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Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs

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Abstract

Different patterns of T-DNA integration in *Arabidopsis* were obtained that depended on whether a root or a leaf-disc transformation method was used. An examination of 82 individual transgenic *Arabidopsis* plants, derived from 15 independent *Agrobacterium*-mediated transformations in which different cointegrate and binary constructs were used, indicated that the transformation method had a significant influence on the type and copy number of T-DNA integration events. Southern hybridizations showed that most of the transgenic plants produced by a leaf-disc method contained multiple T-DNA insertions (89%), the majority of which were organized as right-border inverted repeat structures (58%). In contrast, a root transformation method mostly resulted in single T-DNA insertions (64%), with fewer right-border inverted repeats (38%). The transformation vectors, including cointegrate and binary types, and the plant selectable markers, hygromycin phosphotransferase and dihydrofolate reductase, did not appear to influence the T-DNA integration patterns.

Introduction

Agrobacterium-mediated transformation is the most commonly used method for the production of transgenic plants (for reviews, see [28, 34]). While a great deal is known at the molecular level about the transfer of T-DNA to plants, including the expression of T-DNA-encoded oncogenes (for reviews, see [16, 22, 35]), less is known about the integration of T-DNA into the plant genome [9, 24].

An examination of a wide variety of transgenic dicotyledonous plants transformed by *Agrobacterium tumefaciens* indicates a common trend in the Mendelian segregation of T-DNA loci. In most cases, single segregating loci have been observed, followed by linked and unlinked multiple loci, in tomato [4, 25, 31], tobacco [30], petunia [5], and *Arabidopsis* [8]. In addition, there is a consistent pattern in the copy number of T-DNA insertions detected in these plants. Multiple T-DNA insertions have been commonly observed and are frequently organized as direct repeats in head-to-tail arrays (e.g. RB/LB), as inverted repeats about the right or left T-DNA borders in head-to-head arrays (RB/RB or LB/LB inverted repeat structures), or in even more complex structures [13, 15]. It has therefore been assumed that the segregation pattern, the copy number and the organization of T-DNA insertions are inherent

properties of the *Agrobacterium*-mediated transformation process.

In order to determine the most efficient means of introducing a two-part *Activator/Dissociation* (*Ac/Ds*) transposable element system to *Arabidopsis* [10, 22, 23], we have used two different *Agrobacterium*-mediated transformation methods including a leaf-disc [21] and a root [17, 32] transformation method. A total of 82 independent transgenic plants were produced, and the copy number and the structural integrity of the T-DNA insertions in these plants was determined by Southern analysis. This resulted in the identification of transgenic *Arabidopsis* plants containing intact, single-copy T-DNA insertions, which were subsequently used for *Ac/Ds* transposon mutagenesis studies [10]. Transgenic plants produced by a leaf-disc method mostly resulted in multiple T-DNA insertions, often organized as inverted repeats about the right border (RB/RB inverted repeat) while a root transformation method frequently resulted in single-copy insertions. Furthermore, distinct differences in the orientation of the inserts, including inverted repeat T-DNA structures, correlated with the transformation methods that were used.

The results presented in this paper have important implications for experiments in which *Agrobacterium*-mediated transformation is used to produce transgenic plants. Single T-DNA insertions, obtained in a higher frequency using a root transformation method, as described in this study, are advantageous for the genetic analysis of transgenic plants and, furthermore, may be especially important for studies involving transgene expression. It has recently been reported that single T-DNA insertions result in high levels of transgene expression, whereas multiple T-DNA insertions, organized as inverted repeat structures, result in low expression [12].

Materials and methods

Bacterial strains and plants

The T-DNA cointegrate vectors pGV3850::pDS-DHFR-1 and pGV3850::pDS-DHFR-2 [22], pGV3850HPT::pDs35S-1, and the binary vectors pB-Ac101 and pB-Ac102 [10] have been previously described; pGV3850::pDs35S-2 is a derivative of pGV3850HPT::pDs35S-1 that contains a DHFR marker cassette (V. Fantes *et al.*, manuscript in preparation). The *Arabidopsis* Columbia and Nossen ecotypes were used with *Agrobacterium*-mediated transformations including the cointegrate and binary vectors, respectively. Transgenic *Arabidopsis* plants used for molecular analysis were selected with methotrexate (0.1 μg/ml) or hygromycin sulphate (20 μg/ml), according to the selectable marker in the T-DNA.

Plant transformation methods

The leaf-disc transformation (LDT) method of Lloyd *et al.* [21], including minor modifications (see below), was used with leaf and stem explants, and the root transformation (RT) method of Kemper *et al.* [17], based on a procedure described by Valvekens *et al.* [32], was used to generate transgenic *Arabidopsis* plants; 15 independent transformations were done. The first steps of both transformation methods, including the infection step with *Agrobacterium tumefaciens*, are almost identical: (1) leaves and roots were preconditioned for four days on solid medium containing BAP/NAA (1.0 mg/l benzylaminopurine, 0.1 mg/l naphthaleneacetic acid) for the LDT method, and BAP/NAA/2,4-D (1.0 mg/l benzylaminopurine, 0.1 mg/l naphthaleneacetic acid, 0.5 mg/l2,4-dichlorophenoxyacetic acid) for the RT method, respectively; (2) leaf or root explants were incubated with *A. tumefaciens* and subsequently returned to fresh plates containing BAP/NAA for about three days; (3) after infection, and hence T-DNA integration, leaf-discs and root explants were transferred to fresh plates with appropriate drug selection. Complete details concerning the LDT and RT methods are described by Lloyd *et al.* [21] and Kemper *et al.* [17], respectively.

Molecular analysis

Plant DNA was isolated using a proteinase K method described by Pruitt and Meyerowitz [26] with minor modifications. 5 μg of CsCl-purified DNA were digested with *Hin*d III (Boehringer Mannheim, Germany) and separated by electrophoresis on 0.8% agarose gels. Southern blots [29] were done using nylon membranes (Hybond-N, Amersham, UK) according to Sambrook *et al.* [27]. Hybridization probes were prepared with a Random Primed Labeling Kit (Boehringer Mannheim, Germany). Southern hybridizations were done in succession using a 1.1 kb *Hin*d III/*Bam* HI fragment from the right border (^{32}P-RB) and a 3.0 kb *Eco* RI/*Hin*d III left border fragment (^{32}P-LB). To confirm RB/RB inverted repeat structures the 2.3 kb right-border fragment (^{32}P-RBT) was used (results not shown). Figs. 2–5 are composites of different Southern hybridization results. The differences in signal strength are mostly due to blot to blot variations and, to a minor extent, to differences in the amount of DNA loaded to gels, the amounts transferred, and different exposure times. Unlabelled samples of each of the fragments used as probes were present on each blot to help detect blot to blot differences; in addition, hybridizations were repeated using the same conditions to reduce variation. Filters were stringently washed according to Sambrook *et al.* [27] followed by a final wash with 0.1 × SSC, 0.1% SDS at 68 °C for 30 min.

Results

Molecular characterization of T-DNA structures in Arabidopsis

In order to determine the most efficient means of producing transgenic *Arabidopsis* plants using *Agrobacterium*-mediated transformation, we have tried different transformation methods. A total of 15 independent transformations were done using a leaf-disc method developed by Lloyd *et al.* [21] and a root method by Kemper *et al.* [17]; details concerning the transformation methods are in Materials and methods. A total of 82 transgenic *Arabidopsis* plants, including 27 transformed by the LDT method, and 55 by the RT method, were examined at the molecular level using Southern hybridizations in order to characterize the copy number and the structural integrity of the T-DNA insertions (Figs. 2–4, and Table 1).

Individual hybridization signals are expected with single T-DNA insertions when probed with sequences derived from the left (LB) and right (RB) T-DNA border regions. These signals vary in size depending on the fixed restriction sites in the T-DNA and the nearby random sites in the plant genome. Multiple T-DNA insertions result in a hybridization pattern including two or more signals when probed with the LB or RB, and certain inverted repeat type insertions (see below) result in a predictable hybridization pattern. In this study, the restriction enzyme *Hin*d III was chosen due to the fortuitous location of *Hin*d III sites situated nearby the right and left borders in the various T-DNA vectors (see diagram in Fig. 1A, and [10]). DNA samples isolated from transgenic plants containing the pGV3850::pDsDHFR and pGV3850HPT::pDs35S cointegrate vectors, and the pB-Ac101, pB-Ac102, and pB-Ac103 binary vectors, were digested with *Hin*d III, and probed with LB and RB ^{32}P-labelled fragments (Figs. 2–4).

Multiple T-DNA inserts are common in leaf-disc-derived transgenics

A series of Southern hybridizations of *Hin*d III-digested DNA, isolated from 27 transgenic plants containing cointegrate vectors that were derived from independent leaf-disc transformations, resulted in a hybridization pattern consisting of multiple T-DNA insertions (24/27 = 89%; (Fig. 2A and B). Twenty-five of the transgenic plants were derived from square-cut leaf sections and two were from stem explants. A majority of the transgenic plants contained more than one hybridization signal when probed with either the ^{32}P-RB fragment (Fig. 2A) or the ^{32}P-LB

Table 1. Molecular analysis of transgenic *Arabidopsis*.

Plant		Lane	Vector	T-DNA insertion	
				single[a]	multiple[b]
A. Leaf-disc-derived (Fig. 2)					
1	247	1	pGV3850::pDsDHFR-1	–	+ (RB/RB)
2	239	2	pGV3850::pDsDHFR-1	–	+
3	157	3	pGV3850::pDsDHFR-1	–	+ (RB/RB)
4	162	4	pGV3850::pDsDHFR-1	–	+ (RB/RB)
5	60	5	pGV3850::pDsDHFR-1	–	+ (RB/RB)
6	101	6	pGV3850::pDsDHFR-1	–	+ (RB/RB)
7	161	7	pGV3850::pDsDHFR-1	–	+ (RB/RB)
8	241	8	pGV3850::pDsDHFR-1	–	+ (RB/RB)
9	240	9	pGV3850::pDsDHFR-1	–	+ (RB/RB)
10	160	10	pGV3850::pDsDHFR-1	–	+ (RB/RB)
11	57	11	pGV3850::pDsDHFR-1	–	+ (RB/RB)
12	256	12	pGV3850::pDsDHFR-1	–	+ (RB/RB)
13	62	13	pGV3850::pDsDHFR-1	–	+ (RB/RB)
14	205	14	pGV3850::pDsDHFR-1	–	+ (RB/RB)
15	122.3	15	pGV3850::pDsDHFR-1	–	+
16	52	16	pGV3850::pDsDHFR-1	–	+
17	290	17	pGV3850::pDsDHFR-1	–	+
18	291	18	pGV3850::pDsDHFR-1	–	+
19	292	19	pGV3850::pDsDHFR-1	+	–
20	293	20	pGV3850::pDsDHFR-1	–	+
21	294	21	pGV3850::pDsDHFR-1	–	+
22	159	22	pGV3850::pDsDHFR-1	–	+
23	310	23	pGV3850::pDsDHFR-1	–	+
24	462	25	pGV3850::pDs35S-1	–	+ (RB/RB)
25	474	26	pGV3850::pDs35S-1	+	–
26	481	27	pGV3850::pDs35S-1	–	+
27	404	28	pGV3850::pDs35S-2	+	–
B. Root-derived (Fig. 3)					
1	252	1	pGV3850::pDsDHFR-2	+	–
2	125.2	2	pGV3850::pDsDHFR-2	+	–
3	253	3	pGV3850::pDsDHFR-2	+	–
4	126.4	4	pGV3850::pDsDHFR-2	+	–
5	243	5	pGV3850::pDsDHFR-2	+	–
6	254	6	pGV3850::pDsDHFR-2	+	–
7	302	7	pGV3850::pDsDHFR-2	–	+ (RB/RB)
8	303	8	pGV3850::pDsDHFR-2	+	–
9	304	9	pGV3850::pDsDHFR-2	–	+
10	305	10	pGV3850::pDsDHFR-2	+	–
11	306	11	pGV3850::pDsDHFR-2	–	+
12	307	12	pGV3850::pDsDHFR-2	–	+ (RB/RB)
13	308	13	pGV3850::pDsDHFR-2	–	+ (RB/RB)
14	296	14	pGV3850::pDsDHFR-2	+	–
15	297	15	pGV3850::pDsDHFR-2	–	+
16	298	16	pGV3850::pDsDHFR-2	+	–
17	299	17	pGV3850::pDsDHFR-2	–	+
18	501		pGV3850::pDsD35S-2	+	–
19	502		pGV3850::pDsD35S-2	+	–

Table 1. (Continued)

Plant		Lane	Vector	T-DNA insertion	
				single[a]	multiple[b]
20	503		pGV3850::pDsD35S-2	+	–
21	504		pGV3850::pDsD35S-2	–	+ (RB/RB)
22	505		pGV3850::pDsD35S-2	+	–
23	506		pGV3850::pDsD35S-2	+	–
24	507		pGV3850::pDsD35S-2	–	+ (RB/RB)
25	508		pGV3850::pDsD35S-2	+	–
26	509		pGV3850::pDsD35S-2	+	–
27	510		pGV3850::pDsD35S-2	+	–
28	511		pGV3850::pDsD35S-2	+	–
29	512		pGV3850::pDsD35S-2	+	–
30	513		pGV3850::pDsD35S-2	+	–
31	514		pGV3850::pDsD35S-2	+	–
32	515		pGV3850::pDsD35S-2	–	+ (RB/RB)
33	516		pGV3850::pDsD35S-2	+	–
34	517		pGV3850::pDsD35S-2	–	+
35	518		pGV3850::pDsD35S-2	+	–
36	519		pGV3850::pDsD35S-2	–	+
37	520		pGV3850::pDsD35S-2	+	–
38	521		pGV3850::pDsD35S-2	–	+
39	522		pGV3850::pDsD35S-2	+	–
40	523		pGV3850::pDsD35S-2	–	+
41	524		pGV3850::pDsD35S-2	+	–
42	1.3	1A[c]	pB-Ac101	–	+
43	12.2	1C	pB-Ac101	+	–
44	17.1	2A	pB-Ac101	+	–
45	21.1	3A	pB-Ac101	–	+
46	1.3	2C	pB-Ac102	–	+
47	23.3	3C	pB-Ac102	+	–
48	59.1	4A	pN-Ac102	–	+
49	74.1	4C	pB-Ac102	+	–
50	85.3	5A	pB-Ac102	+	–
51	119.1	5C	pB-Ac102	+	–
52	4.1	6C	pB-Ac103	–	+ (RB/RB)
53	52.1	7C	pB-Ac103	–	+ (RB/RB)
54	75.1	8C	pB-Ac103	+	–
55	79.1	9C	pB-Ac103	+	–

[a] Single T-DNA insertions in an individual transgenic plant.

[b] Multiple T-DNA insertions consist of two or more insertions in the same transgenic plant; inverted repaet type insertions oriented in a head to head, right border/right border orientation are designated RB/RB; see diagram in Fig. 1B.

[c] Numbers and letters (42–55) refer to the Southern blot hybridizations shown in Fig. 4A–D.

fragment (Fig. 2B). In some transgenic plants a high number of T-DNA insertions was observed (Fig. 2A and B, lanes 8, 12, 16). Transgenic plant 239 appears to contain a single T-DNA insertion (Fig. 2A and B, lane 2); however, the thick band observed following a hybridization with a ^{32}P-LB fragment (Fig. 2B, lane 2) is actually composed of two closely migrating bands which can be discerned with a lighter exposure. In addition, another LDT-derived plant, 481, transformed with a different cointegrate construct (pGV3850::pDs35S-1), resulted in a slightly

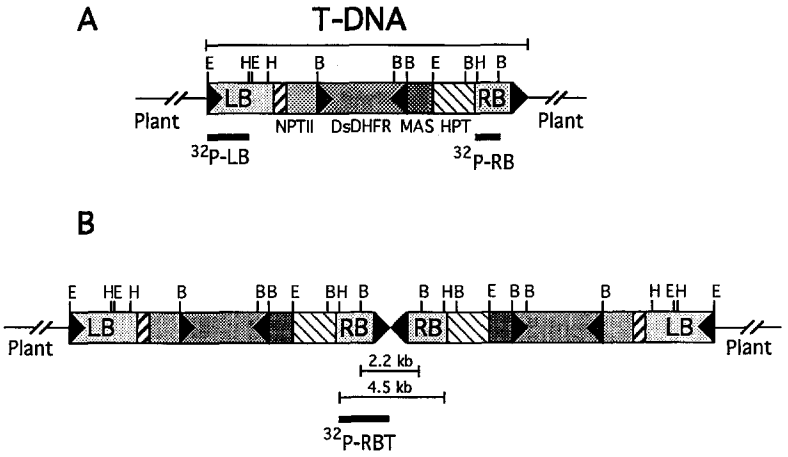

Fig. 1. Schematic diagram shows the pGV3850::pDsDHFR T-DNA construct; not drawn to scale. A represents a single integration event, whereas B shows a right-border/right-border inverted-repeat type insertion. Sequences used for hybridization are shown. Restriction sites shows are: E, *Eco* RI; H, *Hin*d III; B, *Bam* HI. LB, left-border region; RB, right-border region; ^{32}P-LB, ^{32}P-RB and ^{32}P-RBT were used as probes (see Materials and methods for details). MAS-DsDHFR-NPTII = *Ds* transposable element containing a dihydrofolate reductase selectable marker cassette inserted into the leader sequence of an NPTII gene [23]; MAS, mannopine synthase promoter. The striped box adjacent to the NPTII gene represents an OCS poly(A) sequence.

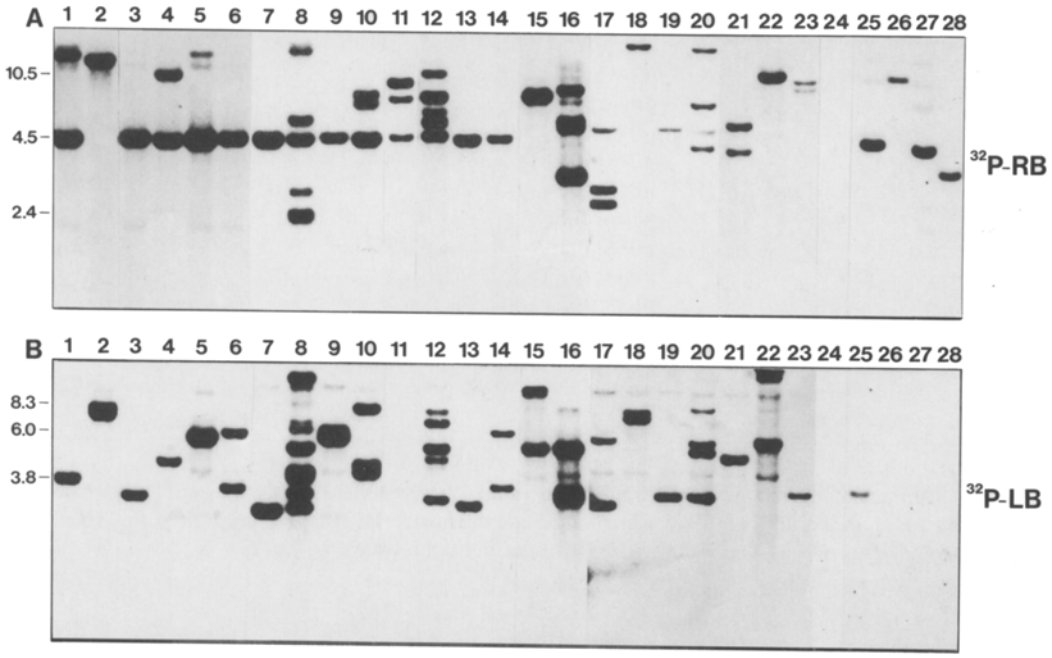

Fig. 2. A. Composites of Southern blot hybridizations of *Hin*d III-digested genomic DNA isolated from LDT-derived transgenic *Arabidopsis* plants containing pGV3850::pDs-DHFR (lanes 1–23), pGV3850HPT::pDs35S-1 (lanes 25–27), pGV3850::pDs35S-2 (lane 28) and Columbia wild type (lane 24). Lane 1, 247; 2, 239; 3, 157; 4, 162; 5, 60; 6, 101; 7, 161; 8, 241; 9, 240; 10, 160; 11, 57; 12, 256; 13, 62; 14, 205; 15, 122.3; 16, 52; 17, 290; 18, 291; 19, 292; 20, 293; 21, 294; 22, 159; 23, 310; 24, wt; 25, 462; 26, 474; 27, 481; 28, 404. ^{32}P-RB was the labelled probe. B. The Southern blots shown in A were stripped and rehybridized with a ^{32}P-LB probe. Sizes shown are in kb.

smaller hybridization signal (4.3 kb) with the same right border probe (Fig. 2A, lane 27); no signal was observed using a ^{32}P-LB probe. While it may initially appear that transgenic plant 481 contains a single T-DNA insertion, repeated hybridizations with *ocs*- and *Ac*-specific probes resulted in a hybridization pattern consistent with multiple insertions, which most likely were not organized as direct or indirect repeats (results not shown).

Upon closer examination, it is readily apparent that a 4.5 kb *Hin*d III band is present in more than half of the leaf-disc derived transgenics containing multiple insertions when DNA samples were probed with the ^{32}P-RB fragment (14/24 = 58%; Fig. 2A). We suspected that these similar-sized 4.5 kb bands were due to an inverted repeat structure of two T-DNAs arranged in a head-to-head orientation centred at their right borders (RB/RB, see diagram in Fig. 1B); this type of inverted repeat has previously been identified in *A. tumefaciens* C58 derivatives containing pGV3850 cointegrate vectors [15]. For example, in LDT-derived transgenic plant 101 a 4.5 kb *Hin*d III band was observed following a Southern hybridization that was probed with the ^{32}P-RB fragment (Fig. 2A, lane 6). A rehybridization of the same blot with the ^{32}P-LB fragment indicated that two signals, ca. 3.8 and 5.9 kb in size, were present in plant 101 (Fig. 2B, lane 6). Taken together, these hybridization results suggest that plant 101 contains a RB/RB inverted-repeat type T-DNA insertion.

In order to confirm that the common 4.5 kb *Hin*d III hybridizing fragment represents a RB/RB inverted-repeat type insertion instead of a similar-sized T-DNA/host fragment, Southern blots were prepared that included LDT-derived DNA samples digested with *Eco* RI/*Hin*d III and *Bam* HI, respectively. The restriction pattern in the RB region expected with RB/RB insertions includes 4.5 kb *Eco* RI/*Hin*d III and 2.2 kb *Bam* HI size fragments (see diagram in Fig. 1B). A Southern hybridization of *Eco* RI/*Hin*d III-digested DNA samples derived from LDT plants 62 and 161, that had presumptive RB/RB insertions (Fig. 2, lanes 7, 13) was probed with the ^{32}P-RB fragment (Fig. 5, lanes 1, 2). In both cases a 4,5 kb *Eco* RI/*Hin*d III band consistent with RB/RB type insertions was observed. A LDT-derived plant (122.3; Fig. 2, lane 15) that was not expected to harbour a RB/RB insertion maintained a large 8.0 kb *Eco* RI/*Hin*d III band (Fig. 5, lane 3). A more definitive Southern hybridization with *Bam* HI-digested DNA derived from LDT plants 62, 157, and 247, probed with the entire right-border fragment (^{32}P-RBT, see Fig. 1B), showed an expected 2.2 kb *Bam* HI fragment that was consistent with RB/RB type T-DNA insertions (results not shown).

Single T-DNA inserts are common with root-explant-derived transgenics

In contrast to the multiple T-DNA insertions observed with nearly all of the transgenic *Arabidopsis* plants derived from the LDT method, the RT method mostly resulted in single T-DNA insertions (35/55 = 64%; see Fig. 3 and Table 1). The hybridization patterns obtained with *Hin*d III-digested root-explant derived transgenics harbouring cointegrate vectors that were probed with the ^{32}P-RB (Fig. 3A) and the ^{32}P-LB (Fig. 3B) labeled fragments are remarkably different than the results obtained with leaf- or stem-explant-derived transgenics (Fig. 2). For example, a single right and left T-DNA border was observed in RT-derived transgenic plants 252, 126.4, 243, and 298 (Fig. 3A and B, lanes 1, 4, 5 and 16). Other RT-derived plants, including 125.2, 303, and 305, only showed a single hybridization signal when probed with either the LB or RB fragment (Fig. 3A and B, lanes 2, 8, 10). Among the root transformants that did contain multiple inserts, most contained only two copies, and these were usually not arranged as RB/RB inverted repeats (see below).

The pattern of single T-DNA insertions observed in RT-derived transgenics was independent of the type of plant vector used. Southern hybridizations of *Hin*d III-digested DNA, isolated from transgenic plants containing the binary vectors pB-Ac101, pB-Ac102 and pB-Ac103, re-

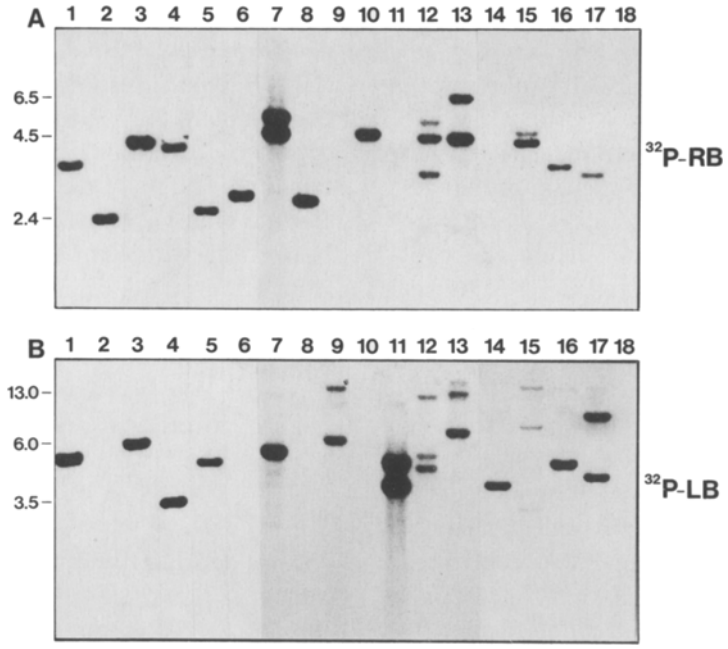

Fig. 3. A. Composites of Southern blot hybridizations of *Hind* III-digested genomic DNA isolated from RT-derived transgenic *Arabidopsis* plants containing pGV3850::pDs-DHFR (lanes 1–17) and Columbia wild type (lane 18). Lane 1, 252; 2, 125.2; 3, 253; 4, 126.4; 5, 243; 6, 254; 7, 302; 8, 303; 9, 304; 10, 305; 11, 306; 12, 307; 13, 308; 14, 296; 15, 297; 16, 298; 17, 299; 18, wt. ^{32}P-RB was the labelled probe. B. The Southern blots shown in A were stripped and rehybridized with a ^{32}P-LB probe. Sizes shows are in kb.

sulted in a hybridization pattern consistent with single T-DNA insertions (Fig. 4A–D). Eight of the fourteen transgenics harbouring the binary vector contained a single T-DNA insertion hybridization signal when probed with either the ^{32}P-RB fragment or the ^{32}P-LB fragment (Fig. 4A/B, lanes 2 and 5; Fig. 4C/D, lanes 1, 3–5, 8, 9). Of the six other transgenics examined that were found to have multiple inserts, four plants contained two independent T-DNA insertions (Fig. 4A/B, lanes 1, 3, 4; Fig. 4C/D, lanes 2) and the remaining two plants contained presumptive RB/RB inverted repeat-type insertions (Fig. 4C, lanes 6 and 7).

Root-derived transgenics contain fewer RB/RB type T-DNA insertions

The frequency of RB/RB inverted repeat type inserts among the multiple insertions (8/21 = 38%) was lower in comparison to those observed with the LDT (14/24 = 58%). The significance of this difference is not altogether apparent, however, in that the multiple insertion sample sizes for both RT and LDT are relatively low. In some RT-derived plants a *Hind* III band of ca. 4.5 kb was observed when probed with the ^{32}P-RB fragment (for example: Fig. 3A, lanes 3, 4 and 7). As described in the preceeding section, *Hind* III bands in this size range could represent RB/RB type inserts (see diagram in Fig. 1). We therefore examined RT-derived transgenic plants that contained putative RB/RB insertions by Southern analysis. Plant DNA samples were digested with *Eco* RI/*Hind* III and hybridized with the ^{32}P-RB fragment (Fig. 5, lanes 4–7). In one of the RT-derived transgenic plants, 253, the previously observed 4.5 kb *Hind* III band (Fig. 3A, lane 3) was reduced to a smaller *Eco* RI/*Hind* III fragment (Fig. 5, lane 5). This smaller-sized band differed from the 4.5 kb *Eco* RI/*Hind* III band that is ex-

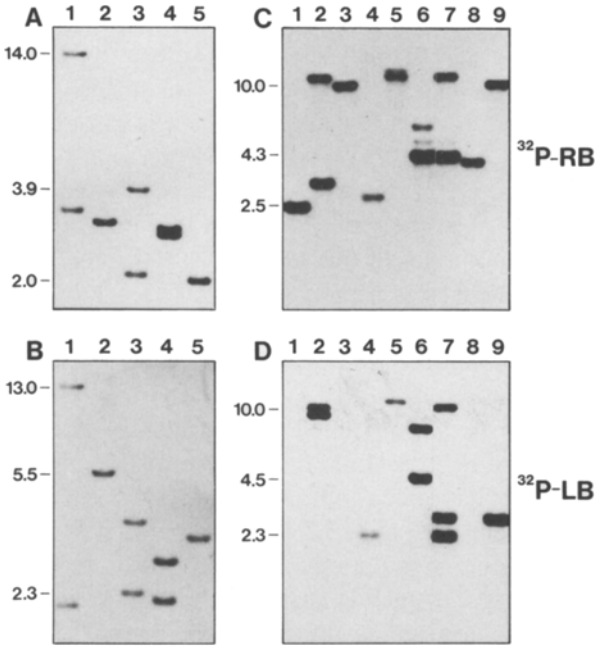

Fig. 4. Composites of Southern blot hybridizations of *Hin*d III-digested genomic DNA isolated from RT-derived transgenic *Arabidopsis* plants containing pB-Ac101, pB-Ac102 and pB-Ac103 (see Table 1 for individual transgenic plants). A and B. Lane 1, 140.1.3; 2, 140.17.1; 3, 140.21.1; 4, 150.59.1; 5, 150.85.3. ^{32}P-RB was the labelled probe for A, and ^{32}P-LB was used in B. C and D. Lane 1, 140.12.2; 2, 150.1.3; 3, 150.23.3; 4, 150.74.1; 5, 150.119.1; 6, 160.4.1; 7, 160.52.1; 8, 160.75.1; 9, 160.79.1. ^{32}P-RB was the labelled probe for C, and ^{32}P-LB was used in D. Sizes shown are in kb.

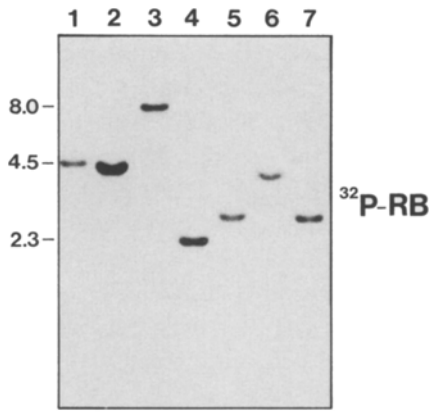

Fig. 5. Composites of Southern hybridizations of *Eco* RI/*Hin*d III-digested genomic DNA isolated from LDT- and RT-derived transgenic *Arabidopsis* plants (listed in Table I; see text). Lane 1, 62; 2, 161; 3, 122.3; 4, 125.2; 5, 253; 6, 126.4; 7, 299. ^{32}P-RB was the labelled probe, sizes shown are in kb.

pected with RB/RB type insertions, and therefore it is most likely that such insertions did not occur in these transgenic plants.

In RT-derived transgenic plant 126.4 an *Eco* RI/*Hin*d III fragment of ca. 4.2 kb (Fig. 5, lane 6) was about the same size as a *Hin*d III band previously identified with the ^{32}P-RB probe (Fig. 3, lane 4) and, similar to the results just described, the single T-DNA insertion in this transgenic plant is probably not arranged in a RB/RB orientation. RT-derived transgenics 125.2 and 299 did not contain presumptive RB/RB insertions (Fig. 3A, lanes 2 and 17), and thus served as controls (Fig. 5, lanes 4 and 7). An additional, and more precise, Southern hybridization that included *Bam* Hi-digested DNA derived from RT transgenic plants 125.2, 157, 253, 294, 297 and 305 indicated that independent insertions had occurred in these plants when probed with the ^{32}P-RBT fragment, while RB/RB type insertions were detected in plants 302, 307, 308 (results not shown).

LTD- and RT-derived transgenics frequently lack left-border T-DNA regions

An examination of the left T-DNA border regions in LDT-derived transgenic plants indicated that complete T-DNA copies were not always transferred. Hybridization with the ^{32}P-LB probe showed fewer left border region copies present than right ones (for example Fig. 2A, lanes 1, 4, 11). A survey of the 27 LDT-derived transgenic plants indicated that approximately two-thirds ($17/27 = 63\%$) did not contain at least 3 kb of the LB region. Since the ^{32}P-LB probe spans 3.0 kb of the left-border region in the pGV3850DsDHFR (1 and 2) and the pGV3850Ds35S (1 and 2) vectors (see diagram in Fig. 1A), it was important to determine how much of the remaining T-DNA was transferred. Therefore, Southern hybridizations of *Eco* RI/*Hin*d III-digested DNA from LDT-derived plants which had lost left borders were probed with the *MAS*

promoter sequence (Fig. 1A). In all cases a 7.2 kb size *Eco* RI/*Hin*d III fragment was observed, which indicated that the terminal end of the transferred T-DNA was located distal to the NPTII gene and that the central region of the T-DNA was structurally intact (data not shown).

RT-derived transgenic plants were also found to have lost left T-DNA borders. For example, RT transgenic plants 125.2, 303 and 305 did not contain left border region sequences (Fig. 3B, lanes 2, 8, and 10). Of the transgenic plants containing the pGV3850DsDHFR and pGV3850Ds35S vectors that were examined for the loss of left border regions, less than half did not contain the terminal left border region (19/41 = 46%) when the 3.0 kb LB fragment was used as a probe.

The lack of right-border T-DNA regions occurs more often with RT-derived transgenics

Right-border region T-DNA sequences were also missing in both LDT- and RT-derived transgenic plants after Southern hybridizations in which a 1.1 kb *Hin*d III/*Bam* HI right-border fragment was used as a probe (Figs. 2A and 3A). The LDT-derived transgenics appear to contain more right-border T-DNA sequences; however, this is due to the increased content of T-DNAs and the occurrence of RB/RB inverted repeats in these plants (see Discussion). The RT-derived transgenics contained more pGV3850DsDHFR (1 and 2) and pGV3850Ds35S (1 and 2) T-DNA insertions without the terminal RB region (15/41 = 37%) than the LDT plants (4/27 = 15%).

Discussion

In the light of the results presented in this study, the patterns of T-DNA insertions reported in the literature are most likely due to the choice of the explant material used in *Agrobacterium*-mediated plant transformations. Recently, van Wordragen and Dons [33] have summarized the results of numerous publications in which *A. tumefaciens* has been used to transform plants. In most plant species (35/36) the tissues used were derived from upper plant parts, and in only one species, *Arabidopsis*, roots were used as the explant source [32].

A variety of transgenic plant species have been examined at the molecular level to determine the copy number and the integrity of T-DNA insertions, and from the results obtained it has been assumed that multiple T-DNA insertions, frequently organized in direct and indirect repeated type structures, are inherent features of the *Agrobacterium*-mediated transformation process. For example, multiple and aberrant T-DNA insertions have been commonly observed in tobacco [30], tomato [15, 25, 31], and petunia [5, 13]. Also, stem inoculations of tomato (4) and *Arabidopsis* seed transformations [7] using *A. tumefaciens* exhibit similar integration patterns. It is important to note that in each of these examples leaf, cotyledons, or stem explants were used with *Agrobacterium*-mediated transformation; in the case of the *Arabidopsis* seed transformation method, the actual T-DNA integration events are thought to occur at a later stage in plant development in upper plant parts [8].

We have provided evidence, based on Southern hybridization results from 82 transgenic plants, that a root transformation (RT) method generated more single T-DNA insertions (64%; 35/55) when compared to a leaf-disc transformation (LDt) method (11%; 3/27). Most of the LDT-derived transgenic plants contained multiple insertions (89%; 24/27), usually organized as right border (RB/RB) inverted-repeat type insertions (58%; 14/24). The RB/RB insertions were confirmed by Southern analyses of DNA isolated from transgenic plants digested with different restriction enzymes sites located closer to the right border. Left-border/left-border (LB/LB) and tandem left-border/right-border (LB/RB) insertions, which have been previously identified in other transgenic plants [3, 9, 11], were observed at a low frequency in some of the transgenics. In contrast to the results obtained with the LDT-derived transgenics, fewer multiple insertions were observed in transgenics derived from root

explants, and most of these contained only two, unlinked insertions. Furthermore, the use of root explants resulted in fewer right border inverted repeat type insertions (38%; 8/21). These results are summarized in Fig. 6.

The plant selectable marker in the binary vectors, and the cointegrate vector pGV3850::pDs35S-1, used in this study are located proximal to the right border [10], while the marker in the pGV3850::pDsDHFR and pGV3850::pDs35S-2 cointegrate vectors are located closer to the central region (see Fig. 1). Since the transfer of T-DNA to plant cells is thought to occur in a polar manner beginning at the right-border [35], full-length and truncated T-DNA insertions were expected. A comparison between the loss of left and right border regions among the RT-derived transgenics indicated that fewer right borders were missing (37%; 15/41) than left borders (19/41; 46%), which was expected because deletions of the right border region, especially with the binary and pGV3850::pDs35S-1 vectors, would delete the plant selectable marker. In contrast, the difference between the loss of left border regions (63%; 17/27) and right border regions (15%; 4/27)

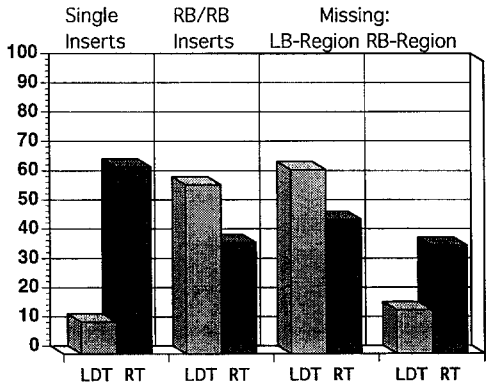

Fig. 6. The bar graph shows the distribution of: single T-DNA insertions found with LDT (11%; 3/27) and RT derived plants (64%; 35/55); right-border/right-border (RB/RB) inverted-repeat type insertions in LDT- (58%; 14/24) and RT-derived plants (38%; 8/21); insertions missing the left-border (LB) region among LDT- (63%; 17/27) and RT-derived plants (46%; 19/41); and T-DNA insertions missing the right-border (RB) region among LDT- (15%; 4/27) and RT-derived plants (37%; 15/41).

among the LDT-derived transgenics is much greater (Fig. 6); that is, the LDT-derived transgenics appear to contain more right border T-DNA sequences. This difference can be explained by the higher frequency of RB/RB tandem insertions observed with the LDT-derived transgenics (58%; 14/24). Since the two left borders are on each end of the insertion, it is possible to lose one or both of the left borders and yet still retain the tandem RBs located in the central region of the insertion (Fig. 1B).

A new series of binary vectors has been developed with plant selectable markers located next to the left border in order to increase the frequency of full-length T-DNA insertions [2]. The use of these binary vectors is expected to result in fewer right-border deletions, which would simultaneously lead to a loss of the selectable marker. Due to the location of the selectable markers in the cointegrate and binary constructs used in this study (as stated above), left border truncated T-DNA insertions were expected. While we have not determined the exact location in the pGV3850::pDsDHFR (1 and 2) cointegrate vectors where most of the left-border region terminations have occurred, an *ocs* poly(A) sequence, located proximal to the left T-DNA border region (Fig. 1), was present in all of the transgenics examined (results not shown). Therefore, the truncated T-DNA insertions in these transgenics probably terminated at a location adjacent to the left-border region.

The T-DNA integration pattern in a large number of transgenic petunia plants has been previously examined. Delores and Gardner [5] used Southern hybridizations to analyse 96 transgenic petunia plants, generated by leaf-disc transformation using a binary vector, and found that: (1) 38% contained single T-DNA insertions; (2) 60% had multiple insertions, frequently organized in repeated arrays; and (3) left and right T-DNA borders were missing in ca. 20% of the transgenic plants. In general, these frequencies of T-DNA integration are similar to our findings for the LDT-derived transgenic *Arabidopsis* plants. However, while the loss of right borders was similar in both petunia and *Arabidopsis*, in our study

about three times more left borders (63%) were lost (see above).

We conclude from the results obtained in this study that the particular transformation method (RT vs. LDT) was responsible for the patterns of T-DNA integration that were observed. That is, the use of two types of transformation vectors, including cointegrate and binary vectors, as well as different *A. tumefaciens* strains and *Arabidopsis* ecotypes, were not directly responsible for the distinct patterns of T-DNA integration observed. Likewise, the dihydrofolate reductase selectable marker (DHFR) and the hygromycin (HPT) plant selectable markers, used with both the RT and LDT methods, did not result in different integration patterns. The DHFR marker has been previously used to produce transgenic petunia [6], *Arabidopsis* and tobacco plants [17, 23]. Of the different T-DNA constructs used with the LDT and RT methods, all contained either *Ac* (transposase-coding sequence only) or *Ds* (non-autonomous) transposable element sequences. It is unlikely that *Ac*/*Ds* was responsible for the results obtained in that the T-DNA analysis was done with individual transgenic plants before any genetic crosses (*Ac* × *Ds*) were done to activate the transposon system.

While it is most likely that the explant tissue used for the transformations is the determinant factor, it is nevertheless possible that other differences in the initial stages of the transformations were responsible for the observed T-DNA integration patterns. The major difference in this study between the root and leaf-disc transformations, through to the stage when the clonal growth of selected callus occurred, was the use of 2,4-D in the RT method (see Materials and methods). We rationalize that 2,4-D was not responsible for the phenomena observed because it was also used in the various leaf-disc transformation methods of tomato [25, 31], tobacco [30], and petunia [5]; in contrast, stem inoculations of tomato [4] and an *Arabidopsis* seed transformation method [7, 8] did not use 2,4-D. As stated above, multiple and aberrant T-DNA insertions, were observed in all of these plants, and the common factor was that upper plant parts were used for the *Agrobacterium*-mediated transformations; otherwise, divergent hormone and tissue culture regimes were employed.

There are indications that the type of T-DNA insertion directly affects the expression of transgenes, which could in part help to explain some of the genetic suppression effects that have been observed in transgenic plants (for review, see [14]). Recently, Hobbs *et al.* [11, 12] examined transgenic tobacco plants, produced by a leaf-disc method in which a binary vector was used, and have shown that transgene expression was positively associated with single T-DNA insertions, while negative results were obtained with inverted repeat type insertions. Therefore, root-derived transgenic plants, which mostly contain single insertions, or multiple insertions consisting of unliked T-DNA copies, should prove useful for studies involving transgene expression.

One possibility for the patterns of T-DNA integration described in this paper is that the tissue specificity of the promoter used to drive the plant selectable marker may be a determinant factor. The commonly used cauliflower mosaic virus (CaMV) 35S and nopaline synthase (NOS) promoters, used to drive the DHFR and HPT markers (Fig. 1), respectively, are developmentally regulated and are more active in lower plant parts, including roots, than in the young or upper plant leaves that are preferentially used for plant transformations [1, 3]. In addition, the bidirectional mannopine synthase (MAS) promoter (Fig. 1) is also frequently used to drive plant selectable markers, and displays a similar pattern of expression in plants [19, 20].

It is therefore possible that in root-derived transformed cells single T-DNA copies provide enough marker gene expression to allow for efficient drug resistance, due to the relatively higher activity of the CaMV35S and NOS promoters in roots. In contrast, multiple T-DNA insertions maybe required in leaf-derived cells to result in a sufficient level of marker gene expression needed for drug resistance. This would help to explain why additional T-DNA copies were found in the LDT-derived transgenics and mostly single copies in the RT-derived plants. Further experiments

using different combinations of tissue-specific promoters will be necessary to determine whether this is the case. In addition, it is possible that tissue-specific factors, associated with the integration of T-DNA by an illegitimate recombination type mechanism [9, 24], are in some way responsible for the observed integration patterns with the RT and LDT; however, speculation in this area is premature as such factors have yet to be identified.

The different T-DNA integration patterns observed in this study, using the root and leaf-disc transformation methods, have important implications for a broad range of basic and applied research in which *Agrobacterium*-mediated transformation is used to produce transgenic plants. The most important observation is the high frequency of single T-DNA insertions obtained with the root transformation method. Transgenic plants that contain single-copy inserts are advantageous for transgene expression, T-DNA insertional mutagenesis, segregation analysis, and T-DNA reporter gene fusions that depend on T-DNA/plant sequence fusions [9, 18].

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