Theor Appl Genet (2000) 100:461–470

Z. Yin · G.-L. Wang

Evidence of multiple complex patterns of T-DNA integration into the rice genome

Received: 22 April 1999 / Accepted: 22 June 1999

Abstract The transfer of the long T-DNA (T-DNA and non-T-DNA) of a binary plasmid from *Agrobacterium* into the rice genome was investigated at both molecular and genetic levels. Out of 226 independent transgenic plants, 33% of the transformants contained non-T-DNA sequences. There was no major difference in the frequency of non-T-DNA transfer among three *Agrobacterium tumefaciens* strains. Four T1 plants containing a single putative long T-DNA insertion were selected for Southern analysis. Three of them were confirmed to have a long T-DNA insertion with a size of greater-than-unit-length of the binary plasmid. This was further confirmed by rescuing the intact binary plasmid from these plants. Our results suggest that long T-DNA transfer by rolling-circle replication from *Agrobacterium* to rice occurs frequently, and that the high frequency of non-T-DNA transfer should be considered when producing transgenic rice for commercial production.

Key words *Agrobacterium tumefaciens* · T-DNA · Non-T-DNA · Long T-DNA transfer · Rice transformation

Introduction

Agrobacterium tumefaciens can transfer part of its tumor-inducing (Ti) plasmid, the T-DNA, to plant genomes during tumorigenesis (reviewed by Zupan et al. 1995; Sheng et al. 1996). T-DNA transfer is mainly mediated by the products of the *Agrobacterium vir* (virulence) genes. The two products of the *virD* locus, VirD1 and VirD2, are thought to function together as an endonuclease that generates a nick between nucleotides 3 and 4 of the bottom strand of the T-DNA borders (Wang et al. 1987). Following cleavage, VirD2 covalently associates with the 5′ end of the T-DNA at the right-border (RB) nick and presumably acts as a "pilot protein" to lead the single-stranded T-DNA-protein complex, designated as the T-strand, from the bacterial cell to the plant cell (Ward and Barnes 1988; Durrenberger et al. 1989). VirD2 also covalently associates with the 5′ end of the remaining bottom strand of the Ti plasmid at the left-border (LB) nick (Durrenberger et al. 1989).

Although T-DNA transfer has been assumed to only involve DNA sequences between the RB and the LB, evidence from earlier work suggests that DNA sequences residing outside the T-DNA (non-T-DNA) may also be occasionally transferred to the plant (Ooms et al. 1982). Non-T-DNA transfer from both wild-type Ti plasmids and binary plasmids may occur frequently in dicotyledonous plants, without species specificity (Ooms et al. 1982; Joos et al. 1983; Virts and Gelvin 1985; Martineau et al. 1994; Ramanathan and Veluthambi 1995; Kononov et al. 1997; Van der Graaff et al. 1997; Wenck et al. 1997). Non-T-DNA sequences are transferred to the plant either independently of the T-DNA, or linked to the T-DNA across either the LB or the RB (Kononov et al. 1997). Long T-DNA transfer, sometimes with a greater-than-unit-length binary plasmid, has been detected in transgenic *Arabidopsis thaliana* plants (Wenck et al. 1997).

Agrobacterium-mediated transformation has been one of the most widely used methods for producing transgenic plants in many dicotyledonous species. However, monocotyledonous plants, in particular cereal crops, were recalcitrant to transformation by *A. tumefaciens* until the 1990s. Fertile transgenic rice was first obtained via *Agrobacterium*-mediated transformation at a low efficiency from immature embryos of a japonica cultivar (Chan et al. 1993). Transgenic rice plants were efficient-

Communicated by R. Nelson

Z. Yin · G.-L. Wang (✉)
Institute of Molecular Agrobiology, 1 Research Link,
The National University of Singapore, Singapore 117604,
Republic of Singapore
e-mail: wanggl@ima.org.sg
Fax: 65-872 7007

Present address: G.-L. Wang,
Department of Plant Pathology, The Ohio State University,
Kottman Hall, 2021 Coffey Road, Columbus,
Ohio 43210-1087, USA
Fax (614) 292 4455

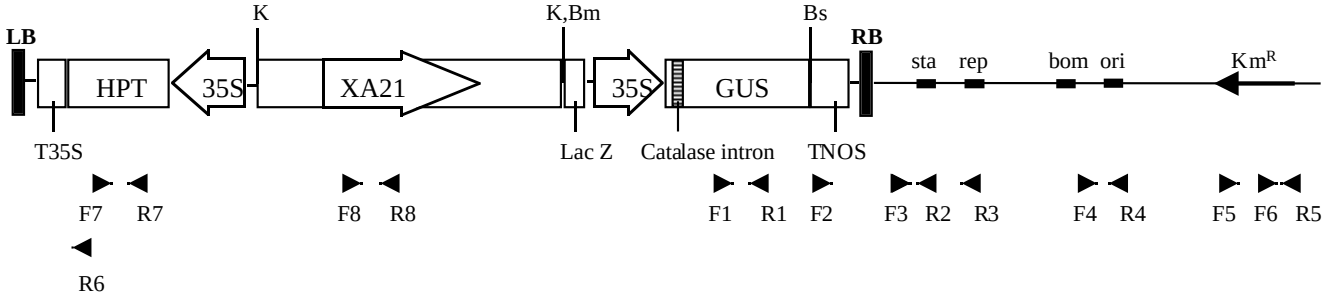

Fig. 1 Diagram of the binary plasmid pCXa21K (not drawn to scale). The lower part of the figure shows the PCR primers listed in Table 1 and the arrowheads indicate the direction of the primers from 5′ to 3′; *LB* left border; *RB* right border; *T35S* CaMV35S promoter; *HPT*, hygromycin phosphotransferase; *XA21* the product of the bacterial blight resistance gene *Xa21*; *Lac Z* β-galactosidase α fragment; *GUS* β-glucuronidase; *TNOS* 3′ signal of nopaline synthase; *sta* and *rep sta* and *rep* regions of pVS1; *bom* and *ori bom* and *ori* regions of pBR322; *Km*^{R} kanamycin-resistance gene active in bacteria; *K Kpn*I; *Bm Bam*HI; *Bs Bst*EII

ly produced from scutella-derived calli of japonica (Hiei et al. 1994), javanica (Dong et al. 1996) and indica (Rashida et al. 1996) rice, and from immature embryos of japonica and indica rice (Aldemita et al. 1996). Isolated shoot apices were transformed, though at a lower frequency (Park et al. 1996). *Agrobacterium*-mediated transformation has also been used to study the tapetum-specific expression of the promoter of the *Osg6B* gene in rice (Yokoi et al. 1997). Recently, efficient *Agrobacterium*-mediated transformation of maize (Ishida et al. 1996), barley (Tingay et al. 1997) and wheat (Cheng et al. 1997) has also been reported. However, these studies focused only on the transfer of the T-DNA region to these plants. Here, we provide evidence that there is a high frequency of non-T-DNA transfer from *Agrobacterium* to rice. The transfer of a greater-than-unit-length binary vector may result from the processing of the T-strand by rolling-circle replication, with initiation from either the RB or the LB. The implication of these findings for engineering transgenic rice plants is discussed.

Materials and methods

A. tumefaciens strains and the binary plasmids

Three *A. tumefaciens* strains, EHA105 (Hood et al. 1993), AGL1 (Lazo et al. 1991) and LBA4404 (Hoekema et al. 1983), were used in this study. LBA4404 contains octopine-type *vir* genes on the helper Ti plasmid, while AGL1 and EHA105 harbor agropine-type "super" *vir* genes on their helper Ti plasmids. *A. tumefaciens* was grown on AB medium (Chilton et al. 1974) at 28°C.

The binary plasmid pCXa21K (21.7 kb) (Fig. 1) was constructed by inserting the 9.9 kb *Kpn*I fragment of pB822 (kindly provided by P. Ronald, University of California, Davis) containing the *Xa21* gene (Song et al. 1995) into the binary vector pCAMBIA1301 (11.8 kb) (R. Jefferson, CAMBIA, Australia). The cloned *Xa21* gene was used as the test transgene since it confers resistance to *Xanthomonas oryzae* pv *oryzae (Xoo)* and has been used in transforming both japonica and indica rice (Wang et al. 1996; Tu et al. 1998; Zhang et al. 1998). pCAMBIA1301 carries a hygromycin-resistance gene (*hpt*) and a reporter gene (*intron-gus*). pCXa21K was introduced into the three *A. tumefaciens* strains by electroporation (Sambrook et al. 1989).

Rice transformation

Vigorously growing embryogenic calli derived from the scutellum of mature embryos of japonica rice cultivar Taipei 309 were inoculated and co-cultured with *A. tumefaciens* strains as previously described (Hiei et al. 1994). After co-culture, the rice tissues were cultured on the NB_0 medium (Li et al. 1993) containing 250 mg/l of cefotaxime, 200 mg/l of ampicillin, 2 mg/l of 2,4-D and 50 mg/l of hygromycin at 26°C in the dark for 3–4 weeks. Hygromycin-resistant calli were subcultured on fresh selection medium for 2 weeks and then transferred to NB_0 medium containing 1 mg/l of 6-BA, 2 mg/l of NAA, 5 mg/l of ABA and 50 mg/l of hygromycin for 3 weeks. Compact, white embryogenic calli showing hygromycin resistance were transferred to the NB_0 medium containing 2 mg/l of 6-BA, 1 mg/l of IAA, 1 mg/l of NAA, 1 mg/l of KT and 50 mg/l of hygromycin and regenerated at 26°C with a 14-h light (about 2000 lx) and a 10-h dark period. Regenerated plantlets were subsequently transplanted to the soil in pots and grown in a greenhouse.

PCR primers and reaction

PCR primers used for screening transgenic plants are listed in Table 1 and the positions of these primers are shown in Fig. 1. Three primer pairs (gus-F/gus-R, Xa21-F/Xa21-R and hpt-F/hpt-R) were designed for the detection of three genes, *gus*, *Xa21* and *hpt*, respectively; two primer pairs (RBF-F/RBF-R and LBF-F/LBF-R) for the T-DNA borders and their flanking sequences on either side; two primer pairs (RBA-F/RBA-R and LBA-F/LBA-R) for the sequences adjacent to the T-DNA borders in the non-T-DNA region; and one primer pair (VB-F/VB-R) for the pVS1 replication region in the non-T-DNA region of pCAMBIA1301, designated as the vector "backbone" (VB) region. The reaction mix (25 µl) for PCR consisted of 10 ng of rice genomic DNA, 0.25 mM each of dNTPs, 50 ng of each PCR primer, 2.5 µl of 10×PCR buffer (100 mM Tris-HCl, pH 8.8 at 25°C, 15 mM $MgCl_2$, 500 mM KCl and 0.1% Triton X-100) and 0.5 units of *Taq* DNA polymerase (Finnzymes OY, Finland). Thermal cycling was done at 94°C for 4 min followed by 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1 min. After the final cycle, the reactions were maintained at 72°C for 5 min before completion. Reactions were conducted using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., USA).

Southern analysis and histochemical staining

Two to five micrograms of rice genomic DNA were digested with an appropriate restriction enzyme and fractionated in a 0.65% agarose gel by electrophoresis. Southern-blot hybridization was carried out using standard procedures (Sambrook et al. 1989). The probes used for the detection of three transgenes, i.e. *hpt*, *Xa21* and *gus*, in the T-DNA region, the sequences adjacent to the T-DNA borders and the pVS1 replication sequence in the non-T-DNA region were the PCR-products amplified from pCXa21K using the primers listed in Table 1.

Table 1 PCR primers used in this study

Primer	Primer sequence (5′–3′)	Nucleotide position on pCXa21K
gus-F[a] (F1)	GGTGGGAAAGCGCGTTACAAG	307–327 bp
gus-R (R1)	GTTTACGCGTTGCTTCCGCCA	1,486–1,506 bp
RBF[b]-F (F2)	AACAGGTATGGAATTTCGCC	1,840–1,859 bp
RBF-R (R2)	GCGTAGGTGGTCAAGCATC	2,844–2,863 bp
RBA[c]-F (F3)	TAAAAGGGCGTGAAAAGGTT	2,407–2,426 bp
RBA-R (R3)	TTGTTCCTCTTGGCGTTCAT	3,345–3,364 bp
VB[d]-F (F4)	TGGCCGTTTTCCGTCTGTCG	5,018–5,037 bp
VB-R (R4)	GAAGCGGCGGGGCGTAGG	6,123–6,140 bp
LBA[e]-F (F5)	TCCGCCCTGCCGCTTCTC	7,552–7,569 bp
LBA-R (R5)	GCCAACAGCTCCCCGACC	8,599–8,615 bp
LBF[f]-F (F6)	TGTCCTTTTCCCGTTCC	8,011–8,028 bp
LBF-R (R6)	GATGGCTGTGTAGAAGTA	8,993–9,010 bp
hpt-F (F7)	TACTTCTACACAGCCATC	8,993–9,010 bp
htp-R (R7)	TATGTCCTGCGGGTAAAT	9,812–9,829 bp
Xa21-F (F8)	ATAGCAACTGATTGCTTGG	16,463–16,481 bp
Xa21-R (R8)	CGATCGGTATAACAGCAAAAC	17,819–17,839 bp

[a] gus-F, forward primer for amplifying the *gus* gene fragment; *hpt*, *hpt* gene fragment; Xa21, *Xa21* gene fragment; F, forward primer; R, reverse primer
[b] RBF, T-DNA right border and its flanking sequences on both sides
[c] RBA, the sequence adjacent to the right border in the non-T-DNA region
[d] VB, the pVS1 replication region in the non-T-DNA region of pCAMBIA1301
[e] LBA, the sequence adjacent to the left border in the non-T-DNA region
[f] LBF, T-DNA left border and its flanking sequences on both sides

GUS activity was assayed according to procedures previously described (Hiei et al. 1994).

Inoculation of rice plants with *Xoo*

Eight-week-old rice plants were inoculated with *Xoo* strain PXO99 (race 6) (provided by H. Leung, IRRI) using a bacterial suspension with a density of 0.5 at D_{600}. At least three fully expanded leaves of a single plant were inoculated using the leaf-clipping method (Kauffman et al. 1973). Inoculated plants were maintained in a greenhouse and the lesion length was measured 2 weeks after inoculation.

Plasmid rescue and DNA-sequence analysis

Plasmid rescue was performed by digesting 1 μg of genomic DNA from transgenic T1 plants with *Bam*HI or *Bst*EII and ligating with *T4* DNA ligase (Boehringer Mannheim, Germany) at 15°C overnight, followed by electroporation into *Escherichia coli* cells (DH10B, Gibco BRL, USA). Transformants were selected for resistance to both hygromycin and kanamycin. One microgram of the rescued plasmid was digested with *Bam*HI, *Bst*EII, *Kpn*I, *Pst*I or *Eco*RI and separated in an 0.8% agarose gel. Southern hybridization was carried out using pCXa21K as a probe. The rescued plasmids were sequenced for DNA sequences flanking the digestion sites of *Bam*HI or *Bst*EII using the ABI PRISM 377 DNA sequencer (Perkin-Elmer, USA). The DNA sequence was analyzed with software Sequencher 3.0 (Genecode, USA).

Genetic analysis

Selfed seeds (T1 generation) of the transformants were surface-sterilized and tested for resistance to hygromycin at a concentration of 70 mg/l as previously described (Hiei et al. 1994). Drug resistance was scored and GUS activity was assayed 10 days after sowing.

Results

Production of transgenic plants

A total of 2,633 independently transformed plants were obtained from co-culture transformation of scutellum-derived calli with three *A. tumefaciens* strains containing pCXa21K. Two hundred and twenty six plants were randomly chosen for planting in a green house. Some morphological aberrations were observed in a few plants, such as a dwarf plant type and white-striped leaves. Twenty five plants (11.0%) produced no seeds, 58 plants (25.7%) showed partial fertility, and 143 plants (63.3%) had full fertility. Fertility of some of the partially fertile T0 plants was fully recovered in the T1 generation. It is not clear whether these phenotypic aberrations were due to tissue culture or to T-DNA insertion in these plants.

All 226 hygromycin-resistant T0 plants were detected for GUS activity and resistance to *Xoo* strain PXO99 (Table 2). Among the 184 *gus*-PCR-positive T0 plants, 171 (92.9%) were positive for GUS activity while the remaining 13 (7.1%) were not stained blue with the substrate X-Gluc. Some of the GUS-positive plants only showed a few blue spots on leaves when stained with X-Gluc (data not shown). After inoculation with the bacterial blight pathogen, 133 (69.6%) of the 191 *Xa21*-PCR-positive T0 plants showed a high level of resistance to *Xoo* strain PXO99 (lesion length <10.0 cm), 37 plants (19.4%) showed partial resistance (10 cm ≤lesion length ≤20 cm), and 21 plants (11.0%) were susceptible (lesion length >20.0 cm). All of the *hpt*-PCR-positive T0 plants showed strong resistance against hygromycin selection since the *hpt* gene was used as the selection marker. One hundred and seventy one plants (75.7%) showed PCR-

positive results for all three transgenes, among which 114 plants (66.7%) were strongly resistant to bacterial blight infection and were also positive for GUS expression.

PCR and Southern analyses of the transferred DNA in transgenic T0 plants

To investigate T-DNA insertion in transgenic rice, the 226 transgenic T0 plants were screened by PCR using six primer pairs that were designed based on the DNA sequence of the T-DNA or non-T-DNA regions (see Table 1 and Fig. 1). All the hygromycin-resistant plants showed specific amplified bands of the *hpt* gene (Table 3, column 3). Based on the presence or absence of the non-T-DNA sequences, the 226 plants could be divided into two major groups: group I (patterns 1–4) with only T-DNA insertion and group II (patterns 5–18) with both T-DNA and non-T-DNA insertions. In group I, plants with pattern 1 carried all three transgenes in the T-DNA region and could therefore be considered to have the intact T-DNA insertion in a normal manner (Table 3, pattern 1). In other plants, only one (Table 3, pattern 2) or two (Table 3, patterns 3 and 4) transgenes in the T-DNA region could be detected, suggesting that these plants carried a truncated fragment of the T-DNA. The absence of the *Xa21* gene in the plants of pattern 4 might be due to the multiple insertion of the truncated T-DNAs, rather than the deletion of the *Xa21* gene within a single T-DNA, as the segregation of the *hpt* gene with the *gus* gene was detected in the progeny (T1 generation) of these plants (data not shown). The transfer of the pCXa21K non-T-DNA sequence to rice in group II was found in 75 transgenic plants (33.2%, Table 3, patterns 5–18), either linked to the T-DNA across the left border (Table 3, patterns 5–7), the right border (Table 3, patterns 8–11), both of the borders (Table 3, patterns 12–15), or independently of the T-DNA (Table 3, patterns 16–18). In group II, both the T-DNA and the non-T-DNA could be truncated. The frequency of non-T-DNA transfer in rice is considerably lower than that reported for *Nicotiana tabacum* (75%, Kononov et al. 1997) and *A. thaliana* (62%, Wenk et al. 1997). Similar to the findings of a previous study on tobacco (Kononov et al. 1997), statistical analysis showed that there were no major differences in the transfer of the non-T-DNA region from *Agrobacterium* to rice among the three *A. tumefaciens* strains tested (Table 4).

Table 2 Transgene expression in T0 plants

Gene expression	Plants	%
Resistance to hygromycin		
hpt-PCR-positive	226	
Resistant	226	100
GUS activity		
gus-PCR-positive	184	
Positive	171	92.9
Negative	13	7.1
Resistance to *Xoo*[a]		
Xa21-PCR-positive	191	
Resistant	133	69.6
Partial resistant	37	19.4
Susceptible	21	11.0

[a] Resistant, lesion length <10 cm; partial resistant, 10 cm ≤lesion length ≤20 cm; susceptible, lesion length >20 cm

Table 3 PCR screening of the transferred DNA in transgenic rice T0 plants

Pattern	LBF[a]	*hpt*	*Xa21*	*gus*	RBF	VB	Plants	(%)
Control[b]	–	–	–	–	–	–		
Group I								
1	–	+	+	+	–	–	109	(48.2)
2	–	+	–	–	–	–	19	(8.4)
3	–	+	+	–	–	–	13	(5.8)
4	–	+	–	+	–	–	10	(4.4)
Group II								
5	+	+	+	+	–	+	1	(0.4)
6	+	+	+	–	–	–	1	(0.4)
7	+	+	–	–	–	+	2	(0.9)
8	–	+	+	+	+	–	1	(0.4)
9	–	+	+	+	+	+	15	(6.6)
10	–	+	+	–	+	+	2	(0.9)
11	–	+	–	–	+	–	1	(0.4)
12	+	+	+	+	+	–	1	(0.4)
13	+	+	+	+	+	+	33	(14.6)
14	+	+	+	–	+	+	2	(0.9)
15	+	+	–	+	+	+	2	(0.9)
16	–	+	+	+	–	+	11	(4.9)
17	–	+	+	–	–	+	2	(0.9)
18	–	+	–	+	–	+	1	(0.4)
Total plants							226	

[a] See corresponding abbreviations in Table 1
[b] Untransformed Taipei 309

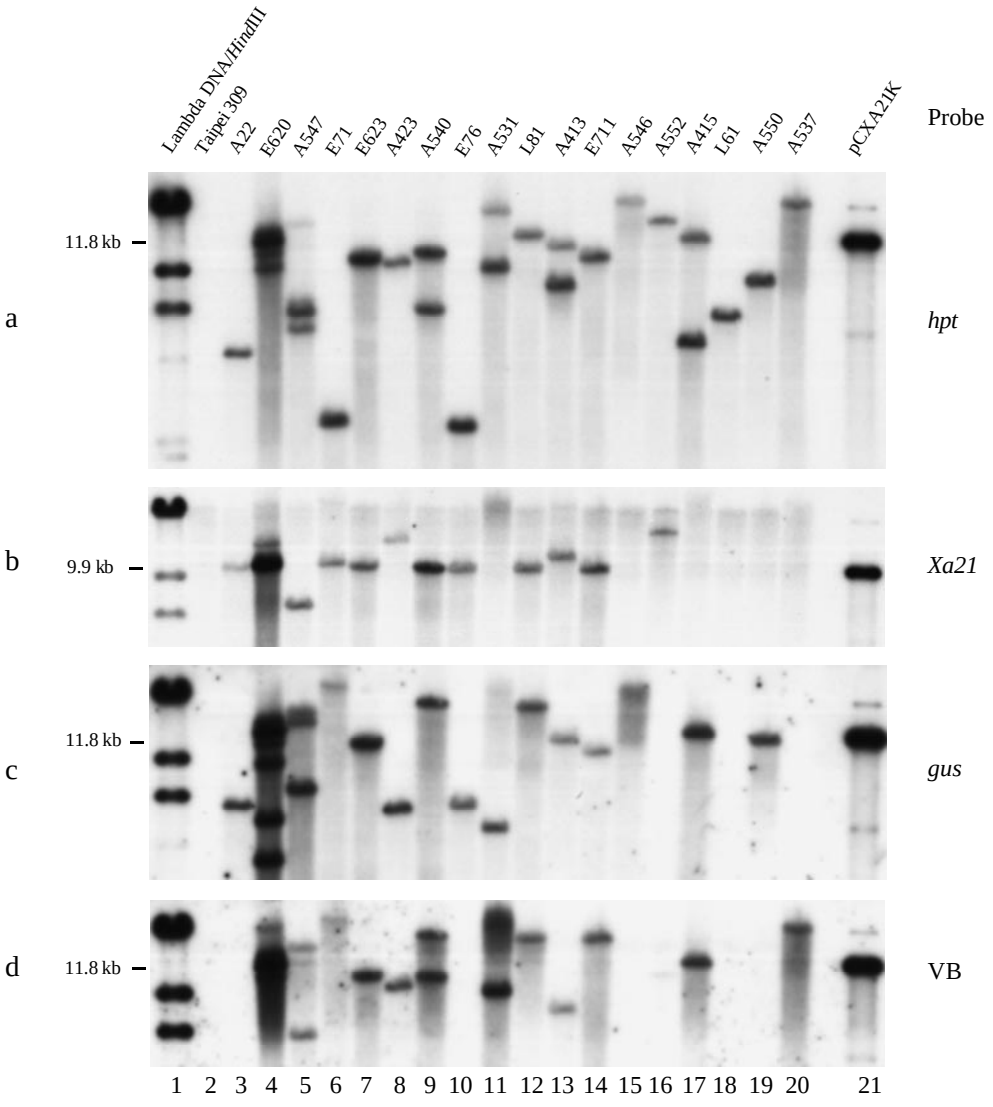

Fig. 2a–d Southern-blot analysis of T0 plants. Two to five micrograms of DNA from a non-transgenic plant (Taipei 309, *lane 2*), transformants (*lanes 3–20*) and 1 ng of pCXa21K were digested with *Kpn*I, fractionated by electrophoresis, transferred to a nylon membrane, and probed with the *hpt* (panel **a**), *Xa21* (panel **b**), *gus* (panel **c**) or VB (panel **d**) probes. *Lane 1* is the lambda-DNA marker digested with *Hind*III

Table 4 Percentage of transgenic plants with a non-T-DNA insertion transformed by three *A. tumefaciens* strains harboring pCXa21K

Strain	Total plants	Plants with non-T-DNA (%)
AGL1	145	47 (32.4)
EHA105	68	24 (35.3)
LBA4404	13	4 (30.8)

The integration of T-DNA and non-T-DNA sequences was also confirmed by Southern analysis of transgenic T0 plants using *hpt*, *Xa21*, *gus* and VB sequences as probes (Fig. 2). Southern analysis with the *hpt* probe showed that 12 of the 18 (66.7%) transgenic plants analyzed contained a single T-DNA insertion. Six plants (33.3%) carried two or more copies of the T-DNA insertion. Eight plants contained the 9.9-kb *Xa21* hybridizing band (Fig. 2, panel b, lanes 3, 4, 6, 7, 9, 10, 12 and 14), while ten plants showed no *Xa21* hybridizing band or else carried a smaller or larger fragment, probably due to DNA deletion or a rearrangement that occurred during the transfer or integration of the T-DNA (Fig. 2, panel b, lanes 5, 8, 11, 13 and 16). When the *gus* gene was used as a probe, only three plants had no hybridizing band. Twelve of the eighteen transgenic plants analyzed showed hybridization with VB, thus confirming the PCR results (Fig. 2, panel d).

Genetic analysis of transgenic plants with a putative long T-DNA insertion

To determine the inheritance pattern of the putative long T-DNA in transgenic plants, the T1 progeny of 26 transgenic plants (Table 3, pattern 13; the other seven T0 plants produced few or no seeds) were tested for resistance to hygromycin and for GUS expression (Table 5). All the plants with pattern 13 contain non-T-DNA sequences linked to both T-DNA borders. Most of them showed a single-gene segregation ratio of 3:1 for resistance to hygromycin (Table 5). In 13 plants, the resis-

Table 5 Genetic analysis of transgenes in transgenic rice with a long T-DNA insertion

Plant	$HYG^{R,a}$		HYG^{S}	Ratio	χ^2
	$GUS^{+,b}$	GUS^{-}			
A45	29	0	13	29:13	0.51
A418	38	0	11	38:11	0.06
A514	7	0	2	7:2	0.04
A520	39	0	15	39:15	0.10
A571	26	0	11	26:11	0.23
A611	5	0	4	5:4	0.93
A629	21	0	28	21:28	25.31*
A89	59	0	13	59:13	1.50
E510	9	0	4	9:4	0.03
E617	12	0	2	12:2	0.38
E74	53	0	14	53:14	0.40
E81	13	0	2	13:2	0.56
E82	26	0	11	26:11	0.23
A46	26	2	6	28:2	0.63
A573	21	3	2	24:2	3.28
A712	30	6	0	36:0	10.70*
E748	3	29	13	32:13	0.19
A519	0	0	45	0:45	131.00*
A543	0	0	18	0:18	50.00*
A566	0	0	44	0:44	128.00*
A72	0	0	27	0:27	77.05*
A817	0	0	1	0:1	0.33
E620	0	0	11	0:11	29.12*
E720	0	0	4	0:4	8.33*
E740	0	0	33	0:33	95.04*
L51	0	0	14	0:14	38.01*

* Significant at the 1% level
[a] HYG^{R}, hygromycin resistant; HYG^{S}, hygromycin sensitive
[b] GUS^{+}, GUS positive; GUS^{-}, GUS negative

tance to hygromycin co-segregated with GUS activity. Plants A46, A573, A712 and E748 showed segregation of the *hpt* gene and the *gus* gene, which may have resulted from multiple T-DNA insertions.

Interestingly, the T1 plants from nine plants (A519, A543, A566, A72, A817, E620, E720, E740 and L51) were sensitive to hygromycin, possibly due to the *hpt* gene silencing in the T1 plants but not in the T0 plants. To confirm whether the transgenes are still present in the progeny, PCR analysis was conducted. The results showed that all the three transgenes and the non-T-DNA sequences were still present in some of the T1 and T2 plants of A566, E740 and L51 (T1 seeds of A519, A543, A72, A817, E620 and E720 were not available for the analysis since all the seeds were used for the hygromycin selection test) (data not shown). Compared with the uniform blue staining for GUS activity assay in the original T0 plants, these plants showed blue spots on the leaves (data not shown). The progeny of A566, E740 and L51 were inoculated with the *Xoo* strain PXO99 and all the plants carrying the *Xa21* gene were highly resistant (data not shown), indicating that the *Xa21* gene was not silenced in these plants. The mechanism of *hpt* and *gus* gene silencing, but not that of the *Xa21* gene, remains to be further investigated.

Long T-DNA transfer with a size of greater-than-unit-length binary plasmid detected by Southern analysis

If long T-DNA contains a greater-than-unit-length binary plasmid, it will release the intact binary plasmid when digested with a restriction enzyme having a single site on the binary plasmid. In this study, four T1 plants (A45–9, A520–5, A89–4 and E74–9) probably carrying a single copy of a long T-DNA insertion were selected for Southern analysis. The DNA extracted from these plants was digested with *Bam*HI, which has only one site on pCXa21K (Fig. 1). Long T-DNAs in these plants should release a *Bam*HI fragment with the same size as pCXa21K (21.7 kb), which would be detected when probed with *hpt*, *Xa21*, *gus*, LBA, VB or RBA (Fig. 3, panels a–f, lane 2). It is expected that when both a 9.9-kb *Kpn*I fragment hybridizing with *Xa21* and an 11.8-kb *Kpn*I fragment hybridizing with the *hpt*, *gus*, LBA, VB or RBA probes should also be detected when the DNA from the transgenic plants is digested with *Kpn*I.

Southern analysis detected the 21.7 kb *Bam*HI fragment in plant A89–4 after probing with the T-DNA (*hpt*, *Xa21* and *gus*) or non-T-DNA (VB, LBA and RBA) probes (Fig. 3, panels a–f, lane 6). This 21.7-kb fragment should represent the contiguous piece of the (*Bam*HI)-*Xa21*-*hpt*-LB-LBA-VB-RBA-*gus*-(*Bam*HI) linkage fragment, which is equivalent to pCXa21K digested by *Bam*HI. A smaller *Kpn*I fragment with a size of 11.8 kb was detected for the contiguous piece of the (*Kpn*I)-*hpt*-LB-LBA-VB-RBA-*gus*- (*Kpn*I) linkage fragment (Fig. 3, panels a, c, d, e, and f, lane 7), and a 9.9-kb *Kpn*I fragment was detected with the *Xa21* probe (Fig. 3, panel b, lane 7). Two hybridizing bands were detected for each of the three transgenes, *hpt*, *Xa21*(excluding the endogenous *Xa21* homologue) and *gus* (Fig. 3, panels a to c, lane 6), indicating that there were two copies of the three transgenes in plant A89–4. As there was only one T-

DNA insertion in this plant (inferred from genetic analysis), the two copies of the three transgenes must come from a single long T-DNA, which presumably starts from the RB, circles through the T-DNA region, skips the LB and continues around to reach the non-T-DNA region, passes the RB and T-DNA again and terminates when reaching the second LB. The long T-DNA transfer would then include two complete copies of the T-DNA region and one copy of the non-T-DNA region (Fig. 4, panel a).

The long T-DNA insertion in plant E74–9 is different from that of plant A89–4. A large *Bam*HI hybridizing band with a size of more than 21.7 kb was detected with each of the probes (Fig. 3, panels a–f, lane 8), suggesting that this fragment could be the contiguous piece of the (*Bam*HI)-*Xa21*-*hpt*-LB-LBA-VB-RBA-RB-*gus*-rice DNA-(*Bam*HI) linkage fragment. A small *Kpn*I fragment with a size of about 13 kb (Fig. 3, panels a, c, d, e and f, lane 9), which could be the contiguous piece of the (*Kpn*I)-*hpt*-LB-LBA-VB-RBA-RB-*gus*-rice DNA-(*Kpn*I) linkage fragment, was detected by the *hpt*, *gus*, LBA, VB and RBA probes, and a 9.9-kb *Kpn*I fragment was detected by the *Xa21* probe (Fig. 3, panel b, lane 9). Another *Bam*HI fragment with a size of about 11 kb was detected with both *gus* and non-T-DNA probes (LBA, VB and RBA) (Fig. 3, panels c–f, lane 8). This fragment could be the contiguous piece of the (*Bam*HI)-rice DNA-LBA-VB-RBA-RB-*gus*–(*Bam*HI) linkage fragment. A relatively large *Kpn*I fragment (about 30 kb) hybridized with both the *gus* and non-T-DNA probes (LBA, VB and RBA) and could be the contiguous piece of the (*Kpn*I)-rice DNA-LBA-VB-RBA-RB-*gus*–(*Kpn*I) linkage fragment (Fig. 3, panels c–f, lane 9). Interestingly, only one copy of the *hpt* and *Xa21* genes (excluding the endogenous *Xa21* homologue) was detected (Fig. 3, panels a and b, lanes 8 and 9), while two copies of the *gus* gene and the non-T-DNA region (Fig. 3, panels c–f, lanes 8 and 9) were detected.

Based on the above results, it is possible that the long T-DNA transfer in the plant E74–9 started at the LB, included the non-T-DNA region and the RB, continued through the T-DNA and the LB, and again through the non-T-DNA region, the RB and *gus*, but terminated before reaching the *Bam*HI site of pCXa21K (Fig. 4, panel b). This conclusion was later confirmed when a predicted 21.7-kb fragment was detected in a *Bst*EII digest of DNA from the plant E74–9 (data not shown). *Bst*EII has only one site in pCXa21K between the *gus* coding region and *nos* terminator (Fig. 1).

A 21.7-kb *Bam*HI fragment hybridizing with probes from both T-DNA genes (*hpt*, *Xa21* and *gus*) and non-T-DNA regions (VB, LBA and RBA) was detected in plant A45–9 (Fig. 3, panels a–f, lane 10). An 11.8-kb *Kpn*I fragment hybridized with the *hpt*, *gus*, LBA, VB and RBA probes (Fig. 3, panels a, c, d, e and f, lane 11) and a 9.9-kb *Kpn*I fragment that hybridized with the *Xa21* probe (Fig. 3, panel b, lane 11) was also detected. Therefore, the 21.7-kb *Bam*HI fragment is considered to be the intact unit of pCXa21K. Another *Bam*HI fragment with a

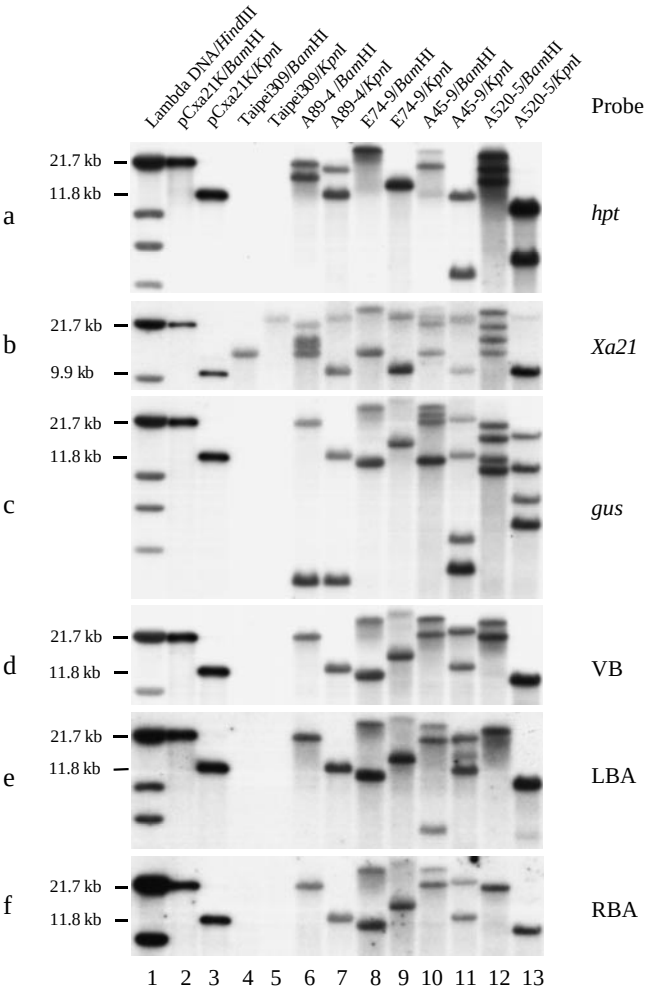

Fig. 3a–f Southern analysis of T1 plants. One nanogram of pCXa21K (lane 2 and lane 3) and 2–5 µg of DNA from a non-transgenic plant (*lane 4* and *lane 5*) and T1 plants of A89–4 (*lane 6* and *lane 7*), E74–9 (*lane 8* and *lane 9*), A45–9 (*lane 10* and *lane 11*) and A520–5 (*lane 12* and *lane 13*) were digested with *Bam*HI or *Kpn*I, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to *hpt* (panel **a**), *Xa21* (panel **b**), *gus* (panel **c**), VB (panel **d**), LBA (panel **e**) or RBA (panel **f**) probes. *Lane 1* is the lambda-DNA marker digested with *Hind*III

size of about 30 kb hybridized with both the non-T-DNA (LBA, VB and RBA) and *gus* probes (Fig. 3, panels c, d, e and f, lane 10). A *Kpn*I fragment with a size of about 24 kb was also detected using the same probes (Fig. 3, panels c, d, e and f, lane 11). The above 30-kb *Bam*HI fragment could represent the contiguous piece of the (*Bam*HI)-rice DNA-LBA-VB-RBA-*gus*-(*Bam*HI) linkage fragment. A third *Bam*HI fragment with a size of about 25 kb was detected with the probes of both *Xa21* and *hpt* (Fig. 3, panels a and b, lane 10). A relatively small *Kpn*I fragment (4.6 kb) hybridizing only with *hpt* was also detected (Fig. 3, panel a, lane 11). The third *Bam*HI fragment (25 kb) is thus proposed to be the contiguous piece of the (*Bam*HI)-*Xa21*-*hpt*-rice DNA-(*Bam*HI) linkage fragment. Based on the results from ge-

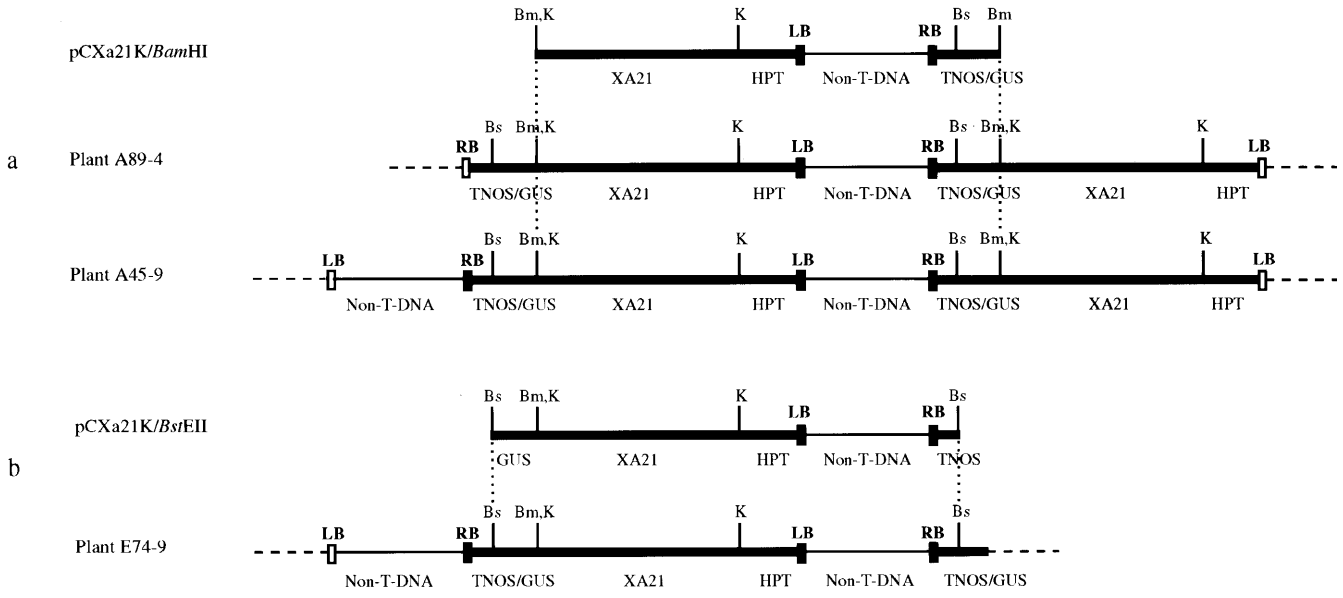

Fig. 4a, b Schematic map of long T-DNA insertions in three transgenic plants and the rescued fragments (not drawn to scale). The region between the *vertical dotted lines* represents the *Bam*HI or *Bst*EII fragment of the intact pCXa21K which was rescued from T1 plants A89–4 and A45–9 (panel **a**) or E74–9 (panel **b**). Linear pCXa21K digested with *Bam*HI (panel **a**) or *Bst*EII (panel **b**) is shown above the corresponding map position of the long T-DNAs. The *bold lines* represent the T-DNA region and the *thin lines* represent the non-T-DNA region. The *dashed lines* represent rice genomic DNA. The *solid rectangles* represent the intact T-DNA borders and the *open rectangles* represent the truncated T-DNA borders due to nick generation inside the border sequences during T-DNA processing. *LB* left border; *RB* right border; *TNOS* 3′ signal of nopaline synthase; *T35S* CaMV35S promoter; *HPT* hygromycin phosphotransferase; *XA21* the product of the bacterial blight resistance gene *Xa21*; *GUS* β-glucuronidase; *Non-T-DNA* non-T-DNA region of pCXa21K; *K Kpn*I; *Bm Bam*HI; *Bs Bst*EII

netic analysis, these three *Bam*HI fragments are assumed to have resulted from a long T-DNA insertion which started from the LB, passed the pCXa21 K first from the non-T-DNA region, then the T-DNA region for nearly two units, and terminated when reaching the LB again (Fig. 4, panel a).

The insertion of T-DNA in plant A520–5 was more complicated. Southern analysis detected three copies of the *hpt* gene (Fig. 3, panel a, lane 12) and four copies of the *Xa21* and *gus* genes (Fig. 3, panels b and c, lane 12). One of the copies (about 30 kb) is the contiguous piece of the (*Bam*HI)-*Xa21*-*hpt*-LBA-VB-rice DNA-(*Bam*HI) linkage fragment (Fig. 3, panels a, b, d and e, lane 12). Another *Bam*HI fragment with a size of 21.0 kb detected by the *gus*, RBA and VB probes could be the contiguous piece of the *gus* fragment linked to the RB flanking sequence (Fig. 3, panels c, d, and f, lane 12). All the T-DNAs contained the intact *Xa21* gene with a size of 9.9 kb for the *Kpn*I fragment (Fig. 3, panel b, lane 13). But none of the T-DNAs carried a fragment with a size greater-than-unit-length of pCXa21K.

In summary, we have evidence showing that the transfer of long T-DNA with a size of greater-than-unit-length of plasmid pCXa21K presumably results from the processing of T-DNA in a rolling-circle replication manner (Erickson and Meyer 1993). Such transfer was found in three of the four transgenic plants analyzed. Long T-DNA transfer can initiate from either of the T-DNA borders and terminate by either reaching the T-DNA borders, or elsewhere, on the binary plasmid during T-DNA processing.

Plasmid rescue from the transgenic T1 plants with a long T-DNA insertion

The long T-DNA insertion with a greater-than-unit-length of the binary plasmid in the three transgenic plants was confirmed by rescuing the intact pCXa21K plasmid from the genomic DNAs. DNA from plants A89–4 and A45–9 was digested with *Bam*HI, and DNA from the plant E74–9 with *Bst*EII. The digested DNA was self-ligated, and plasmids were subsequently rescued from the transformants which were resistant to both kanamycin and hygromycin. Southern analysis of three rescued plasmids showed the same hybridization pattern as that of pCXa21K after digestion with five restriction enzymes (*Bam*HI, *Bst*EII, *Kpn*I, *Pst*I and *Eco*RI, data not shown). The rescued plasmids were sequenced across the junctions of the *Bam*HI or *Bst*EII sites. From plants A89–4 and A45–9, 569 bp and 560 bp were sequenced respectively for the rescued plasmids. These two DNA sequences match perfectly with the region 20,535–21,104 bp or 20,535–21,095 bp on pCXa21K (including the region of the *Bam*HI site at 20,950 bp), respectively. Similarly, 769 bp were sequenced for the plasmid rescued from plant E74–9. The sequence matched perfectly the 1880–2649 bp region on pCXa21 K, which includes the *Bst*EII site at 2049 bp and the right border from 2364 to 2389 bp. No ”filler” DNA was found in the flanking region of the *Bam*HI site or the *Bst*EII site on the rescued

plasmids. These results suggested that the rescued plasmids are the intact pCXa21K portions of the long T-DNA. These results also support the Southern analysis and confirm that a long T-DNA transfer of greater-than-unit-length pCXa21K occurred in all three plants tested.

Discussion

In this study, we investigated the transfer of long T-DNA with a greater-than-unit-length binary plasmid from *Agrobacterium* to rice using the following four approaches: (1) PCR-screening of transgenic plants with putative long T-DNA insertion; (2) looking for plants with a single putative long T-DNA insertion by genetic analysis; (3) confirming the presence of the long T-DNA with a greater-than-unit-length binary plasmid in these plants by Southern-blot analysis; (4) re-confirming the long T-DNA insertion by plasmid rescuing and DNA sequencing. We characterized the T-DNA insertion patterns in four T1 plants and found three of them (A89–4, E74–9 and A45–9) to carry a single copy of long T-DNA with a greater-than-unit-length binary plasmid. Another T1 plant (A520–5) was identified carrying four copies of the truncated T-DNAs, but none of them had the size of a greater-than-unit-length binary plasmid. Our results clearly demonstrated that the transfer of long T-DNA occurs at a high frequency from *Agrobacterium* to rice.

The long T-DNA transfer does not seem to have resulted from the mutation of T-DNA borders, as 48.2% of the transformants still had normal T-DNA insertions (Table 3). As mentioned in a previous study (Wenk et al. 1997), one of the possible reasons for the transfer of long T-DNA may be inefficient nicking or insufficient Vir proteins for the binary system. For normal T-DNA transfer in a binary plasmid system, VirD2 needs to be in a 1:1 ratio with the T-DNA borders, and thus two molecules of VirD2 are needed in a single bacterial cell. According to this hypothesis, one VirD2 molecule tightly associates with the 5′ end of the T-DNA after nicking its right border and then pilots the T-strand from the bacterial cell to the plant cell. The second VirD2 molecule tightly associates with the 5′ end of the non-T-DNA portion of the binary plasmid after nicking of the left border and rejoins with the 3′ end of the replacement strand of the T-DNA, generated by rolling-circle replication after the release of the T-strand (Lanka and Wilkins 1995). Indeed, VirD2 was also found to tightly associate with the 5′ end of the non-T-DNA portion of the Ti plasmid after it cleaves at the LB (Durrenberger et al. 1989) and shown was to participate in ligating the LB nick (Pansegrau et al. 1993). If there is only one VirD2 molecule, or if there are fewer VirD2 molecules than T-DNA borders due to more copies of the binary plasmid than of the helper plasmid in a single bacterial cell (Gallie et al. 1985; Hajdukiewicz et al. 1994), then T-DNA processing will skip the LB (or the RB if the T-DNA processing initiates from the LB). Thus, both the T-DNA and non-T-DNA link with each other and become a new contiguous long T-DNA. Investigations with bacterial conjugation show that a greater-than-unit-length T-strand is seldom found. Erickson and Meyer (1993) constructed plasmids containing two mutated *oriT*s to prevent initiation and termination of transfer at the same *oriT* and found that almost all the transconjugant cells contained greater-than-unit-length plasmids, generated by a rolling-circle mechanism of DNA replication.

Truncated T-DNA transfer was observed in some transformed plants, with frequencies of between 1 and 40% (Herman et al. 1990). Deletion can occur either at one or both the ends of T-DNA insert. In the present study, we found that one type of transformant (Table 3, pattern 2) contained only the *hpt* gene and had lost the *Xa21* and *gus* genes. The other type of transformant (Table 3, pattern 3) carried the *hpt* and *Xa21* genes but had lost the *gus* gene. The frequency of these two types of T-DNA truncations, which are from the right border, is about 14% (Table 3). The deletion of the T-DNA sequence in the left region, including the *hpt* gene, should be present but could not be detected as we used the *hpt* gene as the selection marker. As pointed out by Herman et al. (1990), shortened T-DNA insertions may be generated by breakage at some stage during the transfer or integration process, most probably after the synthesis of a normal T-DNA intermediate.

Agrobacterium-mediated transformation is routinely used in dicot plants and is becoming a popular method for cereals. However, the presence of non-T-DNA in transgenic plants can cause at least two problems. First, the presence of long transferred T-DNA in “gene tagging” experiments will affect our ability to isolate tagged genes using both cloning and PCR approaches. Second, the presence of the antibiotic gene within the non-T-DNA region in transgenic plants may cause environmental problems. The production of marker-free transgenic plants is imperative and possible. Recently, Komari et al. (1996) designed a few ‘super-binary’ vectors that carried two separate T-DNAs in a plasmid and marker-free transgenic progeny were obtained from more than half of the co-transformants by selecting GUS-positive and drug-sensitive plants. However, the transfer of non-T-DNA from *Agrobacterium* to plants may be a part of the normal T-DNA transfer mechanism, and so may be unavoidable. Scientists need to be aware of the presence of non-T-DNA sequences that may contain antibiotic genes, and select plants without unwanted marker gene for field trials.

Acknowledgments We thank Dr. L. Li and Mr. G. Liu for technical assistance, Dr. R. A. Jefferson for providing the binary plasmid pCAMBIA1301, Dr. P. C. Ronald for plasmid pB822, Dr. A. M. Chaudhurg for *A. tumefaciens* strain AGL1, Mr. B. Yang for *A. tumefaciens* strain EHA105, Dr. Y. Hiei for *A. tumefaciens* strain LBA4404, Dr. T. Komari for his useful suggestions on rice transformation using *A. tumefaciens*, Dr. L. Joseph and Dr. J. Lee for critical reading of the manuscript. Z. Yin was supported by the Singapore-China Biotechnology Program (SCBP). The experiments conducted in this paper comply with the current laws in Singapore.

References

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens* –mediated transformation of japonica and indica rice varities. Planta 199:612–617

Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. Plant Mol Biol 22:491–506

Cheng M, Fry JE, Pang SZ, Zhou HP, Hironaka CM, Duncan DR, Conner TW, Wan YC (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens.* Plant Physiol 115:971–980

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

Dong J, Teng W, Buchholz WG, Hall TC (1996) *Agrobacterium* –mediated transformation of Javanica rice. Mol Breed 2:267–276

Durrenberger F, Crameri A, Hohn B, Koukolikova-Nicola Z (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc Natl Acad Sci USA 77:7347–7351

Erickson MJ, Meyer RJ (1993) The origin of greater-than-unit-length plasmids generated during bacterial conjugation. Mol Microbiol 7:289–298

Gallie DR, Novack S, Kado CI (1985) Novel high- and low-copy stable cosmids for use in *Agrobacterium* and *Rhizobium*. Plasmid 14:171–175

Hajdukiewicz P, Svab Z, Maliga P (1994) The small versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:981–994

Herman L, Jacobs A, Van Montagu M, Depicker A (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Mol Gen Genet 224:248–256

Hiei Y, Ohta S, Komari T, Kumashira T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hoekema A, Hirsch PR Hooykaas PJJ, Schilperoot RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgen Res 2:208–218

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol 14:745–750

Joos H, Timmerman B, Van Montagu M, Schell J (1983) Genetic analysis of transfer and stablization of *Agrobacterium* DNA in plant cells. EMBO J 2:2151–2160

Kauffman HE, Reddy APK, Hsieh SPY, Merca SD (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep 57:537–541

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J 11:945–957

Lanka E, Wilkins BM (1995) DNA processing reactions in bacterial conjunction. Annu Rev Biochem 64:141–169

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genome library in *Agrobacterium.* Bio/Technology 9:963–967

Li L, Qu R, Kochko A, Fauquet C, Beachy RN (1993) An improved transformation system for rice using the biolistic method. Plant Cell Rep 12:250–255

Martineau B, Voelder TA, Sanders RA (1994) On defining T-DNA. The Plant Cell 6:1032–1033

Ooms G, Bakker A, Molendijk L. Wullems GJ, Gorden MP, Nester EW, Schilperoort RA (1982) T-DNA organization in homogeneous and heterogeneous octopine-type crown gall tissues of *Nicotiana tabacum*. Cell 30:589–597

Pansegrau W, Schoumacher F, Hohn B, Lanka E (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. Proc Natl Acad Sci USA 90:11538–11542

Park SH, Pinson SRM, Simith RH (1996) T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol Biol 32:1135–1148

Ramanathan V, Veluthambi K (1995) Transfer of non-T-DNA portion of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of T_L-DNA. Plant Mol Biol 28:1149–1154

Rshida H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Rep 15:727–730

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbour, New York

Sheng J, Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8:1699–1710

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804–1806

Tingay S, McEllroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens* –mediated barley transformation. Plant J 11:1369–1376

Tu J, Ona I, Zhang Q, Mew TW, Khush GS, Datta SK (1998) Transgenic rice variety 'IR72' with *Xa21* is resistant to bacterial blight. Theor Appl Genet 97: 31–36

Van der Graaff E, Dulk-Ras A, Hooykaas PJJ (1997) Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. Plant Mol Bio 31:677–681

Virts EL, Gelvin SB (1985) Analysis of transfer of tumor-inducing plasmids from *Agrobacterium tumefaciens*. J Bacteriol 162:1030–1038

Wang GL, Song WY, Ruan DL, Sideris S, Ronald P (1996) The cloned gene, *Xa21,* confers resistance to multiple *Xanthomonas oryzae* pv *oryzae* isolates in transgenic plants. MPMI 9:850–855

Wang K, Stachel SE, Timmerman B, Van Montagu M, Zambryski P (1987) Site-specific nick occurs within the 25-bp transfer promoting border sequence following induction of *vir* gene expression in *Agrobacterium tumefaciens*. Science 235:587–591

Ward ER, Barnes WM (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5′ end of T-strand DNA. Science 242:927–930

Wenck A, Czako M, Kanevski I, Marton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium* -mediated transformation. Plant Mol Bio 34:913–922

Yokoi S, Tsuchiya T, Toriyama K, Hinata K (1997) Tapetum-specific expression of the *Osg6B* promoter-β-glucuronidase gene in transgenic rice. Plant Cell Rep 16:363–367

Zhang S, Song W, Chen L, Ruan D. Taylor N, Ronald P, Beachy R, Fauquet C (1998) Transgenic elite indica rice varieties, resistant to *Xanthomonas oryzae* pv *oryzae*. Mol Breed 4:551–558

Zupan JR, Zambryski P (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol 107:1041–1047