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The impact on biosafety of the phosphinothricin-tolerance transgene in inter-specific *B. rapa*×*B. napus* hybrids and their successive backcrosses

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Abstract There is strong evidence indicating that gene flow from transgenic *B. napus* into weedy wild relatives is inevitable following commercial release. Research should now focus on the transmission, stability, and impact of transgene expression after the initial hybridization event. The present study investigated the transfer of a phosphinothricin-tolerance transgene by inter-specific hybridization between *B. rapa* and two transgenic *B. napus* lines. The expression of the transgene was monitored in the F_1 hybrids and in subsequent backcross generations. The transgene was transmitted relatively easily into the F_1 hybrids and retained activity. Large differences in the transmission frequency of the transgene were noted between offspring of the two transgenic lines during backcrossing. The most plausible explanation of these results is that the line showing least transmission during backcrossing contains a transgene integrated into a C-genome chromosome. Approximately 10% of offspring retained the tolerant trait in the BC_3 and BC_4 generations. The implications of these findings for the stable introgression of transgenes carried on one of the chromosomes of the C-genome from *B. napus* and into *B. rapa* are briefly discussed.

Key words Biosafety · *Brassica rapa* · Inter-specific hybridization · Phosphinothricin-tolerance gene · Transgenic *Brassica napus*

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Introduction

The genus *Brassica* includes several economically important species, such as *B. oleracea*, *B. rapa*, *B. napus*, *B. juncea* and *B. nigra*. The genomic relationships of these *Brassiceae* are described in the so-called triangle of U (1935; Fig. 1). The A-genome occurs in *B. juncea*, *B. napus* and *B. rapa*, which are all grown for oil production. Oilseed rape (*B. napus*) is allotetraploid with the genome constitution AACC (2n = 38). The cytogenetic relationships were confirmed by nuclear DNA content (Verma and Rees 1974), DNA analysis (Erickson et al. 1983), and genome-specific chromosome markers (Hosaka et al. 1990).

The production of transgenic *Brassicas* has raised the question of whether transgene dispersal into natural populations can be expected or not (Metz et al. 1997). In the Netherlands, (semi)spontaneous populations of *B. rapa* are found in the wild. These might be regarded as wild relatives of *B. napus*. In Denmark and Canada, *B. rapa* is a common weed in cultivated areas, mostly in oilseed rape fields.

Crosses between *B. rapa* and *B. napus* are frequently described as successful (U 1935; Palmer 1962; Nwankiti 1971; MacKay 1977; Beversdorf et al. 1980). Spontaneous hybridization with *B. napus* has been observed in agricultural fields (Bing et al. 1991; Jørgensen and Andersen 1994; Jørgensen et al. 1996 a). In the Netherlands spontaneous hybridization also occasionally occurs in nature (De Vries et al. 1992). In addition, under open pollination conditions small amounts of viable seeds from the hybrid plants were obtained, indicating that hybrids are able to survive to the next generation (Bing et al. 1991).

Furthermore, it has been shown that inter-specific hybrids can backcross as female with *B. rapa* (Mikkelsen et al. 1996 a,b), even under field conditions (Jørgensen et al. 1996 a). Most of the hybrids between *B. rapa* and

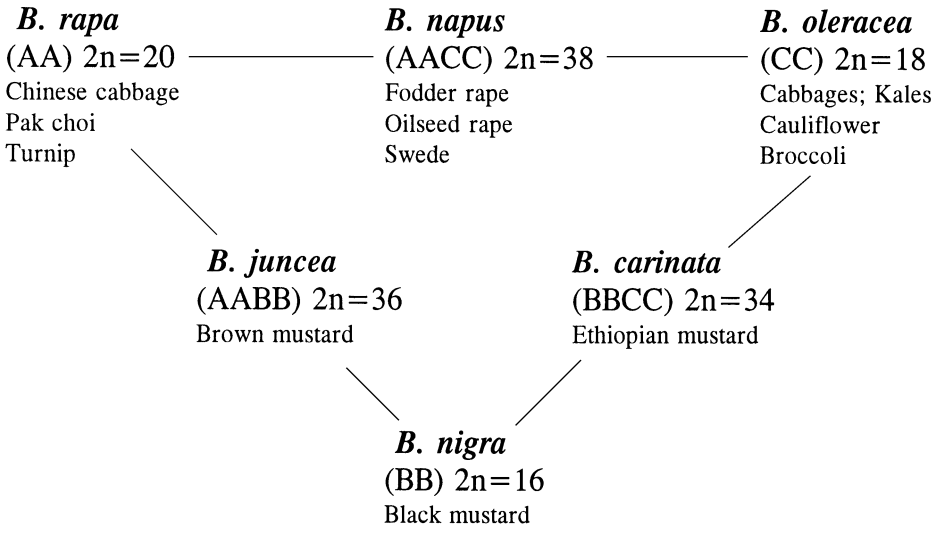

Fig. 1 'U-triangle' representing the genomic relationships among *Brassica* species (redrawn from U 1935)

B. napus possess 29 chromosomes. At metaphase-I of meiosis most pollen mother cells have been shown to contain ten bivalents, presumably between the A-genome chromosomes, and nine univalents, representing the C-genome chromosomes (U 1935; Beversdorf et al. 1980). During the evolution of *Brassica* species, chromosome structure seems to have been sufficiently conserved for the potential occurrence of homoeologous pairing between chromosomes of the A and C genomes. Meiosis in hybrids of *B. napus* and *B. rapa* and in backcross generations provides the opportunity for genomic recombination leading to the introgression of (trans)genes (Quiros et al. 1994; Jørgensen et al. 1996 b; Mikkelsen et al. 1996 a, b). Introgression of traits by breeding has been reported from *B. rapa* into *B. napus* (MacKay 1977, Gowers 1982; Goring et al. 1992). Also, reciprocally, there were reports of the introgression of cold tolerance and black-rot resistance from *B. napus* into Pak choi and Chinese cabbage (Guo et al. 1990; Health et al. 1994).

There is strong evidence that gene flow from *B. napus* and introgression into weedy relatives is inevitable (Timmons et al. 1995, 1996; Mikkelsen et al. 1996 b; Kerlan et al. 1993), consequently, research on transgenic *Brassica* species should now focus on the impact and stability of transgene expression and its fate after inter-specific hybridization (Metz and Nap 1997). In our study, transgenic phosphinothricin-tolerant *B. napus* plants were crossed under controlled conditions with *B. rapa* representatives Pak choi and Chinese cabbage to investigate whether the transgene could be transferred to the inter-specific hybrid and whether it remains active. The expression and fate of the transgene was monitored in successive generations of backcrosses with *B. rapa*.

Materials and methods

Plant material

Transgenic material

Two phosphinothricin (PPT)-tolerant oilseed rape (*B. napus*) transgenic R_1 populations, obtained after selfing two primary transformants of cv Drakkar, were kindly provided by Dr. P. Rüdelsheim (Plant Genetic Systems, Ghent, Belgium). These populations were designated TP2 and TP3, respectively. Both lines were transgenic for a T-DNA insertion containing 3′ocs-*NPTII*-neo and pSsuAra-*bar*-3′*g*7 conferring kanamycin resistance and phosphinothricin – the active ingredient of Basta/Radicale – tolerance, respectively. For both populations it was not known whether the transgene locus was located on chromosomes of A- or of the C-genome (Rüdelsheim personal comminication). The two lines were the result of independent transformation events.

PPT tolerance is conferred by the *bar* gene (De Block et al. 1987; Thompson et al. 1987). This gene encodes an acetyltransferase that inactivates PPT by acetylation of the free NH_2-group. PPT inhibits glutamine synthetase resulting in a rapid accumulation of ammonia leading to cell death (Tachibana et al. 1986).

Non-transgenic material

Non-transgenic oilseed rape cv Drakkar, self-incompatible Pak choi [*B. rapa* Chinensis group (*B. chinensis*), origin China, accession number BC30.02] and self-compatible Chinese cabbage [B. rapa Pekinensis group (*B. pekinensis*), origin China, accession number BC20.08] were used as crossing parents.

Crossing experiments

Twenty individual PPT-tolerant R_1 plants were selfed and two were crossed with non-transgenic *B. napus* to study the genetics of this trait. Crosses of Pak choi and Chinese cabbage were performed with two transgenic, PPT-tolerant, *B. napus* R_1 plants, which were hemizygous for this trait. All plants that were used as female parent were emasculated and the pollinated flowers were

bagged for 3 days prior to pollination to prevent uncontrolled cross-pollination.

The PPT-tolerant inter-specific hybrids were used as male parents and backcrossed with Pak choi or Chinese cabbage, producing BC_1 generation segregation for PPT tolerance. With Pak choi as female and PPT-tolerant backcross plants as male parent, another three rounds of backcrosses were made resulting in BC_2, BC_3 and BC_4 populations segregating for PPT tolerance. A schematic presentation of the crosses is given in Fig. 2.

Crosses were conducted in a pollen cage, placed in the greenhouse, with underpressure to avoid transgenic pollen spread by air flow and a double-door entrance to prevent insects entering the cage, according to regulations ordered by the Dutch Committee of Genetic Modification (COGEM).

Techniques

Phosphinothricin-tolerance test

Plants, at the two- to four-leaf stage were sprayed from an approximately 20-cm distance, using a normal household plant spray, with 0.5% Radicale (150 gl^{-1} PPT), as uniformly as possible. Alternatively, leaf discs of hybrids were tested for the activity of the PPT-tolerance gene non-destructively on MS-10 containing 7.5 mgl^{-1} Radicale and 50 mgl^{-1} chlorophenol red (Metz et al. 1995). This test made it possible to screen the PPT-sensitive plants for the presence or absence of the transgene.

Pollen stainability

Pollen stainability was assessed by staining freshly harvested pollen with Alexander stain (Alexander 1996). Ten samples were collected and 100 pollen grains were counted per sample. Stainability was expressed as the number of red pollen grains per number of pollen grains counted.

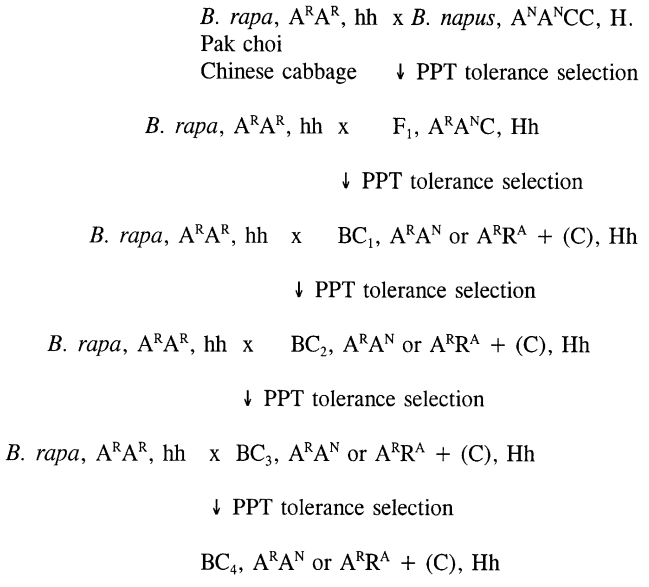

Fig. 2 Crossing scheme. Phosphinothricin (PPT)-tolerant inter-specific hybrid plants were backcrossed to *B. rapa* followed by three more backcrosses of PPT-tolerant plants on *B. rapa*. A^R and A^N represent the A-genome of *B. rapa* and *B. napus*, respectively. (*C*) indicates that only part of the C-genome may be present containing the PPT tolerance. *H*. indicating the presence of PPT tolerance in either a homozygous (*HH*) or a hemizygous (*Ho*) configuration; *hh* indicates the absence of the *bar* gene, implying herbicide sensitivity

Flow cytometry

Preparation of the nuclear samples and flow cytometry were performed as described by Bino et al. (1993). The fluoresence signals are presented as frequency distribution histograms, the DNA amount being expressed as relative C values. The 1C value represents the DNA amount of the unreplicated haploid chromosome complement for oilseed rape AC.

Southern analysis

Southern analysis was conducted according to the method described by Metz et al. (1995). In brief, about 5 µg DNA, extracted from young leaf tissue (Dellaporta et al. 1983), was digested using *Hin*dII and run on a 0.8% agarose-gel overnight. DNA was transferred onto a Hybond N^+ filter (Amersham) by vacuum blotting (Pharmacia Biotech). Probes labelled with ^{32}P were hybridized onto the filter, washed with 2 × SSC (with 1% SDS) and exposed to Kodak X OMAT-AR or Fuji RX films. The hybrid nature of the putative hybrid plants was analysed using *AphA2* (Koncz and Schell 1986) or *bar* (Wilmink 1996) gene fragments as probes.

Statistical analysis

Phenotypic segregation ratios were tested for goodness-of-fit by χ^2 tests.

Results

The original transgenic R_1 PPT-tolerant *B. napus* TP2 and TP3 plants were sprayed with 0.5% Radicale. Selfings of individual tolerant R_1 plants resulted in R_2 populations, and crosses between individual R_1 plants and non-transgenic *B. napus* were made in order to study the genetics of this transgenic trait. Inter-specific hybrid plants, of either Pak choi or Chinese cabbage and PPT-tolerant *B. napus*, and successive backcross populations were analysed for the presence and expression of the *bar* gene conferring PPT tolerance. Furthermore, plants from different generations were analysed using flow cytometry.

Fate of herbicide tolerance

Intra-specific crosses

None of the 54 non-transgenic *B. napus* control plants survived the PPT spray. Fourty six and 29 randomly chosen plants of the primary R_1 transgenic *B. napus* TP2 (92.2) and TP3 (92.3), respectively, were tested for PPT tolerance by spraying with Radicale. These populations displayed a segregation ratio of PPT-tolerant : sensitive plants which did not deviate significantly

Table 1 Number and percentage of the observed and expected phosphinothricin (PPT)-tolerant plants in transgenic R_1 populations (TP) and in intra-specific backcrosses after spraying with 0.5% Radicale (150 gl^{-1} PPT). T and S are PPT-tolerant and -sensitive plants respectively. WT means wild-type *B. napus*

Population (number) cross	Observed			Expected probability	
	Number		Percentage T	$\chi^2_{1:1}$	$\chi^2_{3:1}$
	T	S			
WT	0	54	0		
TP3 (92.3)	22	7	76		0.90–0.95
TP2 (92.2)	35	11	76		0.80–0.90
WT × 92.3.20	23	21	52	0.75–0.90	
WT × 92.2.12	15	18	46	0.60–0.75	

from 3 : 1 (Table 1). Selfed progenies of selected PPT-tolerant R_1 plants yielded, as expected, a 1 : 0 or 3 : 1 segregation (data not shown). This indicated that the PPT-tolerant R_1 plants were either homozygous or hemizygous for a single T-DNA insertion expressing PPT tolerance.

A cross between a non-transgenic 'Drakkar' *B. napus* plant with a hemizygous PPT-tolerant TP2 R_1 plant (92.2.12) yielded 33 plants of which 15 were PPT-tolerant. The same cross using a hemizygous PPT-tolerant TP3 R_1 plant (92.3.20) gave 23 tolerant plants out of 44 plants tested. This is again in agreement with a monogenic (1 : 1) segregation ratio (Table 1).

Inter-specific hybrids

Controlled inter-specific crosses and backcrosses were performed with the transgenic allotetraploid species *B. napus*. The PPT-tolerant inter-specific hybrid or backcross plants were the male, and Pak choi or Chinese cabbage plants were the female, parents. In gene dispersal from the (transgenic) crop to its wild relative, the initial hybridization event is most probably with *B. rapa* as the female parent. Crossing of Pak choi or Chinese cabbage with PPT-tolerant *B. napus* R_1 plants from TP3 (92.3.20) and Pak choi with TP2 (92.2.12), respectively, resulted in viable, fertile hybrids, denoted Pak choi*3, Chinese cabbage*3 and Pak choi*2. They were morphologically intermediate exhibiting traits from both parents. The hybrids had less trichomes on their leaves than *B. rapa*. The glaucous leaves resembled *B. napus* more than *B. rapa*. The inflorescence of the hybrid mirrored the *B. napus* parent and had open flowers rising above the closed flower buds.

The pollen stainability of the inter-specific hybrids was about 60%, while that of the parents was 100%. Backcrosses of the male-fertile hybrids with Pak choi or Chinese cabbage as female parent yielded over 600 seeds. However, selfing of Pak choi*2 and Chinese cabbage*3 hybrids did not yield any seed-bearing siliques. Selfings of Pak choi*3 hybrids were not performed.

Table 2 gives the number and percentage of PPT-tolerant hybrid plants in Pak choi*2 and Chinese cabbage*3. As expected from the selfings and test-cross results, both inter-specific F_1 hybrid progenies segregated 1 : 1 for PPT tolerance : sensitivity. All PPT-tolerant F_1 plants which were tested by Southern blotting with an *AphA2* probe, displayed the presence of the transgene (Fig. 3). All PPT-tolerant Pak choi*2 hybrids showed the two bands characteristic for tolerant plants from TP2. Pak choi*3 and Chinese cabbage*3 hybrids showed the single band of tolerant plants from TP3. The result of the DNA analysis were in agreement with the chlorophenol red test. This indicated that the non-destructive *bar* enzyme activity determination was reliable. All 24 leaf discs of the three PPT-tolerant F_1

Table 2 Number and percentage of the observed and expected phosphinothricin (PPT)-tolerant plants in inter-specific F_1s of transgenic *B. napus* with *B. rapa* (Pak choi, Pc or Chinese cabbage, Cc) and hemizygous PPT-tolerant *B. napus* plants from transgenic R_1 populations TP2 and TP3, and backcrosses (BC_n) onto *B. rapa*, after spraying with 0.5% Radicale (150 gl^{-1} PPT). T and S are PPT-tolerant and -sensitive plants, respectively

Cross	Observed			Expected probability
	Number		Percentage T	$\chi^2_{1:1}$
	T	S		
*Chinese cabbage*TP3*				
Cc × 92.3.20 (F_1)	6	6	50	1.00
Cc × F_1.1 (BC_1.1)	4	5		
Cc × F_1.2 (BC_1.2)	10	11		
Cc × F_1.3 (BC_1.3)	5	5		
Cc × F_1.4 (BC_1.4)	14	17		
BC_1 total	33	38	46	0.50–0.60
*Pak choi*TP3*				
Pc × 92.3.20 (F_1)	1	0	100	
Pc × F_1.1 (BC_1)	4	7	36	0.30–0.40
*Pak choi*TP2*				
Pc × 92.2.12 (F_1)	7	6	54	0.70–0.80
Pc × F_1.1 (BC_1.1)	9	30		
Pc × F_1.2 (BC_1.2)	7	23		
Pc × F_1.3 (BC_1.3)	15	40		
Pc × F_1.4 (BC_1.4)	8	20		
BC_1 total	39	113	26	< 0.0005
Pc × BC_1.1.1 (BC_2.1) Pc × BC_1.3.1 (BC_2.2) Pc × BC_1.3.2 (BC_2.3) Pc × BC_1.4.2 (BC_2.4) BC_2 total	6	111	5	< 0.0005
Pc × BC_2.1.2 (BC_3.1) Pc × BC_2.3.1 (BC_3.2) Pc × BC_2.4.2 (BC_3.3) BC_3 total	33	267	11	< 0.0005
Pc × BC_3.2.1 (BC_4.1)	1	10	9	0.005–0.01

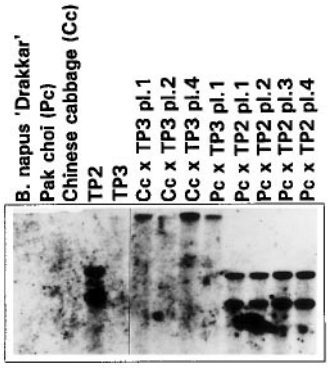

Fig. 3 Southern blot, using an *AphA2* probe, of non-transgenic *B. napus*, Pak choi and Chinese cabbage, plants from two independent transgenic *B. napus* populations (*TP*), and several F_1s of Pak choi or Chinese cabbage with transgenic *B. napus*

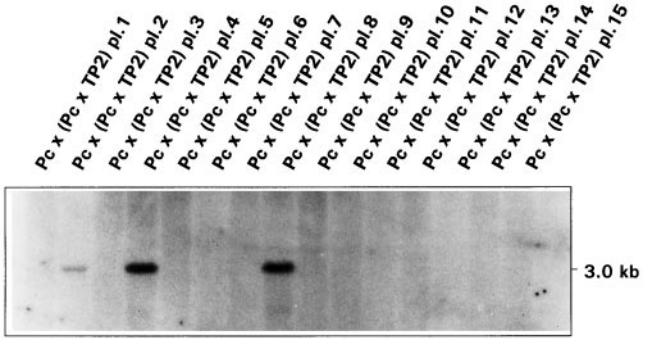

Fig. 4 Southern blot, using a *bar* probe, of 15 randomly chosen Pak choi × (F_1, Pak choi × transgenic *B. napus*) BC_1 plants indicated as Pc × (Pc × TP2)

plants turned the colour of the medium into orange/yellow, showing the presence of the active *bar* gene, while the leaf discs of the PPT-sensitive F_1 plants and the non-transgenic *B. napus* coloured the medium purple.

Backcrosses for indications of the presence of transgenes on chromosomes of the A- or C-genome

PPT tolerance could be transferred to the next generation by backcrossing PPT-tolerant Chinese cabbage*3 hybrids with Chinese cabbage or the PPT-tolerant Pak choi*3 hybrid with Pak choi (Table 2). In the first backcross the segregation ratio observed did not deviate significantly from 1 : 1. This was expected when the PPT tolerance gene was present on one of the chromosomes of the A-genome of plants from TP3. The pollen stainability of Chinese cabbage*3 hybrid plants was about 20%.

By backcrossing PPT-tolerant Pak choi*2 hybrids with Pak choi, PPT tolerance could also be transferred to the next generation. However, only 26% of the progeny expressed PPT tolerance instead of 50%. The PPT tolerance : sensitivity segregation clearly deviated from 1 : 1, having a deficiency in the class of PPT-tolerant plants. This observation is different from the expected segregation if this trait was located on one of the chromosomes of the A-genome. On a Southern blot, using a *bar* probe, three PPT-tolerant Pak choi BC_1 plants out of 15 plants tested showed the TP2 band, which was not found for the 12 sensitive plants (Fig. 4). The pollen stainability of the BC_1 plants was about 40%.

In the successive BC_2 generation, using a PPT-tolerant BC_1 plant as male, only 5% of the plants were PPT-tolerant (Table 2). The male and female (self)fertility of the BC_2 plants was completely restored. The pollen stainability was found to be 100% and, after selfing one of the tolerant BC_2 plants, viable seeds were obtained.

The following BC_3 generation, with a PPT-tolerant BC_2 plant used as male parent, gave only 11% PPT-tolerant plants, while on testing a limited number of plants for the BC_4 only 1 out of 11 plants was found to be PPT-tolerant (Table 2). A random sample of ten PPT-sensitive BC_3 plants was screened for the presence or absence of the PPT-tolerance gene on a Southern blot. The PPT gene was absent in all ten PPT-sensitive plants. As expected, the BC_3 and BC_4 plants that survived a PPT spray resembled the Pak choi parent in morphology.

The number of PPT-tolerant plants to the BC_1 population produced with TP2 differed from those made with TP3. The most plausible explanation for these results is that in TP2 the PPT-tolerance gene is present on one of the chromosomes of the C-genome. This explanation is supported by the low transmission percentages found in the BC_2, BC_3 and BC_4 generations.

Flow cytometric analyses

Flow cytometric analyses of a mixed sample with Pak choi, the Pak choi*2 hybrid and *B. napus* nuclei, showed that the peak of the hybrid was clearly between the peaks of both parents (Fig. 5 A). This confirmed that the DNA content of the Pak choi*2 hybrid was intermediate between the DNA contents of Pak choi and *B. napus*, which are 1.05 pg/2C and 2.45 pg/2C, respectively (Arumuganathan and Earle 1991). This indicated that the inter-specific hybrid had the expected triploid genomic constitution (see also Fig. 2). Compared to this F_1 hybrid, the DNA content of the BC_1 plant showed a shift towards the Pak choi peak (Fig. 5 B). The peaks of the tested BC_2 and BC_3 plants coincided with that of the recurrent Pak choi (Fig. 5 C and D). It was not easy to define the presence of only one or two additional (parts of) C-chromosomes using flow cytometry.

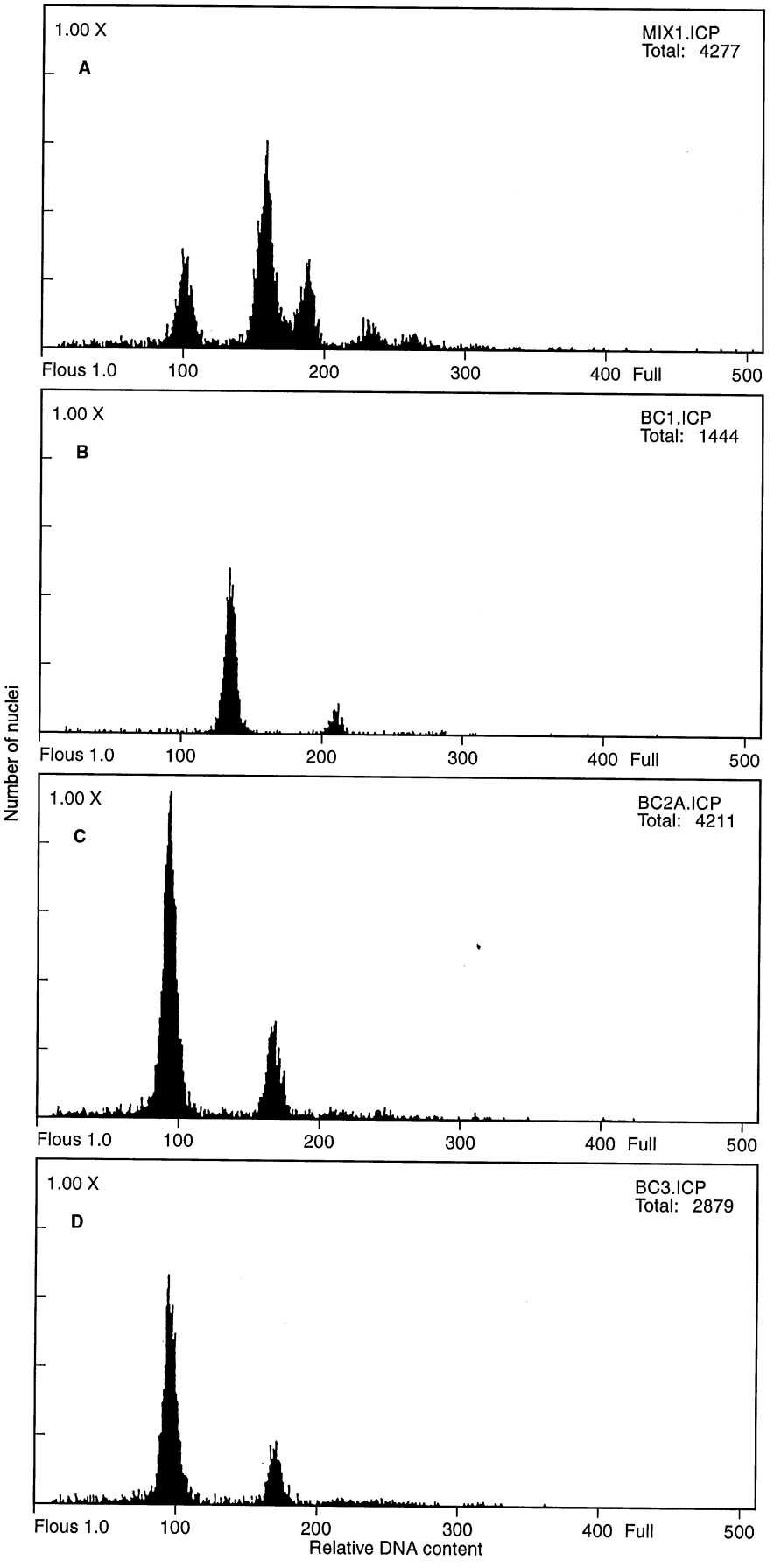

Fig. 5A–D Relative DNA content. Histograms of flow cytometric analyses of nuclei from leaves of a mixed sample of Pak choi, the Pak choi-*B. napus* hybrid and transgenic *B. napus* (**A**), and the subsequent BC_1 (**B**), BC_2 (**C**) and BC_3 (**D**) on Pak choi. Nuclei from leaves show peaks at the 2C and 4C levels. Totals refer to the number of nuclei measured

Discussion

Crosses between transgenic, PPT-tolerant, and non-transgenic *B. napus*, and selfed progenies of PPT-tolerant *B. napus* exhibited a normal Mendelian segregation. In both populations (TP2 and TP3) PPT tolerance was inherited as a stable, single dominant trait. From transmission genetics and the male and female fertility of the transgenic *B. napus*, introduction of the PPT-tolerance gene did not indicate a fitness disadvantage due to the transformation event. Controlled crosses between Pak choi and Chinese cabbage with PPT-tolerant *B. napus* showed that the transgene could be relatively easily transmitted to the inter-specific hybrids and that it was still active in the hybrid. This inter-specific transfer occurred in an expected ratio and was confirmed by several analyses. The flow cytometric analysis indicated that the genomic constitution of the hybrid was probably triploid.

The formation of the *B. rapa* × *B. napus* hybrid in controlled crossing experiments has been reported previously by several researchers (Palmer 1962; Mikkelsen et al. 1996 b; Nwankiti 1971; MacKay 1977). In our study only controlled inter-specific crosses were performed with the allotetraploid species as male parent. In subsequent backcrosses PPT-tolerant (backcrossed) hybrid plants were used as the male parent. The initial hybridization event is most probably with *B. rapa* as female parent. In subsequent generations, however, the hybrid will be more fertile as female rather than as male. It has been found that *B. rapa* × *B. napus* hybrids could be good female parents (Mikkelsen et al. 1996 b). As expected, in agricultural fields and field trials spontaneous hybridization between both species has also been observed (Bing et al. 1991; Jørgensen and Andersen 1994; Jørgensen et al. 1996 a; Mikkelsen et al. 1996 b).

In the literature, hybrids were regularly reported to have good pollen production, but they showed reduced fertility (Beversdorf et al. 1980). Values similar to the pollen stainability of about 60%, found here, were observed in other studies McNaughton 1973; MacKay 1977). Jørgensen and Andersen (1994) reported hybrid pollen stainability that ranged from 16 to 86%, while in a later study (Jørgensen et al. 1996 a) stainability was reduced to 35%. In our hands, the pollen production and fertility of the hybrids were sufficient to obtain viable BC_1 seeds, but not to obtain selfed progenies.

The 'Pak choi and Chinese cabbage BC_1' progeny using TP3 segregated 1 : 1 for PPT tolerance, as expected. In this case the PPT-tolerance gene was probably inserted in the A-genome. Previous studies have reported backcross plants with $2n = 20$ in controlled crosses between (*B. napus* × *B. rapa*) and *B. rapa* (Quiros et al. 1987; McGrath and Quiros 1990), and also (spontaneous) backcrossing under field conditions; in the first backcross generation *B. rapa*-like plants with 20 chromosomes and a high pollen fertility were found (Jørgensen et al. 1996 a; Mikkelsen et al. 1996 a). These data support the hypothesis that a transgene located on the A-genome of *B. napus* can be transferred to *B. rapa* within two backcross generations.

The 'Pak choi BC_1' progeny, using TP2, yielded only 26% PPT-tolerant plants, while 50% was expected as a result of the earlier observed monogenic inheritance. The difference in transmission of PPT-tolerant plants in the BC_1 generations made with TP3 and TP2 must be due to the specific integration position of the transgene. The most plausible explanation for this deficiency in PPT-tolerant plants is the presence of the transgene on one of the chromosomes of the C-genome in TP2. In the backcrosses of the inter-specific hybrid to Pak choi, the C-chromosomes have no homologous partners during meiosis. Due to irregular transmission of the single C-chromosomes to the gametes, a (trans)gene located on the C-genome of *B. napus* is expected to be transmitted at a low frequency in the gametes and to be lost after one or a few generations. In studies with RFLP and isozyme markers, transmission of the C-chromosomes from inter-specific hybrids to BC_1 and F_2 populations was often found to be lower than 50% and varied between individual C-chromosomes (McGrath and Quiros 1990; Chen et al. 1990). Deviations from the expected co-segregation of markers belonging to the same linkage group indicated the possible occurrence of inter-genomic recombination of the breakage of chromosomes (Mikkelsen et al. 1996 a). Transmission of 3 out of 33 *B. napus*-specific RAPD markers from the inter-specific hybrid to the backcross progeny was significantly different from 50%. Also in successive backcross generations with Pak choi, under selective conditions, much lower percentages of PPT-tolerant plants were found than normally expected for monogenic transmission. These results confirm that the transgene must be present on one of the chromosomes of the C-genome. Our results indicate that the transmission of a C-genome-linked transgene is stabilized at about 10%. The underlying mechanisms explaining these results might be inter-genomic recombination between A- and C-genomes, chromosome substitutions, or disomic chromosome additions (Jacobsen et al. 1994; Jørgensen et al. 1996 a). In *Brassica* species, both intra- and inter-genomic recombination have been described (Armstrong and Keller 1982; Attia et al. 1987) and inter-genomic recombination between A- and C-chromosomes has also been reported (Quiros et al. 1987; Chen et al. 1990). Partial homology between the A-, B- and C-genome has been revealed by studies of inter-specific hybrids and marker analysis (U 1935; Hosaka et al. 1990; Kerlan et al. 1993; Frello et al. 1995), which incidently may trigger cross-overs between the chromosomes of these genomes. Additional studies using chromosome-specific (RAPD) markers, which are available for both the A- and C-genomes (Quiros et al. 1991, 1994; Jørgensen et al. 1996 b;

Mikkelsen et al. 1996 a), and molecular cytogenetics using genomic in situ hybridization (GISH), might help to monitor the presence of the C-chromosome carrying the PPT transgene and possibly demonstrate interchanges between the A- and C-genomes.

By studying the possible gene flow from transgenic *B. napus* to its weedy relative *B. rapa*, it was shown that inter-specific hybridization and backcrossing of these hybrids with *B. rapa* occurred spontaneously under field conditions (Jørgensen and Andersen 1994; Jørgensen et al. 1996 a; Mikkelsen et al. 1996 b). Also the results we obtained suggest that gene flow from (transgenic) *B. napus* to *B. rapa* is inevitable. However, the data of our study support the hypothesis of Mikkelsen et al. (1996 a) of possible 'safe' integration sites, chromosome regions with a low probability of transfer to backcross generations with *B. rapa* via homo(eo)logous recombination. Specifically, the presence on chromosomes of either the A-genome or C-genome determined the transmission frequency of the PPT-tolerance gene in subsequent backcross generations. Concerning the aspect of gene dispersal in the specific case of transgenic *B. napus* to *B. rapa*, we suggest that the transgene is selected for its presence on chromosomes of the C-genome. This study showed that this might limit the transfer to *B. rapa*. Integration of the transgene on chromosomes of the C-genome would also reduce the probability of gene transfer to *B. juncea*. It might, however, increase the chances of exchange to some other related species containing (parts of) the C-genome, such as *B. oleracea* and *B. carinata*.

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References

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. Stain Technol 44 : 117–122

Armstrong KC, Keller WA (1982) Chromosome pairing in haploids of *Brassica oleracea*. Can J Genet Cytol 24 : 735–739

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9 : 229–241

Attia T, Busso C, Röbbelen G (1987) Di-genomic triploids for an assessment of chromosome relationships in the cultivated diploid *Brassica* species. Genome 29 : 326–30

Beversdorf WD, Weiss-Lerman J, Erickson LR, Souza Machado V (1980) Transfer of cytoplasmically inherited triazine resistance from bird's rape to cultivated oilseed rape (*Brassica campestris* and *B. napus*) . Can J Genet Cytol 22 : 167–172

Bing DJ, Downey RK, Rakow GFW (1991) Potential of gene tranfer among oilseed *Brassica* and their weedy relatives. In: Proc 8th Int Rapeseed Congress, Saskatoon, Canada, pp 1022–1027

Bino RJ, Lanteri S, Verhoeven HA, Kraak HL (1993) Flow cytometric determination of nuclear replication stages in seed tissues. Annals Bot 72 : 181–187

Chen BY, Heneen WK, Simonsen V (1990) Genetics of isozyme loci in *Brassica campestris* L. and in the progeny of a tri-genomeic hybrid between *B. napus* L. and *B. campestris* L. Genome 33 : 433–440

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Rao Movva N, Thompson C, van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a de-toxifying enzyme. EMBO J 6 : 2513–2518

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1 : 19–21

De Vries FT, Van der Meijden R, Brandenburg WA (1992) Botanical files: a study of the real chances for spontaneous gene flow from cultivated plants to the wild flora of the Netherlands. Gorteria Suppl 1 in *Brassica* amphiploids. Theor Appl Genet 65 : 201–206

Erickson LR, Straus NA, Beversdorf (1983) Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphiploids. Theor Appl Genet 65 : 201–206

Frello S, Hansen KR, Jensen J, Jørgensen RB (1995) Inheritance of rapeseed (*Brassica napus*)- specific RAPD markers and a transgene in the cross. *B. juncea* × (*B. juncea* × *B. napus*). Theor Appl Genet 91 : 236–241

Goring DR, Banks P, Beversdorf WC, Rothstein SJ (1992) Use of the polymerase chain reaction to isolate an S-locus glycoprotein cDNA introgressed from *Brassica campestris* into *B. napus* ssp. *oleifera*. Mol Gen Genet 234 : 185–192

Gowers S (1982) The transfer of characters from *Brassica campestris* L. to *Brassica napus* L.: production of clubroot-resistant oilseed rape (*B. napus* ssp. *oleifera*). Euphytica 31 : 971–976

Guo ZH, Dickson MH, Hunter JE (1990) *Brassica napus* sources of resistance to black rot in crucifers and inheritance of resistance. In: Proc 6th Crucifer Genetics Workshop, Ithaca New York, USA, pp 154–155

Heath DW, Earle ED, Dickson MH (1994) Introgressing cold-tolerant Ogura cytoplasms from rapeseed into Pak choi and Chinese cabbage. HortSci 29 : 202–203

Hosaka K, Kianian SF, McGrath JM, Quiros CF (1990) Development and chromosomal localization of genome-specific DNA markers of *Brassica* and the evolution of amphidiploids and n = 9 diploid species. Genome 33 : 131–142

Jacobsen E, Daniel MK, Bergervoet-van Deelen JEM, Huigen DJ, Ramanna MS (1994) The first and second backcross progeny of the intergeneric fusion hybrids of potato and tomato after crossing with potato. Theor Appl Genet 88 : 181–186

Jørgensen RB, Andersen B (1994) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (*Brassicaceae*): a risk of growing genetically modified oilseed rape. Am J Bot 81 : 1620–1626

Jørgensen RB, Andersen B, Landbo L, Mikkelsen TR (1996 a) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy relatives. Acta Hort 407 : 193–200

Jørgensen RB, Chen BY, Cheng BF, Heneen WK, Simonsen V (1996 b) Random amplified polymorphic DNA markers of the *Brassica alboglabra* chromosome of a *B. campestris-alboglabra* addition line. Chromosome Res 4 : 111–114

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

Koncz C, Schell J (1986) The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204 : 383–396

MacKay GR (1997): The introgression of S alleles into forage rape, *Brassica napus* L. from turnip, *Brassica campestris* L. ssp. *rapifera*. Euphytica 26 : 511–519

McGrath JM, Quiros CF (1990) Generation of alien chromosome addition lines from synthetic *Brassica napus*: morphology, cytology, fertility and chromosome transmission. Genome 33 : 374–382

McNaughton IH (1973) *Brassica napocampestris* L. (2n = 58). 1. Synthesis, cytology, fertility and general condiserations. Euphytica 22 : 301–309

Metz PLJ, Nap JP (1997) A transgene-centered approach to the biosafety of transgenic plants: overview of selection and reporter genes. Acta Bot Neerl 46 : 25–50

Metz PLJ, Nap JP, Stiekema WJ (1995) Hybridization of radish (*Raphanus sativus* L.) and oilseed rape (*Brassica napus* L.) through a flower-culture method. Euphytica 83 : 159–168

Metz PLJ, Jacobsen E, Stiekema WJ (1997) Aspects of the biosafety of transgenic oilseed rape (*Brassica napus* L.). Acta Bot Neerl 46 : 51–67

Mikkelsen TR, Jensen J, Jørgensen RB (1996 a) Inheritance of oilseed rape (*Brassica napus*) RAPD markers in a backcross progeny with *Brassica campestris*. Theor Appl Genet 92 : 492–497

Mikkelsen TR, Andersen B, Jørgensen RB (1996 b) The risks of crop transgene spread. Nature 380 : 31

Nwankiti O (1971) Cytogenetic and breeding studies with *Brassica*. II. Progenies from backcrosses involving primary hybrids between *B. napus* and *B. campestris*. Hereditas 68 : 35–46

Palmer TP (1962) Population structure, breeding system, inter-specific hybridization and allopolyploidy. Heredity 17 : 278–283

Quiros CF, Ochoa O, Kianian SF, Douches D (1987) Analysis of the *Brassica oleracea* genome by the generation of *B. campestris-oleracea* chromosome addition lines: characterization by isozymes and rDNA genes. Theor Appl Genet 74 : 758–766

Quiros CF, Hu J, This P, Chevre AM, Delseny M (1991) Development and chromosomal localization of genome-specific markers by the polymerase chain reaction in *Brassica*. Theor Appl Genet 82 : 627–632

Quiros CF, Hu J, Truco MJ (1994) DNA-based marker maps of *Brassica*. In: Phillips RL, Vasil IK (eds) DNA-based markers in plants. Kluwer Academic Publishers, The Netherlands, pp 199–222

Tachibana K, Watanabe T, Sekizuwa Y, Takematsu T (1986) Accumulation of ammonia in plants treated with bialaphos. J Pesticide Sci 11 : 33–37

Timmons AM, O'Brien ET, Charters YM, Dubbels SJ, Wilkinson MJ (1995) Assessing the risks of wind pollination from fields of genetically modified *Brassica napus* ssp. *oleifera*. Euphytica 85 : 417–423

Timmons AM, Charters YM, Crawford JW, Burn D, Scott SE, Dubbels SJ, Wilson NJ, Robertson A, O'Brien ET, Squire GR, Wilkinson MJ (1996) Risks from transgenic crops. Nature 380 : 487

Thompson C, Movva N, Tizard R, Crameri R, Davies J, Lauwereys M, Botterman J (1987) Characterization of the herbicide resistance gene '*bar*' from *Streptomyces hygroscopius*. EMBO J 6 : 2519–2523

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and the peculiar mode of fertilization. Jpn J Bot 7 : 389–452

Verma SC, Rees H (1974) Nuclear DNA and the evolution of allotetraploid *Brassicae*. Heredity 33 : 61–68.

Wilmink A (1996) Genetic modification of tulip by means of particle bombardment. PhD thesis, University Nijmegen, The Netherlands