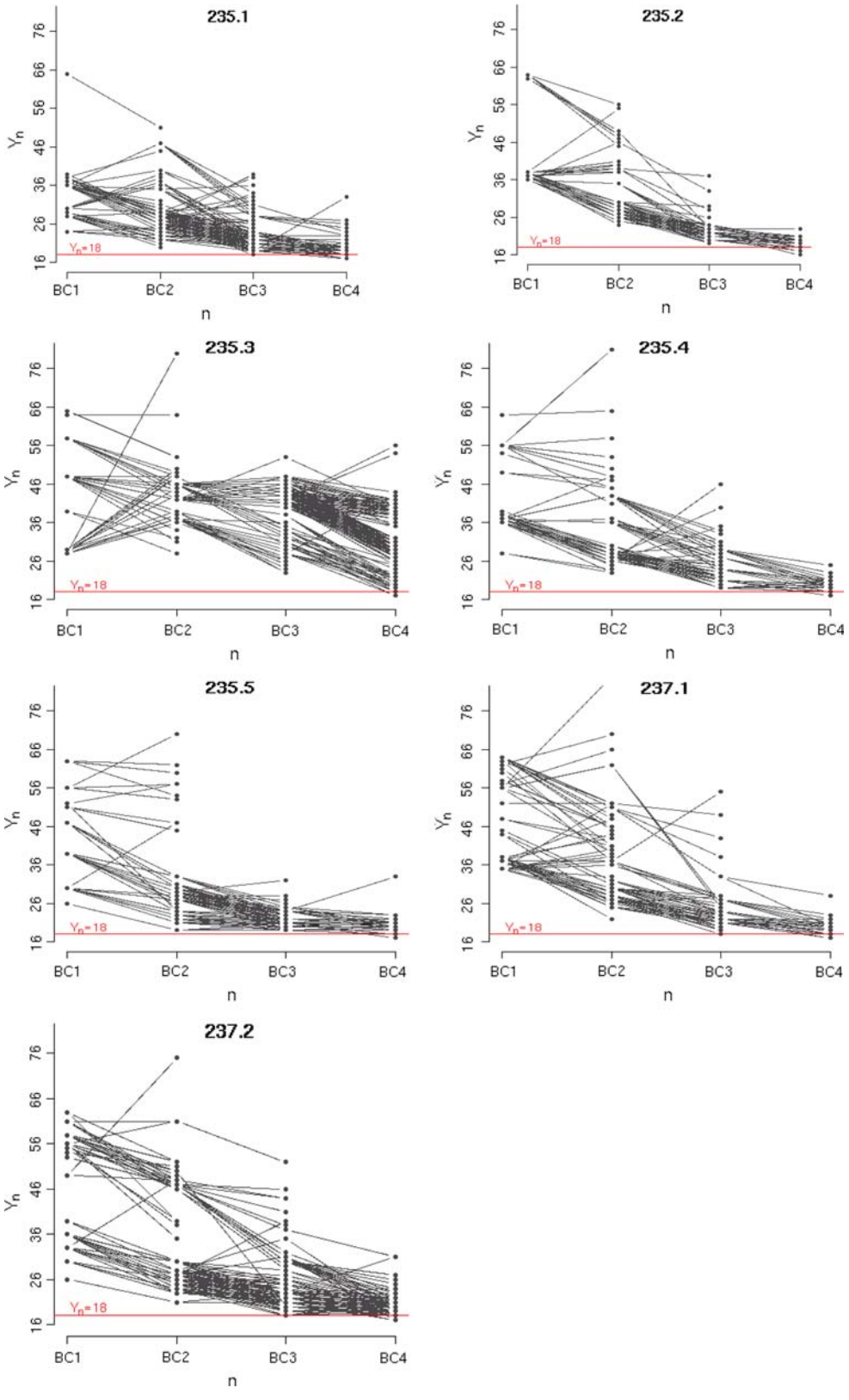

Fig. 4 Chromosome number evolution from BC1 generation to BC4 generation for seven transgenic lines

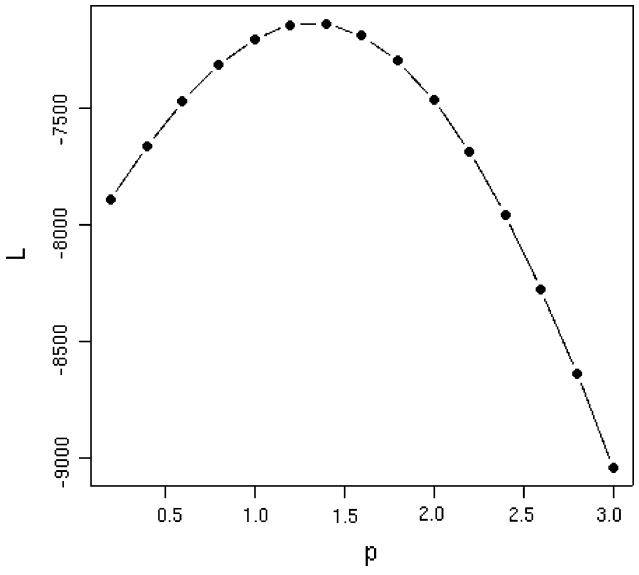

Fig. 5 Profile log-likelihood of variance parameter p. The maximal value of L was reached for $p = 1.4$

line "235.3" were not taken into account in further analyses.

Model construction

The statistical model presented here has been chosen in order to describe the evolution of chromosome numbers across backcross generations, without assumptions on the exact composition of hybrids. The regression approach was applied to fit the relationship between the chromosome number Y_n of a female parent and the expected chromosome number $E(Y_{n+1})$ of its progeny. Since hybrids having the same chromosome number Y_n may carry different chromosomes, a random effect of a parent was introduced into the model in order to control the resulting variability of offspring chromosome number. Model selection consisted in the choice of a regression function, the modelling of the residual variance term and the inclusion of the effects of factors of interest, such as generation G, selection pressure T and transgenic line L. The final regression model has been validated, by means of the residual analysis (data not shown). The definition of the selected model is presented in Material and Methods.

The data set used for fitting the model contained 3,572 pairs of parent and offspring chromosome numbers (Y_n, Y_{n+1}) for three parental generations n: BC1, BC2 and BC3. The value of parameter p accounting for variance heterogeneity was fixed at $p = 1.4$, after fitting it by maximizing the profile likelihood of the model (Fig. 5).

Statistical inference results

The estimated value of the mean slope $\alpha^{(0)} = 0.46$ of the regression function was consistent with the decrease observed in the average chromosome numbers along the generations. The estimated mean chromo-

Table 3 Maximum likelihood estimates of regression model and their 95% asymptotic confidence intervals

Estimation results						
General mean						
α_0	0.46 (0.40, 0.52)					
β_0	18.3 (17.2, 19.4)					
Generation						
n	$BC1$		$BC2$		$BC3$	
$\alpha^{(G)}$	0.36 (0.26, 0.47)		–0.09 (–0.16, –0.02)		–0.27 (–0.34, –0.19)	
$\beta^{(G)}$	–1.84 (–3.98, –0.31)		0.91 (–0.23, 2.05)		0.92 (–0.18, 2.03)	
Oxynil treatment						
i	T			N		
$\alpha^{(T)}$	0.00 (–0.02, 0.02)			0.00 (–0.02, 0.02)		
$\beta^{(T)}$	–0.49 (–0.71, –0.28)			0.49 (0.28, 0.71)		
Transgenic line						
j	235.1	235.2	235.4	235.5	237.1	237.2
$\alpha^{(L)}$	–0.04 (–0.09, 0.01)	–0.08 (–0.13, –0.03)	0.03(–0.03, 0.09)	0.02(–0.04, 0.08)	0.03(–0.02, 0.08)	0.04(0.00, 0.09)
Female parent						
n	$BC1$		$BC2$		$BC3$	
$\tau^{(G)}$	0.17 (0.14, 0.20)		0.05 (0.04, 0.06)		0.03 (0.02, 0.04)	
Residual sd						
σ	0.40 (0.39, 0.41)					

Table 4 Likelihood ratio tests for the absence of generation, selection pressure and transgenic line effect

Factor	Hypothesis (H_0)	Alternative (H_1)	*df*	*P* value
Generation	$\alpha_{BC1}^{(G)} = \alpha_{BC1}^{(G)} = \alpha_{BC3}^{(G)} = 0$ $\beta_{BC1}^{(G)} = \beta_{BC2}^{(G)} = \beta_{BC3}^{(G)} = 0$	Full parameter model (Eq. 2)	4	$<10^{-16}$
Oxynil treatment	$\alpha_T^{(T)} = \alpha_N^{(T)} = 0$ $\beta_T^{(T)} = \beta_N^{(T)} = 0$	Full parameter model (Eq. 2)	2	$<10^{-8}$
Transgenic line	$[\alpha_{235.1}{}^{(L)} = \ldots = \alpha_{237.2}{}^{(L)} = 0]$	Full parameter model (Eq. 2)	5	0.014

some number of 19-chromosome hybrids offspring was equal to $\beta^{(0)} = 18.3$. The estimates of all factorial effects and their 95% confidence intervals are reported in Table 3.

- Generation effect

The likelihood ratio test rejected the hypothesis H_0: "$\alpha_n^{(G)} = 0$, $\beta_n^{(G)} = 0$ for each n" with *P* value less than 10^{-16} (Table 4). Thus, the rate of decrease in chromosome (chromosome numbers equation) was different for at least one generation. The hypothesis H_0: $\alpha_{BC1}^{(G)} = \alpha_{BC2}^{(G)}$, $\beta_{BC1}^{(G)} = \beta_{BC2}^{(G)}$ was rejected with *P* value less than 10^{-11}, revealing differences between the generations *BC*1 and *BC*2. The test of the hypothesis H_0: $\alpha_{BC2}^{(G)} = \alpha_{BC3}^{(G)}$, $\beta_{BC2}^{(G)} = \beta_{BC3}^{(G)}$ was rejected with a *P* value less than 10^{-9}, which confirmed that there were also differences between the generations *BC*2 and *BC*3. Finally, the separate comparison of slope and intercept effects for generations *BC*2 and *BC*3 indicated that $\alpha_{BC2}^{(G)} \neq \alpha_{BC3}^{(G)}$ (*P* value less than 10^{-10}) whereas $\beta_{BC2}^{(G)} = \beta_{BC3}^{(G)}$ (*P* value = 0.24).

The slope effect $\alpha^{(G)}$ decreased significantly along the generations, indicating that the more advanced the generations, the higher the rate of decrease in chromosome number. The intercept effects $\beta_{BC2}^{(G)}$ and $\beta_{BC3}^{(G)}$ were non significantly different, but both were significantly higher than $\beta_{BC1}^{(G)}$. The regression lines fitted for three generations are presented in Fig. 6.

- Selection pressure effect

The hypothesis on the absence of selection pressure H_0: $\alpha_N^{(T)} = \alpha_T^{(T)} = 0$, $\beta_N^{(T)} = \beta_T^{(T)} = 0$ was rejected with *P* value less than 10^{-8}. The offspring of 19-chromosome hybrids treated with oxynil had on average one more chromosome than the offspring of untreated hybrids and this difference was significant: the 95% confidence interval for the difference $\beta^{(T)}{}_T - \beta_N{}^{(T)}$ was (0.55, 1.52). The rate of decrease in chromosome numbers was not significantly different between the two groups, treated and untreated: the 95% confidence interval for the difference $\alpha_T^{(T)} - \alpha_N^{(T)}$ was (– 0.03, 0.04).

- Transgenic line effect

The tests confirmed that the transgenic line effects were significantly different from 0 with *P* value equal to 0.014. The influence of this factor was weaker, compared to generation and selection pressure effect, according to the *P* values calculated for the three factors (Table 4). More detailed inspection of transgenic line effect estimates (Table 3) revealed that the chromosome number of hybrids belonging to the progeny of the line 235.2 decreased faster, compared to the mean rate, whereas the chromosome number of the progeny of 237.2 hybrids decreased at a significantly slower rate. The slope of four other lines did not differ significantly from the mean.

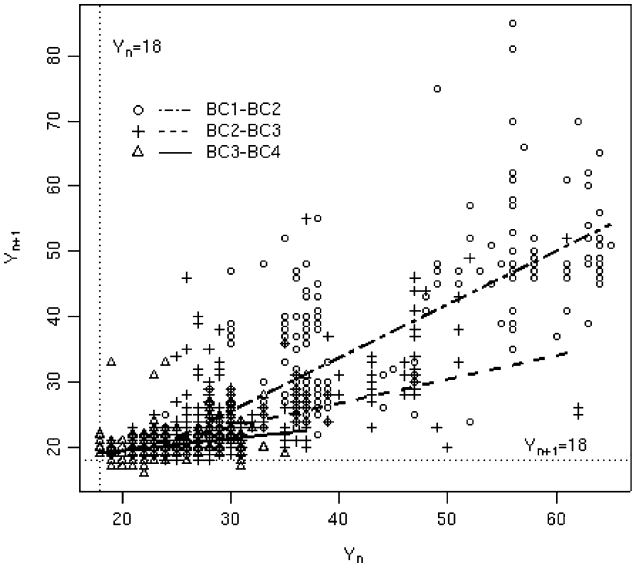

Fig. 6 Fit of regression model taking into account the generation effect. The slope of regression line decreases across the generations, the intercept is different only for BC1–BC2 straight line

Specific investigations confirmed the absence of line effects on the intercept for all the transgenic lines, apart from the line 235.5. As the differences were limited to *BC*2 generation, they were considered as exceptional and the line effect on the intercept of the model was omitted, without altering the effectiveness of the model.

- Female parent effect

The standard deviation τ_n of the female parent random effect was significantly larger than zero along the three generations, confirming that the differences in progeny chromosome numbers were due to the differences in parental genotypes. As expected, parental effect was strongest for the progeny of *BC*1 hybrids: $\tau_{BC1} = 0.17$ against $\tau_{BC2} = 0.05$ and $\tau_{BC3} = 0.03$ for the following generations.

Discussion

In this paper, we have analysed four backcross progenies obtained under field conditions from seven transgenic oilseed rape lines pollinated with *R. raphanistrum* . All the F1 hybrids presented the expected F1 genomic structure (ACRr with 28 chromosomes). From the ''BC1'' generations, we have modelled the evolution of the number of chromosomes and assessed the main factors affecting the structure of the offspring. We observed that this relationship varies between the successive generations and that it is modified when selection pressure is exerted for the presence of the transgene.

''BC1'' generation shows a high range of chromosome number

In our study, the herbicide tolerant ''BC1'' plants showed a surprisingly wide range of chromosome numbers. According to the literature and to previous analyses (Chèvre et al. 1997a), F1 ACRr hybrids should produce mainly unreduced gametes with 28 chromosomes so that a majority of plants with $2n = 37$ chromosomes (ACRrRr) should be expected among the BC1 hybrids (i.e. fertilisation of unreduced 28-chromosomes gametes from the hybrid by reduced 9-chromosomes gametes from *R. raphanistrum*). The production of such unreduced gametes retaining all the chromosomes from the mother-plant is commonplace in interspecific *Brassica* hybrids that contain distantly related genomes (Heyn 1977; Chèvre et al. 1998). In this study, variation was observed among the progenies of each initial transgenic line and within the different progenies. Several plants possessed less than 37 chromosomes (ACRrRr); this may indicate that partial restitution had taken place during meiosis which can be due to intergenomic chromosome pairing, since the A, C and Rr are highly related and able to pair and recombine at meiosis (Kerlan et al. 1993). Alternative explanations include unequal chromosome separation and/or the existence of apomictic hybrids producing a progeny without contribution of the male gametes. Apomictic seed formation was already described on different interspecific hybrids (Chèvre et al. 1998) and is likely to be at the origin of spontaneous haploid plants observed among Brassica species (Prakash and Hinata 1980). In this study, apomixis could explain the origin of the 235.3 ''BC1'' hybrids with 28 chromosomes like the F1 mother-plants. Variation between progenies for the production of putative apomictic offspring suggests that this mechanism is under *R. raphanistrum* genetic control. The hypothesis is confirmed by the analysis of the following generations as we observed a bimodal distribution of the chromosome numbers in some ''BC3'' plant progeny and plants with a high chromosome number in ''BC4''. Additionally, an impact of the initial location of the transgene in the line 235.3 may be suggested as this line only shown a poor seed set fater selfing (data not shown). On the other hand, plants with more than 37 chromosomes may have originated from intercrosses between partially fertile hybrids (Kerlan et al. 1993) since the frequency of partially male fertile plants increased in the advanced generations. Our observation of partially male fertile plants suggest that restorer genes for the Ogu-INRA male sterile system (Pelletier et al. 1983), which has a radish origin, are present in the wild radish populations we have used as pollinator. In fact, mitochondrial orf138 which confers the Ogu-INRA male sterility has been described in wild radish populations (Yamagishi and Terachi 1997) and restorer genes are present in radish populations (Bonnet 1975). Plants with more than 37 chromosomes may have resulted also from pollination by *R.raphanistrum* unreduced gametes as already described for the production of plants with 46 chromosomes (ACRr + RrRr) (Eber et al. 1998; Chèvre et al. 2000; Warwick et al. 2003). This latter assumption is supported by the fact that more than 18 *R. raphanistrum* chromosomes were detected in several plants in the advanced generations (Table 2). Whatever the structure of the ''BC1'' plants, GISH analyses revealed that the wild radish genome is present at least at the diploid stage and that the other chromosomes belong mainly to the oilseed rape genomes.

Fitting a regression model to describe the relationship between parent and offspring chromosome numbers

Our study used a mixed-effects linear model to fit the evolution of the number of chromosomes during the successive generations. We have first envisaged to model the number of oilseed rape chromosomes (that are in addition to the 18 chromosomes of *R. raphanistrum*) as a binomial $B(n,p)$, where n is the number of ''supplementary'' oilseed rape chromosomes in the female parent and p a probability parameter. Such a model, associated with a selection pressure against aneuploid gametes, was proposed by Lu et al. (2002) for *B. napus* × *B. rapa* hybrids. However, there are several reasons why such models did not appear to be well adapted to the data in the present paper. Firstly, some offspring in our study had more chromosomes than their female parents, which is incompatible with the binomial assumption (Fig. 4). Secondly, we found that the binomial fitted the data quite well only if p was allowed to vary between generations: we have notably estimated that p equal to 0.54, 0.28, 0.27 for the BC1, BC2, BC3 generations, respectively (data not shown). Thirdly, the female parent effects identified with the main model showed for the first time that transmission mechanism is more complex than a random selection of chromosomes.

The aim of the model presented in this paper is to describe the relationship between chromosome numbers in the progeny of a plant and the main explicative factors. For this, we have included not only the average behaviour but also the complex variability sources and the resulting variance heterogeneity. Such modelling required trial and error. For example, various transformations on the data and various regression models were considered. The resulting model allows an accurate description of the data and of its variability, and does so without relying on strong mechanistic assumptions. The general emphasis here was on finding a parsimonious empirical model rather than a complex one, while coping with the main sources of variability in the data. This empirical modelling approach is a major help for interpreting such complex data. However, our model does not account for the very peculiar evolution of chromosome number observed in the progeny of line 235.3, which may be suggestive for the segregation of mutation inducing apomixis (see above). If this is true, then one may expect hybrids to go back to mainstream evolution when the mutation is lost (see bimodal distribution on Fig. 4). This point deserves critical evaluation in order to determine whether the idiosyncratic behaviour of line 235.3, which would never have been commercialized given its poor fertility, can be relevant for the general purpose of crop-to-wild gene flow assessment.

Pointing to the main explicative factors influencing chromosome number in successive plant generations

Our model allows statistical inference to be made and LR tests to be performed for biologically relevant hypotheses on factorial effects. Significant differences were detected for the rate of chromosome number decrease between the generations. Two main factors may explain these differences. Firstly, as some hybrids may have originated from selfing or intercrosses between partially fertile hybrid mothers, it is possible that they carry the two homologues of several oilseed rape and/or of *R. raphanistrum* chromosomes, especially when they have a high chromosome number. When this configuration is true, pairing allows a fair transmission of the paired chromosomes to the gametes, explaining that the plants with a higher chromosome number show a slower decrease of the chromosome number in the progeny. If intercrosses occur between hybrids, the probability of occurrence of such plants with homologous chromosome pairs is higher in the first generations close to the hybrids presenting the whole genome of both species. Such homologous pairs are separated progressively in advanced generations through the recurrent pollinations with *R. raphanistrum*. This is in agreement with the individual effect of the female parent but it is important to mention that the plants with the highest chromosome number showed generally a poor fertility. Secondly, it has been established that the transmission rate of additional chromosomes may be different between chromosomes of the same genome and that the male and female transmissions can differ for the same chromosome (Chèvre et al. 1997b). Additionally, it is likely the transmission rate of a specific chromosome is different according to the presence or not of several other additional chromosomes. The selection pressure by the herbicide treatment did not show an effect on the decrease on the chromosome number. However, one supplementary chromosome was detected in the treated plants. Whatever the genomic structure of the plants, only those carrying the oilseed rape chromosome with the transgene will survive after herbicide treatment and that this chromosome will be retained. This difference between treated and untreated plants will disappear only after chromosome recombination between the oilseed rape chromosome carrying the transgene and a *R. raphanistrum* one. Small but

significant differences were detected between the initial transgenic lines. According to their homogeneous genetic background, genetic variability among the wild radish population, especially for the production of unreduced gametes, can explain such differences. However, a transgene position effect is also suspected for the line 235.3. This latter line showed a poor fertility even after selfing (data not shown).

The main motivation for developing the model presented in this paper was first to provide a convenient framework for analysing this data set. At the present time, there are scarce data on intergeneric hybrids and their back-cross offspring, whereas such information is much needed for modelling the risks of gene introgression (Thompson et al. 2003). Consequently, the model developed here turns out to be useful for simulation-based approaches to the evaluation of such risks. This requires other parameters to be estimated, such as fitness, which is the subject of a forthcoming companion paper.

Acknowledgments We thank Bayer Crop Science for providing the original transgenic lines. Our colleagues at the INRA experimental farm (Le Rheu, France) are gratefully acknowledged for their technical assistance and Dr M. Barbetti (University of Western Australia) for his critical reading of the manuscript. This work was supported by a grant of the French Research Ministry (ACI OGM).

References

Arriola PE, Ellstrand NC (1996) Crop-to-weed gene flow in the genus Sorghum (Poaceae): spontaneous interspecific hybridization between jonhstongrass, *Sorghum halepense*, and crop sorghum, *S. bicolour*. Am J Bot 83:1153–1160

Bonnet A (1975) Introduction et utilisation d'une stérilité mâle cytoplasmique dans les variétés précoces européennes de radis. Ann Amélior Plantes 25(4):381–397

Chèvre AM, Eber F, Baranger A, Renard M (1997a) Gene flow from transgenic crops. Nature 389:924

Chèvre AM, Eber F, Barret P, Dupuy P, Brace J (1997b) Identification of the different *Brassica nigra* chromosomes from both sets of *Brassica oleracea–B.nigra* and *Brassica napus–B.nigra* addition lines with a special emphasis on chromosome transmission and self-incompatibility. Theor Appl Genet 94:603–611

Chèvre AM, Eber F, Baranger A, Hureau G, Barret P, Picault H, Renard M (1998) Characterisation of backcross generations obtained under field conditions from oilseed rape–wild radish F1 interspecific hybrids: an assesment of transgene dispersal. Theor Appl Genet 97:80–98

Chèvre AM, Eber F, Darmency H, Fleury A, Picault H, Letanneur JC, Renard M (2000) Assessment of interspecific hybridization between transgenic oilseed rape and wild radish under normal agronomic conditions. Theor Appl Genet 100:1233–1239

Chèvre AM, Ammitzboll H, Breckling B, Dietz-Pfeilstetter A, Eber F, Fargue A, Gomez-Campo C, Jenczewski E, Jorgensen R, Lavigne C, Meier MS, den Nijs H, Pascher K, Seguin-Swartz G, Sweet J, Stewart CN Jr, Warwick S (2004) A review on interspecific gene flow from oilseed rape to wild relatives. In: den Nijs HCM, Bartsch D, Sweet J (eds) Introgression from genetically modified plants into wild relatives. CABI, Cambridge, pp. 235–251

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Eber F, Chèvre AM, Baranger A, Vallée P, Tanguy X, Renard M (1994) Spontaneous hybridization between a male sterile oilseed rape and two weeds. Theor Appl Genet 88:362–368

Eber F, Letanneur JC, Chèvre AM (1997) Chromosome number of oilseed rape (*Brassica napus*)-wild radish (*Raphanus raphanistrum*) spontaneous hybrids and of their progeny estimated by flow cytometry. Cruciferae Newslett 19:17–18

Eber F, Boucherie R, Broucqsault LM, Bouchet Y, Chèvre AM (1998) Spontaneous hybridization between vegetable crops and weeds. 1. Garden radish (*Raphanus sativus* L.) and wild mustard (*Sinapis arvensis* L.). Agronomie 18:489–497

Guéritaine G, Sester M, Eber F, Chèvre AM, Darmency H (2002) Fitness of backcross six of hybrids between transgenic oilseed rape (*Brassica napus*) and wild radish (*Raphanus raphanistrum*). Mol Ecol 11:1419–1426

Hansen BL, Siegismund HR, Jørgensen RB (2001) Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* in a natural population. Genet Res Crop Evol 48: 621–627

Harlan JR (1992) Crops and man. American Society of Agronomy, Madison

Heyn FW (1977) Analysis of unreduced gametes in the *Brassiceae* by crosses between species and ploidy levels. Z Pflanzenzüchtg 78:13–30

Jenczewski E, Ronfort J, Chèvre AM (2003) Crop-to-wild gene flow, introgression and possible fitness effects of transgenes. Environ Biosafety Res 2:9–24

Kerlan MC, Chèvre AM, Eber F (1993) Interspecific hybrids between a transgenic rapeseed (*Brassica napus* L.) and related species: cytogenetical characterization and detection of the transgene. Genome 36:1099–1106

Lindstrom MJ, Bates DM. (1990) Nonlinear mixed effects models for repeated measures data. Biometrics 46:673–687

Lu CM, Kato M, Kakihara F (2002). Destiny of a transgene escape from *Brassica napus* into *Brassica rapa*. Theor Appl Genet 105: 78–84

Metz PLJ, Jacobsen E, Nap JP, Pereira A, Stiekema WJ (1997) The impact of biosafety of the phosphinothricin-tolerance transgene in inter-specific *B. rapa* x *B. napus* hybrids and their successive backcrosses. Theor Appl Genet 95:442–450

Mikkelsen TR, Andersen B, Jorgensen RB (1996) The risk of crop transgene spread. Nature 380:31

Pelletier G, Primard C, Vedel F, Chétrit P, Remy R, Rousselle P, Renard M (1983) Intergeneric cytoplasmic hybridin Cruciferae by protoplast fusion. Mol Gen Genomics 191:244–250

Pinheiro JC, Bates DM (2000) Mixed-Effects Models in S and S-PLUS. Springer, Berlin Heidelberg New York

Prakash S, Hinata K (1980) Taxonomy, cytogenetics and origin of crop Brassicas, a review. Opera Botany 55:3–57

Rieger MA, Potter TD, Preston C, Powles SB (2001) Hybridisation between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic field conditions. Theor Appl Genet 103:555–560

Small E (1984) Hybridization in the domesticated weed-wild complex. In: Grant WF (eds) Plant biosystematics. Academic, Toronto, pp. 195–210

Snowdon RJ, Köhler W, Friedt W, Köhler A (1997) Genomic in situ hybridization in Brassica amphidiploids and interspecific hybrids. Theor Appl Genet 95:1320–1324

Thompson CJ, Thompson BJP, Ades PK, Cousens R, Garnier-Gere P, Landman K, Newbigin E, Burgman MA (2003) Model-based analysis of the likelihood of gene introgression from genetically modified crops into wild relatives. Ecol Model 162:199–209

Tomiuk J, Hauser TP, Bagger-Jørgensen R (2000) A- or C-chromosomes; does it matter for the transfer of transgenes from *Brassica napus*. Theor Appl Genet 100:750–754

Warwick SI, Simard MJ, Légère A, Beckie HJ, Braun L, Zhu B, Mason P, Séguin-Swartz G, Stewart Jr CN (2003) Hybridization between transgenic *Brassica napus* L. and its wild relatives: *B. rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L., and *Erucastrum gallicum* (Willd.) O.E. Schulz. Theor Appl Genet 107: 528–539

Wilkinson MJ, Elliot JL, Allainguillaume J, Shaw MW, Norris C, Welters R, Alexander M, Sweet J, Mason DC (2003) Hybridization between *Brassica napus* and *B. rapa* in a national scale in the United Kingdom. Science 302:457–459

Yamagishi H, Terachi T (1997) Molecular and biological studies on male sterile cytoplasm in the Cruciferae IV Ogura-type cytoplasm found in wild radish, *Raphanus raphanistrum*. Plant Breed 116:323–329