Environ. Biosafety Res. 3 (2004) 45–54

DOI: 10.1051/ebr:2004001

Inheritance of GFP-Bt transgenes from *Brassica napus* in backcrosses with three wild *B. rapa* accessions

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Transgenes from transgenic oilseed rape, *Brassica napus* (AACC genome), can introgress into populations of wild *B. rapa* (AA genome), but little is known about the long-term persistence of transgenes from different transformation events. For example, transgenes that are located on the crop's C chromosomes may be lost during the process of introgression. We investigated the genetic behavior of transgenes in backcross generations of wild *B. rapa* after nine GFP (green fluorescent protein)-Bt (*Bacillus thuringiensis*) *B. napus* lines, named GT lines, were hybridized with three wild *B. rapa* accessions, respectively. Each backcross generation involved crosses between hemizygous GT plants and non-GT *B. rapa* pollen recipients. In some cases, sample sizes were too small to allow the detection of major deviations from Mendelian segregation ratios, but the segregation of GT:non-GT was consistent with an expected ratio of 1:1 in all crosses in the BC_1 generation. Starting with the BC_2 generation, significantly different genetic behavior of the transgenes was observed among the nine GT *B. napus* lines. In some lines, the segregation of GT:non-GT showed a ratio of 1:1 in the BC_2, BC_3, and BC_4 generations. However, in other GT *B. napus* lines the segregation ratio of GT:non-GT significantly deviated from 1:1 in the BC_2 and BC_3 generations, which had fewer transgenic progeny than expected, but not in the BC_4 generation. Most importantly, in two GT *B. napus* lines the segregation of GT:non-GT did not fit into a ratio of 1:1 in the BC_2, BC_3 or BC_4 generations due to a deficiency of transgenic progeny. For these lines, a strong reduction of transgene introgression was observed in all three *B. rapa* accessions. These findings imply that the genomic location of transgenes in *B. napus* may affect the long-term persistence of transgenes in *B. rapa* after hybridization has occurred.

Keywords: transgene / oilseed rape / *B. campestris* / interspecific hybridization / backcross / introgression / green fluorescent protein (GFP) / *Bacillus thuringiensis* (Bt)

INTRODUCTION

Regarding the commercial release of transgenic crops, a major ecological concern is the likelihood of transgene flow from crop plants to their wild relatives, because the subsequent introgression of a transgene may lead to the persistence of the transgene in wild plant populations (Hoffman, 1990; Jørgensen et al., 1996b; Mikkelsen et al., 1996a; Raybould and Gray, 1993; Snow, 2002). The introgression of a transgene, like any other gene, is a multi-generation process (Mikkelsen et al., 1996a); its success may depend upon initial sexual compatibility, interchromosomal recombination, integration and genetic stability in successive generations of wild plants (Jørgensen et al., 1996b; Raybould and Gray, 1993; Scheffler and Dale, 1994). One of the key parameters used in assessing the introgression of a transgene is to investigate whether the transgene follows a normal Mendelian genetic model in subsequent generations of wild plants (Metz et al., 1997; Mikkelsen et al., 1996a).

Transgenic oilseed rape (*Brassica napus*) is a common transgenic crop grown in North America and it

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has been genetically modified to tolerate certain herbicides (*e.g*. glufosinate, glyphosate) (Warwick et al., 2003). Wild *B. rapa*, a closely related species to oilseed rape, is an economically important weed in cultivated fields in North America and Europe (Holm et al., 1997; Metz et al., 1997). This has raised the concern of transgene transfer from transgenic *B. napus* to wild *B. rapa*, because spontaneous occurrence of interspecific hybridization and successive backcrosses with wild *B. rapa* was reported under field conditions (Jørgensen and Andersen, 1994; Jørgensen et al., 1996a; Mikkelsen et al., 1996a; Warwick et al., 2003).

Cytogenetic studies have suggested that *B. napus* is an amphidiploid species ($2n = 38$, AACC), formed from interspecific hybridization between the two primary diploid species, *B. rapa* ($2n = 20$, AA) and *B. oleracea* ($2n = 18$, CC) (U, 1935). This close genetic relationship between *B. rapa* and *B. napus* has led to speculation that the genomic location of a transgene in the *B. napus* genome, specifically on A or C chromosomes, may influence the introgression of the transgene from *B. napus* into *B. rapa*. Metz et al. (1997) compared the transfer rate of a phosphinothricin tolerance transgene to *B. rapa* between two transgenic *B. napus* lines. The significant difference in the transmission frequency of the transgene in subsequent backcross generations between the two transgenic lines suggested that the genomic location of the transgene in *B. napus* might play a role in transgene introgression into *B. rapa*. This was supported by the findings of Lu et al. (2002), who reported that the transmission of a transgene located on a C chromosome into backcross generations was much lower than that on an A chromosome. In contrast, Mikkelsen et al. (1996b) observed that random amplified polymorphic DNA (RAPD) markers were transferred from F_1 hybrids to BC_1 progenies at a frequency of ~ 50% for most markers after interspecific crosses between *B. napus* and *B. rapa*. This result did not support the hypothesis of safe integration sites in the *B. napus* genome. However, the genetic behavior of the RAPD markers was investigated only in F_1 and BC_1 generations in that study. The theoretical analysis by Tomiuk et al. (2000) showed there were no safe locations in the *B. napus* genome with respect to the introgression of a transgene into wild *B. rapa*, because the transmission rate of all *B. napus* chromosomes to the backcross generations of *B. rapa* was similar regardless of A or C chromosomes. Thus, the role of the genomic location of a transgene in *B. napus* in the transgene introgression into *B. rapa* remains unclear.

Most often, the genetic behavior of a transgene in interspecific hybrids and successive backcrosses was investigated in only one or two transgenic *B. napus* lines (Metz et al., 1997; Mikkelsen et al., 1996a). This has impeded a comparison of a large number of independent transformation events of *B. napus* with their role in the transgene introgression into *B. rapa*. Therefore, we have studied the inheritance of transgenes derived from nine independent transgenic *B. napus* lines in successive backcross generations of wild *B. rapa* (*e.g*. BC_3, BC_4). By crossing nine GFP-Bt *B. napus* lines with three wild *B. rapa* accessions respectively, we investigated the genetic behavior of the GFP-Bt transgenes in the interspecific hybrids (F_1) and successive backcross (BC_1-BC_4) populations in *B. rapa*, and assessed the introgression pattern of the transgenes among the nine GFP-Bt *B. napus* lines.

RESULTS

Intraspecific inheritance of GFP-Bt transgenes in *B. napus*

The segregation ratios of GT:non-GT did not deviate significantly from the expected ratio of 1:1 in any of the BC_1 populations derived from the crosses between the nine GT *B. napus* lines and Westar (data not shown). These intraspecific crosses confirmed that the GFP-Bt transgenes in the nine GT *B. napus* lines followed a single dominant gene model.

Segregation of GT in interspecific F_1 and BC_1 generations

We manually crossed each of the three wild *B. rapa* accessions with each of the nine GT *B. napus* lines, resulting in 27 cross combinations. All cross combinations produced viable and fertile F_1 hybrids. A total of 1343 putative F_1 plants were obtained, and the frequency of GT F_1 plants was 99.26%, with 10 plants (0.74%) showing non-GT (confirmed by PCR). The pollen viability of F_1 plants was about 50%. Subsequently, GT F_1 hybrids from each of the cross combinations were crossed with the corresponding wild *B. rapa* accessions to develop BC_1 populations. The result showed that the GFP-Bt transgenes were successfully transferred into the BC_1 generations in all cross combinations. Although in some cases, sample sizes were too small to allow the detection of major deviations from Mendelian segregation ratios, the segregation of GT:non-GT in the BC_1 generations was consistent with an expected ratio of 1:1 in all crosses (Tab. 1).

Table 1. Number of observed GT and non-GT plants in BC_1 progenies developed from the interspecific crosses between three *B. rapa* accessions (2974, 2975 and CA) and nine GFP-Bt *B. napus* lines. Chi-square tests were used to determine whether observed frequencies deviated significantly from expected ratios of 1:1 (NS, not significant, $P > 0.05$).

Cross	Observed in 2974			Observed in 2975			Observed in CA		
	GT	Non-GT	*P*	GT	Non-GT	*P*	GT	Non-GT	*P*
GT1	17	15	NS	17	10	NS	12	12	NS
GT2	36	32	NS	22	20	NS	9	8	NS
GT3	19	14	NS	16	22	NS	12	20	NS
GT4	15	17	NS	20	15	NS	14	17	NS
GT5	18	17	NS	13	16	NS	19	17	NS
GT6	18	21	NS	12	14	NS	12	15	NS
GT7	24	21	NS	24	21	NS	18	16	NS
GT8	17	12	NS	20	13	NS	12	19	NS
GT9	13	20	NS	22	24	NS	12	20	NS

Segregation of GT in BC_2, BC_3 and BC_4 generations

By backcrossing GT BC_1 plants with the corresponding wild *B. rapa* accessions, successive backcross (BC_2, BC_3 and BC_4) progenies were developed to investigate the genetic behavior of the GFP-Bt transgenes. Despite the successful transfer of the GFP-Bt transgenes in successive backcross generations of all three wild *B. rapa* accessions, the frequency of GT hybrids was quite different among the nine GT *B. napus* lines.

In the accession of 2974

The segregation of GT:non-GT of all families in BC_2, BC_3 and BC_4 generations fit into a ratio of 1:1 in GT1, GT2, GT4, GT5 and GT6, and it deviated from 1:1 in GT3, GT7 and GT8 in 2974 (Tab. 2). GT9 was not tested in 2974.

In the accession of 2975

The segregation of GT:non-GT in the BC_2 families fit into a ratio of 1:1 in GT1, GT2, GT3, GT4, GT5, and GT6, but it deviated significantly from 1:1 in GT7, GT8 and GT9 (Tab. 3). In all BC_3 and BC_4 families, the segregation ratio of GT:non-GT remained 1:1 in GT1, GT2, GT3, GT4, GT5, and GT6, while the segregation ratio still deviated from 1:1 in GT7 (Tab. 3). However, although the segregation ratio in family BC_4.1 of GT8 deviated from 1:1, the segregation in family BC_4.2 of GT8 and family BC_4.1 of GT9 started to show a ratio of 1:1 (Tab. 3).

In the accession of CA

The segregation of GT:non-GT in the BC_2 and BC_3 families in GT1, GT2, GT3, GT4, and GT6 showed a ratio of 1:1, whereas the segregation in GT5, GT7, GT8, and GT9 deviated from 1:1 (Tab. 4). In the BC_4 generation, the segregation ratio of GT:non-GT in all BC_4 families in GT1, GT2 and GT3 fit into 1:1, whereas it deviated significantly from 1:1 in GT7, GT8 and GT9 (Tab. 4). BC_4 progenies of GT4, GT5 and GT6 in CA were not tested.

Pattern of transgene genetic behavior in three wild *B. rapa* accessions

Starting with the BC_2 generation, the significant difference in GT:non-GT segregation ratios among the nine GT *B. napus* lines indicated that the introgression of the GFP-Bt transgenes from the nine GT *B. napus* lines followed a different pattern. The genetic behavior of the GFP-Bt transgenes could be grouped into three types. The first type was where the segregation of GT:non-GT showed a ratio of 1:1 in all BC_2, BC_3, and BC_4 generations. This included all families in GT1, GT2, GT4, GT5, and GT6 in 2974, GT1, GT2, GT3, GT4, GT5 and GT6 in 2975, and GT1, GT2, and GT3 in CA (Tab. 5). In the second type, the segregation did not fit into a ratio of 1:1 in any of BC_2, BC_3 or BC_4 generation. This group included all families of BC_2, BC_3 and BC_4 of GT3, GT7 and GT8 in 2974, family BC_4.1 of GT7 and family BC_4.1 of GT8 in 2975, and all families of BC_2, BC_3 and BC_4 of GT7, GT8, and GT9 in CA (Tab. 5). The third type was that the segregation of GT:non-GT

Table 2. Number of observed GT and non-GT plants in BC_2, BC_3, and BC_4 families of the interspecific crosses between the accession of 2974 and GFP-Bt *B. napus* lines. Chi-square tests were used to determine whether observed frequencies deviated significantly from expected ratios of 1:1 (NS, not significant, $P > 0.05$; * indicates significant, $P < 0.01$).

Cross		Observed				Observed				Observed		
	BC_2 families	GT	Non-GT	*P*	BC_3 families	GT	Non-GT	*P*	BC_4 families	GT	Non-GT	*P*
GT1	BC_2.1(2974×BC_1.1)	38	40	NS	BC_3.1(2974×BC_2.1.1)	38	34	NS	BC_4.1(2974×BC_3.1.1)	39	35	NS
					BC_3.2(2974×BC_2.1.2)	37	33	NS				
GT2	BC_2.1(2974×BC_1.1)	41	43	NS	BC_3.1(2974×BC_2.1.1)	29	33	NS	BC_4.1(2974×BC_3.1.1)	38	36	NS
	BC_2.2(2974×BC_1.2)	37	38	NS	BC_3.2(2974×BC_2.2.1)	38	33	NS				
GT3	BC_2.1(2974×BC_1.1)	10	62	*	BC_3.1(2974×BC_2.1.1)	1	60	*	BC_4.1(2974×BC_3.1.1)	11	56	*
	BC_2.2(2974×BC_1.2)	18	56	*	BC_3.2(2974×BC_2.2.1)	2	67	*				
GT4	BC_2.1(2974×BC_1.1)	36	36	NS	BC_3.1(2974×BC_2.1.1)	35	36	NS	BC_4.1(2974×BC_3.1.1)	30	43	NS
	BC_2.2(2974×BC_1.2)	32	39	NS	BC_3.2(2974×BC_2.2.1)	33	39	NS				
GT5	BC_2.1(2974×BC_1.1)	30	36	NS	BC_3.1(2974×BC_2.1.1)	33	39	NS	BC_4.1(2974×BC_3.1.1)	30	40	NS
	BC_2.2(2974×BC_1.2)	37	41	NS	BC_3.2(2974×BC_2.2.1)	29	34	NS	BC_4.2(2974×BC_3.2.1)	35	34	NS
GT6	BC_2.1(2974×BC_1.1)	35	39	NS	BC_3.1(2974×BC_2.1.1)	32	30	NS	BC_4.1(2974×BC_3.1.1)	44	32	NS
GT7	BC_2.1(2974×BC_1.1)	2	12	*	BC_3.1(2974×BC_2.1.1)	6	61	*	BC_4.1(2974×BC_3.1.1)	0	59	*
GT8	BC_2.1(2974×BC_1.1)	16	59	*	BC_3.1(2974×BC_2.1.1)	12	52	*	BC_4.1(2975×BC_3.1.1)	0	57	*
	BC_2.2(2974×BC_1.2)	13	64	*								

Table 3. Number of observed GT and non-GT plants in BC_2, BC_3, and BC_4 families of the interspecific crosses between the accession of 2975 and GFP-Bt *B. napus* lines. Chi-square tests were used to determine whether observed frequencies deviated significantly from expected ratios of 1:1 (NS, not significant, $P > 0.05$; * indicates significant, $P < 0.01$).

Cross		Observed				Observed				Observed		
	BC_2 families	GT	Non-GT	*P*	BC_3 families	GT	Non-GT	*P*	BC_4 families	GT	Non-GT	*P*
GT1	BC_2.1(2975×BC_1.1)	34	47	NS	BC_3.1(2975×BC_2.1.1)	33	30	NS	BC_4.1(2975×BC_3.1.1)	40	34	NS
	BC_2.2(2975×BC_1.2)	32	49	NS	BC_3.2(2975×BC_2.2.1)	39	37	NS	BC_4.2(2975×BC_3.2.1)	31	42	NS
	BC_2.3(2975×BC_1.3)	37	38	NS								
GT2	BC_2.1(2975×BC_1.1)	38	47	NS	BC_3.1(2975×BC_2.1.1)	31	42	NS	BC_4.1(2975×BC_3.1.1)	43	33	NS
	BC_2.2(2975×BC_1.2)	31	43	NS	BC_3.2(2975×BC_2.2.1)	17	19	NS	BC_4.2(2975×BC_3.2.1)	39	36	NS
	BC_2.3(2975×BC_1.3)	45	56	NS								
	BC_2.4(2975×BC_1.4)	41	43	NS								
GT3	BC_2.1(2975×BC_1.1)	36	50	NS	BC_3.1(2975×BC_2.1.1)	35	35	NS	BC_4.1(2975×BC_3.1.1)	41	33	NS
	BC_2.2(2975×BC_1.2)	32	40	NS	BC_3.2(2975×BC_2.2.1)	19	13	NS				
GT4	BC_2.1(2975×BC_1.1)	15	16	NS	BC_3.1(2975×BC_2.1.1)	33	36	NS	BC_4.1(2975×BC_3.1.1)	40	30	NS
					BC_3.2(2975×BC_2.1.2)	36	38	NS	BC_4.2(2975×BC_3.2.1)	39	35	NS
GT5	BC_2.1(2975×BC_1.1)	31	38	NS	BC_3.1(2975×BC_2.1.1)	33	41	NS	BC_4.1(2975×BC_3.1.1)	38	29	NS
	BC_2.2(2975×BC_1.2)	17	18	NS	BC_3.2(2975×BC_2.2.1)	35	35	NS				
GT6	BC_2.1(2975×BC_1.1)	15	14	NS	BC_3.1(2975×BC_2.1.1)	33	44	NS	BC_4.1(2975×BC_3.1.1)	26	41	NS
					BC_3.2(2975×BC_2.1.2)	38	35	NS	BC_4.2(2975×BC_3.2.1)	31	42	NS
GT7	BC_2.1(2975×BC_1.1)	5	70	*	BC_3.1(2975×BC_2.1.1)	11	68	*	BC_4.1(2975×BC_3.1.1)	4	65	*
	BC_2.2(2975×BC_1.2)	17	53	*								
	BC_2.3(2975×BC_1.3)	16	58	*								
GT8	BC_2.1(2975×BC_1.1)	2	70	*	BC_3.1(2975×BC_2.1.1)	10	58	*	BC_4.1(2975×BC_3.1.1)	3	69	*
	BC_2.2(2975×BC_1.2)	3	19	*	BC_3.2(2975×BC_2.2.1)	7	53	*	BC_4.2(2975×BC_3.2.1)	30	42	NS
GT9	BC_2.1(2975×BC_1.1)	23	55	*	BC_3.1(2975×BC_2.1.1)	8	53	*	BC_4.1(2975×BC_3.1.1)	39	30	NS
	BC_2.2(2975×BC_1.2)	20	65	*								

Table 4. Number of observed GT and non-GT plants in BC_2, BC_3, and BC_4 families of the interspecific crosses between the accession of CA and GFP-Bt *B. napus* lines. Chi-square tests were used to determine whether observed frequencies deviated significantly from expected ratios of 1:1 (NS, not significant, $P > 0.05$; * indicates significant, $P < 0.01$).

Cross		Observed				Observed				Observed		
	BC_2 families	GT	Non-GT	*P*	BC_3 families	GT	Non-GT	*P*	BC_4 families	GT	Non-GT	*P*
GT1	$BC_2.1(CA\times BC_1.1)$	36	26	NS	$BC_3.1(CA\times BC_2.1.1)$	31	41	NS	$BC_4.1(CA\times BC_3.1.1)$	31	44	NS
					$BC_3.2(CA\times BC_2.1.2)$	28	44	NS	$BC_4.2(CA\times BC_3.2.1)$	39	43	NS
GT2	$BC_2.1(CA\times BC_1.1)$	43	35	NS	$BC_3.1(CA\times BC_2.1.1)$	29	38	NS	$BC_4.1(CA\times BC_3.1.1)$	25	22	NS
	$BC_2.2(CA\times BC_1.2)$	46	39	NS	$BC_3.2(CA\times BC_2.2.1)$	35	43	NS	$BC_4.2(CA\times BC_3.2.1)$	39	31	NS
	$BC_2.3(CA\times BC_1.3)$	33	35	NS								
GT3	$BC_2.1(CA\times BC_1.1)$	35	42	NS	$BC_3.1(CA\times BC_2.1.1)$	26	39	NS	$BC_4.1(CA\times BC_3.1.1)$	30	36	NS
	$BC_2.2(CA\times BC_1.2)$	32	40	NS	$BC_3.2(CA\times BC_2.2.1)$	44	41	NS	$BC_4.2(CA\times BC_3.2.1)$	25	23	NS
	$BC_2.3(CA\times BC_1.3)$	45	60	NS								
GT4	$BC_2.1(CA\times BC_1.1)$	21	34	NS	$BC_3.1(CA\times BC_1.1.1)$	37	34	NS	Not tested			
GT5	$BC_2.1(CA\times BC_1.1)$	8	42	*	$BC_3.1(CA\times BC_1.1.1)$	13	63	*	Not tested			
GT6	$BC_2.1(CA\times BC_1.1)$	30	42	NS	$BC_3.2(CA\times BC_1.1.1)$	43	55	NS	Not tested			
GT7	$BC_2.1(CA\times BC_1.1)$	27	80	*	$BC_3.1(CA\times BC_2.1.1)$	15	48	*	$BC_4.1(CA\times BC_3.1.1)$	2	59	*
					$BC_3.2(CA\times BC_2.1.2)$	4	65	*	$BC_4.2(CA\times BC_3.2.1)$	2	63	*
GT8	$BC_2.1(CA\times BC_1.1)$	32	67	*	$BC_3.1(CA\times BC_2.1.1)$	8	58	*	$BC_4.1(CA\times BC_3.1.1)$	0	66	*
	$BC_2.2(CA\times BC_1.2)$	24	64	*								
GT9	$BC_2.1(CA\times BC_1.1)$	19	39	*	$BC_3.1(CA\times BC_2.1.1)$	10	59	*	$BC_4.1(CA\times BC_3.1.1)$	7	53	*
					$BC_3.2(CA\times BC_2.1.2)$	18	52	*	$BC_4.2(CA\times BC_3.2.1)$	1	46	*

significantly deviated from a ratio of 1:1 in BC_2 and BC_3 generations, but in the BC_4 generation, it showed a ratio of 1:1. Family $BC_4.2$ of GT8 and family $BC_4.1$ of GT9 in 2975 belonged to this type (Tabs. 3 and 5).

Verification of presence of GFP-Bt transgenes with PCR

A total of 931 BC_1 plants developed from the original 27 interspecific cross combinations were screened for GFP expression, and 463 GFP-expressing BC_1 plants were identified. These plants were further tested for Bt toxin expression. No conflicts between GFP visualization and Bt toxin immunological test were observed, indicating the reliability of using GFP for tagging the Bt gene. We also verified the presence or absence of the GFP-Bt transgenes in GT and non-GT plants by amplifying the GFP and Bt transgenes with PCR. Over 500 plants randomly selected from the BC generations derived from the original 27 cross combinations were tested. No conflict between PCR amplification of the transgenes and GFP visualization or Bt immunological test was observed (data not shown).

Flow cytometric analysis

After analyzing the relationship between chromosome number and the 2C (C is the haploid DNA content per nucleus) histogram mean values among *B. napus*, *B. rapa* and F_1 hybrids, a linear regression was obtained between 2C histogram mean values (x) and chromosome number (y), $y = 0.16x + 4.72$; the correlation coefficient was 0.99. The peak of nucleic DNA content in BC_1 plants containing the GFP-Bt transgenes showed a binomial distribution in all crosses, with most BC_1 plants having 2–7 additional chromosomes. As shown in the genetic analysis, the GFP-Bt transgenes in GT1 followed a dominant gene model in all BC_2, BC_3 and BC_4 generations of the three *B. rapa* accessions, while that of GT7 did not in any of the three backcross generations. However, the distribution of chromosome number among BC_1 progenies developed from these two GT lines showed a similar pattern (Fig. 1). The number of additional chromosomes in the subsequent backcross progenies containing the GFP-Bt transgenes in BC_2, BC_3 and BC_4 generations decreased as the distribution of chromosome number in these three generations shifted towards *B. rapa* (Fig. 1).

Table 5. Summary of deviations from an expected segregation pattern (1:1) of the GFP-Bt transgenes in backcross progeny derived from nine GT *B. napus* lines and three wild *B. rapa* accessions (see Tabs. 1–4 for details). NS, not significant, $P > 0.05$; * indicates that at least one family had significantly fewer transgenic progeny than expected, $P < 0.01$; 0 indicates no data available.

GT line	*B. rapa*	BC_1	BC_2	BC_3	BC_4
GT1	2974	NS	NS	NS	NS
	2975	NS	NS	NS	NS
	CA	NS	NS	NS	NS
GT2	2974	NS	NS	NS	NS
	2975	NS	NS	NS	NS
	CA	NS	NS	NS	NS
GT3	2974	NS	*	*	*
	2975	NS	NS	NS	NS
	CA	NS	NS	NS	NS
GT4	2974	NS	NS	NS	NS
	2975	NS	NS	NS	NS
	CA	NS	NS	NS	0
GT5	2974	NS	NS	NS	NS
	2975	NS	NS	NS	NS
	CA	NS	*	*	0
GT6	2974	NS	NS	NS	NS
	2975	NS	NS	NS	NS
	CA	NS	NS	NS	0
GT7	2974	NS	*	*	*
	2975	NS	*	*	*
	CA	NS	*	*	*
GT8	2974	NS	*	*	*
	2975	NS	*	*	*
	CA	NS	*	*	*
GT9	2974	NS	0	0	0
	2975	NS	*	*	NS
	CA	NS	*	*	*

DISCUSSION

Interspecific hybridization between three wild *B. rapa* accessions and nine GT *B. napus* lines

In this study, manual crosses between the three wild *B. rapa* accessions and the nine GT *B. napus* lines produced interspecific hybrids at a frequency of 99.26% overall. Only a few plants showed non-GT, which might have been generated from unreduced gametes or contamination. Because of the extremely low percentage of non-GT plants (0.74%), it should not significantly affect the analyses of segregation ratios of GT:non-GT in the subsequent backcrosses. The frequency of hybrids in F_1 and BC_1 progenies in this study was higher than that reported by Halfhill et al. (2001). One reason for this could be that more stringent isolation for avoiding pollen contamination was implemented when crosses were made in the present study. Jørgensen and Andersen (1994) reported over 60% hybrids in a field trial when individual plants of *B. rapa* were widely spaced within fields of *B. napus*. However, a lower rate of interspecific hybridization was observed when the *B. rapa* plants were adjacent to the *B. napus* plants or a short distance away in fields (Scott and Wilkinson, 1998). Thus, the frequency of transgene transfer between wild *B. rapa* and *B. napus* might depend on a number of factors, including the origin of wild plants, genome constitution, population structure, mating system of the hybridizing plants, and field experiment designs (Jørgensen and Andersen, 1994; Jørgensen et al., 1996b).

Segregation of GFP-Bt transgenes in BC_1 populations

Although the pollen viability of F_1 plants in this study was about 50%, the pollen production and fertility were sufficient to produce viable BC_1 seeds (~5–6 seeds per pollination). A similar rate of pollen viability was also reported by Metz et al. (1997). In all BC_1 populations of the three *B. rapa* accessions, although sample size was relatively small in some cases, the segregation of GT:non-GT did not deviate from a ratio of 1:1 for all nine GT *B. napus* lines (Tab. 1). This agreed with the findings by Mikkelsen et al. (1996b) who reported that 33 *B. napus* specific RAPD markers were transferred from F_1 into BC_1 population of *B. rapa* at a rate of 50%. Nozaki et al. (2000) recently analyzed chromosome transfer rates in the backcross progenies developed from the cross of AAC × AA by using RAPD markers in 13 synteny groups, which were specific to *B. alboglabra* (CC). Most of the synteny groups were transmitted at a rate of 50%, suggesting that when backcrossed with *B. rapa*, the distribution of C chromosomes of AAC into BC_1 progenies was likely random.

Pattern of transgene introgression in BC_2, BC_3, and BC_4 generations

The GFP-Bt transgenes from some transformation events introgressed into *B. rapa* more easily than others, as summarized in Table 5. The genetic behavior of the

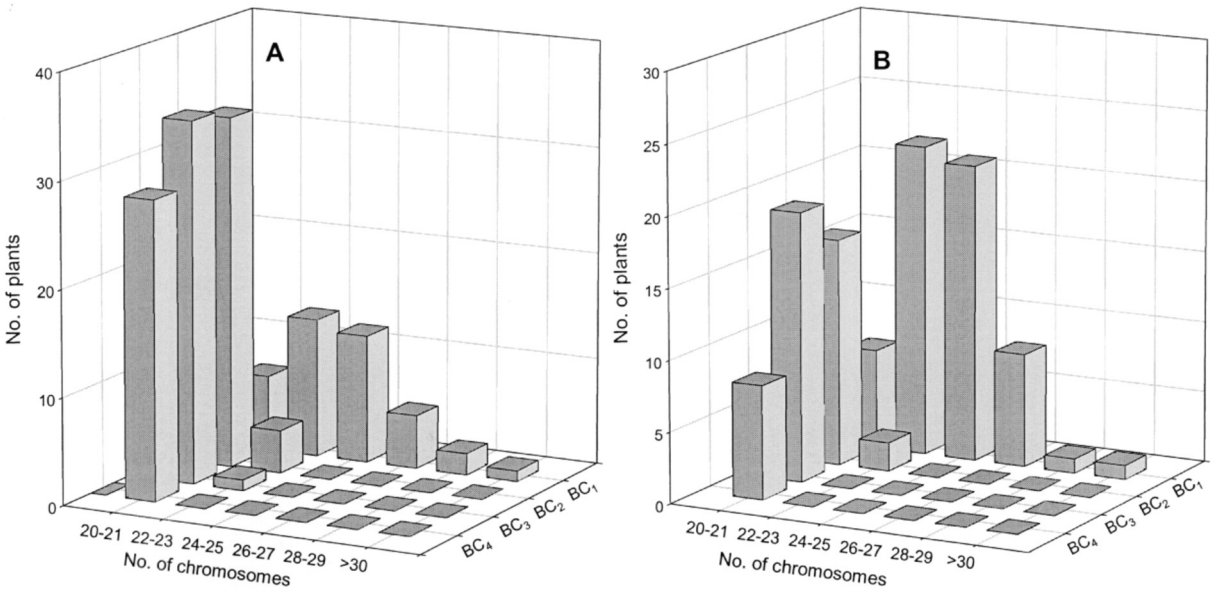

Figure 1. Distribution of estimated chromosome number in backcross progenies containing the GFP-Bt transgenes. BC_1, BC_2, BC_3 and BC_4 progenies were developed from the crosses of the three wild *B. rapa* accessions × GT1 (**A**) and × GT7 (**B**), respectively.

GFP-Bt transgenes in BC_2, BC_3, and BC_4 generations can be grouped into three types. In the first type, the segregation of GT:non-GT showed a ratio of 1:1 in all families of BC_2, BC_3, and BC_4 generations. Snow et al. (1999) also reported 1:1 ratios of transgenic herbicide resistance in BC_3 plants derived from *B. napus* and *B. rapa*. In this study, only high-fertility hybrids were used to develop the backcross generations, and this may be associated with normal Mendelian ratios. With this type of genetic behavior, approximately half of the progeny from crosses between hemizygous transgenic plants and wild plants are expected to inherit transgenes.

In the second type of pattern we observed, the segregation of GT:non-GT did not fit into a ratio of 1:1 in any of the BC_2, BC_3 or BC_4 generations. Metz et al. (1997) observed that the segregation of phosphinothricin tolerance in the BC_2, BC_3 and BC_4 generations of Pak choi (AA) × transgenic TP2 (AACC) significantly deviated from 1:1, with a low frequency of transgenic progeny (<12%). Therefore, the genetic behavior of the transgene in the phosphinothricin tolerant *B. napus* line (TP2) could belong to the second type. Only a small portion of the seeds produced by the wild plants of this type contained transgene(s), because it was likely that transgene(s) were still located on a nonhomologous C chromosome. During the development of successive backcross populations, a transgene on a C chromosome could be lost, such as the observations in family BC_4.1 of GT7 and family BC_4.1 of GT8 in 2974, and family BC_4.1 of GT8 in CA.

The third type of genetic behavior was that the segregation of GT:non-GT deviated significantly from a ratio of 1:1 in BC_2 and BC_3 generations, but in BC_4 generation, the segregation ratio was 1:1, such as family BC_4.2 of GT8 and family BC_4.1 of GT9 in 2975 shown in this study. The mechanism for this is not known, but it may be related to recombination between A and C chromosomes. Chromosome pairings between the A and C genomes were reported in meiotic studies of the amphihaploid (AC), digenomic hybrid (AAC) and resynthesized amphidiploid *B. napus* (AACC) (Attia and Röbbelen, 1986; Attia et al., 1987; Heneen et al., 1995; Nozaki et al., 2000), thus facilitating chromosomal recombination between the two genomes. Consequently, a fragment of C chromosomes might be incorporated into an A chromosome after inter-genomic chromosomal crossover events have occurred (Chen et al., 1997; Heneen and Jørgensen, 2001; McGrath and Quiros, 1991). This would explain the genetic behavior of transgenes in the third type.

Implication of different patterns of transgene introgression

Based on the genetic behavior of the GFP-Bt transgenes among the nine GT *B. napus* lines in BC_2, BC_3 and BC_4

generations, a similar pattern of transgene introgression in all three *B. rapa* accessions was shown for some GT *B. napus* lines. From these observations, we may suggest that (1) the transformation events of GT1, GT2, GT4, and GT6 are easier to introgress into *B. rapa*, whereas the introgression of the transgenes of GT7 and GT8 is more difficult; and (2) the difference in the genetic behavior of the transgenes of GT3 and GT5 shown among the three *B. rapa* accessions indicates that, perhaps due to occasional inter-genomic chromosomal recombination between A and C chromosomes, the introgression of a transgene might be unpredictable in some cases.

A recent nationwide study in the United Kingdom shows that transgene flow from transgenic *B. napus* to wild *B. rapa* is inevitable (Wilkinson et al., 2003). Moreover, transgenic herbicide resistance has already been detected in wild populations of *B. rapa* in Canada (Warwick et al., 2003). In the future, it may be possible to reduce the extent to which transgenes from *B. napus* persist in wild populations by using specific insertion sites in the genome. Further molecular characterization of the genomic locations of transgenes in *B. napus* may allow us to identify specific genomic locations that are more effective than others in reducing the possibility of transgene introgression from *B. napus* to wild *B. rapa*.

MATERIALS AND METHODS

Plant materials

Development of nine transgenic GFP-Bt *B. napus* lines (named GT1-9) was described by Harper et al. (1999). Through transforming a *B. napus* cv. Westar with a transgene construct (*mGFP5er-Bt cry1Ac*), designed to use the GFP (green fluorescent protein) gene to monitor the Bt (*Bacillus thuringiensis*) gene, nine GT *B. napus* lines were developed from nine independent transformation events, each containing the GFP-Bt transgenes at a single locus (Harper et al., 1999). Two wild *B. rapa* accessions, 2974 and 2975, from Milby (45°19'N 71°49'W) and Waterville (45°16'N 71°54'W), Quebec, Canada, respectively, and one accession, named CA, from Irvine (33°40'N 117°49'W), California, USA (courtesy of Art Weiss), were used in interspecific hybridization with the nine GT *B. napus* lines and successive backcross generation development.

Development of backcross progeny in *B. napus*

To investigate Mendelian inheritance of the GFP-Bt transgenes from the nine GT *B. napus* lines in backcross populations of *B. napus*, we crossed the nine homozygous GT lines with their isogenic non-transgenic counterpart, Westar (a *B. napus* cv.), respectively. Then, F_1 plants generated from all nine crosses were back-crossed with Westar to develop BC_1 generations. In each cross combination, over 80 BC_1 plants were screened for GFP expression.

Development of interspecific F_1 and successive backcross populations

Each of the three wild *B. rapa* accessions, 2974, 2975 and CA, was manually crossed with each of the nine GT *B. napus* lines. Buds of *B. rapa* plants were emasculated and pollinated manually 1–2 days before anthesis by pollen from nine GT *B. napus* lines respectively to develop F_1 plants. The pollinated racemes were immediately isolated in glassine bags to avoid pollen contamination. This isolation strategy was also implemented in the following backcross progeny development. After screening for GFP expression, randomly selected individual GT F_1 plants were used as pollen donors and backcrossed with wild plants of 2974, 2975 and CA to develop BC_1 families. For example, families BC_1.1, BC_1.2, and BC_1.3 were developed from three individual F_1 plants from one cross combination respectively. All BC_1 plants from the original 27 cross combinations were screened for GFP expression, then the GFP-expressing plants were screened for Bt toxin expression by immunological tests. One GT plant randomly selected from each BC_1 families, *e.g.* BC_1.1, BC_1.2, or BC_1.3, was backcrossed with wild plants of 2974, 2975 and CA to develop BC_2 family, *i.e.* BC_2.1, BC_2.2, and BC_2.3, respectively. Since no conflicts between GFP expression and Bt toxin immunological test were observed after a total of 931 BC_1 plants were screened, screening for GFP expression only was used to determine the frequency of GT plants in BC_2, BC_3 and BC_4 generations. When developing BC_3 generations, if individual GT BC_2 plants were selected from one family (*e.g.* BC_2.1), the BC_2 plants were designated as BC_2.1.1 and BC_2.1.2, resulting in family BC_3.1 and family BC_3.2 respectively. If selected from different families (*e.g.* BC_2.1 and BC_2.2), the BC_2 plants were designated as BC_2.1.1 and BC_2.2.1. The same strategy was used to develop and designate BC_4 populations. The chi-square test was used to determine whether the GFP-Bt transgene distribution in BC populations followed a Mendelian dominant gene model. Plants of GT *B. napus*, wild *B. rapa*, F_1 and BC progenies were grown in 5-inch pots filled with standard potting soil and maintained in a

growth chamber at 22 ºC/16 ºC (day/night) with 16-h day light under cool-white fluorescent lights.

Pollen viability

Pollen was collected from F_1 and backcross plants of wild *B. rapa* containing the GFP-Bt transgenes and tested for stainability with 1% acetocarmine (McGrath and Quiros, 1990). Five samples per plant were harvested and over 100 pollen grains were counted per sample. Pollen viability was estimated as the number of acetocarmine stained pollen grains per total number of pollen grains counted.

GFP visual detection

Putative 2–3 week-old F_1 hybrids and BC progenies were screened by using a hand-held UV lamp (UVP model-B-100AP, 100W:365nm, UVP, Upland, CA, USA) as described by Halfhill et al. (2001).

Bt toxin detection

Two leaf disks (clipped using a 1.5 ml microtube lid) were ground with 7–8 drops of extraction buffer (EnviroLogix, Portland, ME, USA) in a 1.5 ml microtube, and the supernatants were analyzed by Western blot using Lateral Flow Quickstix (detection limit <10 ppb).

PCR analysis

Leaves from 5–6 week-old plants were harvested and frozen in liquid nitrogen, lyophilized, ground to a fine powder and stored at –20 °C. DNA was extracted followed the procedure described by Somers et al. (1998). For the Bt gene, a pair of specific primers of 5'ATTTGGGGAATCTTTGGTCC3' and 5'ACAGTA-CGGATTGGGTAGCG3' (Stewart et al., 1996), were used to amplify a fragment (590 bp). A fragment (400 bp) of the *mGFP5er* gene was amplified with a pair of primers of 5'TACCCAGATCATATGAAGCGG3' and 5'TTGGGATCTTTCGAAAGGG3' (Halfhill et al., 2001). The two fragments representing the GFP gene and the Bt gene respectively can be amplified simultaneously in a PCR reaction (Halfhill et al., 2001). Each 20 µl PCR reaction contained 10 ng template DNA, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer, and 1 U of *Taq* DNA polymerase (BRL, Mississauga, ON, Canada). The cycle protocol was 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min and a final 72 °C for 7 min. The reaction products were analyzed on 2.0% (w/v) agarose gels in 1 × TAE by electrophoresis at 106 V for 2.5 h. Gels were stained with ethidium bromide and photographed on a digital gel-documentation system.

Estimation of chromosome number by flow cytometry analysis

Randomly selected GT F_1 and BC progenies developed from the crosses between the nine GT *B. napus* lines and the three wild *B. rapa* accession were used for flow cytometry analysis. About 1 cm^2 of a fully expanded young leaf from individual plants was chopped with a sharp razor blade in 1–2 ml nuclei extraction buffer (solution A, High Resolution Kit for Plant DNA, Partec, Germany). After filtration through a 30-µm nylon sieve, a 6–7 ml staining solution containing the dye 4,6-diamidino-2-phenylindole-2HCl (DAPI, solution B) was added. The analyses were performed by a PAS flow cytometry (Partec, Germany). For each sample, a minimum of 4000 particles (total count) were analyzed. The 2C (C is the haploid DNA content per nucleus) histogram mean value was evaluated using a DPAC software (Partec, Germany).

ACKNOWLEDGEMENTS

We are grateful to Drs. Scott Brown and Suzanne Lesage for their insightful suggestions on a previous version of the manuscript. We also thank all reviewers for their constructive comments that improved the manuscript. This research was aided by the Canadian Biotechnology Strategy Fund and the National Water Research Institute, Environment Canada.

Received June 23, 2003; accepted November 17, 2003.

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