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ORIGINAL PAPER

Insights into recognition of the T-DNA border repeats as termination sites for T-strand synthesis by *Agrobacterium tumefaciens*

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Abstract The recognition of the T-DNA left border (LB) repeat is affected by its surrounding sequences. Here, the LB regions were further characterized by molecular analysis of transgenic plants, obtained after *Agrobacterium tumefaciens*-mediated transformation with T-DNA vectors that had been modified in this LB region. At least the 24-bp LB repeat by itself was insufficient to terminate the T-strand synthesis. Addition of the natural inner and/or outer border regions to at least the LB repeat, even when present at a distance, enhanced the correct recognition of the LB repeat, reducing the number of plants containing vector backbone sequences. In tandem occurrence of both the octopine and nopaline LB regions with their repeats terminated the T-strand synthesis most efficiently at the LB, yielding a reproducibly high number of plants containing only the T-DNA. Furthermore, T-strand synthesis did not terminate efficiently at the right border (RB) repeat, which might indicate that signals in the outer RB region inhibit the termination of T-strand synthesis at the RB repeat.

Keywords *Agrobacterium tumefaciens* · *Arabidopsis thaliana* · Left border · Plant transformation · Right border · T-DNA integration · Vector backbone

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Introduction

Agrobacterium tumefaciens-mediated transformation is a preferred method to introduce genes into numerous dicotyledonous and some monocotyledonous plants (Komari et al. 2004). In most cases, only a small number of T-DNA copies integrate into the plant genome with minor rearrangements in comparison with direct transformation methods (Travella et al. 2005). The only sequences needed *in cis* are two imperfect border repeats, designated the left border (LB) and right border (RB) repeats. Both repeats are recognized in the bacterium by the virulence protein complex VirD1/VirD2. VirD2 produces a single-stranded nick in the bottom strand of the repeat, usually between the third and fourth base (Gheysen et al. 1998). The single-stranded T-DNA is generated

by displacement of the bottom strand, in a unidirectional manner, beginning from the free 3′ end at the RB nick (Miranda et al. 1992). The VirD2 protein linked to the 5′ end of the T-DNA acts as a pilot molecule to guide the T-strand from the bacterium into the plant cell (reviewed in Gelvin 2000, 2003; Zhu et al. 2000; Zupan et al. 2000; Tzfira and Citovsky 2002).

Until the mid-nineties, only the sequences between the RB and LB repeats were believed to be transferred and integrated into the plant genome. However, early reports stated that in octopine strains the two adjacent T-DNAs T_L and T_R often produced physically linked T-strands, indicating that the middle border repeats were not recognized as a termination signal of T-DNA production (Stachel et al. 1987; Veluthambi et al. 1988). Also for the nopaline LB, there was evidence for false or inefficient recognition (Joos et al. 1983). Martineau et al. (1994) found that binary vector sequences coming from outside the T-DNA were frequently integrated into the plant genome. This report triggered scientists to investigate the T-DNA inserts in plants in more detail. From then on, the frequent occurrence of vector backbone was confirmed in different plant species and with both cointegrate and binary T-DNA vectors and with both octopine and nopaline border regions (Ramanathan and Veluthambi, 1995; van der Graaff et al. 1996; Kononov et al. 1997; Wenck et al. 1997; Wolters et al. 1998; Jakowitsch et al. 1999; De Buck et al. 2000; Yin and Wang 2000; McCormac et al. 2001; Kim et al. 2003; Afolabi et al. 2004; Breitler et al. 2004; Cotsaftis et al. 2002; Huang et al. 2004; Kuraya et al. 2004; Vain et al. 2004; Fu et al. 2006; Lange et al. 2006).

Two mechanisms can account for the transfer of vector backbone into the plant genome. First, the LB repeat can mistakenly be recognized as initiation site for T-strand production because VirD2 is known to covalently bind to both border repeats (Ramanathan and Veluthambi 1995; van der Graaff et al. 1996). In this case, the vector backbone is transferred first to the plant. Second, correct initiation at the RB but inefficient recognition and nicking at the LB repeat results in ''read-through'' vector backbone transfer. In its most pronounced manner, the integrated DNA contains two T-DNAs separated by the entire vector backbone (Wenck et al. 1997; De Buck et al. 2000; Yin and Wang 2000).

The 24-bp LB and RB repeats are very similar; therefore, recognition as initiation or termination sites of T-strand synthesis has been suggested to depend on surrounding sequences (Jen and Chilton 1986; Wang et al. 1987). For Ti plasmids that contain the nopaline border regions, the RB region has been demonstrated to be more active than the LB in promoting T-DNA transformation although an overdrive sequence was not identified (Jen and Chilton 1986). Additionally, both octopine and nopaline LB regions are not efficiently recognized as termination signal for T-DNA transfer (Jen and Chilton 1986; van Haaren et al. 1987; Wang et al. 1987; Wenck et al. 1997; De Buck et al. 2000). In octopine Ti plasmids, an overdrive sequence, present in the outer RB region, leads to an increased production of T-strands and virulence (Peralta et al. 1986; van Haaren et al. 1987). When present next to the overdrive, the LB region can take over the function of the RB (van Haaren et al. 1987). How the overdrive exactly acts is unknown, but VirC1 and VirC2 might play a role (Toro et al. 1988, 1989). The inner LB region could affect termination efficiency of the T-strand production, because fewer transformants had vector backbone sequences when transformed with a T-DNA vector containing a LB repeat embedded in natural border sequences than with a vector without the inner border region (De Buck et al. 2000). The inner LB region has been reported to negatively influence the recognition of the border repeat as initiation signal for T-strand synthesis (Wang et al. 1987). Termination at a second RB, albeit at low frequency, was demonstrated by using a double RB vector, in which a selectable marker was surrounded by two RB repeats (Lu et al. 2001; Huang et al. 2004), indicating that the RB repeat, when surrounded by its natural border sequences, is not as efficiently recognized as termination signal of T-strand synthesis as the LB repeat.

For several reasons, integration of vector backbone sequences is unwanted. For crops, regulatory authorities demand that genetically modified plants are devoid of unnecessary DNA, especially vector backbone sequences that con-

tain bacterial selectable markers or bacterial origins of replication. For research purposes, such as T-DNA and promoter tagging, integration of vector backbone sequences is undesirable as well (Smith et al. 2001; Eamens et al. 2004).

To reduce integration of vector backbone sequences, Hanson et al. (1999) developed a method to enrich for transformants with only the T-DNA sequences. By incorporating a lethal gene into the non-T-DNA portion of the vector, the number of tobacco plants containing vector backbone sequences in the transgenic population was highly reduced (Hanson et al. 1999). However, with this system, transformants with only limited amounts of vector backbone sequences could still be detected (Eamens et al. 2004). Vain et al. (2004), who examined whether addition of extra *vir* genes to the backbone of pGreen in the pGreen/pSoup dual binary system could have a positive effect on vector backbone integration, demonstrated that additional vir combinations can affect the presence of backbone sequences, in both a positive or a negative manner. Recently, Kuraya et al. (2004) showed that the presence of four copies of the LB repeat positively affected prevention of read-through at the LB in rice transformants.

Here, we investigate the role of the LB and RB regions in the recognition of the border repeats as termination and initiation signals of T-strand production. A better understanding of the initiation and termination processes can lead to the development of more efficient T-DNA vectors and the generation of transformants without integrated vector backbone sequences.

Results

Modification of the LB region to enhance termination at the LB repeat

To analyze the influence of the border regions on the functionality of the LB repeat as a termination site of T-strand synthesis, several binary vectors (pL) that differed exclusively in their LB region were constructed (Fig. 1). Vector pLM contained at least only the octopine LB repeat; in vector pL0 and pL5, the LB repeat was flanked by the outer or inner LB region, respectively, and in vectors pL2 and pL4, the LB repeat was embedded in natural outer and 0.1-kb and 0.6-kb inner border region sequences, respectively. In vectors pL1 and pL6, three LB repeats in tandem were present that were flanked by the outer LB region and both outer and inner LB regions, respectively. The LB region of pL3 vector was identical to that of the pL2 vector, but outside the octopine LB region contained an additional in tandem oriented nopaline LB repeat surrounded by its inner and outer border region (Fig. 1).

For each construct, at least 17 transgenic *Arabidopsis thaliana* plants were selected after floral dip transformation and analyzed for vector DNA content. *A. tumefaciens* strains GV3101 (pGV2260) with T-DNA vectors pLM, pL0, and pL3 were used in three, with pL1 and pL2 in two and with T-DNA vectors pL4, pL5 and pL6 in one independent transformation series. The presence of vector backbone sequences linked to the T-DNA at the LB and RB repeats was analyzed via polymerase chain reaction (PCR). The PCR primers (Table 1) were designed so that the presence of vector backbone sequences linked or unlinked to the LB or RB T-DNA ends could be detected. PCR reactions LBint and RBint (Fig. 1) indicated whether the T-DNA was present without large truncations at the LB or RB. PCR products LB100, LB1000 and RB100, RB1000 were diagnostic for linkage of 100-bp and 1000-bp vector backbone sequences to the left and right T-DNA end, respectively (Fig. 1). LBout and RBout detected vector sequences from outside the T-DNA adjacent to the LB and RB repeats, respectively (Fig. 1). The absence of PCR product LB100 and the presence of products LBout and LBint revealed that T-strand synthesis had started at the LB.

In none of the samples, *Agrobacterium* contamination could be detected with primers specific for the *virD2* and *picA* genes (data not shown). An overview of the PCR results obtained for the different vectors is presented in Tables 2–4. First, we determined the number and percentage of transformants containing only the T-DNA without vector sequences. These plants gave a PCR fragment for the reactions LBint and RBint, but not for the reactions LB100, LB1000,

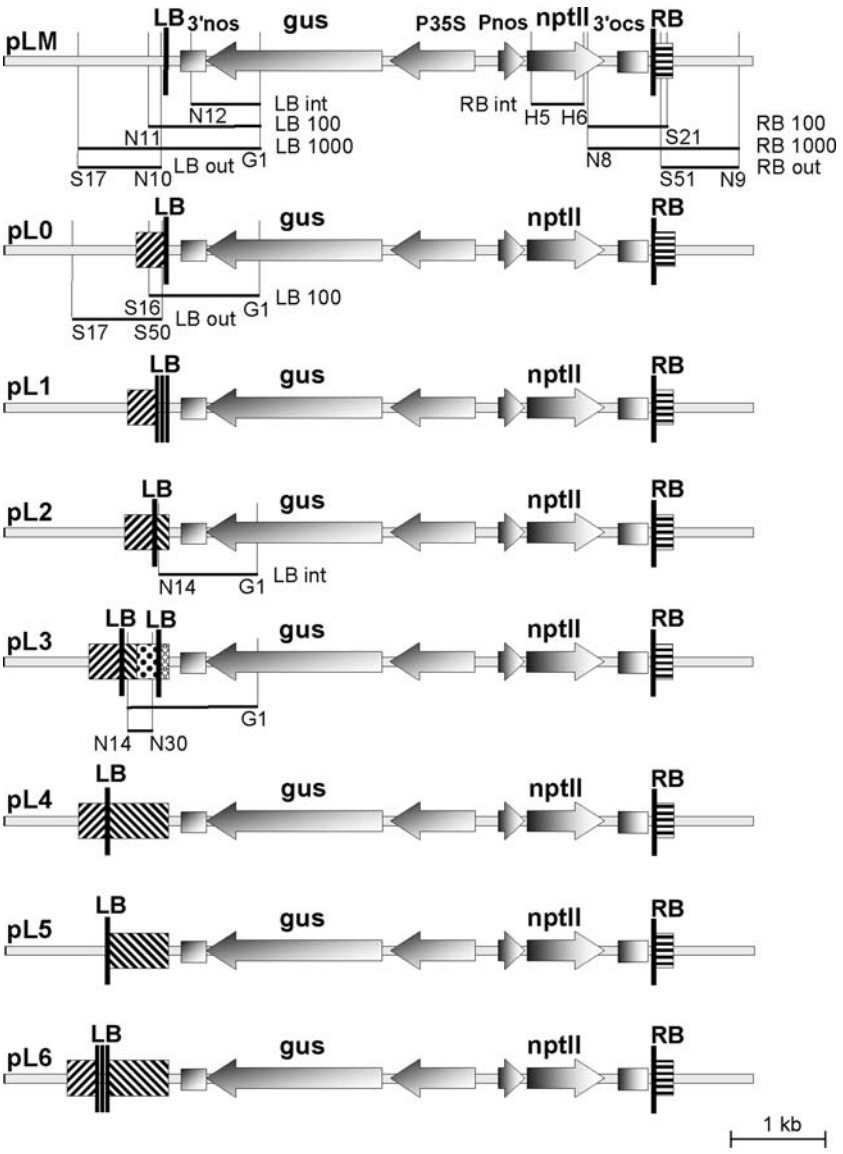

Fig. 1 Schematic outline of the T-DNAs in the different binary pL vectors. The 24-bp RB and LB repeats are represented as vertical black lines, the octopine LB and RB regions by hatched and horizontal bars, respectively, and the nopaline LB regions by dotted bars. The primer combinations used for detection of the different parts of the vectors are given below the T-DNAs and the primer names at the end of the vertical bars, indicating the hybridization place. Abbreviations: nptII, neomycin phosphotransferase II gene; gus, β-glucuronidase gene; LB, left border, RB, right border; 3′nos, 3′ end of the nopaline synthase gene; 3′ocs, 3′ end of the octopine synthase gene; P35S, cauliflower mosaic virus 35S promoter; Pnos, nopaline synthase promoter

RB100, RB1000, LBout and RBout (Fig. 1; Table 2). Second, integration of the PCR data allowed us to divide the transformants with vector backbone sequences into three different classes (see below, Tables 3 and 4).

Frequency of transformants without integrated vector backbone sequences using pL vectors with different LB regions

Initially, when evaluating the different pL constructs, we focused on the frequency of transformants without integrated vector backbone sequences in the genome because this is the main goal during generation of transgenic plants (Table 2). At least the LB repeat by itself was in most transformants not correctly recognized, because in the three transformation series, only 17%, 0% and 30% of the transformants, obtained with the pLM construct, had no vector backbone sequences.

Of the L0 transformants, containing only the LB outer border region, 25%, 36%, and 52% had no integrated vector sequences in their genome. In parallel, 23% of the L5 transformants, harboring a construct containing only the inner

Table 1 Location and sequence of primers

Primer	Vectors	Location	Primer sequence
B1	pE	On T-DNA, in *bar* cassette	CGTTCTGGGCTCATGGTAGAT
G1	pL	On T-DNA, in *gus* gene	ATCACCTGCGTCAATGTAAT
H5	pL	On T-DNA, in *npt*II gene	ATGTGGATTGAACAAGATGGA
H6	pL/pE	On T-DNA, in *npt*II gene	CATGGGTCACGACGAGATCCT
N8	pL	On T-DNA, in *npt*II gene	GCTTTTCTGGATTCATCGACTGTG
N9	pL	In vector backbone, 1000 bp from RB repeat	ATCACAAAAATCGACGCTCAAGTC
N10	pLM/pL5	In vector backbone, next to LB repeat	CGATTACTTCGCCAACTATTGC
N11	pLM/pL5	In vector backbone, 100 bp from LB repeat	GTGCATAATAAGCCCTACAC
N12	pL	On T-DNA, in 3'*nos*	CTAGTAACATAGATGACACC
N14	pL	In inner LB region, next to LB repeat	CAGCAATGAGTATGATGGTC
N27	pE	In octopine outer LB region	CTCGATTGTACCTGCGTTCA
N28	pE	In nopaline outer LB region	GCCTGTATCGAGTGGTGATT
N30	pL3	In nopaline outer LB region	GGCAGCTCGGCACAAAATCA
O1	pE	On T-DNA, in *npt*II cassette	GGAGAATTAAGGGAGTCACGTTATG
O2	pE	On T-DNA, in *npt*II cassette	ATCATGGGCCGGATCTTTGAT
P1	pE	On T-DNA, in *bar* cassette	GACCAAAGGGCTATTGAGACT
S16	pL	In outer LB, 100 bp from LB repeat	TCCGTTGCCCCGTCGCTCACCGTGT
S17	pL	In vector backbone, 1000 bp from LB repeat	CGAGCCAGCCACGATCGACATTGAT
S21	pL/pE	In outer RB, 100 bp from RB repeat	GGCAGTTCATCAGGGCTAAATCAAT
S50	pL	In outer LB, next to LB repeat	TTGCACCCGGTGGAGCTTGCATGTT
S51	pL	In outer RB, next to RB repeat	CGCTGTGTATGTTTGTTTGATTGTT

Table 2 Number of primary L transformants containing only the T-DNA without vector backbone sequences obtained after transformation with the different pL vectors (Fig. 1)

T-DNA Vector	Experiment 1[a,b]	Experiment 2[a,b]	Experiment 3[a,b]
PLM	4/23 (17%)	0/17 (0%)	6/20 (30%)
pL0	9/36 (25%)	12/33 (36%)	15/29 (52%)
pL1	7/19 (38%)	14/31 (45%)	ND
pL2	12/24 (50%)	9/32 (28%)	ND
pL3	15/22 (68%)	14/23 (61%)	13/22 (59%)
pL4	ND	9/21 (43%)	ND
pL5	ND	8/35 (23%)	ND
pL6	ND	14/27 (52%)	ND

[a] Transformants containing no integrated vector backbone sequences gave a PCR fragment for the reactions LBint and RBint, but not for the reactions LB100, LB1000, RB100, RB1000, LBout and RBout

[b] Total number of transformants without vector backbone sequences. The percentage of transformants are indicated in parentheses

ND, not determined

border region, had no vector backbone sequences. These results suggest that the presence of either of the border regions have a positive effect on the correct processing of T-DNAs to the plant cell. Whether or not both inner and outer border regions should be present in a transformation plasmid is not so clear because 50% and 28% of the L2 and 43% of the L4 transformants had no vector backbone sequences integrated into their genome. Both the pL2 and pL4 constructs used during the floral dip transformantion contain both the inner and outer LB regions, but the frequencies of plants without vector backbone sequences were in the same range as those for pL0 and pL5, with only one of the LB regions. Using constructs pL1 and pL6 with three border repeats in tandem, plants without vector backbone sequences were identified in 38% and 45% of the L1 and in 52% of the L6 transformants. Finally, after transformation with the construct pL3, containing the octopine and nopaline border regions in tandem, 68%, 61% and 59% of the plants harbored only the T-DNA (Table 2).

In summary, only the pLM vector yielded low frequencies of plants with only the T-DNA, whereas the pL3 vector consistently resulted in a high frequency of transformants with only the T-DNA. No significantly different frequencies of vector transfer among all other T-DNA vectors could be observed.

Table 3 Origin of vector backbone in primary L transformants obtained after transformation with the different pL vectors (Fig. 1)

T-DNA vector	Transformation series	Plants with vector	Mode of T-DNA-vector linkage[a]		
		Total	Linkage at LB[b]	Start at LB[c]	Only neighboring RB seq[d]
pLM	1	19/23 (83%)	18 (95%)	0/1	1/1
	2	17/17 (100%)	15 (88%)	2/2	0/2
pL0	1	27/36 (75%)	18 (67%)	5/9	4/9
	2	21/33 (64%)	18 (86%)	1/3	2/3
pL1	1	12/19 (62%)	6 (50%)	5/6	1/6
	2	17/31 (55%)	15 (88%)	2/2	0/2
pL2	1	12/24 (50%)	10 (83%)	2/2	0/2
	2	23/32 (72%)	22 (96%)	0/1	1/1
pL3[e]	1	7/22 (32%)	4 (57%)	3/3	0/3
	2	9/23 (39%)	7 (78%)	2/2	0/2
pL4	2	12/21 (57%)	12 (100%)	0/0	0/0
pL5	2	27/35 (77%)	25 (93%)	1/2	1/2
pL6	2	13/27 (48%)	9 (69%)	2/4	2/4

[a] Percentages are calculated from the number of plants with linkage at LB, compared to the total amount of plants containing vector backbone

[b] PCR products found for reactions LBint, RBint, LB100 and/or LB1000

[c] PCR products amplified for reactions LBint, RBint and LBout but not for reactions LB100 and LB1000. The numbers of plants with vector DNA near the LB outer region that was not linked to the vector is identified relative to the number of transformants with vector but without a read-through product

[d] PCR products found for the reactions LBint, RBint, RBout, RB100 and/or RB1000, but not for the reactions LB100, LB1000 and LBout

[e] For this construct, vector sequences were defined as all sequences outside the octopine border repeat, so excluding sequences from the nopaline outer border region. Only for one plant from the first series, read-through at the nopaline LB and termination at the octopine LB could be detected (with primers N14 and G1; data not shown). In both series, initiation at the nopaline LB and termination at the octopine LB was observed in one plant (with primers N14 and N30; data not shown). These data might indicate that most T-strands terminated at the nopaline LB repeat. However, we cannot exclude that part of the sequences between the two LB repeats were deleted by exonuclease activity during integration, so that the annealing place of the primer is removed

Vector backbone transfer by read-through of the LB or by initiation at the LB repeat

In the transformants of series 1 and 2 with vector backbone sequences, their origin was analyzed. A first subclass consisted of plants in which a LB100 and/or LB1000 read-through product could be detected, indicating that the LB was not recognized as a termination signal for T-strand synthesis (Table 3, Linkage at LB). This subclass was observed in 50–100% of the transformants obtained with the different constructs in both transformation series; thus, skipping the LB repeat was a major cause of vector backbone sequence transfer and nor the presence of inner or outer LB regions in addition to the LB repeat nor multiple LB repeats can completely prevent read-through of the LB and vector backbone transfer to the plant cell. The second subclass consisted of plants in which sequences coming from outside the LB were present but unlinked to the left T-DNA end, implying T-strand initiation at the LB repeat. It should be noted that initiation at the LB cannot be detected via PCR analysis in plants that also contain the LB100 read-through product. Therefore, plants containing vector initiated at the LB will be underestimated. This number is relatively low (Table 3, Start at LB), but vector transfer initiated at the LB occurs with all different pL constructs.

A small third subclass consisted of plants in which only vector sequences adjacent to the RB, but not to the LB, were present. These integrations probably result from truncation or exonuclease activity upon T-DNA vector read-through products or LB initiated vector products (Table 2,

Table 4 Distribution of T-DNA termination sites within the vector, at the RB or upstream of the RB in plants containing vector backbone linked at the LB or started at the LB

Plasmid[a]	Transformation Series	Read-through at RB[b,c]	Stop in vector[d]	Stop at RB[e]
pLM	1	12/18 (67%)	6/6	0/6
	2	15/17 (88%)	1/2	1/2
pL0	1	22/23 (95%)	1/1	0/1
	2	18/19 (95%)	1/1	0/1
pL1	1	9/11 (82%)	1/2	1/2
	2	15/17 (88%)	1/2	1/2
pL2	1	10/12 (83%)	2/2	0/2
	2	19/22 (86%)	2/3	1/3
pL3	1	4/7 (57%)	3/3	0/3
	2	7/9 (78%)	1/2	1/2
pL4	2	9/12 (75%)	2/3	1/3
pL5	2	18/26 (69%)	5/8	3/8
pL6	2	10/11 (91%)	1/1	0/1

[a] Transformants obtained with the plasmid in the first and second transformation series are indicated with (1) and (2), respectively

[b] Number of primary transformants considered that gave a positive result for LBout PCR analysis

[c] An RB100 fragment could be detected

[d] No fragments were detected for PCR RB100-RB1000 and RBout

[e] No fragments with RB100-RB1000 but presence of fragment for PCR RBout (Fig. 1)

only neighboring RB sequences present). Only a small number of plants belong to this subclass, because sequences from outside the LB were present in almost all plants with vector sequences.

Does the RB repeat function as T-DNA strand termination site when the LB was incorrectly recognized?

In the literature, in most cases in which linkage between vector backbone and the T-DNA at the LB and RB could be detected via PCR, the complete vector backbone was present (Wenck et al. 1997; De Buck et al. 2000; Yin and Wang 2000). Here, all transformants containing vector sequences from outside the LB (LBout) were analyzed to determine whether the complete vector backbone was present in the plant genome linked or unlinked to the RB or whether transfer terminated before the RB (Table 4). It should be noted that vector transfer termination before or at the RB cannot be detected via PCR analysis in plants that contain vector sequences linked to the RB. In both transformation series, most of the plants had the vector backbone sequences linked to the T-DNA at the RB (57–95%), indicating that termination of T-strand synthesis in the vector backbone is not very efficient. In the remaining plants, termination in the vector backbone was detected in the majority of plants, whereas in both transformation series only in a few plants termination at or within 20 bp of the RB itself was detected (Table 4).

Functionality of LB and RB as termination signals when present within a T-DNA

To further investigate the positive effect of natural LB regions on the correct recognition of the LB repeat, different LB regions or an RB region were inserted into a T-DNA. In this manner, the functionality of an additionally central border was investigated in the presence of a 2-kb downstream LB repeat embedded in its natural border context (Fig. 2). Vector pELB contained a central octopine LB repeat surrounded by its inner and outer border region, whereas in vector pEOct, the inner border region of the additional LB was absent. Vector pE3 harbored a nopaline LB repeat surrounded by its outer and inner border regions, whereas in pENop, at least only the nopaline LB repeat was formed. In the vectors pE4 and pENopI, the extra nopaline LB repeat was surrounded by its outer or inner border region, respectively. Vector pE2/5 is similar to vector pE3, except for the additional presence of the inner and outer octopine border regions. Finally, vector pERB contained an extra octopine RB repeat followed by its outer border region. As a control, a T-DNA construct lacking a central border between the selectable markers was used (pEM, Fig. 2).

The vectors were transferred into the *A. tumefaciens* GV3101 strain with either the octopine pGV2260 or nopaline pMP90 *vir* plasmid. The use of different *vir* plasmids allowed us to determine whether the origin of the Vir proteins has an influence on the recognition of the different border repeats. Upon *Arabidopsis* floral dip transformation with these strains (Fig. 3), the

delineation of the transferred T-DNA was analyzed in at least 16 transformants (only three transformants for pEM (pMP90)). The T-DNA with the neomycin phosphotransferase II (*npt*II) cassette, starting at the RB and stopping at the central LB repeat was designated as T-DNA A and can be regarded as correctly processed T-DNA; the T-DNA containing the bialaphos/phosphinothricin resistance (*bar*) gene cassette starting at the central LB was indicated as T-DNA B; the T-DNA harboring both the *npt*II and *bar* cassette, whereby the T-strand synthesis started at the RB and skipped the central LB, is referred to as T-DNA AB (Fig. 2). Transformants with both T-DNA A and T-DNA B in an unlinked fashion were designated to contain A + B (Fig. 3). The primary transformants selected on kanamycin were analyzed via PCR for the presence of the different T-DNAs (Fig. 2 and 3).

All of the transformants obtained with the control vector pEM contained T-DNA AB (Fig. 3). Using vector pELB and pEOct with an octopine LB region, in combination with the octopine *vir* plasmid, only T-DNA A could be detected in 50% and 38% of transformants, respectively. When both constructs were combined with the nopaline *vir* plasmid, 30% and 43% of the plants only contained he correctly processed T-DNA A (Fig. 3). Read-though T-DNA AB was observed in 31–35% of the ELB and EOct transformants, whereas unlinked copies of T-DNA A and T-DNA B were present in 17–31% of transformants. These results indicate that the extra internal LB region was not only recognized as termination signal, but also as initiation signal, for T-DNA transfer. Additionally, the *vir* region did not seem to influence significantly the frequency of T-strand termination and this was true for all constructs used (Fig. 3).

Regardless of which modified LB repeat was introduced, more or less the same ratio between correct termination versus read-through versus initiation at the central border repeat was observed (Fig. 3). This result was especially suprising when only at least a nopaline LB was inserted between the selectable markers in the T-DNA construct pENop: solely T-DNA A was present in 50% and 48% of the transformants using an *Agrobacterium* with octopine-type and nopaline-type *vir* plasmid, respectively. Read-through T-DNA AB was observed in 24–29% of the ENop transformants, whereas unlinked copies of T-DNA A and T-DNA B were found in 18–29%

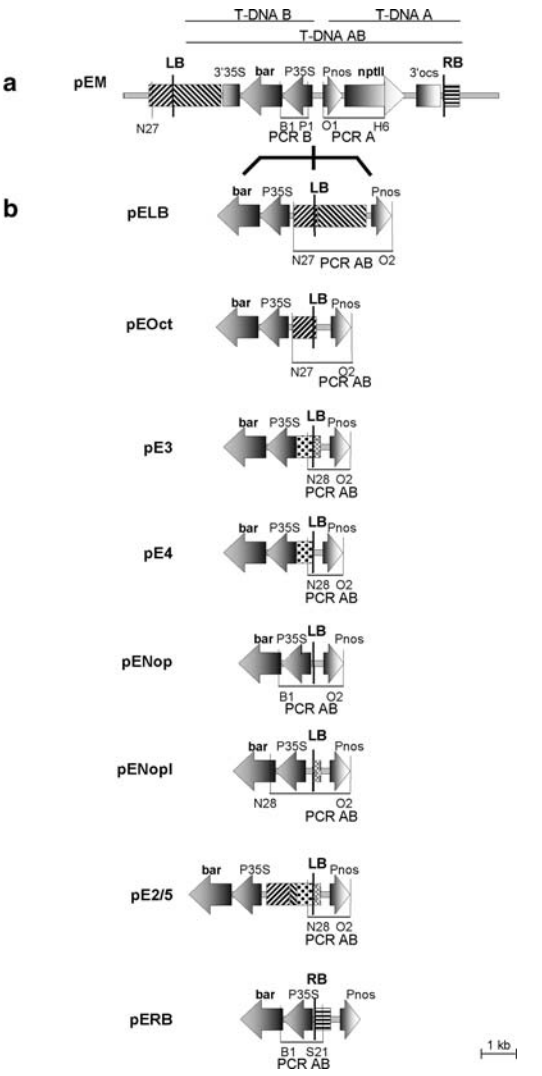

Fig. 2 Schematic outline of the T-DNAs of the different binary pE vectors. (**a**) The control vector pEM. (b) Derivatives of vector pEM with an additional border region between the *nptII* and *bar* expression cassettes. For the sake of simplicity, only the central part of the T-DNA with the additional border region area was drawn. The 24-bp RB and LB repeats are represented as vertical black lines, the octopine LB and RB regions by hatched and horizontal bars, respectively, and the nopaline LB regions by dotted bars. The possible T-DNAs are indicated above the vectors and the primer combinations used for detection of the different T-DNAs are shown below the T-DNAs (PCR products are called PCR A, PCR B, or PCR AB). Abbreviations, see Fig. 1

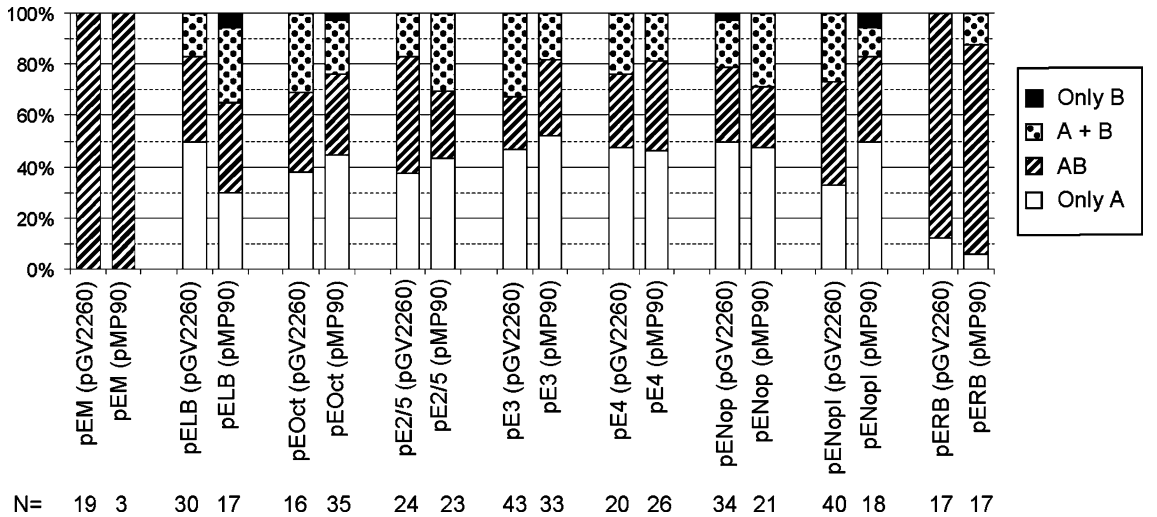

Fig. 3 Detection of the different T-DNAs in primary E transformants obtained after transformation with the *A. tumefaciens* strains containing pE vectors (Fig. 2). For each T-DNA vector in combination with the pGV2260 or pMP90 *vir* plasmid, a schematic representation is given on the percentage of plants containing only T-DNA A (white bars), only T-DNA B (black bars), T-DNAs A and B in a linked (striped bars) or unlinked (dotted bars) fashion. The total number of primary transformants (n) for each

of transformants. Addition of an extra inner and/or outer nopaline border (pE3; pE4 and pENopI) or even the combination of both octopine and nopaline border regions (pE2/5) did not change dramatically the frequencies by which read-through over the LB occurred or by which the extra LB was recognized as a termination or initiation signal (Fig. 3).

In contrast to transformants with the central LB-containing T-DNA, T-DNA A was found in only 12% and 6% of the transformants with the T-DNA construct that had a central RB repeat with outer border region (pERB), whereas 88% and 82% had the read-through T-DNA AB, when using *Agrobacterium* strains with an octopine- and a nopaline-type *vir* plasmid, respectively. These data confirm that the RB is not efficiently recognized as termination signal for T-DNA transfer. Furthermore, because the termination frequency at the RB is even lower than that at the LB repeat by itself (construct pENop), the data suggest that the RB outer border region suppresses T-strand synthesis termination.

Discussion

The results presented provide clear evidence of long-range enhancing effects of the inner and outer LB regions on the activity of at least the border repeat. Up to 100% of the LM transformants with only the LB repeat as LB possessed integrated vector backbone sequences. Therefore, it is clear that at least the octopine LB is inefficiently recognized as termination site for T-strand synthesis. Nevertheless, the frequency of vector integration decreased when transformed with T-DNA vectors that had inner and/or outer LB regions besides the border repeat. Therefore, it can be concluded that the presence of one LB region, albeit inner or outer, has a positive effect on the correct recognition of the LB repeat as a termination site. However, a clear additive effect of the presence of both inner and outer LB regions, as found and reported by De Buck et al. (2000), could not be demonstrated. The frequencies obtained with the above described vectors are very comparable with those (22–79%) published for a number of other vectors, e.g. pBin, pPZP and pCambia derivatives, carrying a nopaline outer LB region of 239 bp or longer and an inner region of 0 to 57 bp (Kononov et al. 1997; Wenck et al. 1997; Wolters et al. 1998; Yin and Wang, 2000; Cotsaftis et al. 2002; Vain et al. 2003, 2004; Afolabi et al. 2004; Breitler et al. 2004; Fu et al. 2006).

Although at least the octopine LB repeat was very inefficiently recognized as T-strand

termination site when present on a binary vector, it was recognized as efficiently as an octopine LB repeat embedded in natural octopine or nopaline border regions when located 2 kb upstream of a LB region within a T-DNA. One of the possible explanations is that efficient recognition of at least the repeat within a T-DNA is due to the presence of the 2-kb downstream LB regions. The finding that the LB regions might function at a distance and the observation that absence of the VirC2 protein in yeast results in an increase in vector backbone transfer (Michielse et al. 2004) might suggest that sequences surrounding the LB repeat are detected by VirC2. We hypothesize that the LB region is recognized by Vir proteins that enhance correct recognition of the LB repeat as a termination site of T-strand synthesis. However, our data could also indicate that the nopaline LB repeat in the pENop construct is more efficiently recognized as termination site than the octopine LB repeat in construct pLM.

Not only the influence of the inner and outer border regions, but also the effect of multiple LB repeats on vector backbone transfer was analyzed. In 38% and 45% of the L1 and in 52% of the L6 transformants, no vector backbone sequences were identified. Comparison of these frequencies with the numbers obtained for the analogous pL0 (25%, 36% and 52%) and pL4 constructs (43%) with only one border repeat showed that the presence of three repeats has no major positive effect on T-strand termination, in contrast with the results obtained by Kuraya et al. (2004). By using a vector with only one LB repeat, more than 90% of the rice transformants had vector backbone sequences, whereas vectors with two to four LB repeats in tandem gave fewer than 10% of the transformants with integrated vector backbone sequences (Kuraya et al. 2004). The reason for the different results is not clear but it demonstrates that efficient termination at multiple LB repeats cannot be generalized and probably also depends on the type of binary vector, *Agrobacterium* strain and cocultivation conditions. For instance, Kuraya et al. (2004) combined a T-DNA vector with additional *virC*, *virG* and *virB* genes and immature embryos during cocultivation, while we used the floral dip transformation method. Also for rice transformation, a high number of the transformants is known to carry a single insert, whereas most transformants obtained by floral dip contain multiple T-DNAs (De Buck et al. 2004). In agreement, Wenck et al. (1997) described that after vacuum infiltration of flowering *A. thaliana* plants, the number of transformants with vector backbone was higher than that compared to transformants obtained after *A. thaliana* root transformation.

In all three transformation series, the pL3 T-DNA construct with an adjacent in tandem oriented nopaline or octopine LB region was most efficient in preventing the transfer of vector backbone sequences to the plant genome. Because the frequencies of plants with only T-DNA (68%, 61% and 59%) were even higher than for constructs pL1 (38% and 45%) and pL6 (52%) with three LB repeats, it is tempting to speculate that the presence of the octopine border regions had a positive effect on the termination at the nopaline LB region or that termination of T-strand synthesis at the nopaline LB was more efficient than at the octopine border region.

When comparing the frequencies of vector backbone integration in the independent transformation series, large variations could be observed with the same transformation vector (Table 2). This variation is intriguing and shows the need for more studies to determine the impact of different experimental conditions. For instance, until now, it is not determined whether differences in the physiology of the agrobacteria and the developmental stage of the plants can contribute to these large variations in T-strand termination at the LB repeat.

Read-through at the LB repeat seems to be the main cause of vector backbone integration in transformants obtained with all pL vectors. However, the frequencies of vector transfer initiated at the LB might be higher than detected, because we cannot exclude that multiple T-DNA copies are present in the genome, of which one contains linked vector sequences at the LB and others contain vector sequences linked at the RB. This problem could have been overcome if we had concentrated our work on single-copy T-DNA transformants. However, the aim of this analysis was to develop a T-DNA vector that

prevents transfer of vector backbone sequences during plant transformation procedures. Additionally, selection for single-copy transformants would enrich for transformants lacking vector backbone sequences, because often two T-DNAs are linked by the entire vector backbone. Therefore, all transformants, containing one or more T-DNA copies, were analyzed.

That read-through as well as initiation at the LB can be responsible for transfer of vector backbone sequences to the plant genome was clearly demonstrated when the pE T-DNA constructs were used that contained an extra border region in between the LB and RB regions. In all LB repeat contexts, the central LB repeat terminated equally efficiently ranging from 30% to 50% in 14 different transformation series and independent of the octopine or nopaline *vir* plasmid used. In 21–46% of the transformants, read-through products were observed as AB-linked T-DNAs, while in 17–33%, the presence of T-DNA B downstream of the LB repeat resulted from initiation at this internal LB repeat. However, when the RB region was present within the T-DNA, fewer than 12% of the transformants contained only T-DNA A. These data are in agreement with the findings of Huang et al. (2004), who observed RB-RB T-DNAs in only 12.4% of the transformants. Because termination at least at the LB repeat (pENop) within a T-DNA occurs, but rarely does at the RB repeat when flanked by its outer border region, we assume that motifs in the outer RB region inhibit the termination of T-strand production at the RB repeat. These observations point to a clear distinction between events occurring at the LB and RB repeats in *Agrobacterium*. Although what we know about the process biochemically does not allow their distinction and although at least the LB and RB repeats have been described as functionally identical (Wang et al. 1987), the border regions are far from interchangeable and the flanking regions may therefore have functionally different *in cis*- acting effects, which are until now still unidentified. Indeed, also Jen and Chilton (1986) and Wang et al. (1987) demonstrated that the LB region is much less active in promoting T-DNA transformation and suggested that the large difference in activity between the RB and LB regions may be due to differences in the border repeats or the neighbouring border sequences. The observations make it clear that initiation and termination of T-strand synthesis are complex mechanisms.

In conclusion, further insights into the functionality of the LB and RB regions as termination signals for T-strand synthesis were obtained from this analysis. To our knowledge, we present the first evidence for long-range enhancing effects of the LB regions on the recognition of at least an LB repeat as termination signal of T-strand synthesis. Furthermore, a long-range effect of the LB regions could not be observed on the RB repeat when flanked by its outer border region. These data indicate that the RB region might suppress the termination reaction. Although at least the LB and RB repeats are almost identical it seems that the surrounding regions have evolved to enhance the desired function of the border repeats.

Materials and methods

Vector construction

T-DNAs of the different plasmids used are shown in Figs. 1 and 2, and were constructed through standard recombinant DNA technology (Sambrook et al. 1989) in *Escherichia coli* DH5á.

pL vectors

The vectors pCDVIB, pCDVIB1, and pCDVIB3 were generous gifts from CropDesign N.V. (Ghent, Belgium). These binary vectors contained the 3″(9)-*O*-aminoglycoside adenylyltransferase (aad), which confer resistance to spectinomycin, and the pBR322 and the pVS1 replication origins in *E. coli* and *A. tumefaciens*, respectively. The borders used were octopine type, unless stated otherwise. The RB region consisted of the 24-bp border repeat and a 0.2-kb outer border region. The vectors differed exclusively in their LB region. In the various starting vectors, a 5-kb fragment from pXD610 was cloned (De Loose et al. 1995) that contained a neomycin phosphotransferase II (*npt*II) and a *β*-glucuronidase (*gus*) gene. The vectors, designated

pL0, pL1, pL2 and pL3, were constructed from pCDVIB, pCDVIB1, pCDVIB2 and pCDVIB3, respectively (Fig. 1). In vector pL0, the LB region consisted of an LB repeat and a 0.3-kb outer border region; pL1 contained three LB repeats in tandem and the 0.3-kb outer border fragment; in vector pL2, the LB repeat was embedded in a 0.3-kb outer border and a 0.1-kb inner border fragment; and pL3 had the octopine LB surrounded by the 0.3-kb outer border and 0.1-kb inner border region and an additional nopaline LB repeat embedded in 0.23-kb outer and 60-bp inner border regions.

Vector pLM was constructed by removing the LB repeat and outer LB region of pCDVIB with *Nhe*I and *Nco*I and replacing it with an adaptor containing the 24-bp octopine LB repeat (Gheysen et al. 1998) and 4 bp coming from the inner border region (constructed by annealing of two complementary synthetic nucleotides 5′CTAGCGTTAACGGCAGGATATATTCAATTGTAAATGGCTC-3′ and 5′-CATGGAGCCATTTACAATTGAATATATCCTGCCGTTAACG-3′), with pCDVIBm as result. In pCDVIBm, the 5-kb pXD610 fragment was inserted as described above to give pLM.

Vectors pL4, pL5 and pL6 were constructed by inserting a 0.6-kb PCR fragment, which contained the inner LB region from pXD610 (De Loose et al. 1995), digested with *Bam*HI-*Mfe*I or *Bam*HI (primers 5′-CGCGGATCCTCAACAGCATCAATCACG-3′ and 5′-GGAAATTATCTGCCTAACCGGCTCAG-3′ or 5′-GCAGGATCCCCGGGAAATCTACATGGATC-3′), into the *Bam*HI-*Mfe*I or *Bam*HI sites of pCDVIB, pCDVIBm, and pCDVIB1, respectively, yielding upon insertion of the *gus* and *npt* cassettes, pL4, pL5 and pL6 (Fig. 1). A more detailed description of the constructs and the DNA sequence of all vectors can be obtained upon request. All plasmids were transferred to *A. tumefaciens* GV3101 (pGV2260), through electroporation.

pE vectors

In vector pL4, the *gus* cassette was replaced by a bialaphos/phosphinothricin resistance (*bar*) gene with the CaMV 35S promoter and terminator; the vector obtained was designated pEM. In this pEM vector, between the *npt*II and *bar* cassettes, an additional border was inserted using the restriction sites *Hin*dIII and *Xba*I. Vector pELB was obtained by inserting a *Hin*dIII/*Xba*I-digested PCR fragment with the LB region of vector pXD610 obtained with primers 5′-CTCCAAGCTTTCAACAGCATCAATCACG-3′ and 5′-GACTCTAGAGCTAGCTTGCTTGGTCGT-3′. In the same manner, vector pERB was made with primers 5′-CTCCAAGCTTAAATCCATTCCCATTCC-3′ and 5′-GCTCTAGACTGGCAGGATATATACC-3′. To obtain vector pEOct, the extra octopine LB region was inserted as an *Nhe*I/*Nco*I-digested fragment from pCDVIB in the *Hin*dIII/*Xba*I-digested pEM vector together with an adaptor (obtained by annealing of two complementary synthetic nucleotides 5′-CATGGACTAGTGAGA-3′ and 5′-AGCTTCTCACTAGTC-3′). The vector pE2/5 was constructed by inserting two PCR fragments in the *Hin*dIII/*Xba*I-digested pEM vector. The first PCR fragment, containing the octopine outer LB region was amplified with the primers 5′-GACTCTAGACAGGCAGCAACGCTCTGT-3′ and 5′-CATGCCATGGCCGCTGTTGCTTTGCA-3′ from pCDVIB3 and digested with *Xba*I and *Nco*I. The pCDVIB3 vector was also used to amplify the second PCR fragment, harboring the octopine inner LB region and the nopaline LB region. This second PCR fragment was obtained by using the primers 5′-GACGAGCTCCATGGCCGGGAAATCTA-3′ and 5′-CTCCAAGCTTCGGCGTTAATTCAGTACA-3′ and was digested with *Hin*dIII and *Nco*I. The PCR fragment with the nopaline LB region, amplified from pL3 with primers 5′-GACTCTAGACAGGCAGCAACGCTCTGT-3′ and 5′-CTCCAAGCTTCGGCGTTAATTCAGTACA-3′, was digested with *Hin*dIII and *Xba*I and inserted into pEM, resulting in vector pE3. Similarly, the *Hin*dIII/*Xba*I-digested PCR fragment with the nopaline LB repeat and nopaline LB outer border region, amplified from pL3 with primers 5′-GCTCTAGACAGGCAGCAACGCTCTGT-3′ and 5′-CTCCAAGCTTGTTTACACCACAATATATCCTGCCA-3′, was inserted into pEM, delivering vector pE4. Inserting an adaptor (obtained by annealing of two complementary synthetic

nucleotides 5′-CTAGAGCGTAGGAGCTCGT-GGCAGGATATATTGTGGTGTAAACA-3′ and 5′-AGCTTGTTTACACCACAATATATC-CTGCCACGAGCTCCTACGCT-3′) into pEM resulted in vector pENop. This adaptor contained at least the nopaline border repeat. Vector pENopI was obtained by ligating an adaptor (5′-AGCTTAAATTGACGCTTAGACAACTT-AATAACACATTGCGGACGTTTTTAATGT-ACTGAATTAACGCCGAATTA-3′and 5′-AG-CTTAATTCGGCGTTAATTCAGTACATTA-AAAACGTCCGCAATGTGTTATTAAGTT-GTCTAAGCGTCAATTTA-3′) in the *Hind*III-digested pENop vector. All vectors were electroporated into *A. tumefaciens* strains GV3101 (pGV2260) and GV3101 (pMP90).

Arabidopsis thaliana transformation

Plants of *Arabidopsis thaliana* (L.) Heynh. (ecotype Colombia) were transformed with *A. tumefaciens*, carrying the different binary vectors, by minor modifications of the floral dip method (Clough and Bent 1998). Transformants were selected on Murashige and Skoog agar medium with vancomycin (500 mg/l), kanamycin (50 mg/l), or 10 mg/l glufosinate-ammonium Pestanal (Sigma-Aldrich, St Louis, MO, USA).

PCR analysis

DNA was isolated from leaf material of primary transformants as described by De Neve et al. (1997) or using the Puregene® Genomic DNA Isolation Kit (Puregene-BIOplastics, Landgraaf, The Netherlands), depending on the amount of leaf material that could be harvested.

Primary transformants were screened for the presence of different parts of the binary vectors by performing different PCR reactions, essentially as described by De Buck et al. (2000). The sequence and the annealing site of the different primers are presented in Table 1 and Figs. 1 and 2 for pL and pE constructs, respectively.

For the analysis of the pL vectors, primer combinations were chosen so that the vector backbone that was linked to the T-DNA at the LB or RB with a minimum length of 100 bp or 1000 bp would result in a diagnostic PCR fragment (LB100, LB1000 and RB100, RB1000; Fig. 1). Also PCR analyses with primers inside or outside the T-DNA were performed (LBint, LBout and RBint, RBout; Fig. 1). To make sure that the DNA sample was not contaminated with *Agrobacterium* cells, a PCR reaction was done on each sample with primers specific for the chromosomal gene *pic*A (Yusibov et al. 1994) or for the *vir*D2 gene (primers VirD2S 5′-GCTCA-AGTWATCATTTCGCATTGTGCC-3′ and VirD2AS 5′-CTGACCCAAACATCTCGGCTGCCCA-3′).

For screening of the transformants obtained after transformation with the T-DNA pE vectors, PCR analysis was performed as described above and primer combinations diagnostic for the different possible T-DNAs were used (Table 1; Fig. 2). In some of the transformants of the different series only T-DNA B was present as determined by PCR analysis. However, because the transformants were selected for the selection marker on T-DNA A, we presume that the absence of the T-DNA-specific PCR product is due to truncations at the primer annealing sites.

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