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Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus

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Summary. Hypocotyl explants of three *Brassica napus* varieties were infected with two nopaline type *Agrobacterium* strains each carrying a distinct disarmed T-DNA containing different selectable markers. Selection was done for only one of the markers, after which the regenerated plants were screened for the presence of the second marker. High co-transformation frequencies of both T-DNA's were obtained (39%–85% of the transformants). Where the two T-DNA's were integrated linked, they were usually present in an inverted orientation relative to each other; in all of the cases observed the two right borders were adjacent. Tandem orientations occurred less frequently. The T-DNA's were mainly integrated as intact copies and deletions did not often occur. The co-transformation system described favors a genetically linked integration of the two T-DNA's (78%), although in a single transformed plant both linked and unlinked copies of both T-DNA's may be present.

Key words: *Agrobacterium tumefaciens* – Co-transformation – *Brassica napus* T-DNA integration – T-DNA organization

Introduction

The introduction of foreign genes into the plant genome is a basic technique used to study gene expression in plants. This technology is of growing importance in plant physiology studies and will soon be taken up in the breeding programs of major crop species. One of the restrictions in the use of current transformation techniques is that only a few genes can be transformed at the same time and that selectable marker genes have to be used, which results in transgenic plants with sometimes undesirable antibiotic resistance genes.

The introduction of multiple genes into one plant can be done by retransforming an already transformed plant or by crossing two independently transformed plants. Both methods are slow, time consuming, and more sensitive to somaclonal variation, and in most cases the introduced genes will not be linked. 'Co-transformation' has been evaluated by several groups using direct gene transfer (Christou and Swain 1990; Schocher et al. 1986; Tagu et al. 1988; Uchimiya et al. 1986). Protoplasts were electroporated with a mixture of two separate plasmids containing different genes of which at least one was a selectable marker gene. Transformed calli were obtained by selecting for the resistance gene located on one plasmid and then screened for the presence of the gene(s) derived from the other plasmid. High co-transformation frequencies were obtained (20%–25%). However, the genomic organization of the integrated genes was not studied.

Depicker et al. (1985) studied the co-transformation of unlinked genes into plant cells by co-cultivating tobacco protoplasts and *Agrobacterium tumefaciens*. In these experiments the foreign genes were located on different T-DNA's carried by one or two distinct *Agrobacterium* strains. They found that if the two T-DNA's were carried by different *Agrobacterium* strains, the co-transformation frequency was equal to the product of the frequencies of each single transformation, which indicates that co-transformation was the result of two independent transformation events. However, it the two T-DNA's were contained within one bacterium, the co-transformation frequency was much higher than the product of the frequencies of each single transformation, indicating that plant cells are more likely to acquire several T-DNA's from one bacterium than one from each of several bacte-

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ria. The Mendelian segregation of the T-DNA's could not be studied because wild-type T-DNA's were used, meaning that all the co-transformants were tumors that could not be regenerated to plants.

McKnight et al. (1987) infected leaf disks of *Nicotiana tabacum* with two *Agrobacterium tumefaciens* LBA4404 strains each carrying a different T-DNA on identical binary vector plasmids. One of the T-DNA's carried the nopaline synthetase gene, while the other T-DNA carried the *neo* gene. Of the 16 plants obtained after kanamycin selection, 3 contained nopaline. In subsequent backcrosses with wild-type plants, they found that the markers for kanamycin resistance and for the production of nopaline were genetically unlinked. No Southern analyses were done on the double transformants.

In this paper the co-transformation of two distinct disarmed T-DNA's each carried by a different *Agrobacterium* strain was studied. Derivatives of the nopaline type *Agrobacterium tumefaciens* strain c58 were used. The co-transformations were done on hypocotyl explants of *Brassica napus*. In the regenerated plants the molecular and genomic organization of the co-transferred T-DNA's was studied in detail.

Materials and methods

Agrobacterium strains

The *Agrobacterium* strains used were C58C1Rif®(pMP90)-(pGSFR781A) (De Block 1990; Fig. 1) and C58C1Rif(pMP90)-(pTAD7) (Declercq et al. 1990; Fig. 1)

Plant materials

The *Brassica napus* cultivars used were 'Westar' (Allelix, Canada) and 'Drakkar' (INRA, France), both spring varieties, and the winter variety 'R8494' (Hilleshog, Sweden).

Co-transformation

The transformation of *Brassica napus* cultivars used were 'Westar' (Allelix, Canada) and 'Drakkar' (INRA, France), both spring varieties, and the winter variety 'R8494' (Hilleshog, Sweden).

Co-transformation

The transformation of *Brassica napus* was done as described by De Block et al. (1989), but instead of one *Agrobacterium* strain, two were added at the same time to the infection plates (end concentration of each *Agrobacterium* strain in the infection medium is 2×10^6 bacteria/ml). The selection was done on 50 mg/l kanamycin-SO_4 or 20 mg/l phosphinotricin (ammonium salt).

Plant DNA isolation and Southern analysis

DNA was isolated from the leaf tissue as described previously (Dellaporte et al. 1983), except that 50 mg polyvinylpolypyrrolidone was added per 15 ml of extraction buffer. Five micrograms of DNA was digested with restriction enzymes as described by the manufacturer, electrophoresed in an 1% agarose gel, transferred to nylon Hybond-N filters, and hybridized with multiprime-labeled DNA (Amersham) as described in the Amersham manual for the use of Hybond-N filters. The filters were subsequently hybridized with a purified BamHI fragment containing the *bar* gene from the plasmid pGSFR780A (De Block et al. 1989), a purified PvuII-HindIII fragment containing the *neo* gene from the plasmid pSPT18-neo, and a 150-bp purified fragment of the 3'end of the *at2S1* gene (Figs. 1, 2). Between each hybridization the hybridized probe was removed from the filters using the NaOH method as described in the Amersham manual for the use of Hybond-N filters.

Vernalization

The winter cultivar of *Brassica napus* was vernalized at 4 °C for 12 weeks at low light intensity (500 lux).

Screening of seedlings for kanamycin and phosphinotricin resistance

The double transformants containing both the *neo* and *bar* gene were selfed and/or backcrossed. The mature seeds were sterilized and germinated in vitro as described earlier (De Block et al. 1989). After 2 weeks the hypocotyls of the seedlings were cut in 7-mm segments, which were then placed on medium A4 (De Block et al. 1989) containing 50 mg/l kanamycin-SO_4 or 20 mg/l phosphinotricin (ammonium salt). The hypocotyl segments derived from seedlings containing either the *neo* or the *bar* gene remained green and formed callus and shoots on the kanamycin- or phosphinotricin-containing medium, respectively. The hypocotyl segments derived from seedlings lacking the *neo* or the *bar* gene bleached and did not form callus or shoots on the kanamycin- or phosphinotricin-containing medium, respectively.

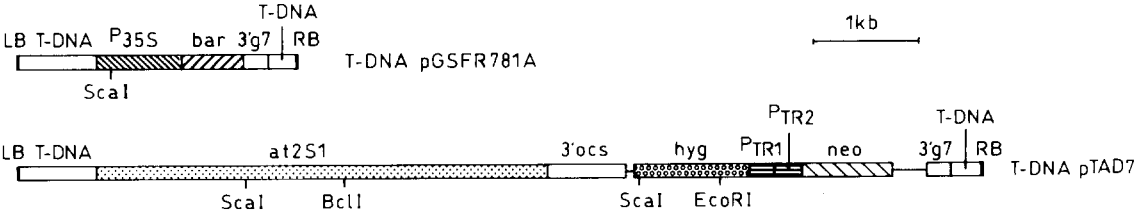

Fig. 1. Schematic representation of the T-DNA's of pGSFR781A and pTAD7. pGSFR781A contains the *bar* gene under the control of the 35S promoter (Odell et al. 1985). pTAD7 contains the *hyg* gene (Van den Elzen et al. 1985; Waldron et al. 1985), the *neo* gene and the native *Arabidopsis* gene *at2S1* encoding for the 2S1 storage protein (Krebbers et al. 1988). The *hyg* and the *neo* genes are under the control of the promoters TR1' and TR2' (Velten and Schell 1985), respectively. 3'g7 and 3'ocs are fragments encoding termination and polyadenylation signals, and are derived from the T-DNA gene 7 (Velten and Schell 1985) and the octopine synthase gene, respectively. The genes are inserted between the T-DNA border repeats (*RB* right border, *LB* left border)

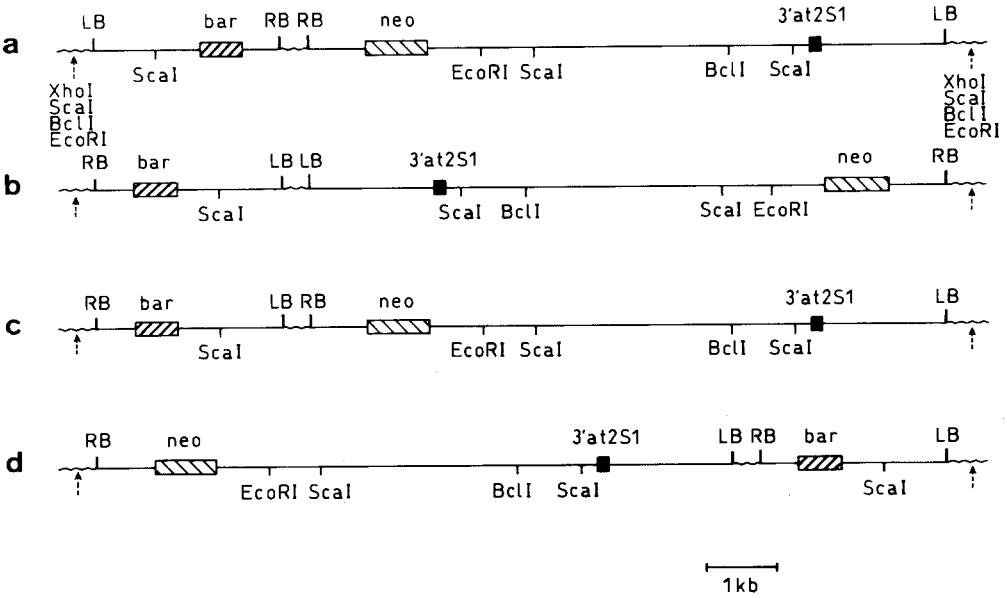

Fig. 2a–b. Possible integration patterns in the plant genome of the T-DNA's of the plasmids pGSFR781A and pTAD7 with respect to each other. The restriction sites and the purified fragments of the *neo*, *bar*, and *at2S1* genes used as probes in the Southern analyses are indicated. **a** Inverted orientation around the right border; **b** inverted orientation around the left border; **c** and **d** the two possible direct orientations

Table 1. Transformation frequencies of the *neo* and *bar* gene

B. napus variety	Percent transformation[a]					
	Total	PPT^R Km^S	PPT^S Km^R	PPT^RKm^R Selected on		
				PPT	Km	Total
R8494	18.0	5.4	2.5	2.5	7.6	10.1
Westar	16.9	0	2.6	5	9.3	14.3
Drakkar	34.1	10.9	9.9	3.4	9.9	13.3

R, resistant; S, sentitive

[a] Transformation rates were measured as the percentage of infected hypocotyl explants that gave rooted transformed shoots. The numbers are an average of the frequencies obtained in two independent transformation experiments. Each experiment was started with 100 explants of 'R8494', 200 of 'Westar', and 200 of 'Drakkar'

Results

In the study of co-transformation three questions are of particular interest. First, what is the frequency of co-transformation of the two T-DNA's when explants are used as starting material? Secondly, are both T-DNA's integrated into the genome without rearrangements? Finally, how are the T-DNA's organized in the plant genome; are they closely linked or not?

To distinguish the two T-DNA's they were marked with two different selectable genes, *neo* or *bar*. The *bar* gene encodes the enzyme phosphinotricin acetyl transferase, which inactivates the herbicide phosphinotricin (Glufosinate) by acetylation (De Block et al. 1987; Murakami et al. 1986; Thompson et al. 1987). Transgenic *Brassica napus* plants expressing a chimeric *bar* gene are resistant to high doses of phosphinotricin (De Block et al. 1989). The T-DNA containing the *bar* gene was 2610 bp in size (Fig. 1). To evaluate the susceptibility of the system to possible T-DNA rearrangements, the second T-DNA used carried not only the *neo* gene, conferring resistance to kanamycin, but was marked with two extra genes. These were the *hyg* gene, conferring hygromycin resistance, and the a*t2S1* gene, encoding a 2S albumin seed protein from *Arabidopsis thaliana* (Krebbers et al. 1988). The structure of the 8900-bp T-DNA is shown in Fig. 1.

Co-transformation of the neo and bar genes occurs at a high frequency

Hypocotyl segments of one winter variety ('R8494') and two spring varieties ('Westar' and 'Drakkar') of *Brassica napus* were infected with the two derivatives of the disarmed *Agrobacterium* strain C58C1Rif(pMP90) (Koncz and Schell 1986) that carry the two T-DNA's (Fig. 1). Half of the hypocotyl explants were placed on kanamycin selective medium (50 mg/l) and the other half on phosphinotricin selective medium (20 mg/l). Transgenic plants were obtained with the same frequency as if a single *Agrobacterium* strain was used (Table 1; De Block et al. 1989). The selected plants were screened for the presence of both T-DNA's by placing leaf discs of

these plants on both kanamycin or phosphinotricin selective medium (De Block et al. 1989). The co-transformation frequencies obtained were much higher than would be expected if the two events were independent. For 'R8494', the overall co-transformation frequency was 10.1% or 56% of the total transformants; for 'Westar' the corresponding figures were 14.3% and 84.6%; for 'Drakkar', 13.3% and 39%. Based on the transformation frequencies of the two markers independently, the expected overall co-transformation rates would have been the product of the frequencies with which kanamycin-respectively phosphinotricin-resistant transformants were obtained. This would have been 0.8% [(5.4% + 2.5%) × (2.5% + 7.6%)] for 'R8494', 0.6% [(0% + 5%) × (2.6% + 9.3%)] for 'Westar', and 2.8% [(10.9% + 3.4%) × (9.9% + 9.9%)] for 'Drakkar' (Table 1).

Molecular and genetic analyses of the double transformants

To determine how many copies of each T-DNA were present in the double transformants, and how these were organized in the plant genome, Southern analyses were done on eighteen randomly chosen double transformants (3 'R8494', 4 'Westar', 11 'Drakkar'). Ten of these were derived from kanamycin selection and eight from phosphinotricin selection. The possible integration patterns of the T-DNA's of plasmids pTAD7 and pGSFR781A in the plant genome with respect to each other are depicted in Fig. 2. In summary, the two T-DNA's can be integrated independently of each other and are located at different loci on the same or different chromosomes. Alternatively, they could integrate at the same locus in either tandem or inverted orientations relative to each other. In the latter case either the right or left borders could be adjacent. Southern analyses of the plant DNA's cut with ScaI, EcoRI, BclI, and XhoI and hybridized with the *bar* gene, the *neo* gene, and the 3-end of the *2S1* gene were carried out. The kind of data obtained in the Southern analyses are summarized in Table 2 for some of the 'Drakkar' transformants.

To determine the precise genotype of the eighteen double transformants, they were selfed or backcrossed (with pollen of a non-transformed control plant). With the exception of one transformant the genotype of all the transformants could be determined. Table 3 summarizes for the same 'Drakkar' transformants as described in Table 2 the kind of genetic data obtained from these crosses. In combination with the data obtained in the molecular analyses the precise genomic organization of the T-DNA's could be determined; these data are summarized in Table 4.

Of the 63 integrated copies examined, only 27 were found to be unlinked to other copies. Of the 16 cases where the two T-DNA's were determined to be linked, the two T-DNA's were in an inverted orientation about the right border in 13 of them, while in the other 3 cases the two T-DNA's were in a tandem orientation. Few deletions were observed. There is an extensive deletion in the pTAD7-T-DNA of the transformants B82-5 and B85-203. Two out of the five pTAD7-T-DNA's of the transformant B85-201 also have a deletion at the left border. There is a deletion of about 1 kb at the right border repeat of the pTAD7 and pGSFR781A-T-DNA's in transformants B85-1 and B71-100. The pGSFR781A-T-DNA of the transformant B82-202 has a deletion of about 1 kb at his left border. Finally, the T-DNA's that are integrated at the same locus are often separated by a few hunderd to a few thousand base pairs (8 out of 16 cases) of non-T-DNA.

Table 2. Examples of the kind of data obtained in Southern analyses for some of the 'Drakkar' double transformants

Plant DNA	Restriction enzyme	Number of hybridizing bands with probes			Common hybridizing bands with probes *neo* and *bar*	
		bar	*neo*	3′ at 2S1	Amount	MW (kb)
B85-1	ScaI	1	1		1	3.9
	EcoRI	1	1	1	1	13.1
	BclI	1	1		1	7.9
B85-4	ScaI	1	2		0	
	EcoRI	1	2	2	0	
	BclI	1	2		0	
	XhoI	1	1		0	
B85-7	ScaI	2	2		2	5.5
						4.9
	EcoRI	2	2	2	2	7.5
						6.5
	BclI	2	2		2	11.3
						9.5
B85-8	ScaI	1	1		1	4.9
	EcoRI	1	1	1	1	5.5
	BclI	1	1		1	8.6
B85-14	ScaI	1	3		1	4.9
	EcoRI	1	3	3	1	7
	BclI	1	3		1	8.1
B85-23	ScaI	1	2		1	6.7
	EcoRI	1	2	2	1	12.5
	BclI	1	2		1	8.3
B85-101	ScaI	1	1		0	
	EcoRI	1	1	1	0	
	BclI	1	1		0	
B85-111	ScaI	1	1		1	5.1
	EcoRI	1	1	1	1	7.9
	BclI	1	1		1	11.3
B85-203	ScaI	2	2		0	
	EcoRI	2	2	1	0	
	BclI	2	2		1	8.9
	XhoI	2	2		1	11.3

Table 3. Examples fo the kind of data obtained in the genetic analysis for some of the 'Drakkar' double transformants[a]

Cross		Number[b] of seedlings tested	Number of seedlings				Proposed genotype[c]	χ^2	P
			PPT^SKm^S	PTT^RKm^S	PPT^SKm^R	PPT^RKm^R			
B85-1	Drakkar	110	51	0	0	59	b1 n1	0.58	10–50
B85-4	Drakkar	128	26	38	30	34	n1 b2	2.45	10–50
B85-7	Drakkar	119	26	0	0	93	b1 n1 b2 n2	0.68	10–50
B85-8	Drakkar	154	68	0	0	76	b1 n1	0.44	50–90
B85-14	Drakkar	280	27	0	114	139	b1 n1 n2 n3	2.6	10–50
B85-23	B85-23	158	8	0	8	128	b1 n1 n2	2.77	10–50
B85-101	Drakkar	208	45	48	55	60	n1 b2	2.65	10–50
B85-111	Drakkar	144	76	0	0	68	b1 n1	0.44	50–90
B85-203	Drakkar	144	32	29	0	83	b1 n1 b2	2.81	10–50

The transformants B71-. are derived from the experiment with the winter variety 'R8494'
The transformants B82-. and B85-. are derived from the experiments with the spring varieties 'Westar' and 'Drakkar', respectively
[a] The transformants correspond with these listed in Table 2
[b] All seeds are derived from one plant
[c] Composed with the information obtained from the Southern analyses (see Table 2). b: pGSFR781A T-DNA; n: pTAD7 T-DNA; b1 n1 . . . : linkage group 1; b2 n2 . . . : linkage group 2; b3 n3 . . . : linkage group 3

Table 4. Summary of the structural and genetic organization of the T-DNA insertions in the double transformants

Transformant	Selected on[a]	Genotype[b]	Remarks
B71-6	Km	b1 * b1 > < n1 b1 n2	
B71-50	Km	b1 ⩾ n1 * n1 b2	Orientation of b1 ⩾ n1 as depicted in Fig. 2c
B71-100	PPT	b1 n1 > < b1 n2	Deletion of about 1 kb at RB repeat of n1 > < b1
B82-3	Km	n1 b2	
B82-5	Km	n1 ⩾ b1	Deletion of about 4 kb at LB of pTAD7 T-DNA Orientation of tandem as depicted in Fig. 2d
B82-102	PPT	n1 b2	
B82-202	PPT	(n) n1 > < b1	
B85-1	Km	n1 > < b1	Deletion of about 1 kb at RB repeat of n1 > < b1
B85-4	Km	n1 * n1 b2	
B85-7	Km	n1 > < b1 n2 > < b2	
B85-8	Km	n1 > < b1	
B85-14	Km	n1 > < b1 n2 n3	
B85-23	Km	n1 > < b1 n2	
B85-101	PPT	n1 b2	
B85-111	PPT	n1 > < b1	
B85-201	PPT	? 2 × n > < b	F1 segregation too complex to be interpreted; 2 of the pTAD7 T-DNA's have a deletion of about 4 kb
B85-203	PPT	n1 n1 ⩾ b1 n2	Deletion of about 4 kb at LB of pTAD7 T-DNA of tandem Orientation of tandem as depicted in Fig. 2d
B85-206	PPT	(n) n1 > < b1 b1 b2	

RB, Right border; LB, left border
The transformants B71-. are derived from the experiment with the winter variety 'R8494'. The transformants B82-. and B85-. are derived from the experiments with the spring varieties 'Westar' and 'Drakkar', respectively
[a] PPT, 20 mg/l phosphinotricin (ammonium-salt); Km, 50 mg/l kanamycin-SO_4
[b] n, pTAD7 T-DNA; b, pGSFR781A T-DNA; b1 n1 . . . , linkage group 1; b2 n2 . . . , linkage group 2; b3 n3 . . . , linkage group 3 ⩾, tandem in direct orientation; > <, linked T-DNA's in inverted orientation around the right border; *, linked T-DNA's of which orientation is not determined; ( ), from the crosses carried out it is not possible to decide if this copy belongs to linkage group 1 or if this copy is a silent copy belonging to another linkage group

Discussion

We studied in *Brassica napus* the co-transformation of two distinct T-DNA's each delivered by a different *Agrobacterium tumefaciens* strain. Co-transformation is an efficient process, and the frequency of its occurrence is much higher than the product of two independent events. Depicker et al. (1985) found that if two distinct T-DNA's are located in different *Agrobacterium* strains, the co-transformation frequency is the product of the frequency of each single transformation, indicating that co-transformation is the result of independent transfor-

mation events and that every plant cell is equally competent to be stably transformed. These data are contradicted by our results. However, Depicker et al. used tobacco protoplasts, while in this study *Brassica napus* hypocotyl explants were used as the starting material. Little is known about the influence of plant material and tissue culture conditions before and during infection on the competence of a plant cell to be transformed and how these factors can influence the final T-DNA integration pattern. It has been shown that efficient transformation only occurs when there is DNA synthesis and/or cell division (An 1985; Firoozabody and Galbräith 1984; Kudirka et al. 1986). Moreover, Kartzke et al. (1990) found that for naked DNA transformation of tobacco protoplasts the integration pattern is determined by the stage of the cell cycle. It is possible that this also holds for the *Agrobacterium tumefaciens* transformation system. Van Lijsebettens et al. (1986) found that T-DNA rearrangements occur less frequently in primary tumors induced on tobacco plants than in transformed cell lines obtained using the protoplast co-cultivation system. In summary, the manner of T-DNA integration may be influenced by the state of the plant material, the cell type (the explant), and the tissue culture conditions.

In 57% of the cases in which transformed plants contained the two T-DNA's as linked copies, 81% were in an inverted orientation relative to each other, while 19% were in a tandem orientation. Jones et al. (1987) and Jorgensen et al. (1987) made a detailed analysis of the T-DNA structures in tomato and *Petunia* plants transformed by *Agrobacterium tumefaciens* C58 derivatives. They concluded that inverted repeat structures both around the right or left border are the predominant pattern of T-DNA organization in plants transformed by derivatives of the nopaline strain C58. These joint integrations are much less frequently found in plants transformed by *Agrobacterium tumefaciens* derivatives of the octopine strain LBA4404. Spielmann and Simpson (1986) studied plants transformed with this strain and found only 2 linked integrations that occurred as inverted repeats out of 22 integration events.

McKnight et al. (1987) conducted co-transformation experiments using the strain LBA4404, which contained a binary vector with a disarmed T-DNA derived from the nopaline type *Agrobacterium tumefaciens* strain T37. Although they did not do Southern analyses and obtained only three double transformants, their results indicated that the two T-DNA's in all three double transformants were genetically unlinked. This would imply that if multiple T-DNA's are to be inserted at a single locus nopaline type *Agrobacterium* strains (virulence functions) would give a greater chance of success, while if unlinked insertions are desired, octopine type *Agrobacterium* strains (virulence functions) would be advantageous. This concept is now being tested.

Two possible models have been proposed to explain the occurrence of integrated T-DNA copies in an inverted or tandem linked orientation. Because no multimeric T-DNA forms can be detected in *vir*-induced *Agrobacterium*, it is thought that they arise in the plant cell. The first model proposes that the T-DNA repeats are the result of replication and repair of the T-DNA during insertion into the plant DNA. The second model explains the linked arrangement of the T-DNA's as the result of ligations that occur in the plant cell before integration; this is analogous with plasmid concatenation observed in the plant genome after direct DNA transformation (Riggs and Bates 1986; Schocher et al. 1986). The first model can be excluded because the linked T-DNA's of the co-transformants described in this paper are composed of two distinct T-DNA's derived from different *Agrobacterium* strains. Although the second model is more acceptable, it does not explain why the tandem T-DNA's occur preferentially in an inverted orientation, and, in particular, most frequently around the right border. The second model can also not explain the occurrence of plant DNA in between the linked T-DNA's.

Matzke et al. (1989) found that in sequentially transformed tobacco plants the presence of one T-DNA could affect the state of methylation and expression of genes on a second, unlinked T-DNA. Our selection scheme did not allow for the detection of this suppressive interaction because only those transformants expressing both resistant markers were studied further. However, in those double transformants suppressive interaction between the T-DNA's was never observed in subsequent generations. Experiments are now under way which study the interaction between both T-DNA's at the expression level in the initially selected plants. Plants expressing only the selectable marker gene located on one T-DNA will be screened by PCR for the presence of the second T-DNA. It will be interesting to see if this interaction at the expression level between two T-DNA's also holds if they are not sequentially but simultaneously introduced into the plant genome.

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