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High-frequency linkage of co-expressing T-DNA in transgenic *Arabidopsis thaliana* transformed by vacuum-infiltration of *Agrobacterium tumefaciens*

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Abstract The efficiency of co-expression and linkage of distinct T-DNAs present in separate *Agrobacterium tumefaciens* was analysed in *Arabidopsis thaliana* transformed by the vacuum infiltration method. Co-expression was monitored by the synthesis of three bacterial proteins involved in the production of polyhydroxybutyrate (PHB) in the plastids. Out of 80 kanamycin-resistant transgenic plants analysed, 13 plants were co-transformed with the two distinct T-DNAs and produced PHB. Of those, 7 lines had a kanamycin-resistance segregation ratio consistent with the presence of a single functional insert. Genetic linkage between the distinct T-DNAs was demonstrated for all 13 PHB-producing lines, while physical linkage between the distinct T-DNAs was shown for 12 out of 13 lines. T-DNAs were frequently linked in an inverted orientation about the left borders. Transformation of *A. thaliana* by the co-infiltration of two *A. tumefaciens* containing distinct T-DNAs is, thus, an efficient approach for the integration and expression of several transgenes at a single locus. This approach will facilitate the creation and study of novel metabolic pathways requiring the expression of numerous transgenes.

Key words *Arabidopsis thaliana* · T-DNA · Co-transformation · Linkage · *Agrobacterium tumefaciens*

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Introduction

The expression of several transgenes is frequently required in either basic or applied studies in plant biology and biochemistry. For example, the creation of a novel metabolic pathway for the synthesis of polyhydroxyalkanoates in the plastids involved the coordinate expression of up to four transgenes (Nawrath et al. 1994a; Poirier et al. 1992; Valentin et al. 1999). Different strategies have been used to introduce several genes into a single plant. Crosses between transgenic plants expressing a single gene is time-consuming and laborious whenever more then two transgenes need to be combined and homozygocity for all loci must be obtained. Some binary vectors are sufficiently versatile to include two transgenes within a single T-DNA, but the lack of convenient restriction sites and the size of the T-DNA quickly become limiting when considering the introduction of three or more transgenes. Alternatively, binary vectors have been developed which contain two different T-DNAs on a single plasmid (Komari et al. 1996). Transformation of tobacco and rice with these vectors revealed a rate of co-transformation with the two T-DNAs of 47–85%. Among co-transformed plants, over 50% had the two T-DNAs unlinked, enabling the segregation of each transgene in subsequent generations. Independent segregation of two T-DNAs is advantageous in the development of transgenic lines expressing a transgene without the presence of a linked antibiotic resistance gene. However, when two or more transgenes need to be co-expressed in plants, linkage is desired to simplify the creation of homozygous lines and the combination of additional transgenes by crossing.

Transformation of plants with several genes can be relatively easily achieved through biolistic methods by precipitating a mixture of DNA onto particles. Up to 12 different DNA fragments have been transformed into soybean embryogenic clones by this method (Hadi et al. 1996). However, for most dicotyledons and a growing number of monocotyledons, infection with *Agrobacterium tumefaciens* is the method of choice for transforma-

tion. Co-transformation of plants with a mixture *Agrobacterium* containing distinct T-DNAs has been demonstrated in a few plant species (De Block and Debrouwer 1991; De Buck et al. 1998; De Framond et al. 1986; De Neve et al. 1997; Denis et al. 1995; Depicker et al. 1985; Komari et al. 1996; McKnight et al. 1987). The frequency of linkage of co-introduced T-DNA is variable, depending on the plant, bacteria and transformation protocol used. For example, while early studies of co-transformation of tobacco using protoplasts or leaf disks revealed no linkage between transgenes (De Framond et al. 1986; Depicker et al. 1985; McKnight et al. 1987), later studies reported linkage frequency of 40%–60% (De Block and Debrouwer 1991; De Buck et al. 1998; De Neve et al. 1997; Denis et al. 1995; Komari et al. 1996).

In recent years, several methods have been developed for the transformation of *Arabidopsis thaliana* using protocols which do not involve tissue culture, such as *A. tumefaciens* vacuum-infiltration and floral dip (Bechtold et al. 1993; Chang et al. 1994; Clough and Bent 1998; Feldmann and Marks 1987; Katavic et al. 1994). These methods are simple, rapid and avoid problems of somaclonal variation and transgenic infertility often associated with tissue culture and plant regeneration from calli (Larkin and Scowcroft 1986; Scholl et al. 1981). In the study presented here, we wished to examine the potential of the vacuum-infiltration method for the expression of three genes involved in polyhydroxybutyrate synthesis through the integration and linkage of two distinct T-DNA contained in separate *A. tumefaciens*. We were able to show here that all 13 transgenic lines co–expressing the three genes have linked copies of the distinct T-DNAs.

Materials and methods

DNA constructs

The structure of the T-DNAs in the binary vectors pBI-TPSS-Thio, pBI-TPSS-Red and pBI-TPSS-Syn containing, respectively, the *phaA, phaB* or *phaC* genes from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) fused to the chloroplast transit peptides of the small subunit of Rubisco (TPSS), have been described in detail (Nawrath et al. 1994b). A T-DNA harbouring both the *phaA* and *phaB* genes was constructed by isolating the *Cla*I-*Eco*RI fragment from pBI-TPSS-Thio containing the CaMV35 S promoter, TPSS-*phaA* gene fusion and nopaline synthase terminator. The fragment was made blunt-end by the Klenow fragment of the *Escherichia coli* DNA polymerase I and was ligated into the *Eco*RI site of the vector pBI-TPSS-Red, which was also made blunt-end. The resulting vector was named pBI-TPSS-TR. The binary vectors pBI-TPSS-Syn and pBI-TPSS-TR were transferred into *A. tumefaciens* C58 pGV3101 by electroporation and selected on agar plates containing Luria broth and 50 µg/ml kanamycin. Both T-DNAs harboured the neomycin phosphotransferase II (NPTII) gene conferring kanamycin resistance to plants.

Plant transformation

A. thaliana, accession Columbia, was transformed by vacuum infiltration. Briefly, *A. tumefaciens* containing either pBI-TPSS-Syn or pBI-TPSS-TR were grown separately in Luria broth supplemented with 50 µg/ml kanamycin until an OD_{600} of 1.0. At this point, equal volumes of both cultures were mixed, centrifuged and the pellet resuspended in infiltration medium to a final OD_{600} of 0.8. The infiltration media contained half-strength Murashige and Skoog (MS) salts (Murashige and Skoog 1962) adjusted to pH 5.7, 5% sucrose, 44 n*M* benzylamino purine and 0.005% (V/V) Silwet L-77. *A. thaliana* plants were grown in soil under continuous fluorescent light and the primary bolts were cut. Plants (T0) were infiltrated with the *A. tumefaciens* mixture when the first flowers of the secondary bolts were opening. Infiltration was done in a vacuum chamber by immersing the above-ground parts of the plant in the *A. tumefaciens* solution and creating a vacuum of 650 mm Hg for 10 min. After infiltration, plants were placed under a plastic dome for 1 day before resuming growth under 60% humidity and constant fluorescent light until seeds were collected (T1 seeds). Transgenic plants were obtained by plating surface-sterilised T1 seeds on plates containing MS salts, 1% sucrose, 0.75% agar and 50 µg/ml kanamycin for 1 week. Segregation analysis of transgenes in the various transgenic lines was done on the same media.

Polyhydroxybutyrate analysis

Polyhydroxybutyrate (PHB) produced in transgenic plants was quantified by gas chromatography. Briefly, a single leaf was cleared by repeated washes with methanol at 55°C and placed in a glass tube containing 1 ml chloroform and 1 ml of 3% sulphuric acid in methanol. The samples were heated at 100°C for 4 h, cooled down to room temperature and 2 ml of an aqueous solution of 0.9% NaCl added; the mixture was then vortexed and centrifuged. The lower chloroform phase containing the methyl ester of 3-hydroxybutyric acid was recovered and quantified by gas chromatography as previously described (Poirier et al. 1992). Bacterial polyhydroxybutyrate (Sigma, St. Louis) was used as a standard.

Synthesis of PHB in transgenic plants was also visualised by epifluorescence microscopy. One-week-old plants growing in media containing MS salts, 1% sucrose, 0.75% agar and 50 µg/ml kanamycin were gently pulled out of the petri dishes and immersed in an aqueous solution of 0.1% Nile Blue A for 5 min. The plants were then rinsed under tap water, immersed for 1 min in 8% acetic acid and rinsed again in water. Whole seedlings were put on microscope slides, and the roots were examined under epifluorescence microscopy using a 530–560 nm excitation filter and 580 nm barrier filter.

Molecular analysis

Plant DNA was isolated by the CTAB procedure as described by Rogers et al. (1988). Southern analysis was done using nylon membranes (Amersham, Buckinghanshire, UK) and random-primed [^{32}P]-labelled probes, as described by Sambrook et al. (1989) Filters were washed for 1 h at 65°C in 0.1×SSC and 0.1% SDS. Before the filters were re-hybridised with a different probe, they were washed for 1 h at 85°C in 0.05×SSC, and the absence of signal was verified by autoradiography.

Results

Isolation of transgenic plants co-transformed with two T-DNAs

It has previously been demonstrated that PHB synthesis in plastids of *A. thaliana* requires the expression of three proteins, namely the 3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase, encoded, respectively, by the *phaA, phaB* and *phaC* genes from *R. eutropha* (Nawrath et al. 1994a). All genes were modified by addition of the

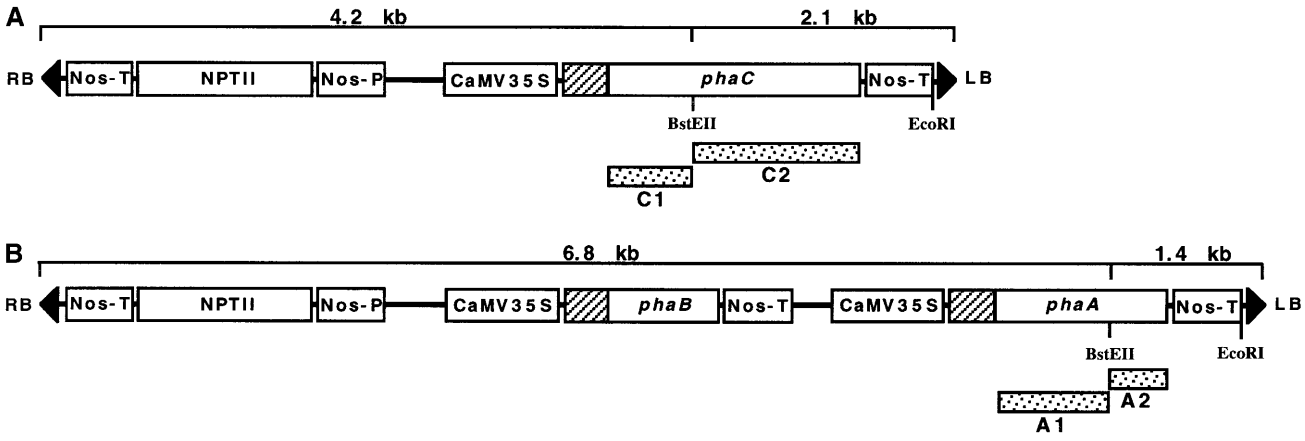

Fig. 1A, B Maps of the T-DNAs used to introduce the *pha* genes of *R. eutropha* into *A. thaliana*. **A** T-DNA of pBI-TPSS-Syn containing the *phaC* gene, **B** T-DNA of pBI-TPSS-TR containing the *phaA* and *phaB* genes. *NPTII* Neomycin phosphotransferase II gene, *Nos-P* nopaline synthase promoter, *Nos-T* nopaline synthase transcriptional terminator, *CaMV 35 S* cauliflower mosaic virus 35 S promoter, *RB* right border, *LB* left border. The *hatched boxes* adjoining the *pha* genes represent sequences encoding the plastid transit peptide of the small subunit of Rubisco, which is fused in frame with the *pha* gene sequences. The *dotted boxes* represent the various DNA fragments which have been used as probes for Southern analysis

sequence encoding the plastid signal peptide from the small subunit of Rubisco (Nawrath et al. 1994b). Combination of all three genes in a single transgenic plant had previously been achieved by successive crosses between plants transformed with each gene and detection of the hybrids by Western analysis of the expressed proteins (Nawrath et al. 1994a). No plants homozygous for all three genes could be obtained because of the complexity of the segregation of all T-DNAs.

In a first step to express all three *pha* genes at a single locus, a T-DNA containing both *phaA* and *phaB* genes was constructed. Figure 1 shows the structure of the T-DNAs in the binary vectors pBI-TPSS-TR and pBI-TPSS-Syn. The binary vectors were transferred independently to *A. tumefaciens* C58 pGV3101. A solution containing an equal proportion of both *A. tumefaciens* clones was used to transform *A. thaliana* by vacuum infiltration. Four independant infiltrations were done on a total of 32 plants. Seeds from every infiltrated plant (T0) were harvested, and transgenic plants (T1) were selected on media containing kanamycin and transferred to soil. After 3 weeks of growth, a leaf sample was analysed for the presence of PHB. Since PHB can only be synthesised in the plastids through the co-expression of all three bacterial proteins, only plants co-transformed with both T-DNAs and co-expressing all three *pha* genes were selected. Of 80 kanamycin-resistant (Km^R) T1 transgenic plants analysed, 13 had a detectable level of PHB (≥20 µg/g fresh weight).

Segregation analysis

Segregation of the Km^R phenotype was analysed for the T2 seeds of plants co-expressing the three *pha* genes (Table 1). Seven lines (nos. 1, 6, 26, 31, 32, 67, 71) had

Table 1 Segregation of the kanamycin resistance marker in T2 seeds of co-expressing plants

Transgenic lines	Km^R	Km^S	Segregation pattern[a]	χ^2	*P*	PHB^+/Km^R [b]
1	91	27	3:1	0.282	0.50–0.70	48/48[c]
6	87	32	3:1	0.227	0.50–0.70	46/46[c]
10	117	3	63:1	0.686	0.30–0.50	58/59[d]
23	111	1	63:1	0.327	0.50–0.70	58/58[d]
26	89	32	3:1	0.135	0.70–0.80	45/45[c]
31	88	27	3:1	0.142	0.70–0.80	46/46[c]
32	171	46	3:1	1.673	0.10–0.30	49/49[c]
34	115	2	63:1	0.016	0.80–0.90	49/49[d]
36	108	7	15:1	0.005	0.90–0.95	44/44[c]
61	62	0	>63:1	0.984	0.30–0.50	44/44[d]
67	90	31	3:1	0.025	0.80–0.90	41/44[c]
69	112	0	>63:1	1.778	0.10–0.30	56/57[d]
71	145	64	3:1	3.523	0.05–0.10	41/42[c]

[a] The segregation ratio having the highest probability, as determined by χ^2 analysis, is given
[b] Number of Km^R plants producing PHB over the total number of Km^R plants
[c] Genetic linkage of TPSS-TR and TPSS-Syn T-DNAs at each locus determined by the $\chi 2$ analysis
[d] Genetic linkage of TPSS-TR and TPSS-Syn T-DNAs at two or more loci determined by the χ^2 analysis

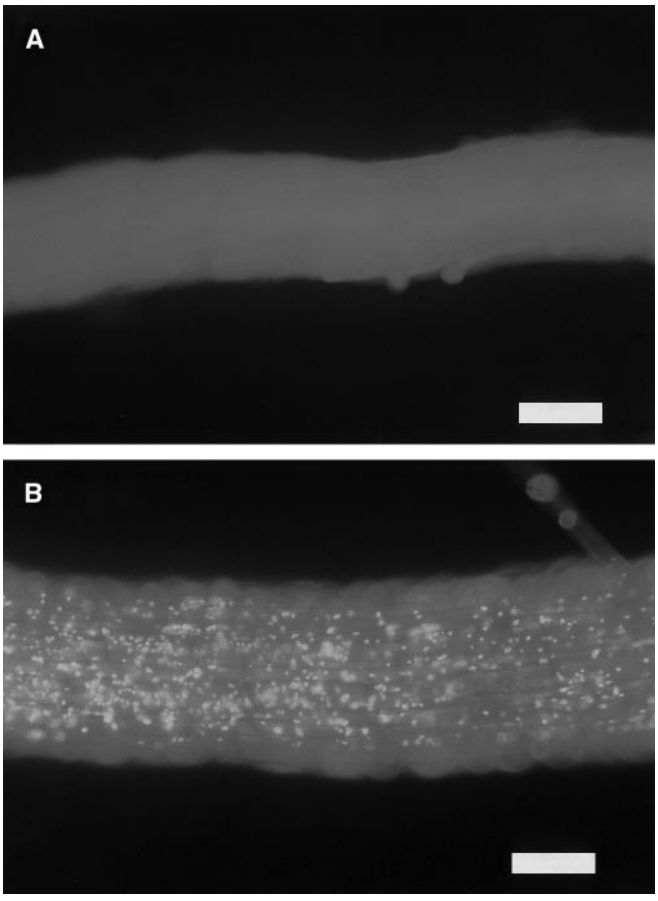

Fig. 2A, B Visualisation of agglomerations of PHB inclusions by epifluorescence microscopy of roots stained with Nile Blue A. **A** Root from a wild type plant, **B** root from a transgenic plant producing PHB. *Bars*: 50 u*M*

a segregation ratio in accordance with the presence of a single functional insert, while 1 line (no. 36) and 3 lines (nos. 10, 23, 34) had a segregation ratio indicating the presence of two and three functional inserts, respectively. Two lines (nos. 61, 69) had a segregation ratio indicating the presence of at least three functional inserts.

A first test for genetic linkage between two distinct T-DNAs was done by analysing PHB synthesis in transgenic Km^R T2 plants. In these plants, PHB synthesis could be quickly determined by the detection of agglomerations of PHB inclusions by epifluorescence microscopy (Fig. 2, Table 1). In 5 lines with a single functional insert (nos. 1, 6, 26, 31, 32) all Km^R plants produced PHB, indicating genetic linkage between the distinct T-DNAs and co-expression of the *phb* genes. Lines nos. 71 and 67, which also contained a single functional insert, had 1 out of 42 Km^R plants and 3 out of 44 Km^R plants, respectively, with no detectable synthesis of PHB. These Km^RPHB^- events are too rare to fit an independent segregation of two unlinked T-DNAs with only one of the two T-DNAs containing a functional NPTII gene. The presence of these Km^RPHB^- seedlings can be explained either by contamination of the seed stock, silencing effect or that although genetically linked, the distance between the two T-DNAs is sufficient to allow detectable cross-over events.

For plant line no. 36, which contained two functional inserts, all Km^R plants produced PHB, indicating genetic linkage and co-expression of the distinct T-DNAs at both loci. For plants having three or more functional inserts (lines nos. 10, 23, 34, 61, 69), the ratio of Km^R plants producing PHB is consistent with genetic linkage and co-expression of the distinct T-DNAs at two or more loci.

Detection of linkage by Southern analysis

Linkage of T-DNAs was also analysed in all co-expressing transgenic plants by Southern blot. Genomic DNA was digested by *Bst*EII, an enzyme cutting only once in each T-DNA, and thus generating fragments of variable length for each T-DNA/genomic DNA junctions (Fig. 1). Furthermore, physical linkage of two T-DNAs at the right or left borders can be detected by the presence of fragments of predictable lengths.

Southern analysis was performed for all transgenic plants by hybridisation with the C1, C2, A1 and A2 probes, with all filters being successively hybridised with at least two different probes (Fig. 1). Identification of fragments hybridising to two probes could only be made unambiguously for fragments smaller then 6 kb. Thus, linkage involving right borders could not be identified clearly, except for linkage between the right border of TPSS-Syn and the left border of TPSS-TR.

Representative autoradiographs are shown in Fig. 3, and results are summarised in Table 2. The number of insertions for each T-DNA was variable in the different lines, ranging from 1 to 12. Strikingly, all lines except 1 (line no. 67) showed physical linkage between the TPSS-TR and TPSS-Syn T-DNAs at their left borders, as detected by the presence of a 3.5-kb fragment hybridising to both C2 and A2 probes (Fig. 3B, D, arrow a). Numerous lines also showed physical linkage of two identical T-DNAs at their left borders, detected as a 2.8-kb fragment for TPSS-TR (Fig. 3B, arrow b) and 4.2-kb fragment for TPSS-Syn (Fig. 3D, arrow f). Linkage of TPSS-TR and TPSS-Syn T-DNAs in a tandem configuration was also detected in 7 lines (Fig. 3B, C, arrow d). Some fragments of variable lengths could also be shown to hybridise to probes derived from both TPSS-TR and TPSS-Syn T-DNAs, indicating some rearrangements and deletions of the linked T-DNAs (Fig. 3B, C, arrow c). A 3.8-kb fragment hybridising to both C1 and C2 probes (Fig. 3C, D, arrow e) and a 6.8-kb fragment hybridising to probe C2 (Fig. 3D, arrow g) were detected in several lines. It is unknown how such distinct fragments could be generated in several independent lines, but the presence of multiple copies of the NPTII genes as well as of the CaMV35 S promoter, Nos promoter and Nos terminator may promote recombinations at different sites, leading to rearrangements and deletions.

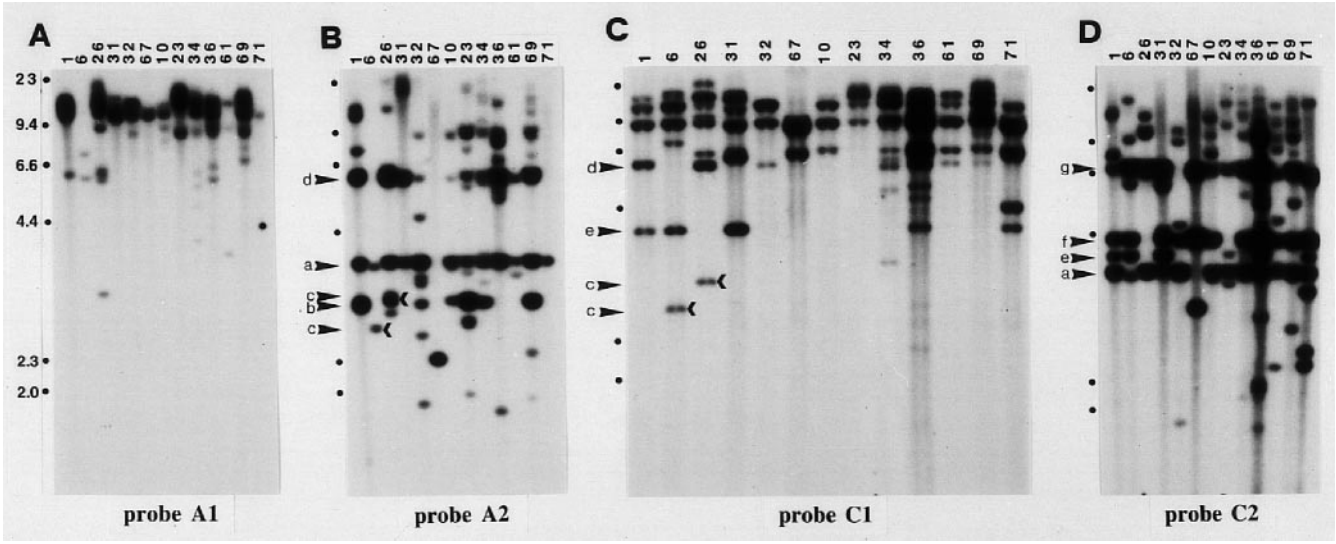

Fig. 3A–D Southern blot analysis of plants co-transformed with TPSS-TR and TPSS-Syn T-DNAs. Plant genomic DNA was digested with the restriction enzyme *Bst*EII and analysed using a series of probes described in Fig. 1. Hybridisation was done with: **A** probe A1, **B** probe A2, **C** probe C1 and **D** probe C2. The location of DNA size markers are shown to the *left* of each autoradiograph by *black dots*, with sizes in kilobases indicated at the *extreme left* of the figure. The *number* at the *top of each lane* refers to the transgenic line. *Arrow a* 3.5-kb fragment hybridising to probes A2 and C2, *arrow b* 2.8-kb fragment hydridising to probe A2, *arrow c* fragments (indicated by <) hydridising to probes A2 and C1, *arrow d* 5.6-kb fragment hydridising to probes A2 and C1, *arrow e* 3.8-kb fragment hydridising to probes C1 and C2, *arrow f* 4.2-kb fragment hydridising to probe C2, *arrow g* 6.8-kb fragment hydridising to probe C2

Table 2 Summary of Southern analysis of co-expressing plants

Transgenic lines	Linkage A2–C2[a]	Linkage A2–C1[b]	Linkage A2–A2[c]	Linkage C2–C2[d]
1	+	+	+	+
6	+	+		+
26	+	+		
31	+			+
32	+	+	+	+
67				+
10	+		+	+
23	+		+	
34	+	+	+	+
36	+	+		+
61	+	+		+
69	+	+	+	+
71	+			+

[a] Left border of TPSS-Syn linked to left border of TPSS-TR, fragment of 3.5 kb hybridising to the A2 and C2 probes
[b] Right border of TPSS-Syn linked to left border of TPSS-TR, fragment of 5.6 kb hybridising to the A2 and C1 probes except for line no. 6 with a fragment of 2.6 kb and line no. 26 with fragments of 2.9 and 5.6 kb hybridising to the A2 and C1 probes
[c] Two TPSS-TR linked to each other via the left borders, fragment of 2.8 kb hybridising to the A2 probe only
[d] Two TPSS-Syn linked to each other via the left borders, fragment of 4.2 kb hybridising to the C2 probe only

Line no. 67 was unique among all transgenic plants analysed in that no fragments were detected which could hybridise successively to probes derived from TPSS-TR and TPSS-Syn (Fig. 3). Clearly, in this line, the two distinct T-DNAs are not physically linked. These results combined with the segregation analysis of the kanamycin resistance and PHB biosynthesis in the T2 generation indicates that TPSS-TR and TPSS-Syn T-DNAs, although genetically linked, are inserted at some distance of each other.

Discussion

From a total of 80 transgenic plants obtained by the vacuum infiltration method using an equal mixture of two distinct *A. tumefaciens*, 13 plants were isolated which had co–integrated the two distinct T-DNAs and co-expressed the *pha* genes to produce PHB. The observed rate of 16% co-expression (13/80) is probably an underestimation of the T-DNA co-transformation frequency, since our selection method would have missed all plants which were co-transformed by the two T-DNAs but that either did not contain all three *pha* genes because of deletions or did not express all three genes to a sufficient level required for detectable accumulation of PHB. Nevertheless, it is expected that the rate of T-DNA co-transformation in *A. thaliana* infiltrated with separate *A. tumefaciens* is within the range of co-transformation frequencies observed for transformation of tobacco, rice, rapeseed and *A. thaliana* using techniques requiring infection of protoplasts or tissue explants, followed by calli production and plant regeneration through *in vitro* culture (De Block and Debrouwer 1991; De Buck et al. 1998; De Framond et al. 1986; De Neve et al. 1997; Denis et al. 1995; Depicker et al. 1985; Komari et al. 1996; McKnight et al. 1987).

Although the precise mechanism of *A. thaliana* transformation by the various methods involving inoculation of seeds or whole plants with *A. tumefaciens* (referred to

as *in planta* transformation, vacuum infiltration or floral dip) is unknown, it has been observed that the transformed progeny are typically hemizygous and that unique insertion patterns are obtained for different transformants obtained from a single inoculated plant, characteristics which are also found in transformants obtained in this study (Fig. 3, lines nos.23 and 26 were obtained from the same infiltrated plant) (Bechtold et al. 1993; Chang et al. 1994; Clough and Bent 1998; Feldmann and Marks 1987; Katavic et al. 1994). These results have suggested that transformation occurs after the divergence of anther and ovary cell lineages and that the likely targets for heritable transformation are either mature gametophytes, gametophyte-progenitor tissues or recently fertilised embryos (Feldmann and Marks 1987). These potential target cells appear therefore to be equally competent for T-DNA co-transformation by separate *A. tumefaciens* as protoplast or somatic cells from leaf, hypocotyl or root tissues.

The majority of plants co-expressing the *pha* genes had complex T-DNA integration patterns, with three or more copies of each T-DNA (Fig. 3). It is likely that the selection for co-expression favours the isolation of events where the target cell was simultaneously infected by several bacteria, thus leading to a bias population of transformants having complex integration patterns. Despite this complexity, 7 lines segregated for a single Km^R functional insert (Table 1). This implies that T-DNA integration does not necessarily lead to expression of the introduced genes due to factors such as DNA rearrangements or methylation.

Genetic linkage of the distinct T-DNAs has been shown for all 13 co-expressing lines. Physical linkage of the distinct T-DNAs was observed for 12 lines with at least one pair of T-DNAs linked in an inverted configuration through their left borders. Linkage of the same T-DNA through an inverted configuration about the left borders was also frequent, and tandem linkage was detected in several lines. Linkage through an inverted configuration about the right borders could not be adequately studied with the combination of vectors, enzymes and probes used. The high frequency of linkage of two T-DNAs about their left borders is striking since some studies have shown that inverted repeats implicating the right borders are more frequent than for the left borders (De Block and Debrouwer 1991; De Neve et al. 1997; Grevelding et al. 1993), although others have found equal proportion of both types of repeats (Jorgensen et al. 1987).

This study reports a high rate of genetic and physical linkage between distinct T-DNAs in plants co-transformed with two separate *A. tumefaciens* and co-expressing the different transgenes. Previous co-infection experiments using protoplast or tissue explants have shown a rate of T-DNA linkage in co-transformed plants ranging from 0–60% for tobacco, 0% for rice, 45% for *A. thaliana* and 67% for rapeseed (De Block and Debrouwer 1991; De Framond et al. 1986; De Neve et al. 1997; Denis et al. 1995; Komari et al. 1996; McKnight et al. 1987). Several factors could be involved in the modulation of linkage frequency, including *Agrobacterium* strains, plant species, target cells transformed and transformation protocols. The present work was done with an *A. tumefaciens* strain derived from C58. This strain was chosen, in part, because interbacterial conjugation of binary vectors cannot be detected with it, ensuring that linkage of the T-DNA must have arisen in the plant cells (Offringa et al. 1990). It has been suggested earlier that the use of a nopaline-type strain (e.g. C58) might favour more linked co-transformation compared to an octopine strain (e.g. LBA4404) (De Block and Debrouwer 1991). It is interesting to speculate whether the type of target cells transformed by the vacuum infiltration method (i.e. mature gametophytes, gametophyte-progenitor tissues or recently fertilised embryos) may also be an important factor favouring the linkage of T-DNAs.

The presence of several co-expressing lines with a single functional insert is highly valuable since it allows the investigator to easily obtain plants homozygous for all three genes as well as to move the introduced genes into various genetic backgrounds by crosses to different plants. For example, PHB-producing lines can be crossed to various *A. thaliana* mutants affected in starch or lipid biosynthesis in order to study the influence of carbon partitioning on PHA biosynthesis (Poirier 1999). In conclusion, the present study demonstrates that *A. thaliana* transformation by vacuum co-infiltration of two *A. tumefaciens* containing distinct T-DNAs is an efficient approach for the expression of several transgenes into a single locus.

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References

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci 316:1194–1199

Chang SS, Park SK, Kim BC, Kang BJ, Kim DU, Nam HG (1994) Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation *in planta*. Plant J 5:551–558

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

De Block M, Debrouwer D (1991) Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor Appl Genet 82:257–263

De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thalinana* root explants and tobacco protoplasts. Mol Plant-Microbe Interact 11:449–457

De Framond AJ, Back EW, Chilton WS, Kayes L, Chilton M-D (1986) Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F_1 generation. Mol Gen Genet 202:125–131

De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 11:15–29

Denis M, Renard M, Krebbers E (1995) Isolation of homozygous transgenic *Brassica napus* lines carrying a seed-specific chimeric 2 S albumin gene and determination of linkage relationships. Mol Breed 1:143–153

Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol Gen Genet 201:477–484

Feldmann KA, Marks MD (1987) Agrobacterium-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non tissue culture approach. Mol Gen Genet 208:1–9

Grevelding C, Fantes V, Kemper E, Schell J, Masterson R (1993) 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. Plant Mol Biol 23: 847–860

Hadi MZ, McMullen MD, Finer JJ (1996) Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep 15:500–505

Jorgensen R, Snyder C, Jones JDG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:471–477

Katavic V, Haughn GW, Reed W, Martin M, Kunst L (1994) *In planta* transformation of *Arabidopsis thaliana*. Mol Gen Genet 245:363–370

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

Larkin PJ, Scowcroft WR (1986) Somaclonal variations – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60:197–214

McKnight TD, Lillis MT, Simpson RB (1987) Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. Plant Mol Biol 8:439–445

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473–497

Nawrath C, Poirier Y, Somerville C (1994a) Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc Natl Acad Sci USA 91:12760–12764

Nawrath C, Poirier Y, Somerville C (1994b) Plastid targeting of the enzymes required for the production of polyhydroxybutyrate in higher plants. In: Doi Y, Fukuda K (eds) Biodegradable plastics and polymers. Elsivier Science, Amsterdam, pp 136–149

Offringa R, De Groot MJA, Haagsman HJ, Does MP, Van den Elzen JM, Hooykass PJJ (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation. EMBO J 9:3077–3084

Poirier Y (1999) Production of new polymeric compounds in plants. Curr Opin Biotechnol 10:181–185

Poirier Y, Dennis DE, Klomparens K, Somerville C (1992) Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–523

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer Academic Pub, Dordrecht, the Netherlands, pp 1–10

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

Scholl RL, Keathley DE, Baribault TJ (1981) Enhancement of root formation and fertility in shoots regenerated from anther- and seedling-derived callus cultures of *Arabidopsis thaliana*. Z Pflanzenphysiol Bd 104:225–231

Valentin HE, Broyles DL, Casagrande LA, Colburn SM, Creely WL, DeLaquil PA, Felton HM, Gonzalez KA, Houmiel KL, Lutke K, Mahadeo DA, Mitsky TA, Padgette SR, Reiser SE, Slater S, Stark DM, Stock RT, Stone DA, Taylor NB, Thorne GM, Tran M, Gruys K (1999) PHA production, from bacteria to plants. Int J Biol Macromol 25:303–306