The Maternal Chromosome Set Is the Target of the T-DNA in the *in Planta* Transformation of *Arabidopsis thaliana*

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ABSTRACT

In planta transformation methods are now commonly used to transform *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. The origin of transformants obtained by these methods has been studied by inoculating different floral stages and examining gametophytic expression of an introduced β-glucuronidase marker gene encoding GUS. We observed that transformation can still occur after treating flowers where embryo sacs have reached the stage of the third division. No GUS expression was observed in embryo sacs or pollen of plants infiltrated with an Agrobacterium strain bearing a GUS gene under the control of a gametophyte-specific promoter. To identify the genetic target we used an insertion mutant in which a gene essential for male gametophytic development has been disrupted by a T-DNA bearing a Basta resistance gene (B^R). In this mutant the B^R marker is transferred to the progeny only by the female gametes. This mutant was retransformed with a hygromycin resistance marker and doubly resistant plants were selected. The study of 193 progeny of these transformants revealed 25 plants in which the two resistance markers were linked in coupling and only one plant where they were linked in repulsion. These results point to the chromosome set of the female gametophyte as the main target for the T-DNA.

UNDER natural conditions, plant cell transformation by pathogenic strains of Agrobacteria occurs after contact between the bacteria and the cell walls of wounded plant tissues. A majority of bacterial virulence genes are transcriptionally activated by plant exudates containing sugars and phenolic compounds that are produced by wounded tissues (Stachel *et al.* 1985; Winans 1992; Hooykaas and Beijersbergen 1994; Zupan and Zambryski 1997). These genes are involved in T-DNA processing and transfer to the plant cell nucleus (Zambryski *et al.* 1989). After infection by *Agrobacterium tumefaciens* or *A. rhizogenes*, transformed cells develop either a crown gall or a hairy root, respectively.

In vitro transformation methods artificially reproduce these conditions, the excision of explants from the plant playing the role of wounding under natural conditions. A large variety of plant species and tissues can be used, the production of transformed plants being essentially limited by the regeneration capacity of the explant (Gheysen *et al.* 1998).

In planta transformation was first described in Arabidopsis (Feldmann and Marks 1987; Feldmann 1991). In this pioneer work, seeds were treated with a suspension of Agrobacterium during the early stages of germination and plants were grown under natural conditions. These plants were then allowed to produce seeds by self-pollination. Among these seeds, some gave rise to entirely transformed plantlets that could be selected easily by germination on selective medium. Due to the low frequency of transformants, this first method was difficult to reproduce. Improvements were obtained by treatment of entire plants instead of seeds (Chang *et al.* 1994; Katavic *et al.* 1994). The infiltration method proposed by Bechtold *et al.* (1993) was quickly adopted by laboratories because of its higher transformation frequency and its reproducibility. In this protocol, adult plants are vacuum-infiltrated by the bacterial suspension so that tissues and reproductive organs are invaded by the bacteria. This method has been extended recently to another *Brassicaceae*, *Brassica rapa L., ssp Chinensis* (Pakchoi; Liu *et al.* 1996) and *Medicago truncatula* (Harrison *et al.* 1999). A simplification of this method was proposed by Clough and Bent (1998), who replaced the vacuum treatment by dipping inflorescences in a bacterial suspension containing a surfactant (Silwet L-77), allowing the penetration of the bacteria into the intercellular spaces.

In these methods, although transformation or transient expression may affect several somatic tissues (Rossi *et al.* 1993; Vaucheret 1994; English *et al.* 1997; Kapila *et al.* 1997), the transformants obtained after self-pollination are systematically hemizygous for T-DNA insertions (Feldmann 199l; Bechtold *et al.* 1993). It is

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therefore considered that the "efficient transformation events" occur late in the development of the plant and concern either the germinal lines (male or female), or the egg cells before their first division, or the young embryo cells although no chimeras have been detected so far (Feldmann *et al.* 1994).

The discovery of the cellular target(s) of Agrobacterium in the *in planta* transformation method would be an important step toward answering several questions. What can be learned about plant-Agrobacterium interactions from this system? Are the signals involved in this case different from those occurring in nature? Is this method transposable to a wide range of other species in other botanical families? Can we direct gene transfer either to male or female parental genomes? Can we imagine natural spontaneous creation of transgenic plants through this process?

Recently, Ye *et al.* (1999) described the staining pattern conferred by a constitutively expressed GUS construct in infiltrated plants and their progeny. In this report, ovules were predominantly stained but also some pollen grains and some embryo sectors. It is difficult to determine from these experiments if all of these transformation events represent "efficient transformation", resulting in transformed progeny.

In this report we address the question of the T-DNA target(s) in the *in planta* transformation. This question can be divided into two subquestions: what is the genetic target (maternal and/or paternal chromosomes) and what are the cellular targets, *i.e.*, when and where in the plant development? The former question can be addressed by the use of plant material in which male and female counterparts do not play the same role. Desfeux *et al.* (1998 and personal communication) and Ye *et al.* (1999) used male sterile and fertile counterparts in crosses after infiltration of one parent or the other. Both found transformants only when the male sterile parent was infiltrated. This is an indication that transformation occurred in female organs but these results cannot rule out T-DNA transfer into male gametes after pollination, during pollen tube elongation, or during fertilization. In this report, we make use of a mutant deficient in male gametogenesis previously obtained in our laboratory by insertional mutagenesis (Bonhomme *et al.* 1998).

The distribution of transformed seeds after inoculation, the effect of different inoculation methods, the effect of varying the floral stages at which bacteria were applied, and the expression pattern of a reporter gene in transformed seeds were all studied to more precisely identify the cellular target for transformation. The results obtained clearly demonstrate that the genetic target for T-DNA transformation is almost exclusively the female chromosome set. The cellular target is less clearly defined but corresponds most probably to the female gametophyte.

MATERIALS AND METHODS

Materials: WS, wild-type *Arabidopsis thaliana* (L.) Heyn, ecotype Wassilevskija *Ttd8* and *Emb506* are Arabidopsis (WS ecotype) mutants, identified in the Versailles T-DNA collection (Bechtold *et al.* 1993). This population results from *in planta* transformation with the Agrobacterium strain MP5-1, carrying a T-DNA construct with two selection markers, *bar* and *nptII*, conferring, respectively, phosphinothricin (Basta) resistance (B^R) and kanamycin resistance (K^R; Bouchez *et al.* 1993).

Ttd8 is a tagged gametophytic mutant defective in T-DNA transmission via pollen, which was selected on the basis of a 1:1 K^R:K^S ratio upon selfing (Bonhomme *et al.* 1998). *Ttd8* is thus a male gametophytic mutant, with a male transmission of T-DNA of 0.46%. The female transmission of the T-DNA is variable from plant to plant, from 43 to 55%, with an average of 45.8% (defined as *c* coefficient, see below). Pollen tube growth experiments *in vitro* suggested that the mutation is responsible for a defect in pollen tube elongation (Bonhomme *et al.* 1998).

Emb506 is an embryo-defective tagged mutant selected by screening immature siliques of T1 plants showing 25% of aborted seeds (Albert *et al.* 1999). This embryo-lethal mutant is blocked at the transition from globular to heart stages.

The C58C1(pMP90) (Koncz and Schell 1986) strain of *A. tumefaciens* containing different binary vectors was used for all the transformation experiments:

SLJ6585 (p6585) (Jones *et al.* 1992) was used for transformation of both *Ttd8* and *Emb506*. This vector carries *hpt* and *nptII* genes conferring hygromycin resistance (H^R) and K^R, respectively (a gift of J. Jones *et al.*).

pGKB5 (MP5-1) (Bouchez *et al.* 1993), bearing the *bar* gene conferring B^R and the *nptII* (K^R) gene, was used to transform WS to determine the ultimate transformable stage of floral buds.

pJD121 (Drouaud *et al.* 2000) was used to transform WS to detect expression of a reporter gene in gametophytes of infiltrated plants. This vector bears the *bar* (B^R) and the *uidA* genes under the control of a rapeseed Skp1-like gene promoter driving GUS expression in the male and female gametophytes.

Plant transformation protocol: Seeds of lines segregating *Ttd8* and *Emb506* mutants were sown on sand, subirrigated with water containing Basta herbicide (7.5 mg/liter phosphinothricin). The germination was synchronized by a treatment at 4° for 64 hr, and the trays were placed in the greenhouse (16-hr day photoperiod, 15° night/23° day temperature cycle). Two-week-old Basta-resistant plantlets were transferred to soil until flowering.

C58C1(pMP90)(p6585) Agrobacteria were grown in Luria Bertani medium supplemented with 50 mg/liter rifampicin, 100 mg/liter gentamycin, and 40 mg/liter tetracycline for 14 hr at 28°. MP5-1 C58C1(pMP90)(pDFJ48-GUS) and C58C1 (pMP90)(pJD121) Agrobacteria were grown with 50 mg/liter rifampicin, 100 mg/liter gentamycin, and 200 mg/liter kanamycin for 14 hr at 28°. Vacuum infiltration of flowering WS, *Ttd8*, and *Emb506* Basta-resistant plants was performed as described in Bechtold and Pelletier (1998). Floral dipping transformation was performed as described in Clough and Bent (1998). Floral spraying of MP5-1 Agrobacterium suspension in infiltration medium (Bechtold and Pelletier 1998) was performed with a spray gun until all flowers were uniformly wet. Plants were treated twice at 1-wk intervals.

WS Basta-resistant plants were selected as described above.

WS kanamycin-resistant transformants were selected *in vitro* on Arabidopsis medium (Bechtold and Pelletier 1998) containing 100 mg/liter kanamycin.

Double primary transformants (T1) *Ttd8H* and *Emb506H* were selected *in vitro* on Arabidopsis medium containing 25 mg/liter hygromycin (H25). One-week-old plantlets were sprayed in the petri dish with sterile Basta herbicide (300 mg/liter phosphinothricin) (H25 + B300). These transformants were then cultivated in the greenhouse. They were either self-pollinated (T2 generation: *Ttd8H* T2 or *Emb506H* T2) or backcrossed to WS (BCT1: *Ttd8H* × WS or *Emb506H* × WS) for progeny analysis. The BCT2 was obtained by backcrossing to WS H^RB^R-selected plants of *Ttd8H* T2 or *Emb506H* T2.

H^R and B^R segregation analysis in BCT1, T2 generation, and BCT2: The progeny of self-pollinations and backcrosses were sown on hygromycin-containing medium and on nonselective medium as described above. Germination was synchronized by cold treatment at 4° for 64 hr. One-week-old plantlets were sprayed with Basta as described above (B300, H25 + B300). Segregation for resistant/sensitive plantlets was analyzed before Basta treatment on H25 and on B300 and H25 + B300 1 wk after the Basta spraying.

Estimation of the recombination frequency between H^R and B^R markers: These selective treatments allowed us to assess the frequency of each phenotypic class as follows.

Growth on H25 allowed us to observe the frequency $[H^R]$ of hygromycin-resistant and the frequency $[H^S]$ of hygromycin-sensitive plantlets, giving, respectively, $a = [H^R] = [H^RB^R] + [H^RB^S]$ and $1 - a = [H^S] = [H^SB^R] + [H^SB^S]$.

Growth on H25 + B300 allowed us to observe the frequency $[H^RB^R]$ of hygromycin-resistant and Basta-resistant plantlets and the frequency $[H^RB^S]$ of hygromycin-resistant and Basta-sensitive plantlets among the hygromycin-resistant plantlets, giving, respectively, $b = [H^RB^R]/([H^RB^R] + [H^RB^S])$ and $(1 - b) = [H^RB^S]/([H^RB^R] + [H^RB^S])$. Therefore, $ab = [H^RB^R]$ and $a(1 - b) = [H^RB^S]$.

Growth after B300 spraying allowed us to identify the frequency $[B^R]$ of Basta-resistant plantlets and the frequency $[B^S]$ of Basta-sensitive plantlets, giving, respectively, $c = [B^R] = [H^RB^R] + [H^SB^R]$ and $1 - c = [B^S] = [H^RB^S] + [H^SB^S]$. c represents the female transmission of the B^R marker and this last condition allowed us to determine this parameter for each *Ttd8H* line. It must be noted that c is different from the usual transmission efficiency (TE), which would be the ratio between $[B^R]$ and $[B^S]$.

Thus $[H^RB^R] = ab$, $[H^RB^S] = a(1 - b)$, $[H^SB^R] = c - ab$ and $[H^SB^S] = (1 - c) - a(1 - b)$. $[H^RB^R]$ and $[H^RB^S]$ are directly observed frequencies and $[H^SB^R]$ and $[H^SB^S]$ are calculated from the above formulas.

The recombination frequency (p) between B^R and H^R loci was estimated for *Ttd8H* × WS, *Ttd8H* T2, *Emb506H* × WS, and *Emb506H* T2 through the observed and calculated frequencies of the two phenotypic classes, $[H^RB^S]$ and $[H^SB^R]$, respectively, according to the above formula, and as described in Tables 1 and 2.

Molecular hybridization experiments: Total genomic DNA was extracted from plantlets or leaves (Dellaporta *et al.* 1983). Three micrograms of DNA were digested with restriction endonucleases (Amersham, Piscataway, NJ); the fragments were separated on a 0.8% agarose gel in 1 × TBE buffer and blotted onto nylon membranes (Genescreen +) as described by Ausubel *et al.* (1990), using 0.4 N NaOH as transfer solution. Hybridization experiments were carried out in 0.5 m Na_2HPO_4 pH 7.2, 7% SDS, and 1 mm EDTA at 65°. Probes were purified and labeled with ^{32}P-dCTP using the Pharmacia Oligolabeling kit. Washes were performed according to Ausubel *et al.* (1990).

GUS staining: Histochemical assays for GUS activity in flowers and siliques were conducted according to the protocol described by Fourgoux-Nicol *et al.* (1999), modified as described below. Fresh or frozen flowers and longitudinally incised siliques were fixed as described and GUS staining was carried out by vacuum infiltration (three times for 10 min) in the GUS staining solution (1 mm X-Gluc; Duchefa, Haarlem, The Netherlands; 0.1 m potassium phosphate buffer pH 7.0, 0.2% Triton) and incubated one night at 37°. After clearing

TABLE 1

***Ttd8H* Punnet square**

			Male:	Selfing							
				H^RB^S			H^SB^S			H^RB^R	H^SB^R
Female				C (p)	R ($1 - p$)	I (0.5)	C ($1 - p$)	R (p)	I (0.5)	(0)	(0)
H^RB^S	C	$(1 - c)p$			$[H^RB^S]$			$[H^RB^S]$			
	R	$(1 - c)(1 - p)$									
	I	$(1 - c)/2$									
H^SB^S	C	$(1 - c)(1 - p)$			$[H^SB^S]$			$[H^SB^S]$			
	R	$(1 - c)p$									
	I	$(1 - c)/2$									
H^RB^R	C	$c(1 - p)$			$[H^RB^R]$			$[H^RB^R]$			
	R	cp									
	I	$c/2$									
H^SB^R	C	cp			$[H^RB^R]$			$[H^SB^R]$			
	R	$c(1 - p)$									
	I	$c/2$									
				Backcross							

Ttd8H Punnet square (for selfing and T1 backcrosses) including the gamete frequencies in coupling or repulsion phase according to the recombination rate (p) and to the female B^R transmission rate (c; see materials and methods). C, coupling; R, repulsion; I, independence.

TABLE 2

Theoretical segregation values for *Ttd8H* and *Emb506H* backcrosses and T2 generations

	Linkage	$[H^RB^R]$	$[H^RB^S]$	$[H^SB^R]$	$[H^SB^S]$	p
Ttd8H × WS	C	$c(1-p)$	$(1-c)p$	cp	$(1-c)(1-p)$	$[H^RB^S]/(1-c)$
	R	cp	$(1-c)(1-p)$	$c(1-p)$	$(1-c)p$	$1-[H^RB^S]/(1-c)$
	I	$c/2$	$(1-c)/2$	$c/2$	$(1-c)/2$	0.5
Ttd8H T2	C	$c(1+p^2-p)$	$(1-c)(2p-p^2)$	$c(p-p^2)$	$(1-c)(1+p^2-2p)$	$[H^RB^S]/(1-c)-[H^SB^R]/c$
	R	$c(1+p^2-p)$	$(1-c)(1-p^2)$	$c(p-p^2)$	$(1-c)p^2$	$1-([H^RB^S]/(1-c)-[H^SB^R]/c)$
	I	$3/4c$	$3/4(1-c)$	$c/4$	$(1-c)/4$	0.5
Emb506H × WS	C	$(1-p)/2$	$p/2$	$p/2$	$(1-p)/2$	$[H^RB^S]+[H^SB^R]$
	R	$p/2$	$(1-p)/2$	$(1-p)/2$	$p/2$	$1-([H^RB^S]+[H^SB^R])$
	I	1/4	1/4	1/4	1/4	0.5
Emb506H T2	C	$2/3(p^2-p+1)$	$(2p-p^2)/3$	$(1-p^2)/3$	$2/3(p-p^2)$	$3[H^RB^S]-3/2[H^SB^R]$
	R	$2/3(p^2-p+1)$	$p^2/3$	$(1-p^2)/3$	$2/3(p-p^2)$	$1-(3[H^RB^S]-3/2[H^SB^R])$
	I	6/12	3/12	2/12	1/12	0.5

Theoretical values of the frequencies of four phenotypic classes ($[H^RB^R]$, $[H^RB^S]$, $[H^SB^R]$, $[H^SB^S]$) in backcross (*Ttd8H* × WS, *Emb506H* × WS) and selfed (*Ttd8H* T2, *Emb506H* T2) progeny of *Ttd8H* and *Emb506H* T1 according to the recombination rate (p) between Basta and the hygromycin resistance gene for coupling (C) and repulsion (R) linkage and independence (I). c corresponds to the female transmission rate of the Basta resistance gene in *Ttd8H*. The last column indicates how p can be calculated from data in the other columns.

with 70% ethanol, the samples were examined under a binocular microscope.

RESULTS

Toward a better definition of the stage of T-DNA transfer

Observations about *in planta* transformation: Classical protocols, either with vacuum or surfactant infiltration, give rise to transformation events that occur in general at a frequency of 10^{-3}–10^{-2} (number of resistant plantlets per seed germinated in standard selection conditions). These low frequencies and the observation that primary transformants obtained by the different *in planta* transformations are hemizygotes suggest that the male or female germinal lines, or the egg cell, or the zygote are the probable targets of Agrobacterium. On the other hand, transformation events are not randomly (Poisson) distributed on a per silique or per plant basis as shown in Table 3 and Figure 1. It appears that a large proportion of plants or fruits do not produce any transformants, while among the others, more than expected produce a larger quantity of resistant plants. It appears therefore that favorable conditions may increase Agrobacterium transformation in some plants or flowers.

Transformation without vacuum was tested and compared in the same experiment with vacuum infiltration on three samples of 54 plants each. After immersing WS plants in a concentrated MP5-1 suspension (without Silwet L-77), 2 transformants were obtained in their progeny. Twenty-six transformants were obtained after

TABLE 3

Distribution of transformants per silique

T/Silique	0	1	2	≥3
P1	24	2	1	1
P2	21	3	0	0
P3	20	3	0	0
P4	15	4	1	0
P5	17	1	3	0
P6	19	4	0	0
P7	23	4	0	0
P8	21	2	1	0
P9	21	0	2	2
P10	23	2	0	0
Total *S*	204	25	8	3
Theo	192	43.2	4.8	0.2

The number of siliques harvested on the principal floral stem and showing 0, 1, 2, or >3 transformants per silique (*T*/Silique) for 10 plants inoculated (P1 to P10) by floral dipping. The distribution of the total number of siliques (Total *S*) for the four classes is compared to the theoretical random distribution (Theo) [Poisson law, mean 0.22 *T*/Silique, $y = (0.22^x/x!)e^{-0.22}$] and shows a significant difference ($\chi^2 = 15.55$; the upper limit for ddl = 3 and $P = 0.05$ is 7.82).

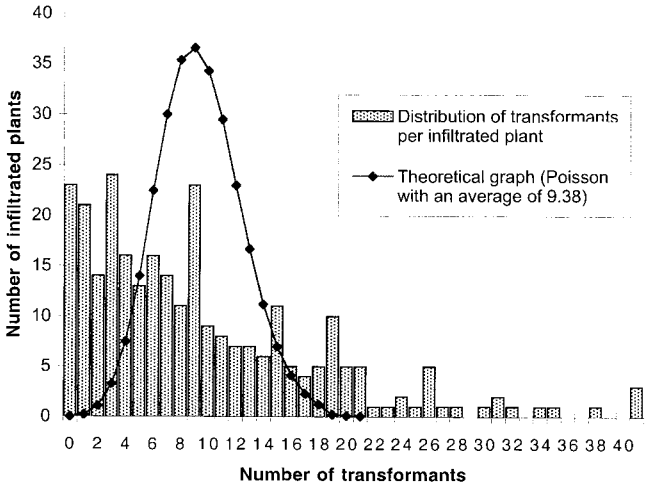

Figure 1.—Distribution of transformants per infiltrated plant. Distribution of the number of transformants among the progeny of 279 individual vacuum-infiltrated plants compared to a random distribution with the same mean. The mean of the distribution is 9.38. In total, 2631 transformants were selected. The curve corresponds to a random distribution (Poisson law) with the same mean ($y = (9.38^x/x!)e - 9.38$).

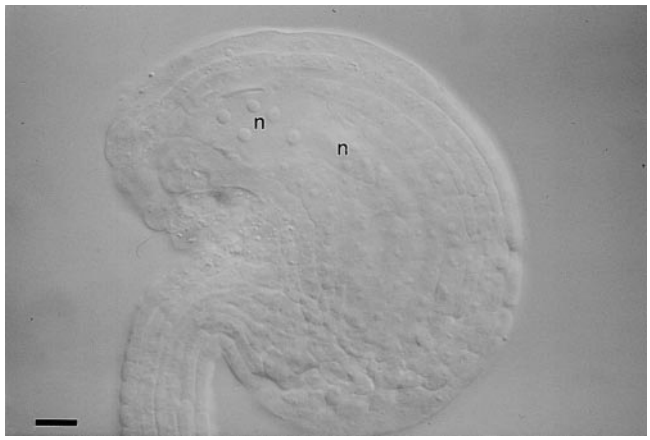

Figure 2.—Ovule development in WS buds of 2 mm. Ovule observed with differential interference contrast microscopy (×500) after clearing treatment (Motamayor *et al.* 2000) in a 2-mm bud from a WS plant, showing a representative embryo sac containing eight nuclei (n) that will migrate to form the seven-celled (mature) embryo sac. Bar, 10 μm.

repeatedly spraying flowers with bacterial suspension whereas 292 were obtained after vacuum infiltration. Hence there is an increase of ~10-fold in the transformation frequency with a treatment that maintains the bacterial population (repeated spraying) and a further 10-fold increase with a treatment that permits penetration of the Agrobacteria into the tissues, namely vacuum infiltration.

Transformation of buds after meiosis: The purpose of this experiment was to determine the latest stage of bud development at which it is possible to obtain transformants by infiltration with Agrobacterium. In Arabidopsis, the different stages of development of the gametophytes are precisely correlated with the size of the floral bud (http://www.isv.cnrs-gif.fr/EMBO/manuals/index.html, module 1, arabidopsis gametogenesis, pps. 1–4). The microspores (male meiosis products) and meiosis-mononucleate embryo sacs are found in floral buds of 1–1.25 mm in our conditions (stage 11, Smyth *et al.* 1990); trinucleate pollen grains and four-nuclei to seven-celled embryo sacs (Figure 2) are found in floral buds of 2 mm (stage 12, Smyth *et al.* 1990). All buds <1 mm were removed from one set of plants and buds <2 mm were removed from another set of plants, just before inoculation using the floral dip method, and plants were grown until the remaining buds (>1 mm or >2 mm) had formed mature siliques. All new buds, stems, or inflorescences formed after the treatment of these plants were eliminated. In these conditions, it is possible to obtain transformants among the seeds derived from these inoculated buds. Table 4 shows that the frequency of transformants obtained from such plants is reduced compared to control plants or to plants for which only small buds (<1 mm) are kept before infiltration. In this case, these buds as well as those derived from inflorescences formed after the treatment were potential sites of transformation events by the Agrobacteria population remaining in the plant (Bechtold *et al.* 1993). Transformation can therefore occur in buds bearing trinucleate pollen grains and four-nuclei (Figure 2) to mature embryo sacs. No transformants were obtained by infiltration of flower buds after pollination (pistil size >2.3 mm).

Infiltration with Agrobacteria bearing a reporter gene expressed in both male and female gametophytes: Transformed Arabidopsis plants bearing a construct comprising a *uidA* reporter coding sequence under the control of a Skp1-like rapeseed promoter (isolated in

TABLE 4

Transformation frequencies for different bud or pistil sizes

		Bud size (mm)			Pistil size (mm)
Experiment	*T*	<1	>1	>2	>2.3
1	11.6	1.64	0.26	1.05	—
2	6.8	1.94	0.93	0	—
3	13.5	13.6	—	1.17	—
4	5.6	—	0.07	0.22	—
5	1.6	—	—	—	0

Frequency of Basta-resistant plantlets (for 10^3 plated seeds) obtained in five independent experiments from MP5-1 floral-dipped WS plants with controlled bud sizes. <1 mm, buds >1 mm were removed before infiltration; >1 (or 2) mm, buds <1 (or 2) mm were removed before infiltration; pistil >2.3 mm, all buds were removed, only open flowers, already self-pollinated, were inoculated. *T*, control plants with all buds intact. —, not analyzed.

TABLE 5

Transformation frequency with pJD121:GUS staining and Basta selection

	GUS+				Basta+			
	Uff	‰	Ff	‰	SA	‰	NSA	‰
I	0/14,550	0	0/28,250	0	142/37,746	3.76	10/44,005	1.7
II	0/12,050	0	0/36,400	0	213/39,176	5.44	30/39,426	0.86

Frequency of positive GUS staining (GUS+) and Basta-resistant (Basta+) plantlets [number observed and per 10^3 (0/00) ovules or seeds] obtained in two independent experiments (I and II) from pJD121 floral-dipped WS plants. Uff, unfertilized flowers (2- to 2.3-mm pistil); Ff, fertilized flowers (2.3- to 3-mm pistil). SA, sampled area corresponds to the area where flowers and siliques were sampled for GUS coloration; NSA, not sampled area corresponds to the rest of the plant.

the laboratory by differential screening of cDNA libraries) express GUS activity almost exclusively in male and female gametophytes. In male gametophytes, GUS staining is visible in microspores and pollen grains while in female gametophytes, GUS staining is visible in megaspores and until at least the seven-cell stage of mature embryo sacs (Drouaud *et al.* 2000). We used this construct in a vector containing the *bar* gene to select transformants in standard infiltration experiments. During the days following infiltration, young flower buds just at the anther dehiscence stage and young developing siliques were regularly removed and tested for GUS expression. On the same stems from which these buds were removed, the remaining siliques were collected at maturity and the seeds were sown on Basta for the selection of transformants. Histological tests revealed no staining in microspores, pollen grains, or in developing embryo sacs before fertilization. As a positive control, seeds harvested on the same plants gave a normal proportion of transformants (Table 5). A sample (18) of these transformants was grown until flowering to observe GUS expression in female and male gametophytes: 17 gave a strong expression in microspores and 9 in ovules. These results indicate that most probably premeiotic cells are not the major targets of Agrobacteria: in this case one would have observed staining of one or the other (or both) gametophytes in infiltrated plants as observed in transformed plants.

Determination of the genetic target of the T-DNA

Principles of the genetic approach: A male gametophyte-deficient mutant, named *Ttd8*, was obtained in our laboratory by insertional mutagenesis using *in planta* (vacuum infiltration) transformation (Bonhomme *et al.* 1998). In this mutant, the T-DNA, which contains both Basta and kanamycin resistance genes, is almost fully transmitted through the female gametes (although a

TABLE 6

***Ttd8H in planta* transformation hypotheses**

Male transformation hypothesis				
Male gamete genotypes		Female	Embryo	B and H
Before T	After T	gamete genotypes	(H^RB^R)	linkage
		(B^R)	($-H^R/B^R-$)	Repulsion
($-$)	($-H^R$)	($-$)		
Female transformation hypothesis				
Female gamete genotypes		Male	Embryo	B and H
Before T	After T	gamete genotypes	(H^RB^R)	linkage
(B^R)	(B^RH^R)		($B^RH^R/--$)	Coupling
($-$)	($-H^R$)	($-$)		

Expected linkage between B^R and H^R markers in *Ttd8H* [H^RB^R] T2 progeny according to two hypotheses: Agrobacterium transforms (T) male chromosomes (in meiocytes, developing male gametes or sperm cells) and Agrobacterium transforms female chromosomes (in megaspores, immature embryo sacs, or egg cells). *Ttd8H* pollen bearing the B^R marker is not transmitted to the progeny. ($-$), the loci corresponding to B^R and H^R in the wild type. Zygotic transformation would result in both types of linkage.

slight defect in the transmission rate is observed) and rarely transmitted through the male gametes (<0.5%). The mutation blocks pollen tube elongation (Bonhomme *et al.* 1998). After self-pollination, this plant line gives equal numbers of wild-type and heterozygous mutant-type plants. As described in Table 6, if the *Ttd8* plants (hemizygous for the T-DNA) are retransformed by infiltration with an Agrobacterium strain containing a T-DNA conferring hygromycin resistance, the phase of genetic linkage (coupling or repulsion) between the two T-DNAs in double-transformed plants indicates, respectively, the female or male parental origin of the secondary transformation event. If Agrobacterium transforms male mother cells or gametophytes and if H^R and B^R are genetically linked, only repulsion linkage will be observed in H^RB^R progeny because coupling linkage with the B^R marker will not be transmitted through the male gametes. If Agrobacterium transforms the female mother cells or gametophytes, only coupling linkage will be observed in H^RB^R progeny since the B^R marker is transmitted only through female gametes. If Agrobacterium transforms the zygote, both coupling and repulsion linkage will be observed with equal frequency.

The hemizygous state of the treated *Ttd8* plants allows determination of linkage directly in the next generation upon backcross to the wild type. In fact, most of the transformants were self-pollinated (for practical reasons; see below) and linkage analysis necessitated one further generation (see materials and methods).

As a control we used heterozygous plants for an embryo-lethal mutation (*Emb506*) tagged with the MP5-1 T-DNA. This mutant was selected from the same collection as *Ttd8* and described by Albert *et al.* (1999). This control was chosen because after self-pollination and selection on Basta, the progeny are composed only of hemizygous plants as in the case of *Ttd8*, allowing a direct reading of the genetic linkage in the progeny obtained by backcrosses to the wild type. When these plants (hemizygous for the first insertion) are transformed with the H^R vector, and since both gametes participate equally in the transmission of the T-DNA to the progeny, we expect the same frequency of coupling and repulsion linkage of the two T-DNAs whatever the target of the T-DNA in the *in planta* transformation.

Production of double transformants: More than 600 *Tdt8* and 600 *Emb506* plants were infiltrated with Agrobacteria bearing the p6585 vector. The frequency of transformation with p6585 was 4.5 transformants for 1000 tested seeds for *Ttd8* and 7.5 transformants for 1000 seeds for *Emb506*. About 40% of *Ttd8H* and 66% of *Emb506H* hygromycin-resistant plants were Basta-resistant as expected, according to the initial segregation of the B^R marker in these mutants.

The progeny of 193 *Ttd8H* and 125 *Emb506H* hygromycin- and Basta-resistant plants were studied further for linkage analysis between the H^R and B^R markers.

Linkage analysis between H^R and B^R markers: The recombination frequency between both markers is easily obtained after pollination of *Ttd8H* and *Emb506H* plants with the wild type. In the case of independence, the four phenotypes (H^RB^R, H^SB^R, H^RB^S, and H^SB^S) are equally represented. A coupling linkage leads to an overrepresentation of H^RB^R and H^SB^S phenotypes, and repulsion leads to an overrepresentation of H^SB^R and H^RB^S phenotypes (Table 7 for *Ttd8H*).

Too many double transformants were obtained at the same time to cross all plants, and it was necessary to make use of the self-pollinated progeny for many of them. In this case it is also possible to detect and to measure linkage directly in coupling phase. Repulsion can be confused with the segregation of multiple independent secondary insertion loci, and analysis of the next generation was necessary.

The recombination frequency (p) was calculated as described in Tables 1 and 2. The formulas defining p in coupling phase are complementary to one of the formulas defining p in repulsion phase. The parameter L used in Table 7, simply defined by the formulas for p in coupling phase (Tables 1 and 2), consequently takes values between 0 and 0.5 for linkage in coupling and between 0.5 and 1 for linkage in repulsion.

Table 7 gives some examples of results obtained for different double transformants in parallel with theoretical proportions. In this table the double transformant 11-38 is an example of tight linkage between H^R and B^R markers without any detected recombination. The double transformants 11-20 and 16-21 represent two cases of linkage in coupling with calculated L values of 0.09 and 0.29, respectively. In backcrosses of H^RB^R individuals of the T2 to WS (BCT2), the types of segregation correspond to those expected in theory: essentially a 50:50 ratio for H^R:H^S associated with an excess of H^RB^R *vs.* H^RB^S and one plant with a 100:0 H^R:H^S ratio giving equal proportions of H^RB^R and H^RB^S. There is a greater deviation than expected for the last ratio, as if the L value was an overestimation of the (female) recombination frequency in the BCT2. The double transformant 4-2 is an example of independence between the H^R and B^R loci, the BCT2 generation confirming the BCT1 and T2 generation data. The double transformant 17-11 is the only transformant showing a linkage in repulsion ($L = 0.76$), confirmed by the BCT2 generation where the three possible H^R:H^S segregations with their corresponding H^RB^R:H^RB^S segregations are clearly observed. The last example (transformant 11-9) illustrates the case of multiple insertions (more than two independent loci) of the H^R marker. A linkage in repulsion would have given a similar L value and the BCT2 generation was necessary for discrimination (compare with the theoretical value for $L = 1$). This result was confirmed by Southern analysis.

Table 8 is the compilation of the results obtained for the two types of double transformants *Ttd8H* and

TABLE 7

Examples of *Ttd8H* segregation compared to theoretical segregations

Line		*L*	BCT1 $H^R:H^S$	BCT1 $H^RB^R:H^RB^S$	BCT1 $B^R:B^S$	T2 generation: $H^R:H^S$	BCT2 $H^RB^R:H^RB^S$	BCT2 NbT2	BCT2 $H^R:H^S$	BCT2 $H^RB^R:H^RB^S$	*I*
C	**Theo**	**0**	**50:50**	**100:0**	**50:50**	**50:50**	**100:0**	**1**	**50:50**	**100:0**	**1**
	11-38	0	—	—	158:173	271:286	271:0		—	—	1
	Theo	**0.1**	**50:50**	**90:10**	**50:50**	**55:45**	**83:17**	**9**	**50:50**	**90:10**	**1**
								1	**100:0**	**50:50**	
	11-20	0.09	—	—	178:163	253:196	210:43		25:25	23:1	—
									8:5	7:1	
									33:29	29:4	
									27:27	26:1	
									8:10	7:1	
									31:15	28:3	
									4:12	3:1	
	Theo	**0.3**	**50:50**	**70:30**	**50:50**	**65:35**	**61:39**	**6**	**50:50**	**70:30**	**1**
								3	**100:0**	**50:50**	
								1	**50:50**	**30:70**	
	16-21	0.29	—	—	189:207	223:131	164:59*		30:19	25:5	—
									25:17	21:4	
									34:24	29:5	
									17:23	14:3	
									26:26	21:5	
									162:0	73:89	
I	**Theo**	**0.5**	**50:50**	**50:50**	**50:50**	**75:25**	**50:50**	**7**	**50:50**	**50:50**	**1**
								3	**100:0**	**50:50**	
	4-2	0.55	48:36	17:20	97:143	246:86	125:121		29:25	12:17	—
									60:45	31:29	
									8:12	2:6	
									38:27	21:17	
									38:25	20:18	
									39:36	23:16	
									29:27	13:16	
									18:14	6:12	
									52:0	24:28	
									39:0	15:24	
R	**Theo**	**0.75**	**50:50**	**25:75**	**50:50**	**85:15**	**46:54**	**7**	**50:50**	**30:70**	**1**
								2	**100:0**	**50:50**	
								1	**50:50**	**70:30**	
	17-11	0.76		—	353:385	1207:154	495:712		94:104	15:79	1
									55:38	7:48	
									115:117	29:86	
									131:114	17:114*	
									66:63	14:52	
									102:0	54:48	
									60:0	23:37	
									127:99*	106:21*	
	Theo	**1**	**50:50**	**0:100**	**50:50**	**100:0**	**50:50**	**10**	**50:50**	**0:100**	**1**
	Theo	**0.88**	**75:25**	**—**	**50:50**	**94:6**	**50:50**	**6**	**100:0**	**50:50**	**2**
	2							**2**	**75:25**	**50:50**	
	T-DNA							**2**	**50:50**	**50:50**	
	11-9	0.97	37:6	—	133:109	373:11	178:206		11:0	4:7	4
									23:0	7:16	
									11:0	3:8	
									44:16	23:21	
									50:16	26:24	
									54:9	30:24	

Ttd8H segregation: theoretical (Theo) values (calculated with $c = 0.5$) in percentage for different linkage values (*L*) [coupling (C), independence (I), repulsion (R)] of the three observed proportions ($H^R:H^S$, $H^RB^R:H^RB^S$, and $B^R:B^S$). Observed segregations for different double transformants of *Ttd8H*, for backcrosses in the T1 generation (BCT1), T2 generation, and backcrosses to T2 individuals (BCT2). *L* is obtained from the results of the T2 selfing progeny. In BCT2, NbT2 corresponds to the approximate number of each segregation type among 10 backcrossed [H^RB^R] T2 progeny (*i.e.*, three different genotypes among H^RB^R phenotypes). *I* corresponds to the number of T-DNA insertions in theory for theoretical examples and estimated by Southern analysis for presented transformants. —, not analyzed; *, observed segregation significantly different from the theoretical segregation ($P = 0.05$, ddl = 1, $\chi^2 > 3.84$).

TABLE 8

Linkage analysis results for *Ttd8H* and *Emb506H*

	Linkage	C	I	R	*T*	mH	NA
Ttd8H	No. of lines	25	128	1	154	24	15
	%	16.2	83.1	0.7	79.8	12.4	7.8
Emb506H	No. of lines	15	73	10	98	14	13
	%	15.3	74.5	10.2	78.4	11.2	10.4

Number and percentage of *Ttd8H* and *Emb506H* lines for which *L* could be estimated, for three classes of recombination rate (*p*) corresponding to linkage in coupling (C) or repulsion (R) or independence (I). *T*, total number and percentage of lines for which *p* was estimated; mH, number and percentage of multiple insertions of the *hpt* gene; NA, number and percentage of lines that were not analyzed because of low amounts of seeds or abnormal segregations.

Emb506H. For single-locus insertions of the H^R marker, the independence between H^R and B^R loci was tested in the T2 generation using a χ^2 test with the theoretical proportions $[H^RB^R] = 3/8$, $[H^RB^S] = 3/8$, $[H^S] = 2/8$ for *Ttd8H* and $[H^RB^R] = 6/12$, $[H^RB^S] = 3/12$, $[H^S] = 3/12$ for *Emb506H* (d.f. = 2, $P = 0.01$, $\chi^2 = 9.21$). In BCT1, the theoretical proportions for *Ttd8H* × WS and *Emb506H* × WS are $[H^RB^R] = 1/4$, $[H^RB^S] = 1/4$, $[H^S] = 2/4$. For significant values of the χ^2 test, segregation in the T2 generation and in BCT1 allowed us to determine coupling or repulsion linkage (*L* value), which were confirmed by BCT2 (according to Table 7) and Southern analysis (see below). The maximum-likehood estimation of *p* was not used since only one phenotypic class $[H^RB^S]$ is directly observed, the other $[H^SB^R]$ being calculated. Calculating the exact value of the recombination frequency was not the purpose of this work. Only the presence of genetic linkage was examined.

The other transformants were classified either as multiple insertions or were not analyzed because of a low number of seeds or abnormal segregations of markers.

Considering only the cases of insertions of the H^R marker at a single locus, the frequencies of detectable linkages in coupling and in repulsion are equivalent in the case of the *Emb506H* line. This result confirms that there is no bias due to the presence of a T-DNA integrated by a first transformation event for the integration of a second T-DNA in an independent transformation experiment. There are almost exclusively linkages in coupling (25 out of 26) and only one linkage in repulsion for the case of the male gametophytic mutant.

One surprising result was the number of transformants without apparent recombination ($p = 0$) in coupling phase. Three out of 15 lines for *Emb506H* and 3 out of 25 for *Ttd8H* have been observed. For *Emb506H*, we did not observe such tight linkage ($p = 0$) among 10 lines in repulsion phase.

Molecular analysis of (H^R) T-DNA integrations: Southern analyses were performed to confirm the data obtained by the genetic analysis essentially for transformants with $p \geq 0.7$. Figure 3 presents some examples of transformants with simple or complex hybridization patterns, which are in agreement with the genetic data indicating single or multiple integration loci. *Eco*RI restriction digests produce one internal T-DNA fragment

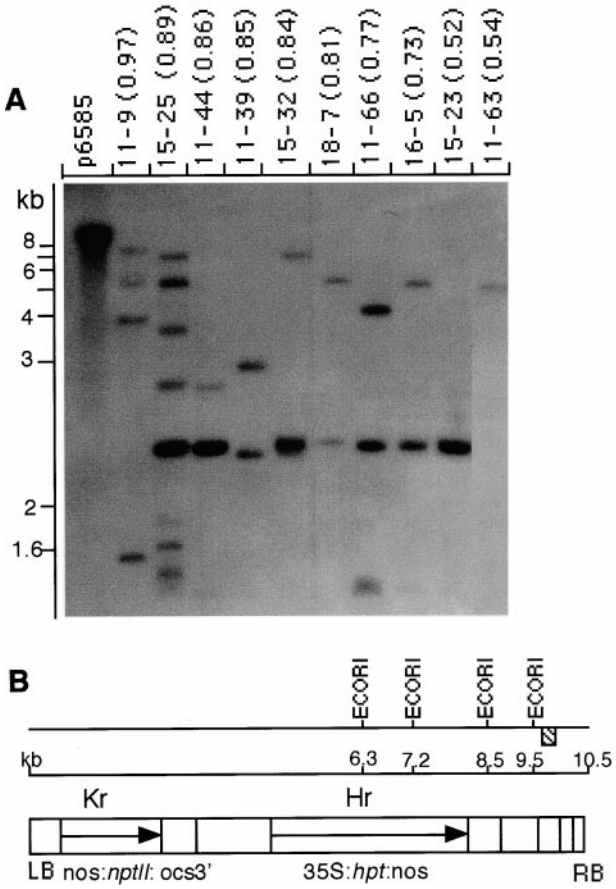

Figure 3.—Southern blot analysis. (A) Southern analysis of 10 *Ttd8H* plants presenting different *L* values (in parentheses). Genomic DNA was digested with *Eco*RI and hybridized with the probe shown in B. *Eco*RI produces one band at 2272 bp internal to the T-DNA for a RB-RB tandem. Other bands correspond to junction fragments between plant DNA and the T-DNA. p6585 corresponds to undigested plasmid DNA. (B) T-DNA map showing the *Eco*RI restriction sites. The hatched area corresponds to the 200-bp probe used in hybridization experiments.

in the case of tandem insertions. The expected sizes for *Eco*RI digests are 2.3 kb for RB-RB inverted repeats and 7.7 kb for RB-LB direct repeats. LB-LB tandem insertions cannot be detected with this probe. No hybridization was observed when using the probe on digested *Ttd8* DNA.

The 11-9, 15-25, and 11-66 *Ttd8H* lines for which, respectively, the *L* values are 0.97, 0.89, and 0.77, presented multiple T-DNA copies. 11-44, 11-39, 18-7, and 16-5 presented at least two T-DNA copies in agreement with their *L* value (~0.8). 15-23 and 11-63 presented only one T-DNA copy in agreement with their *L* value (~0.5).

The fragment of 2.3 kb, corresponding to a RB-RB tandem copy, is present in 8 lines out of 10 as previously described for vacuum infiltration with the MP5-1 vector (Bechtold *et al.* 1993). Multiple insertions of RB-RB tandems also lead to only one fragment of 2.3 kb, so that Southern analysis with only the RB probe cannot identify the exact T-DNA copy number. Our purpose with these analyses was to confirm the presence of multiple H^R insertions and not to determine the exact number of T-DNA insertions: multiple T-DNA copies could be linked and segregate as one locus.

DISCUSSION

The transfer of T-DNA occurs most probably during gametophytic development: The absence of homozygous transformants after *in planta* transformation excludes the possibility of early transformation events in the meristem or bud primordia, which could concern both male and female germ lines.

The experiments described here, where we looked for the latest stage at which the infiltration by Agrobacteria can succeed, indicate more precisely that the T-DNA transfer can occur while both male and female gametophytes are close to maturity. The frequency of transformants per 10^3 collected seeds after infiltration of 1- or 2-mm long buds is relatively low compared to the frequency obtained from younger buds or even from those not yet formed at the time of the infiltration. This difference could be interpreted as a consequence of the processes necessary for the Agrobacteria first to reach their target cells and second to encounter the environmental conditions where virulence genes are induced. These demands may not be met immediately after infiltration. Another interpretation is that transformation is more efficient in younger buds. This would mean that several developmental stages are propitious to T-DNA transfer and consequently that several target cells could be concerned.

The absence of detectable expression of a reporter gene under the control of a promoter specifically active in male and female gametophytes, whereas the same infiltrated plants produce a large number of transformed seeds, favors the hypothesis of a late T-DNA transfer. One can easily imagine that if the T-DNA transfer had occurred in somatic tissues of the young ovule or young anther, GUS expression in the gametophytes resulting from meiosis would have been observed. This is not the case. Although based on negative results, these experiments strongly suggest that the T-DNA transfer is postmeiotic and may even occur after gametophyte development if we suppose that integration or transient expression of the reporter gene during this development would have given a signal in the pollen grain (Touraev *et al.* 1997) or in the embryo sac. These results appear partially contradictory with those published recently by Ye *et al.* (1999), who observed the expression of GUS gene under the control of a constitutive promoter in ovules and in developing embryos. Further studies are required to determine whether these discrepancies are due to the conditions of transformation, the promoter used, or the staining method itself.

The frequency of T-DNA transfer is not affected by a previous transformation event and the integration of a second T-DNA occurs at random in the genome: The second transformation by the hygromycin-resistance vector of the *Emb506* and *Ttd8* lines leads to a frequency of hygromycin-resistant transformants similar to the frequency observed with the wild type. Genetic analysis shows that single-locus and multilocus insertions of this H^R vector occur with the same apparent frequency as in the wild-type (WS) line used to build up the Versailles collection: ~15% of the first 32,000 lines studied showed segregations corresponding to two or more independent loci.

The observed proportion of H^R insertions genetically linked to B^R insertions in both lines is in agreement with their theoretical probabilities. These probabilities can be calculated from the estimation, in centimorgans, of the total size of the Arabidopsis genome (500 cM; Hauge *et al.* 1993) and of the target corresponding to a range of considered linkage values. If, for example, we consider the target corresponding to recombination frequencies (*p*) in coupling and repulsion phase, of between 0 and 0.3, the target length in centimorgans can be estimated by twice (both sides of the first T-DNA) the value given by the Kosambi formula (Kosambi 1944), *i.e.*, $2 \times 46 = 92$ cM. Such a linkage is therefore expected in ~18% of the transformants. The observed values are, respectively, 25.5% for *Emb506H* and 18% for *Ttd8H* (only 21 lines among 26 with a significant linkage have a recombination frequency in the range 0–0.3). Thus we consider these values to be in agreement with a random insertion hypothesis, although this does not take into account the positions of the first T-DNA insertions (*Ttd8*, chromosome *I*, 95 cM, S. Bonhomme, personal communication; *Emb506*, chromosome *V*, 72 cM, Albert *et al.* 1999).

Only one bias in these linkage analyses was observed. It concerns an abnormally high number of tight linkages (no detected recombination) in coupling in both lines, *Emb506H* and *Ttd8H*. One interpretation that remains to be tested by the physical mapping of some H^R inser-

tions is rearrangements of the chromosome in the region including both T-DNAs. These rearrangements have already been observed in transformants obtained with the same *in planta* transformation method (Nacry *et al.* 1998; Laufs *et al.* 1999). These rearrangements, when they result in inversions, could act as suppressors of crossing over and give an overrepresentation of linkage in coupling phase without any detectable meiotic recombination between both markers. Another possibility could be pairing between the two T-DNAs that share DNA sequences in common (35S, nos promoters, *nptII* . . .), leading to "targeted" insertions. This hypothesis seems highly unlikely considering the very low frequency of homologous recombination in plant cells (Masson and Paszkowski 1997).

The large predominance of coupling linkage observed in mutants where the first T-DNA is not transmitted by the male gamete points to the female chromosome set as the genetic target: Among 25 transformants where H^R and B^R markers are linked, 15 are in coupling and 10 are in repulsion in the *Emb506H* population. These numbers are similar and only the internal distribution is different with several transformants showing 0% recombination in coupling phase (see above). On the contrary, almost all linkages (25 out of 26) are in coupling phase in the *Ttd8H* population.

As explained previously, one can conclude from these results that the genetic target is preferentially the female chromosome set. The exception, in which both insertions are linked in repulsion, can be explained in one of two ways. First it could result from two simultaneous events: the rare (0.46%) transmission of the B^R marker through the male gamete and the insertion of the H^R T-DNA into the homologous female chromosome (17%). The probability of this event appears too low (8.10^{-4}) to have occurred once among 154 transformants (91.10^{-4}). Alternatively, one can imagine that the T-DNA can integrate into the male chromosome set in a few instances (one in 26) although it preferentially integrates into the female chromosome set. This situation is conceivable if the target cell is the zygote, where after entering, the male nucleus has more condensed chromatin than the egg cell nucleus until karyogamy and would be therefore less accessible for the T-DNA (Faure *et al.* 1993), or even an embryonic cell that ultimately participates in the formation of the inflorescences of a mosaic plant as suggested by Ye *et al.* (1999).

The egg-cell hypothesis: The high variability observed in transformation frequency among plants and fruits produced in homogeneous conditions indicates that unknown factors affect the response of individual plants and individual flowers. These variations may result from differences in the amount and viability of the bacterial population, the defense reaction of the plant, the presence and local concentration of virulence-inducing substances, etc. The per-silique heterogeneity is particularly striking, keeping in mind that each transformant results from a unique transformation event, even in the cases where several are obtained in the same fruit (N. Bechtold, unpublished data). These observations show that each ovary is a particular environment where multiple transformation events are possible.

We observed that *in planta* transformation can be carried out by applying the Agrobacterium suspension externally, as a spray. In this case transformation occurs at a very low, but reproducible, frequency. This may indicate that a possibility exists for the bacteria to enter plant tissues and reach their targets via a "natural" pathway. The bacteria could passively use the pathway of the pollen tube to enter the ovary since it has been observed that inert latex particles (6 μm) applied on a stigma can spontaneously reach the ovules in different species such as *Vicia faba*, *Hemerocallis flava*, and *Raphanus raphanistrum* (Sanders and Lord 1989). One can imagine that bacteria enter the egg cell along with the male nucleus and deliver their T-DNA inside the cell, a possibility that has been previously demonstrated experimentally by microinjecting Agrobacterium into mesophyll cells (Escudero *et al.* 1995). In this case, Agrobacterium mutants impaired in cell wall attachment functions (Douglas *et al.* 1982; Matthysse 1987; Thomashow *et al.* 1987) should be able to transform by infiltration.

The transfer of the T-DNA implies in any case the induction of *vir* genes. Pollen tube growth in the transmitting tissue of the style toward the ovules is associated with enzymatic activities for the degradation of cell wall carbohydrates and acidic polysaccharides contained in the extracellular matrix of the central septum (Wu *et al.* 1995; Lennon *et al.* 1998). Small molecules are produced (amino acids, sugars, organic acids) that participate in pollen tube growth and guidance (Wilhelmi and Preuss 1997; Lush *et al.* 1998). They may also produce favorable conditions for attraction of the bacteria and *vir* gene induction (Ankenbauer and Nester 1990; Cangelosi *et al.* 1990; Shimoda *et al.* 1990).

The *in planta* transformation method was first described in Arabidopsis. The results presented here demonstrate that the T-DNA is transferred to the maternal chromosome set and indicate that this transfer could occur in the ultimate stages of the development of the female organs. The physiological mechanisms on which this T-DNA transfer depends remain to be explored by other approaches and eventually with another model plant to extend this method to a wider range of species.

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