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A- or C-chromosomes, does it matter for the transfer of transgenes from *Brassica napus*

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Abstract. Introgression of genes from allotetraploid *Brassica napus* into its diploid wild relative *B. rapa* is generally considered to be inevitable. As a means to minimize a potential ecological risk in environments where *B. rapa* is growing, the insertion of transgenes into chromosome regions of *B. napus* with a very low probability of transfer to backcross generations with *B. rapa* has been proposed. Recently, the progeny of four backcross generations between transgenic herbicide-tolerant *B. napus* and *B. rapa* was studied in selection experiments (Metz et al. 1997). The rapid decrease in the frequency of herbicide-tolerant plants was explained by selection against the *C*-chromosomes of *B. napus* in favor of the homeologous *A*-chromosomes. Obviously, such *C*-chromosomes could be potential candidates as *safe* integration sites for transgenes. We considered these safety aspects using a simple population genetic model. Theory and experiments, however, do not favor the chromosomes of *B. napus* as safe candidates with respect to the introgression of transgenes into wild populations of *B. rapa*.

Key words Safe integration · Chromosome variation · Oilseed rape · Gene transfer · Risk assessment,*Brassica rapa*

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Introduction

Field release of transgenic cultivars can pose ecological risks if such crop plants hybridize with their wild relatives (Tiedje et al. 1989; Ellstrand and Hoffman 1990; Kareiva et al. 1991; Williamson 1993; Jørgensen and Andersen 1994; Klinger and Ellstrand 1994; Mikkelsen et al. 1996a, b). Oilseed rape (*Brassica napus*) is one of the genetically modified crops which has various wild relatives; for example, *B. rapa* (syn. *B. campestris* L.) is one of the most predominant weedy relative in Denmark. Genetic studies using isozymes, randomly amplified polymorphic DNA (*RAPD*)-markers and transgenes have shown that genes of oilseed rape can be transferred into *B. rapa* in few generations (Jørgensen and Andersen 1994, 1997; Mikkelsen et al. 1996a). Although F_1-hybrids (Jørgensen and Andersen 1994) and the first backcross generation (BC_1) with *B. rapa* (Lee and Namai 1992) have, on average, a reduced fitness, *B. rapa*-like plants with a high fitness can be found as early as in the first backcross generation (Mikkelsen et al. 1996a, b; Snow et al. 1999; Hauser et al. 1998a, b). The occurrence of fertile, transgenic weed-like plants after only two generations of hybridization and backcrossing suggests a rapid spread of transgenes from oilseed rape into its wild relative *B. rapa* (Mikkelsen et al. 1996a).

The chance that transgenes of crop plants escape from fields might be reduced through their insertion into a chromosomal region with a low probability of gene transfer to backcross generations (Mikkelsen et al. 1996b). In a comparative study of two transgenic herbicide-tolerant varieties of oilseed rape, the frequency of herbicide-tolerant plants within one variety decreased considerably during few backcross generations with *B. rapa* (Metz et al. 1997). This result led to the conclusion that *oleracea*-like chromosomes can be used for a *safe* integration of transgenes in the genome of oilseed rape (Metz et al. 1997). On the other hand, based on RAPD-markers of the *oleracea*-like genome of *B. napus*, Mikkelsen et al. (1996 b) found transmission rates from the F_1- into the BC_1-generation in crosses with *B. rapa* that were close to 50% for most markers. The lowest frequency, however, was 26%, and this rather did not support the hypothesis of *safe* integration sites in *B. napus* (Mikkelsen et al. 1996b).

In this paper we consider pro and contra arguments with respect to safe integration sites of the genome of transgenic oilseed rape. Experimental results (Metz et al. 1997) are reanalyzed and considered within the framework of a simple population genetic model. Our conclusion is that hitherto existing experimental results do not indicate any specific chromosomes to be more safe candidates for an integration of transgenes.

The model

Oilseed rape *Brassica napus* originates from hybridization between diploid species, *B. rapa* (*AA*, $2n=20$) and *B. oleracea* (*CC*, $2n=18$), and it is allotetraploid (*AACC*, $2n=38$). Hybrids between *B. napus* and *B. rapa* are triploid (*AAC*, $2n=29$), and the offspring from the subsequent backcross with *B. rapa* has varying numbers of *C*-chromosomes (*AA*+0–9*C*). The two parental genomes have close structural similarities in which, differences between the homeologous *A*- and *C*-chromosomes are mainly due to chromosomal rearrangements, – e.g. translocation, inversion and gene duplication (Lydiate et al. 1993, 1995; Chen et al. 1997a, b). Furthermore, the similarity of both sets of chromosomes facilitates recombination between homeologous *A*- and *C*-chromosomes in *B. napus* and in plants from backcrosses with *B. rapa*.

It is certain that, genes introgressing from oilseed rape interact with the genetic background that they experience during backcrosses with *B. rapa*. To illustrate such a scenario we assume that chromosomal rearrangements, i.e. translocations between *A*- and *C*-chromosomes, occurred during the evolution of *B. napus.* Figure 1 shows the changes undergone by two pairs of chromosomes that originate from *B. rapa* (A^RA^R) and *B. oleraceae* (C^OC^O). Hybridization between *B. rapa* and *B. oleraceae* (Fig. 1a) and alloploidization result in a line of *B. napus* ($A^RA^R\ C^OC^O$). Translocation between homeologous chromosomes of *B. napus* generates differences between the translocated chromosome A^N and its corresponding *B. rapa*-chromosome, A^R (Fig. 1c). Consequently, we now have three different chromosomes, A^R, A^N and C^N, where pairs of chromosomes with identical centromeres can pass correctly the meiotic process. For simplicity we neglect any structural polymorphism of chromosomes within *B. napus* and *B. rapa*, i.e. homologous chromosomes having identical structures. F_1-hybrids between *B. napus* and *B. rapa* have balanced chromosome sets with respect to the diploid *B. rapa* genome and in addition the hybrids have the haploid *C*-genome of *B. oleraceae* (Fig. 1d). During subsequent backcross generations, chromosome combinations can occur that are genetically unbalanced if A^N-chromsomes are not correctly combined with their corresponding C^N-chromosomes. Such a genetic unbalance may affect fit-

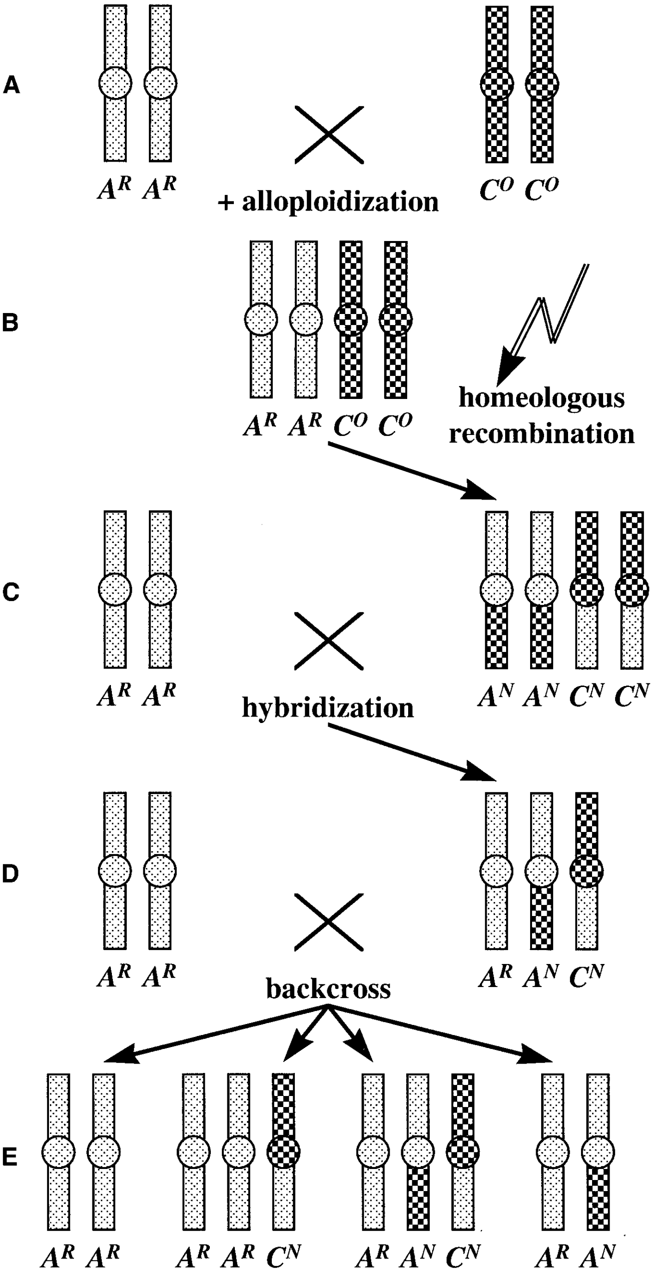

Fig. 1a–e The backcross process from *B. napus* to its wild relative *B. rapa* is demonstrated for two pairs of homologous chromosomes. **a** Chromosomes of parental species of oilseed rape are marked: *B. rapa* with *dots* (◎) and *B. oleraceae* with *squares* (⊗). **b** Hybridization and subsequent alloploidizatin results in allotetraploid *B. napus*. **c** Chromosomal rearrangements in *B. napus* occur through homeologous recombination. Only the karyotype is considered that has identical translocated chromosomes. **d** The triploid F_1-hybrid results from the backcross of *B. napus* and *B. rapa*. In meiosis, chromosomes pair according to their centromere and unpaired chromosomes are passed our or lost by chance. **e** Four chromosome combinations occur in the backcross generation, BC$_1$. One set has a homologous pair of *B. rapa*-chromosomes (left); two sets have three homeologous chromosomes that maintain the diploidy of the *B. rapa* genome (*middle*); one set is unbalanced, i.e. there is neither a complete pair of homologous *B. rapa* nor of *B. oleraceae* chromosomes (*right*)

Table 1 Fitness values related to the total number of null alleles at three loci of transgenic plants

Fitness	Number of null alleles
w_3	None (fitness is affected only by the transgene, at all loci two functional alleles are present)
w_2	One (at one locus only one functional allele is present)
w_1	Two (two loci are heterozygous, i.e. one functional allele plus one null allele; at one locus two functional alleles are present)
w_0	Three (all three loci are heterozygous)

ness of plants (Fig. 1e, right karyotype). In meiosis, unpaired chromosomes can pass or be lost by chance. *A*- and *C*-chromosomes being either paired or unpaired are transmitted from one to the next generation with a probability of 0.5.

The persistence of a transgene during backcrosses from *B. napus* into *B. rapa* is analyzed here with a simple population genetic model. We assume that the fitness of *B. napus* plants is determined by many loci that are located on different *A*- or *C*-chromosomes and that during backcrossing genetically unbalanced plants occur, i.e. aneuploidy can be found (Fig. 1). In a formal sense we consider "missing" alleles to result from the loss of homologous chromosome segments as null alleles that can reduce fitness. The fitness of backcrossed transgenic plants depends on the number of functional alleles at loci. Finally, our simulation follows the crossing design applied by Metz et al. (1997) where only transgenic plants are selected for further backcrosses with *B. rapa*.

For demonstrating more clearly that Metz et al.'s (1997) conclusions in favor of *C*-chromosomes are misleading we consider the special case where the transgene is located on an *A*-chromosome of *B. napus* A^{NT} that is not completely structurally identical with its homologue counterpart of *B. rapa*, i.e. $A^{NT} \neq A^{R}$. Genetic imbalance occurring through the pairing of structurally different *A*-chromosomes during backcrossing, i.e. A^{NT}/A^{R} where at some loci only one functional allele is present, can only be compensated for by three non-transgenic loci that are unlinked and randomly dispersed throughout the *C*-chromosomes of *B. napus*. Consequently, by selecting backcrossed transgenic plants, we can vary fitness through the presence or absence of functional alleles at three loci (Table 1). We assume that with an increasing number of loci at which only one functional allele is present, fitness is increasingly reduced. More than two functional alleles at a locus do not affect fitness. Obviously, the loss of transgenic plants in backcross generations with *B. rapa* results from imbalanced chromosome sets in hybrid plants.

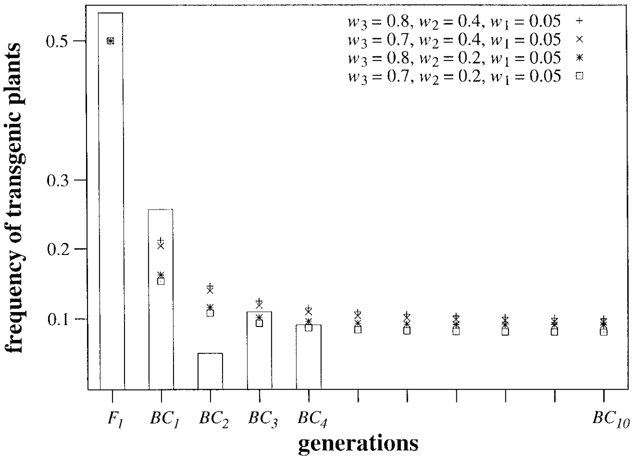

Fig. 2 Frequency changes of transgenic oilseed rape plants in a selection experiment during backcrosses with *B. rapa* genomes. There is artificial selection in favor of plants having the transgenic A^{NT}-chromosome, and natural selection acting against those having the A^{NT}-chromosome. Transgenic plants with no null allele have a relative disadvantage of w_3 (0.8 or 0.7), with one null allele having fitness w_2 (0.4 or 0.2), two null alleles with w_1=0.05 and three null alleles with w_0=0 (see Table 1). The *columns* represent the frequency data of transgenic plants obtained from Metz et al. (1997): comparing the data with the results of the first parameter set, χ^2=11.50, *df*=5, *P*=0.04, and with the second parameter set, χ^2=7.81, *df*=5, *P*=0.17 (see text for further details)

Results

Assuming the same fitness for all plants, i.e. $w_3=w_2=w_1=w_0=1$, simulations showed the expected loss of half of the A^N- and C^N-chromosomes from one to the next generation and the frequency of transgenic plants was equal to 50% in all backcross populations. On the other hand, variation in relative fitness coefficients with $1 \geq w_3 > w_2 > w_1$ and w_0=0 gave a rapid decrease in the frequency of transgenic plants at the beginning of the backcross process. In the first backcross generation, the frequency of transgenic plants fell below 50%, and thereafter frequency decreased until it reached an equilibrium value. Even if the fitness of the transgenic plants with a complete set of functional alleles was equal to the fitness of *B. rapa*, e.g. w_3=1, w_2=0.4 or 0.2, w_1=0.05 and w_0=0, the frequency of transgenic plants decreased to about 25% in the first backcross generation and then converged to a value close to 11%. Following the best case in our risk analysis, we further assumed that genomic manipulations reduce the fitness of transgenic plants, i.e. w_3 <1. Figure 2 shows the results of four parameter sets w_3=0.8 or 0.7, w_2=0.4 or 0.2, w_1=0.05 and w_0=0. Transgenic plants without any combined effects through other fitness-related loci experience a disadvantage of 20% and 30%, respectively, and a loss of one or two functional alleles seriously reduces the relative fitness of transgenic plants. In the BC_1-generation, only about 20% of the total number of plants were transgenic, and after two generations (BC_2) less than 15% of the plants carried the transgene. The frequency of transgenic plants converged to a value close to 9% (Fig. 2).

Selection strongly acted against plants that lost at least one functional allele, which reduced considerably the frequency of transgenic plants in the first backcross generations. In our simulation study two counteracting selection regimes were chosen, natural selection that favored non-transgenic genotypes and artificial selection that favored transgenic plants. This design indeed generates a stable genetic equilibrium.

Results of simulations were compared with experimental data given by Metz et al. (1997). Their data closely fit to those of our model using, for example, a parameter set w_3=0.7, w_2=0.4, w_1=0.05 and w_0=0, where differences between observed and expected data are not significant (Fig. 2, χ^2=7.806, d.f.=5, p=0.167).

Discussion

In the debate about the risk of released transgenic oilseed rape, some confusion may have arisen from observations of offspring originating from backcrossing allotetraploid *B. napus* into its diploid parental species *B. rapa*. The number of *C*-chromosomes varies among individuals of the BC_1-generation, i.e. the number ranges from zero to nine *C*-chromosomes. A decreasing number of *C*-chromosomes during subsequent backcross generations may have led to the conclusion that *C*s are more likely to be eliminated than *A*s. However, excluding selection, every *A*- and *C*-chromosome is transferred from one to the next generation with a probability equal to 0.5. Furthermore, even the observation of a non-binomial distribution of *C*-chromosomes in backcross generations does not favor them *per se* as *safe* integration sites. Cytogenetic studies of diakinesis chromosomes of *B. campestris*-*alboglabra* addition lines demonstrated that chromosomes can form multivalents in backcross offspring (Chen et al. 1997a). Assuming no selection, we considered the very simple case where units of pairs or triplets of *C*-chromosomes were transferred to the next generation. The distribution of *C*-chromosomes in the BC_1-generation was similar to that described by Fantes and Mackay (1978), i.e. observed frequencies of low and high numbers of *C*-chromosomes in the BC_1 generation are higher than the expected frequencies from the corresponding binomial distribution (Fig. 3), but the transmission rate of chromosomes still remains the same.

In their study of transgenic herbicide-tolerant *B. napus* Metz et al. (1997) assumed that two lines, TP2 and TP3, differed with respect to segregation into herbicide-tolerant and sensitive plants after hybridization with *B. rapa*. The large decrease in the frequency of transgenic plants in one line during four backcross generations with the *B. rapa* variety ‘Pak choi’ was explained by the location of the transgene on a *C*-chromosome. Based on these findings Metz et al. (1997) argued that transgenes can more safely be integrated into *C*-chromosomes than into *A*-chromosomes of *B. napus*. Different segretation ratios for the two lines assumed by Metz et al. (1997), however, cannot be proven statistically (‘Pak choi’**TP*3 – 1:1; ‘Pak choi’*TP2 – about 1:3; see Table 2 in Metz et al. (1997); χ^2=0.734, df=1, *P*=0.392).

The operation of homeologous recombination and epistasis proposed by Metz et al. (1997) in order to interpret their results are also the basis of our model. Differently to the conclusion given by Metz et al. (1997) we, however, showed that a large decrease in the frequency of transgenic plants within the first backcross generations can also easily be explained by selection against transgenic *A*-chromosomes of *B. napus* during the backcross process. Therefore, without more detailed genetic information about transgenic lines of *B. napus*, no decision can be made in favor of the *A*- or *C*-genome as the safer candidate with respect to the introgression of transgenes into wild populations of *B. rapa*.

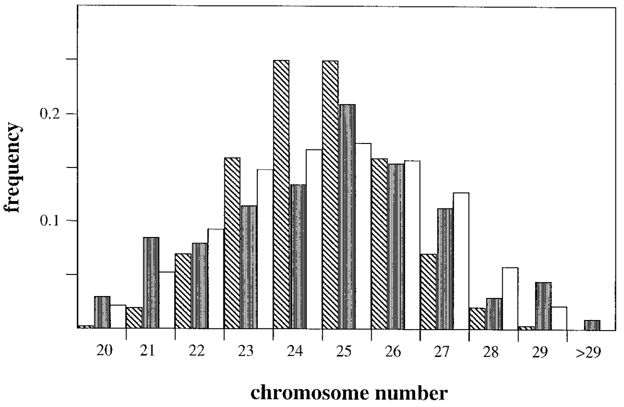

Fig. 3 The number of chromosomes in backcrosses (*B. napus*×*B. rapa*)×*B. rapa*. The expected frequencies assuming a binomial distribution are given by the *slashed columns* (▨). The data of Fantes and Mackay’s (1978) study are represented by the *stripped columns* (▥), and the *white columns* show a chromosome distribution assuming a high association among *C*-chromosomes (see text for further details)

The safety of an integration site depends on the frequency of homologous and homeologous recombination events and the persistence time of transgenic *A*- or *C*-chromosomes in backcross populations. Recombination and chromosomal persistence can be specific for different varieties of *B. napus*, e.g. unbalanced chromosomal rearrangements with respect to the genome of *B. rapa* can be less abundant when the oilseed rape parent in backcrosses with *B. rapa* is a resynthesized new line than an old traditional variety. Thus, genes of recently resynthesized varieties can introgress more easily into *B. rapa*. If transgenes coding for herbicide tolerance, however, have once become established in a population of *B. rapa*, they can hardly be removed from an agricultural ecosystem where the respective herbicide is used. Seed dormancy of backcrossed plants and recurrent selection for herbicide tolerance can maintain the transgenes despite a possible selective disadvantage under natural conditions (see also Tomiuk and Loeschcke 1993). The frequency of about 10% transgenic BC_4 plants that is observed in Metz et al. (1997) study is high and indicates that a large resource of transgenic herbicide tolerance can unintentionally be built up from which genes can introgress permanently into *B. rapa* populations. As a consequence, weedy herbicide tolerant *B. rapa* can cause perpetually control problems in agricultural areas.

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