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Thematic Issue on Horizontal Gene Transfer

Transformation of *Acinetobacter baylyi* in non-sterile soil using recombinant plant nuclear DNA

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To provide estimates of horizontal gene transfer from transgenic crops to indigenous soil bacteria, transformation frequencies were obtained for naturally transformable *Acinetobacter baylyi* BD413 using a chromosomally integrated plant transgene. The transgene comprised sequences for two phenotypic markers: kanamycin resistance (*npt* II) and green fluorescent protein (*gfp*), expressed from their own bacterial promoters. Recipient bacteria carried a copy of these two genes, with deletions in their 3'-termini abolishing the marker activity, these genes were integrated into a 16S rRNA gene in the bacterial chromosomal genome or carried on a broad host range plasmid. Successful recombination between the plant transgene and the bacterial genome resulted in restoration of the markers, allowing detection through antibiotic selection and fluorescence. Transformation parameters of increasing complexity, without any enrichment steps, were used to approach the field conditions, while still obtaining measurable transformation frequencies. In pure culture filter experiments, transformation was detected using ground, chopped and whole leaves, as well as whole sterile seedlings, and ground roots. In sterile soil microcosms, transformation was detected using pure plant DNA (3.6×10^{-8} transformants per recipient) and ground leaves (2.5×10^{-11}). Transformation was also detected for the first time in non-sterile soil using pure plant DNA (5.5×10^{-11}). Since the same constructs were used throughout, these data allow predictions of even more complex environmental systems where measurable frequencies are not easily obtainable.

Keywords: *Acinetobacter baylyi* / natural transformation / recombinant plant DNA / transferring plant DNA to bacteria

INTRODUCTION

With the increased deployment of genetically modified crops, estimated at 68–81 million hectares world-wide in 2004 (James, 2005; Stokstad, 2004), there is potential for horizontal gene transfer (HGT) from crops to bacteria by natural transformation. Frequencies are likely to be highest in soil, due to the huge populations of indigenous bacteria, and the large reservoir of DNA released from roots and other parts of the crop post-harvest. Estimates of HGT made using available data for transformation of naturally transformable soil bacteria put the frequency of recombinants at over a trillion overall in the transgenic crops grown world-wide (Heinemann and Traavik, 2004). Natural bacterial transformation is dependent on the availability of DNA, competence of the bacteria to take up the DNA, and sufficient regions of homology for integration into the bacterial genome *via* RecA-dependent homologous recombination (Day, 2002). Although much of the DNA released by decaying plant material is degraded within cells, substantial amounts are also released into the soil (Ceccherini et al., 2003). Plant DNA persists in soil for up to 2–3 years (Gebhard and Smalla, 1999; Paget et al., 1998), and its persistence is improved by absorption to soil particles (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994).

More than 40 species of naturally competent bacteria have been identified (Lorenz and Wackernagel, 1994). Nineteen of these are soil bacteria, including *Acinetobacter baylyi* BD413 (Vaneechoutte et al., 2006), and competence in this species can be enhanced by compounds naturally present in the rhizosphere of crop plants (Nielsen and van Elsas, 2001). As little as 20 bp of flanking homology can mediate homologous recombination (Watt et al., 1985), albeit at reduced frequency (Simpson et al., 2007). However, attempts to detect gene transfer from plant DNA to bacteria in non-sterile soil have so far been unsuccessful, making estimates of HGT in the field difficult. Laboratory and microcosm studies of increasing complexity have therefore been used in an attempt to make field-estimates of HGT. In laboratory experiments,

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Table 1. Restoration by natural transformation of deletion inactivated *npt*II and *gfp* genes on filters from various sources of donor DNA.

Donor DNA	Recipient (BD413)[1]	Number of transformants (mL^{-1}; arithmetic mean[2])	Transformation frequency (transformants/ recipient; geometric mean[3])	Number of replicates[4]
PC2-SP6, PCR product (5 μg)	pUCP26-BC2-SP6	$6.6 \pm 2.7 \times 10^4$	3.3×10^{-4}	10 (10, 100)
Arabidopsis DNA (0.5 μg)	pUCP26-BC2-SP6	155 ± 67	1.5×10^{-8}	6 (6, 100)
Ground leaves (0.1–0.3 g)	pUCP26-BC2-SP6	106 ± 39	2.7×10^{-9}	14 (12, 86)
Chopped leaves (0.1–0.3 g)	pUCP26-BC2-SP6	50 ± 25	4.5×10^{-9}	9 (9, 100)
Whole seedling (8–10 seedlings)[5]	pUCP26-BC2-SP6	1.3 ± 1.0	4.6×10^{-10}	2 (1, 50)
PC2-psbA, PCR product (5 μg)	BC1-psbA	$1.0 \pm 0.8 \times 10^6$	1.7×10^{-4}	7 (7, 100)
Arabidopsis DNA (0.5 μg)	BC1-psbA	150 ± 70	2.7×10^{-8}	8 (8, 100)
Ground leaves (0.1–0.3 g)	BC1-pbA	94 ± 19	3.5×10^{-9}	10 (10, 100)
Ground roots (0.1–0.3 g)	BC1-psbA	16 ± 1.0	2.7×10^{-8}	4 (4, 100)
Chopped leaves (0.1–0.3 g)	BC1-psbA	19 ± 7	1.1×10^{-9}	6 (5, 83)
Whole seedling (8–10 seedlings)[5]	BC1-psbA	1.2 ± 0.6	3.5×10^{-10}	5 (3, 60)

[1] The deleted gene cassette is plasmid encoded in pUCP26-BC2-SP6 and chromosomally encoded in BC1-psbA, called BC2 and BC1, respectively in the text for clarity.
[2] Mean values ± standard error of the mean.
[3] Minimum significant difference for transformation frequency ($P = 0.05$) = 1.85 $\log_{10}$ units.
[4] Total number of replicate transformation experiments (number of experiments giving transformants, percentage of experiments giving transformants).
[5] Seedlings were about 1 cm height after growth for 14 days.

using pure bacterial cultures, gene transfer from the plant genome to naturally competent *A. baylyi* BD413 has been achieved using purified DNA from a range of transgenic plants at frequencies ranging from 5.4×10^{-9} (Gebhard and Smalla, 1998) to 2.2×10^{-5} (Nielsen et al., 2000), in the latter case following nutrient addition to stimulate competence development. Transformation was also achieved at similar frequencies compared to purified plant DNA using either leaf homogenates harboring transgenic plant nuclear DNA (1.5×10^{-10}; Gebhard and Smalla, 1998) or crushed leaves from transplastomic plants (6.4×10^{-6}; Kay et al., 2002). Even with intact transgenic leaves and intact plants (Tepfer et al., 2003) transformation from the plant nuclear DNA was achieved, albeit after amplification (4.6×10^6). To study HGT some researchers have used experimental systems that aim to be closer to the environmental conditions in soil. For example, transformation of *A. baylyi* BD413 using plant genomic DNA was achieved in sterile soil microcosms at frequencies of 1.4×10^{-8} after incubation with added nutrients for 24 h (Nielsen et al., 2000). Clearly there is substantial variation in the frequencies at different levels of complexity reflecting the different methodologies used by the various laboratories engaged in this work.

In the work presented here, we have aimed to make two major contributions to this field. First, to use the same methodology without any transformant amplification or competence development step to obtain transformation frequencies from plant nuclear transgenes to naturally competent *A. baylyi* BD413, covering and extending the whole range of complexities used by other laboratories. This is important as it allows us to make direct comparisons between levels of complexity. Second, to present data on the first successful transformation of naturally competent bacteria using plant DNA in non-sterile soil. Combining these two datasets allows us to make improved estimates of HGT in the field. This opens the way for further investigations into the factors that facilitate or inhibit gene exchange by natural transformation in field environments and to examine consequences of transfer for particular transgenes.

RESULTS

Transformation on filters was successful using plant DNA, ground and chopped leaves, ground roots and whole seedlings

A. baylyi BD413 containing inactivated versions of *npt*II and *gfp* (constructs BC2 and BC1) were successfully transformed using *Arabidopsis* genomic DNA containing intact *npt*II and *gfp* genes (construct PC2) (Tab. 1). This

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Table 2. Restoration by natural transformation of deletion inactivated *npt*II and *gfp* genes in soil microcosms.

Microcosm	Donor DNA	Recipient (BD413)[1]	Number of transformants (mL^{-1}; arithmetic mean[2])	Transformation frequency (transformants/ recipient; arithmetic mean[3])	Number of replicates[4]
Sterile soil	Pure DNA (3–5 µg)	BC1	136 ± 66	3.6×10^{-8}	3 (3, 100)
Sterile soil	Ground leaves (0.5 g)	BC1	0.09 ± 0.09	2.5×10^{-11}	9 (1, 11)
Non-sterile soil	Pure DNA (8 µg)	BC1-rif	1.02 ± 0.26	5.5×10^{-11}	15 (2, 13)

[1] The deleted gene cassette is chromosomally encoded in BC1-psbA, called BC1, in the text for clarity; BC1-rif is a spontaneous rifampicin resistant mutant of BC1.
[2] Mean values ± standard error of the mean.
[3] Minimum significant difference for transformation frequency ($P = 0.05$) = 0.095 $\log_{10}$ units.
[4] Total number of replicate transformation experiments (number of experiments giving transformants, percentage of experiments giving transformants).

uses 2390 bp of flanking homology to restore a 440 bp deletion in the recipient DNA. Note that transformation frequencies are expressed as transformants per recipient throughout this work. As expected, PCR product gave the highest transformation frequencies ($1.7–3.3 \times 10^{-4}$; Tab. 1) for both the recipient with the deleted gene cassette in a plasmid (BC2) and on the chromosome (BC1). These values were significantly higher than all the other values in Table 1. Transformation frequencies of 1.5×10^{-8} for BC2, and 2.7×10^{-8} for BC1 were achieved using 0.5 µg of transgenic *Arabidopsis* DNA (a saturating concentration, data not shown). At least 10-fold more copies of the deleted gene cassette are present in the plasmid bearing recipient (BC2) than in the chromosomal transformant, but transformation frequencies were not significantly different ($P = 0.798$), indicating that copy number of the target homologous sequence in the recipient has little effect on transformation frequency. However, transformants were easier to detect using plasmid-bearing recipients, as fluorescence was increased by higher expression levels resulting from the raised gene copy number of *gfp*. The effect of donor copy number on transformation frequency was also investigated. Independent transgenic *Arabidopsis* lines varying 40-fold in copy number (with 1, 10 and 40 copies) were used as sources of donor DNA but no significant difference ($P = 0.349$) was found in transformation frequency, hence these data were pooled.

Transformants were detected (Tab. 1) using both ground transgenic *Arabidopsis* leaves (at frequencies of 2.7×10^{-9} with recipient BC2 and 3.5×10^{-9} with recipient BC1) and chopped transgenic *Arabidopsis* leaves (at frequencies of 4.5×10^{-9} BC2 and 1.1×10^{-9} for BC1) as a source of donor DNA. These transformation frequencies were not significantly lower than those obtained using pure plant DNA for either recipients BC1 or BC2. Transformation was also detected using ground transgenic *Arabidopsis* sterile roots (at a frequency of 2.7×10^{-8}) as donor DNA and BC1 as recipient, again at a frequency that was not significantly different from the frequencies using any of the other sources of plant donor DNA (Tab. 1). The transformation frequency when whole, surface-sterilized, transgenic *Arabidopsis* seedlings were placed on nitrocellulose filters as donor DNA, was 4.6×10^{-10} for BC2 and 3.5×10^{-10} (Tab. 1). When these values for whole seedlings are taken together these transformation frequencies were about 50-fold lower than those for pure plant DNA ($P < 0.001$) and 9-fold lower than for all the plant parts taken together ($P < 0.001$). When all the data in Table 1 were taken together, there was no significant difference between the transformation frequencies into the deleted cassette in either BC1 (chromosomally encoded) or BC2 (plasmid encoded). This was the case with both two-way ANOVA excluding the ground root data ($P < 0.001$) or with orthogonal decomposition after one-way ANOVA with all the data ($P = 0.039$). Control experiments with only recipients added to the filters during transformation never gave confirmed transformants.

Transformation was successful in sterile soil using plant DNA and ground leaves

Transformants were obtained in sterile soil microcosms using recipient strain BC1, with 3 to 5 µg of purified transgenic *Arabidopsis* DNA (Tab. 2), at a frequency (3.6×10^{-8}) which was not significantly different from experiments using purified plant donor DNA on filters (see above and Tab. 1). Transformants were also obtained using 500 mg of ground sterile transgenic *Arabidopsis* leaves as donor DNA; however, at a significantly lower frequency (2.5×10^{-11}; Tab. 2) by about 1000-fold. Control experiments using sterile water instead of plant DNA or ground leaves never gave confirmed transformants.

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Transformation in non-sterile soil was successful using plant DNA

For experiments in non-sterile soil, the BC1 recipient strain was transformed with a DNA lysate of a rifampicin resistant strain of *A. baylyi* BD413, and transformants selected on LB rifampicin plates. Rif-resistant transformants, to be used as recipients in later experiments, were screened by PCR, to ensure that they had retained the inactivated *npt*II/*gfp* gene cassette. To determine if the addition of rifampicin resistance to the recipient strains affected the transformation frequency, transformation experiments were carried out on nitrocellulose filters using PC2 PCR product as donor DNA. A mean transformation frequency of 7.5×10^{-5} was obtained, which is not significantly different to the transformation frequency obtained for the original BC1 recipient (1.7×10^{-4}; Tab. 1).

Transformants were detected using 8 μg of purified transgenic *Arabidopsis* DNA in non-sterile soil microcosms in 2 out of 15 replicates, with a mean transformation frequency of 5.5×10^{-11} (Tab. 2). This was significantly ($P < 0.001$) lower by 650-fold than results for sterile soil microcosms using plant DNA, but not different from the transformation frequency with ground leaves in sterile soil (Tab. 2). The restoration of the *npt*II/*gfp* cassette in all the fluorescent transformants detected in these microcosm experiments was confirmed by PCR and colony morphology. Also, laboratory contamination can be ruled out, as this construct had not been previously produced in the laboratory. Transformants were not detected from ground leaves in non-sterile soil microcosms ($\leqslant 1.4 \times 10^{-11}$; n = 14; Tab. 3). As with sterile soil microcosms, similar control experiments in non-sterile soil never produced transformants.

To compare all of our results together, Figure 1 has been compiled by combining the transformation frequencies from all our experiments. This shows, as expected, that the frequencies using PCR product as donor DNA were higher than all the other frequencies. Also transformation frequency from ground leaves in sterile soil and from pure plant DNA in non-sterile soil was lower than most other experiment types. Furthermore, although there were some differences between transformation frequencies in some of the other experimental types, there did not appear to be a consistent trend with experimental complexity.

DISCUSSION

Using transgenic *Arabidopsis* carrying two transgene markers, and appropriately modified recipient strains of *A. baylyi* BD413, has enabled us to generate a progressive data-set of increasing complexities and for the first time obtain measurable frequencies using plant DNA in non-sterile soil (Tab. 2). To compare our results with those of others it is necessary to be careful, because comparable studies have used different approaches to detect transformation in soil (Nielsen et al., 1997; 2000; Tepfer et al., 2003; Tab. 3). Nielsen et al. (2000) added nutrients to soil microcosms to stimulate competence development, which increased numbers of transformants by 200–1000-fold (Nielsen et al., 1997). However, Tepfer et al. (2003) added kanamycin to microcosms, which they reported as amplifying the sensitivity of transformant detection by up to 10^5-fold. We have not used these or similar methods in our soil microcosm experiments, and Table 3 shows that we have been able to estimate transfer frequencies in five different types of experimental conditions for the first time.

Transformation frequencies obtained using purified transgenic *Arabidopsis* nuclear DNA on filters ($1.5–2.7 \times 10^{-8}$) were within the range of frequencies (4×10^{-6}–5.4×10^{-9}; Tab. 3) published by other laboratories for a variety of plants (De Vries and Wackernagel, 1998; De Vries et al., 2001; Gebhard and Smalla, 1998; Nielsen et al., 2000; Tepfer et al., 2003) on filters or in liquid. Using our constructs, we investigated whether donor copy number affects transformation frequency using independent transgenic *Arabidopsis* lines varying 40-fold in copy number. However, no significant differences in transformation frequency were obtained. Recently other laboratories (Ceccherini et al., 2003; Kay et al., 2002; Tepfer et al., 2003) investigated the effect of donor copy number using transplastomic plants, in which the copy number of the transgene is assumed to be over 5000-fold higher than single copy nuclear integrations, and obtained no consistent differences in transformation frequency (Tab. 3). Thus it is not surprising that we saw no effect with only a 40-fold increase in donor copy number. We also investigated whether transformation frequency was affected by copy number of the recipient gene (over a ten-fold range) and found it was not. This indicates that estimates of HGT frequency made for a nuclear-integrated plant transgene are likely to be generally applicable irrespective of copy number either in the donor or recipient target.

However, large differences in transformation frequencies of cultures of *A. baylyi* BD413 using plant DNA carrying nuclear-integrated transgenes without amplification or competence development are reported from different laboratories. Gebhard and Smalla (1998) reported frequencies of 5.4×10^{-9}, while De Vries et al. (2001) report frequencies of $1–7 \times 10^{-8}$ (Tab. 3). Our data indicate that these differences probably reflect diverse methodologies, rather than any direct relationship to copy number. Transformation frequencies using DNA from plant parts were more consistent. Our frequencies obtained with roots

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Table 3. Comparison of transformation frequencies of *A. baylyi* using plant DNA from studies with increasing levels of complexity, approaching field conditions.

	Results from transformation experiments performed in/on			
Donor DNA	Liquid	Filters	Sterile soil	Non-sterile soil
Studies without amplification[1]				
Pure plant DNA	De Vries and Wackernagel (1998), De Vries et al. (2001) (N) $1.0–7.0 \times 10^{-8}$ Kay et al. (2002) (P) 4.1×10^{-6}	Gebhard and Smalla (1998) (N) 5.4×10^{-9} Nielsen et al. (2000) (N) 5.7×10^{-7} This study (N) $1.5–2.7 \times 10^{-8}$	Nielsen et al. (2000)[2] (N) 1.4×10^{-8} This study (N) 3.6×10^{-8}	Nielsen et al. (2000)[2] (N) not detected, but suggested $10^{-10}–10^{-11}$ This study (N) 5.5×10^{-11}
Ground/crushed leaf homogenate	Tepfer et al. (2003)[3] (P) 3×10^{-6} Kay et al. (2002) (P) $2–6.4 \times 10^{-6}$ Ceccherini et al. (2003) (P) 7.7×10^{-4}	Gebhard and Smalla (1998) (N) 1.5×10^{-10} This study (N) $2.7–3.5 \times 10^{-9}$	This study (N) 2.5×10^{-11}	This study (N) $\leq 1.4 \times 10^{-11}$
Chopped/whole leaves	–	This study (N) $1.1–4.5 \times 10^{-9}$	Tepfer et al. (2003)[3] (P) 1.2×10^{-8}	–
Roots	–	This study (N) 2.7×10^{-8}	–	–
Whole plant	–	This study (N) $3.5–4.6 \times 10^{-10}$	–	–
Studies using amplification[1]				
Pure plant DNA	Tepfer et al. (2003)[3] (N) $12–630 \times 10^{-7}$			
Ground/crushed leaves-homogenate	Tepfer et al. (2003)[3] (N) $4.6–590 \times 10^{-6}$	–	–	–
Chopped/whole leaves	Tepfer et al. (2003)[3] (P) 5.9×10^{-2} Tepfer et al. (2003)[3] (N) 4.6×10^{-6} Tepfer et al. (2003)[3] (P) $1.2–19 \times 10^{-7}$	–	Tepfer et al. (2003)[3] (P) 1×10^{-4}	–
Roots	Tepfer et al. (2003)[3] (N) $\leq 2.4 \times 10^{-4}$	–	–	–
Whole plant	Tepfer et al. (2003)[3] (P) $\leq 3 \times 10^{-6}$	–	–	–

N = nuclear; P = plastid; –: not yet done.

[1] Amplification is when enrichment is used to enhance the number of transformants recovered. In most experiments Tepfer et al. (2003) added kanamycin to the transformation medium and incubated for 16 h prior to plating on selective medium and stated that this raised the transformant count by a factor of $\leq 10^5$.

[2] Nielsen et al. (2000) added nutrients to soil microcosms to stimulate competence development (which is equivalent to our filter transformation protocol), but in our soil microcosm experiments nutrients were not added.

[3] Values from Tepfer et al. (2003) calculated from numbers of transformants and recipients given in paper.

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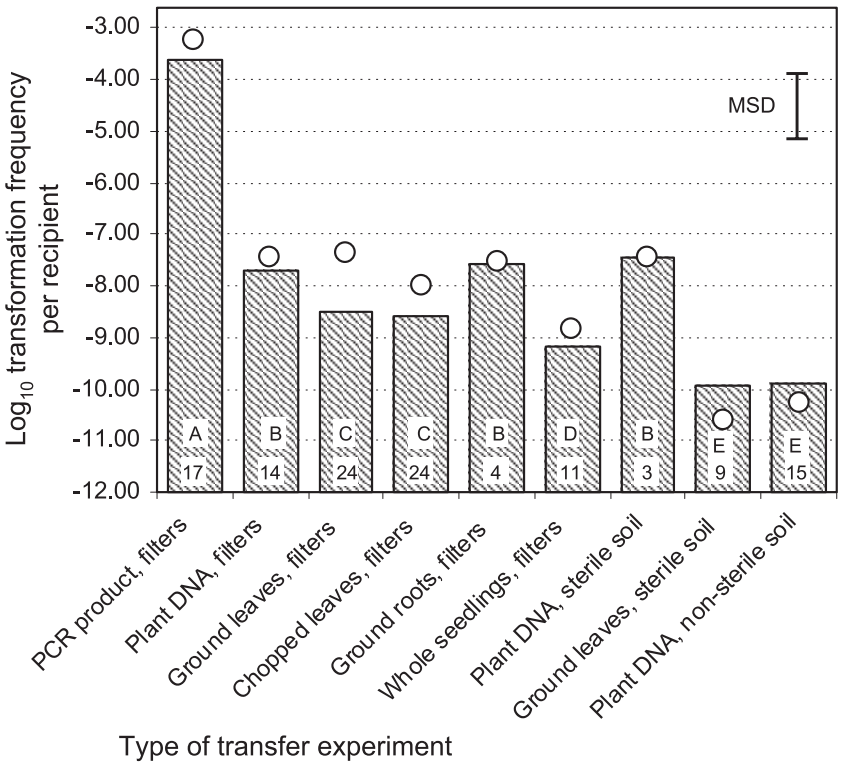

Figure 1. Combined mean transformation frequencies for all the experiments described here. Data for recipients with the deleted gene cassette in plasmid and chromosome are combined. Shaded vertical bars are geometric means and open circles are arithmetic means. The minimum significant difference (MSD) bar ($P = 0.05$) relates to the geometric means. Numbers or replicates are given at the base of each shaded vertical bar. Letter in the bars refer the results of the sum of squares simultaneous test procedure for comparing groups of means, where A ≠ B–E, B ≠ D, C ≠ E, C = D and D = E (where ≠ indicates a significant difference at $P < 0.001$ and = indicates no significant difference at $P > 0.05$).

(2.7×10^{-8}), or whole seedlings ($3.5–4.6 \times 10^{-10}$) were very similar to those of Tepfer et al. (2003), allowing for their amplification enhancement (≈10^5-fold), of about 2×10^{-9} for roots and 5×10^{-11} for leaves. Furthermore, our transformation frequencies ($1.1–4.5 \times 10^{-9}$) with chopped or crushed leaves on filters were only slightly higher than that found by Gebhard and Smalla (1998; Tab. 3) using homogenate.

Sterile soil microcosms were used to obtain transformation frequencies using both purified plant genomic DNA and ground leaves. Although frequencies obtained with the purified donor DNA (3.6×10^{-8}) appear comparable to those of Nielsen et al. (2000; Tab. 3), we did not stimulate competence development by adding nutrients to the microcosms. We obtained measurable frequencies (2.5×10^{-11}) for the first time from a nuclear integrated transgene in sterile soil using ground leaf material without amplification. In our study, this is 1 400-fold lower than using purified DNA in sterile soil. This contrasts with the data for genomic DNA reported by Tepfer et al. (2003) for ground leaves compared to purified DNA in sterile liquid cultures, where the transformation frequency was about 10-fold higher using ground leaves (Tab. 3). This difference may be due to the amplification step they used or its variability. Overall, these comparisons show that the frequencies reported in this study are consistent with those of others where direct comparisons are possible.

Using the same constructs and methodology, we are able to report for the first time measurable transformation frequencies using a purified DNA from a transgenic plant in non-sterile soil. The frequency we obtained of 5.5×10^{-11} is just 650-fold lower than that for sterile soil, reflecting the increased complexity of the medium and competition from indigenous bacteria. This decrease in transformation frequency is in the same order as the decreases between ground leaves and plant DNA in sterile soil, and so probably reflects an overall trend of 100–1000-fold decreases as experimental complexity increases in soil. Attempts by others to measure frequencies in non-sterile soil using plant DNA have not been successful (Gebhard and Smalla, 1999; Nielsen et al.,

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Table 4. Bacterial strains and plasmids.

Strains or plasmids	Description	Source
Strains		
BD413	Wild type *Acinetobacter* sp. BD413	Williams et al. (1996)
BD413-BC1	Containing deleted *npt*II/*gfp* cassette integrated in 16S rDNA gene	Simpson et al. (2007)
BD413-BC1-Rif	BD413-BC1 with rifampicin resistance in chromosome	This paper
BD413-BC2	Containing deleted *npt*II/*gfp* cassette on pUCP26 plasmid	Simpson et al. (2007)
BD413-PC1	Intact *npt*II/*gfp* cassette integrated into 16S rDNA gene	Simpson et al. (2007)
BD413-Rif	BD413 with rifampicin resistance	Williams et al. (1996)
E. coli JM109	For cloning	Promega
Agrobacterium tumefaciens GV3101	For plant transformation	Hellens et al. (2000)
Plasmids		
pGEM-PC1	pGEM T easy vector containing intact *npt*II/*gfp* cassette with *Acinetobacter* 16S rDNA flanking sequences	Simpson et al. (2007)
pGEM-PC2	pGEM T easy vector with intact *npt*II/*gfp* cassette	Simpson et al. (2007)
pGPTV-HPT	Binary plant vector with hygromycin resistance	Becker et al. (1992)

2000). An important difference between the results reported here and previous studies is the selection method employed. The combination of the rifampicin resistance with the two other antibiotic selectable markers in our current study has provided a method to reduce dramatically background numbers of bacteria. Our concentration step using centrifugation to enhance transformant recovery is also an improvement. We believe these factors were crucial in our ability to detect the new transformants. This is in accord with previous work in our laboratory showing that rifampicin resistance in recipients enables both transconjugants and transformants to be selected from complex environments with competing indigenous bacteria (Lilley et al., 1994; 1996; Williams et al., 1996).

Predictions of the numbers of HGT events occurring as a result of the widespread deployment of transgenic crop plants have assumed a transformation frequency of 10^{-16}–10^{-17} (Heinemann and Traavik, 2004), based on extrapolations from experiments with sterile cultures and sterile soil. Since transformation frequency with ground leaves in sterile soil was in the order of 1000-fold lower than with purified DNA in our experiments, it seems feasible that the transformation frequency from post-harvest damaged leaf material in the field would be in the order of 10^{-14} ($\approx 5.5 \times 10^{-11}/1000$). Furthermore, by extrapolation from the trends in Figure 1, a further frequency drop by about 1000-fold to 10^{-17} for HGT from whole plants to bacteria in the field seems possible. Of course these are only estimates, and other important factors include nutrient levels (Nielsen and van Elsas, 2001), levels of homology between transgene and recipient bacteria (Simpson et al., 2007), and agronomic practice influencing availability of the transgenic plant material post-harvest. Furthermore, it is known that few bacteria express natural competence in the environment (Lorenz and Wackernagel, 1994), although root exudates and other nutrients can enhance competence development (Nielsen et al., 1997). So it is possible that most estimates of transfer frequencies in soil overestimate the real frequency of transformation to indigenous bacteria. However, our data at least provide additional baseline data from which to study these factors and inform global estimates of HGT. So our results help confirm that the Heinemann and Traavik (2004) estimate is reasonable.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study are shown in Table 4. Cloning of plant constructs was carried out in *Escherichia coli* JM109 (Promega, Madison, Wisconsin, USA). Cultures were grown in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl) in an orbital shaker (200 rpm) and on LB agar plates (1.5% agar). *E. coli* cultures were incubated at 37 °C. *A. baylyi* BD413 and *Agrobacterium tumefaciens* GV3101 were grown at 30 °C.

Molecular methods

PCR amplification was carried out using the MJ Research DNA engine (Genetic Research Instrumentation, Braintree, Essex, UK), using 30 cycles, with 30 s denaturing

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Table 5. PCR primers.

Primer	Sequence 5' to 3' (Restriction sites underlined)	Restriction site
nptIID	CAGTGAATTCCTTTGACGTTGGAGTCC	*Eco*RI
GFP1	GTGCGTCGACCTCTACTAGTGATCTCAATGAATATTGGTTGAC	*Sal*I
GFP2	CGCTCCGCTTATTTGTATAGTTCATCCATGC	*Aci*I
16S1	ACGCTGAATTCGCTATTAGGTGACACTATAG	*Eco*RI
16S2	CGATGTCGACGCGGTGTGTACAAGGC	*Sal*I

at 94 °C, 30 s annealing at 55 °C and 1 min extension per 1 kb product for *Taq* DNA polymerase (Promega), or 2 min extension per 1 kb product *Pfu* DNA polymerase (Promega). When amplifying genes for cloning, *Pfu* DNA polymerase was used, and *Taq* DNA polymerase was used for screening by colony PCR. PCR primers are listed in Table 5. PCR products were purified using the Qiagen (Qiagen Ltd, Crawley, UK) PCR purification kit. Plasmid minipreps were carried out on 1.5 mL overnight culture using the GenElute plasmid miniprep kit (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). Restriction digests were performed using enzymes from Promega and NEB (New England Biolabs, Hitchin, UK).

Production of transgenic plants containing donor DNA constructs

Constructs were assembled as described in Simpson et al. (2007). Two donor DNA constructs were used, PC1 and PC2 (Fig. 2a). Both comprised intact copies of *gfp* and *npt*II. The *npt*II gene was driven in all cases by its endogenous promoter, while in PC2 the *gfp* gene was driven either by an SP6 or a *psb*A promoter. In PC1 the *gfp* and *npt*II genes were flanked by 16S rRNA sequences. For transformation of *Arabidopsis,* these constructs were cloned into the binary plant vector pGPTV-HPT (Becker et al., 1992) to create pGPTV-HPT-PC1 and pGPTV-HPT-PC2. The PC2 and PC1 inserts (Fig. 2a) were PCR-amplified using primers nptIID and GFP1 for PC2-psbA, nptIID and GFP2 for PC2-SP6 and 16S1 and 16S2 for PC1 (Tab. 5). The PCR products were digested with *Eco*RI and *Sal*I, and ligated into *Eco*RI/*Sal*I digested pGPTV-HPT vector. The resulting plasmids (pGPTV-HPT-PC1 and pGPTV-HPT-PC2) were transformed into *A. tumefaciens* GV3101, and used to transform *Arabidopsis thaliana cv.* Columbia plants by the floral dip method (Clough and Bent, 1998).

Transformed plants were grown for 3 to 4 weeks until siliques (seedpods) began to split, at which time they were harvested and dried before the seeds were collected. Transgenic plants were selected by growing seeds on agar supplemented with hygromycin at 30 $\mu g.mL^{-1}$. Surviving seedlings were transferred to compost after 3 weeks, and grown on for seed collection.

The transgene copy number was estimated by semi-quantitative PCR. DNA was extracted as described below and concentration determined by agarose gel electrophoresis, quantified using Gene Tools program (Syngene, Cambridge, UK). DNA concentrations were equalised to 5 $\mu g.mL^{-1}$, and 0.5, 1 and 5 µL of template were used for PCR to ensure a linear product relationship. A standard curve was prepared by adding a known amount of PCR product to wild-type *Arabidopsis* DNA, and used to determine copy number. Template concentration was adjusted to ensure that the product values were in the linear part of the standard curve.

Production of modified recipient bacterial strains

Recipient *A. baylyi* BD413 used in all experiments contained either construct BC1 integrated into the bacterial genome or BC2 carried on the broad host range plasmid pUCP26. Both BC1 and BC2 comprise deleted copies of *npt*II and *gfp* flanked in BC1 by 16S rRNA gene sequences from *A. baylyi* BD413, which were used to integrate this construct into the bacterial genome (Simpson et al., 2007; Fig. 2b). To improve selection of transformants, BC1 was also transformed with a lysate of a rifampicin resistant strain (BD413-Rif), as described below. BC1 transformants (to be used as recipients in later transformation experiments) were selected for resistance to rifampicin (100 $\mu g.mL^{-1}$) and gentamicin (15 $\mu g.mL^{-1}$), and the presence of the deleted *nptII*/*gfp* cassette was confirmed by PCR (nptIID, GFP1 or GFP2). The transformation efficiency of the rifampicin resistant recipient strain carrying the BC1 construct was tested to ensure that efficiency of transformation had not been altered compared to the wild-type strain on filters using PCR product and pure plant DNA.

Preparation of donor DNA

Plant DNA was extracted essentially as described in Doyle and Doyle (1987). Transgenic *Arabidopsis* leaves

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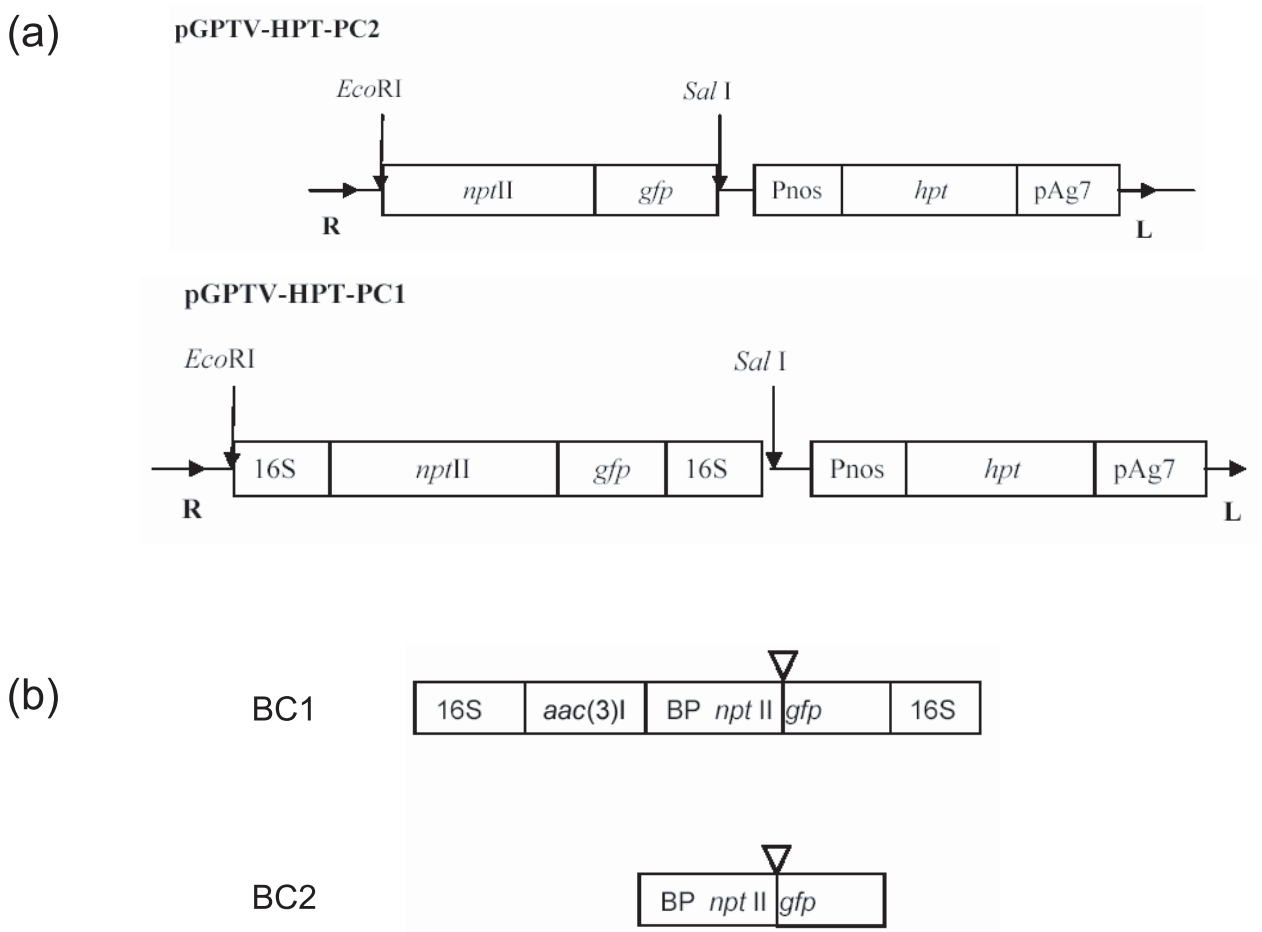

Figure 2. Constructs used in the transformation experiments. Abbreviations: *gfp* = green fluorescent protein; BP = bacterial promoter; *npt* II = neomycin phosphotransferase II; *aac*(3)I = gentamicin resistance gene; 16S = *Acinetobacter baylyi* BD413 16S rDNA flanking sequence. (a) Donor constructs pGPTV-HPT-BC1 and pGPTV-HPT-BC2 for integration into the *Arabidopsis* genome. (b) Recipient constructs BC1 and BC2. BC1 was integrated into the *A. baylyi* BD413 genome *via* the 16S rRNA homologous flanks, whereas BC2 was transformed into *A. baylyi* BD413 on a broad host plasmid pUCP26. PC2 restores a 440 bp deletion in BC1 or BC2 using 2390 bp of flanking homology, whilst PC1 restores the same deletion in BC1 using 2587 bp of flanking homology.

were ground in liquid nitrogen, before the addition of 2 X CTAB solution (2% CTAB, 1.4 M NaCl, 100 mM Tris/HCl pH 8 and 20 mM EDTA) at 1 mL per gram leaf tissue. RNase was added (20 $\mu g.mL^{-1}$), and the tubes were vortex mixed, before incubation at 65 °C for 60 min. Chloroform:iso-amyl alchohol (24:1) was added, and after vortex mixing, the tubes were centrifuged for 5 min at 14 000 rpm in Eppendorf 541C centrifuge. The aqueous layer was transferred to a clean tube, and the DNA was ethanol precipitated, resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA and stored at –20 °C until used.

Plants were grown in compost with 16 h light at 20 °C. Leaves used as donor DNA were surface-sterilized in ethanol followed by five rinses with sterile distilled water and either chopped, or ground. Between 0.1–0.3 g of leaf material was used in individual filter transformations, and 0.5 g in microcosm experiments. Seeds of transgenic *Arabidopsis* were also germinated on sterile agar, on Murashige & Skoog medium (Murashige and Skoog, 1962) and grown in 90 mm petri dishes at an angle of 45° at 20 °C with 18 h light. Material from seedlings (8–10) grown aseptically in sterile medium was used as donor DNA in transformation experiments as ground roots, ground plants and whole seedlings. Fresh material from these second generation plants was used in all experiments.

Bacterial lysates were produced by resuspending the cells from 1 mL of overnight culture in 500 µL sterile saline citrate (0.15 M NaCl, 0.015 M Sodium citrate and 0.05% SDS), and incubating at 60 °C for 1 h with

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occasional shaking. The resulting lysates were stored at 4 °C until used.

Transformation experiments on filters

Transformation experiments were carried out on sterile nitrocellulose filters (Williams et al., 1996). A 25 mm diameter, 0.45 μm pore size nitrocellulose filter was placed on a nutrient agar plate. Recipient cultures were grown overnight in LB broth with shaking (120 rpm) at 30 °C; cell numbers were determined by OD_{670} and diluted to 10^9 per mL. Typically, 100 μL of donor DNA was added to the filter and allowed to dry before 100 μL of recipient culture was applied. Bacterial lysate, PCR product, purified transgenic *Arabidopsis* DNA and ground or chopped leaves as well as ground root and whole seedlings were used as donor DNA. The plates were incubated at 30 °C for 24 h. Each filter was aseptically removed, placed in 3 mL of sterile quarter strength Ringers Solution (Fisher Scientific, Loughborough, UK), and vortex mixed for 1 min. Ten-fold serial dilutions to 10^{-7} were plated as triplicate 20 μL drops. To detect lower transformation frequencies, 200 μL to 3 mL (concentrated to 100 μL) of mixture was spread on a selective plate. This concentration procedure is effective and yields appropriately increased proportions of transformants (Rochelle et al., 1988; Williams et al., 1996). Recipients were counted on LB + gentamicin (BC1) or LB (BC2). Transformants were counted on LB + kanamycin + gentamicin (BC1) or LB + kanamycin (BC2), and always showed GFP fluorescence. Kanamycin and gentamicin were used at concentrations of 50 $\mu g \cdot mL^{-1}$ and 15 $\mu g \cdot mL^{-1}$, respectively. Plates were incubated at 30 °C for 48 h before colonies were counted and fluorescence was monitored. All colonies of putative recipients or transformants resembled the morphology of *A. baylyi*. Samples of putative transformants and recipients were confirmed by colony PCR (nptIID, GFP1 or GFP2; Tab. 5), which amplifies almost the entire *npt*II and *gfp* genes in transformants and the deleted derivatives in recipients (Fig. 2). Counts of recipients routinely reached 8×10^{10} per mL enabling low transfer frequencies to be estimated.

Controls for all filter transformation experiments were carried out as follows. Recipient culture (100 μL) was added to a nitrocellulose filter without addition of donor DNA, and the sterility of the donor DNA lysate was also always checked by plating (100 μL) onto LB. To establish that the transformation protocol was working effectively, control chromosomal integration transformation experiments were carried out using a lysate of a rifampicin resistant *Acinetobacter* sp. BD413. Transformation to rifampicin resistance occurred at an average frequency of $9.33 \pm 1.2 \times 10^{-3}$ (n = 3).

Microcosm transformation experiments

Transformations were carried out in sterile and non-sterile soil (well integrated sandy loam from a Cardiff garden). For sterile soil microcosms, 1 g autoclaved soil was placed in a glass universal tube, autoclaved for 15 min and stored at room temperature until used. For non-sterile microcosms 1 g non-sterile soil was added to a sterile universal, 0.5 mL of overnight bacterial culture was added, and plate counts on this culture were used to determine the starting count. The donor material was either pure DNA (100 to 400 μL, 30 to 50 $ng \cdot \mu L^{-1}$) or ground leaves (0.5 g). Tubes were incubated without shaking at 20 °C overnight, then 5 mL Ringers solution added and shaken for 15 min using a lab-line multi-wrist shaker (Whatman). The sediment was allowed to settle. Then recipients and transformants in the supernatant were enumerated by plating at 30 °C for 48 h. Plating and concentration (for transformants) was as described in the preceding section. Recipients were counted on LB + gentamicin (BC1) or LB + gentamicin + rifampicin (BC1-Rif). Transformants were counted on LB + kanamycin + gentamicin (BC1) or LB + kanamycin + rifampicin (BC1-Rif; gentamicin resistance confirmed by plating separately), and always showed GFP fluorescence. Antibiotic concentrations were as follows: kanamycin (50 $\mu g \cdot mL^{-1}$), gentamicin (15 $\mu g \cdot mL^{-1}$) and rifampicin (100 $\mu g \cdot mL^{-1}$). All colonies of putative recipients or transformants resembled the morphology of *A. baylyi*. Transformants were identified by viewing with blue light (to detect fluorescence due to GFP expression) and storing at 4 °C before viewing again, if fluorescence was not already visible. Plates were stored for up to 3 weeks and fluorescence was monitored weekly. Final transformant confirmation was by colony PCR (nptIID, GFP1 or GFP2; Tab. 5) on all colonies and samples of recipients confirmed with the same PCR primers. Counts of recipients routinely reached 8×10^{10} per g soil enabling very low transfer frequencies to be estimated.

Control experiments were done with sterile water (100 to 400 μL) in place of DNA or plant parts. The efficacy of the transformation protocol was confirmed for all experiments as described in the previous section.

Statistics and analysis

All transformation frequencies are expressed as transformants per recipient. Transfer frequencies were compared using one-way and two-way analysis of variance (ANOVA) and the data were transformed by $\log_{10} x + 1$ (where 1 was an appropriately very low transformation frequency, 1×10^{-10}) to achieve normality of residuals and homogeneity of variance. In most cases means are

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presented as geometric means to better reflect the statistical tests done. However, in transformation experiments giving transformants in few replicate experiments, arithmetic means are used, as these better reflect the likely real frequency. Comparisons between means were made after ANOVA by appropriate *a priori* and *a posteriori* tests with orthogonal decomposition and the sum of square simultaneous test procedure for comparing groups of means and the Tukey-Kramer minimum significant difference (MSD) test for individual means (Fry, 1993). Significant differences are given at $P < 0.05$ unless stated otherwise. Statistical analysis was carried out using Minitab version 14.2 (Minitab Inc., State College, Pennsylvania, USA).

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