Thematic Issue on Horizontal Gene Transfer

Detection of potential transgenic plant DNA recipients among soil bacteria

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The likelihood of gene transfer from transgenic plants to bacteria is dependent on gene number and the presence of homologous sequences. The large number of transgene copies in transplastomic (transgenes contained in the chloroplast genome) plant cells as well as the prokaryotic origin of the transgene, may thus significantly increase the likelihood of gene transfer to bacteria that colonize plant tissues. In order to assess the probability of such transfer, the length of homologous DNA sequences required between the transgene and the genome of the bacterial host was assessed. In addition, the probability that bacteria, which co-infect diseased plants, are transformable and have sequences similar to the flanking regions of the transgene was evaluated. Using *Acinetobacter baylyi* strain BD143 and transplastomic tobacco plants harboring the *aadA* gene (streptomycin and spectinomycin resistance), we found that sequences identical to the flanking regions containing as few as 55 nucleotides were sufficient for recombination to occur. Consequently, a collection of bacterial isolates able to colonize tobacco plant tissue infected by *Ralstonia solanacearum* strain K60 was obtained, screened for DNA sequence similarity with the chloroplastic genes *accD* and *rbcL* flanking the transgene, and tested for their ability to uptake extracellular DNA (broad host-range pBBR1MCS plasmids) by natural or electro-transformation. Results showed that among the 288 bacterial isolates tested, 8% presented DNA sequence similarity with one or both chloroplastic regions flanking the transgene. Two isolates, identified as *Pseudomonas* sp. and *Acinetobacter* sp., were able to integrate exogenous plasmid DNA by electro-transformation and natural transformation, respectively. Our data suggest that transplastomic plant DNA recipients might be present in soil bacterial communities.

Keywords: GMO / transplastomic / transgene / natural transformation / recombination / hybridization / DNA / antibiotic resistance gene / soil / tobacco / bacteria / *Acinetobacter* / *Ralstonia*

INTRODUCTION

The integration and expression of extracellular DNA in the genome of bacteria due to horizontal gene transfer (HGT) by natural transformation is thought to be a major mechanism by which bacteria have evolved and are able to adapt to changing environmental conditions. With the recent development and increasing use of transgenic plants in modern agriculture, this unique ability to ac-

quire foreign genes by natural transformation has, however, raised questions about the possibility that bacteria in contact with plant tissues will integrate and then disseminate transgenes from the plant (James and Krattiger, 1996; Nielsen et al., 1998). Although putative HGT events have been suggested based on comparative DNA sequence analyses (Binnewies et al., 2006), the transfer of plant transgenes to soil and gastrointestinal bacteria has not been observed under natural conditions. While gene transfer from bacteria to plants is well documented (Broothaerts et al., 2005), the lack of direct evidence of HGT from transgenic plants to bacteria has, in fact, intensified the debate regarding the probability of such an

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event and its impact on bacterial communities, and has also revealed the technical limitations in monitoring such events in complex environments (Davison, 2004; de Vries and Wackernagel, 2004; Heinemann and Traavik, 2004; Nielsen and Townsend, 2004; Nielsen et al., 1998).

For transformation to occur under natural conditions, several conditions have to be met. Key requirements have been identified, such as the necessity of direct contact between the recipient cell and extracellular DNA, the ability of the putative bacterial host to be naturally or electro-transformable and the presence of DNA sequence homology between the exogenous DNA and the recipient cell (Cérémonie et al., 2006; de Vries and Wackernagel, 1998; 2004; Gebhard and Smalla, 1998; Kay et al., 2002b; Nielsen et al., 1998). While assumptions have been made that the likelihood that all of these conditions are encountered is very low (Heinemann and Traavik, 2004; Nielsen et al., 1998), independent research has shown that each of these steps can be met individually, suggesting that environmental conditions could be conducive for HGT. Recent data has shown that during the decay process of plant tissues, DNA was rapidly degraded, yet a significant proportion escaped degradation and retained its biological activity (*i.e.*, ability to transform bacteria) (Ceccherini et al., 2003; Pontiroli et al., 2007). Interestingly, research by the same group revealed not only that *Acinetobacter baylyi* strain BD413, a naturally transformable bacterium isolated from soil (Juni and Janick, 1969), was able to grow opportunistically on *Ralstonia solanacearum*-infected plant tissues, but that it could also be naturally transformed *in planta* by transgenic DNA (Kay et al., 2002a; 2002b). While these specific experiments were conducted with *Acinetobacter* sp., opportunistic colonization of decaying plant tissues is most likely accomplished by a diversity of bacteria present in soil and on plant surfaces. To date, almost 90 naturally transformable bacterial species have been identified (de Vries and Wackernagel, 2004). Although they represent only about 2% of identified culturable prokaryotic species (de Vries and Wackernagel, 2004), according to Wackernagel and co-workers, many bacteria remain to be examined for natural transformation (de Vries and Wackernagel, 2004; Lorenz and Wackernagel, 1994) as well as electro-transformation (Cérémonie et al., 2004; 2006; Demanèche et al., 2001). However, and regardless of the system considered, HGT from plants to the genome of potential recipient bacteria has never been detected without the use of artificial constructs with significant sequence similarity to the plant transgene in order to facilitate homologous recombination (Kay et al., 2002b; Nielsen et al., 2000; Tepfer et al., 2003). In the absence of homologous sequences in the recipient cells of the naturally transformable bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. strain BD413,

transformation by transgenic potato plant DNA harboring the *nptII* gene dropped by over 10^8-fold, suggesting a very low probability that non-homologous DNA fragments would be integrated into environmental bacteria by illegitimate recombination events (de Vries et al., 2001). On the other hand, these authors later reported that the presence of homologous sequences between the foreign DNA and the recipient strain could facilitate integration of flanking region by illegitimate recombination (de Vries and Wackernagel, 2002; Meier and Wackernagel, 2003). Long stretches of nucleotides that lacked sequence homology were integrated into recipient genomes when they were linked on one side to a small piece of DNA with homology to resident DNA serving as a recombination anchor (Meier and Wackernagel, 2003).

In order to assess the probability of HGT between plant and soil bacteria under natural conditions, an integrative approach was chosen, where the parameters described above and identified as critical barriers preventing HGT were taken into account. First, using *Acinetobacter baylyi* strain BD413 and transplastomic tobacco plants harboring the *aadA* gene (streptomycin and spectinomycin resistance), our objective was to determine the minimum length of homology required between foreign DNA and recipient cells for recombination to occur. Results revealed that homologous sequences of only a few nucleotides were sufficient for HGT to occur. Consequently, a strategy was designed to screen for and assess the presence of potential recipients of transgene DNA among opportunistic soil bacteria capable of colonizing *Ralstonia*-infected plant tissues. We hypothesized that transformable bacteria that are able to colonize infected plant tissues may be in direct contact with plant DNA in a nutrient-rich environment fostering metabolic activity. Potentially, the development of a competent state, in addition the presence of homologous DNA sequences, may serve as recombinational anchors to facilitate integration of foreign DNA. This paper attempts to identify and isolate potential bacterial candidates for HGT of plant transgenes based on the presence and length of sequences similar to transgene flanking regions and transformability.

RESULTS AND DISCUSSION

Effect of homologous sequence length on transformation frequency

In order to determine the effect of the length of homologous sequences between exogenous donor DNA and recipient bacterial cell DNA on gene transfer, plasmid-based genetic constructs containing fragments of different lengths of the transgene flanking regions were

designed. While it has been reported that recombination could occur with sequence homology of *ca* 200 bp (de Vries and Wackernagel, 2004), to our knowledge, studies assessing the possibility of recombination for shorter sequence homologies in *Acinetobacter* have not been reported. Therefore, plastid sequences ranging from 55 bp to 438 bp were cloned into plasmid pMMB190 (Fig. 1A). The *rbcL* flank and *accD* flank in plasmids pBAB2, pRA438, pRA282, pRA110 and pRA55 were 1149 and 1425, 140 and 298, 147 and 135, 22 and 88, and 39 and 16 nucleotides, respectively. Transformants of *A. baylyi* strain BD413 harboring the different plasmids were detected for all constructs when plasmid pCLT100 was used as donor DNA. While transformation frequencies decreased drastically with the decreasing sizes of homologous flanking regions, recombination events could still be detected when as few as 39 and 16 bp served as recombinational anchors on each side of the transgene (Fig. 1B). Our data corroborate those obtained for other bacterial species suggesting that only a few nucleotides are needed for recombination (Bertolla et al., 1997; de Vries and Wackernagel, 2004; Khasanov et al., 1992). These results confirm that transformation frequencies could dramatically increase with increasing length of homologous sequences. Experiments performed with a larger transgene (*i.e.*, 2.53 kb instead of 1.35 kb) confirmed the significance of transgene size on transformation frequencies since, on average, transformation frequencies were reduced by 50-fold. However, for the larger transgene, no transformants could be detected when homologous flanking regions were smaller than 110 bp (data not shown). Transformation frequencies were also significantly reduced when experiments were performed with purified plant DNA and crushed leaves. Transformants were only obtained with *A. baylyi* strain BD413 harboring plasmids pBAB2 and pRA438 when purified plant DNA was used, and only with cells harboring pBAB2 when experiments were conducted with crushed leaves (Fig. 1B). Hypothetically, transformation frequencies obtained with crushed leaves with sequence similarity less than 100 bp would be of *ca* 10^{-13} transformant per recipient cell. While such hypothetical transformation frequencies are low, and therefore could be considered unlikely to occur under field conditions (Heinemann and Traavik, 2004), the natural occurrence in soil of transformable bacteria presenting significant sequence similarity in their genome, which could lead to the recombination of the transgene at higher frequencies, has not been investigated. Nucleotide-nucleotide Basic Local Alignment Search Tool (blastn; http://www.ncbi.nlm.nih.gov/BLAST/) was run to find regions of local similarity between *accD* or *rbcL* sequences and bacterial genomes. This revealed that a large number of bacteria belonging to all classes within the taxonomic tree harbor sequences similar to the flanking regions of the plant transgene (*Nicotiana tabacum accD* and *rbcL* genes). Identical sequence ranged from 30 to 55 nucleotides and from 50 to 172 nucleotides for the *accD* and *rbcL* genes, respectively (data not shown). These observations suggest that while HGT from plants to bacteria has never been detected experimentally without the use of artificial constructs, significant and sufficient sequence similarity between environmental bacteria and plant DNA sequences might exist for homologous recombination.

Collection of bacterial isolates colonizing infected plant tissues

While screening for sequence similarity could have been performed using bacteria directly isolated from soil, an *in planta* enrichment procedure was used to select for opportunistic bacteria able to colonize decaying tobacco plant tissues, and thus, be exposed to significant amounts of transgenic plant DNA (Ceccherini et al., 2003). Bacteria were first extracted from two soils using a Nycodenz® gradient separation method adapted from Courtois et al. (2001). A direct estimation by microscopy counts of the total number of bacterial cells extracted indicated significant differences between the two soils used. As reported before, soil from Montrond provided a higher density of bacteria compared to soil from La Côte Saint-André, for which the Nycodenz ring was more difficult to obtain (Bertrand et al., 2005; Cérémonie et al., 2004). The average number of bacteria extracted from Montrond soil was 20-fold higher than that from La Côte Saint-André and reached *ca* 10^9 cells.g^{-1}. Based on estimated bacterial cell numbers in each suspension, concentrations of bacterial suspensions extracted from soil were adjusted to *ca* 10^7 cells.mL^{-1} before mixing in a suspension of *R. solanacearum* strain K60, which was also adjusted to *ca* 10^7 CFU.mL^{-1}, and co-inoculated in plant tissues. Based on colony counts, *R. solanacearum* strain K60 cells and either Montrond cells or La Côte Saint-André cells ($7.1 (\pm 0.3) \times 10^6$, $1.2 (\pm 1.6) \times 10^6$ and $1.6 (\pm 3.5) \times 10^5$ CFU, respectively) were injected into individual tobacco leaves or stems.

The total number of CFU increased from the inoculum concentration of *ca* 10^6 per g of fresh plant tissue to $8.5 (\pm 2.4) \times 10^9$ and $5.3 (\pm 7.6) \times 10^9$ CFU.g^{-1} for K60/Montrond and K60/La Côte Saint-André mixtures, respectively, after 5 days incubation at which time plant pathogenic symptoms had developed. While population sizes of *Ralstonia* and non-*Ralstonia* cells recovered from infected plant tissues were not determined precisely, they usually did not differ by more than 50-fold. Similar experiments performed with a mixture of *Ralstonia* and
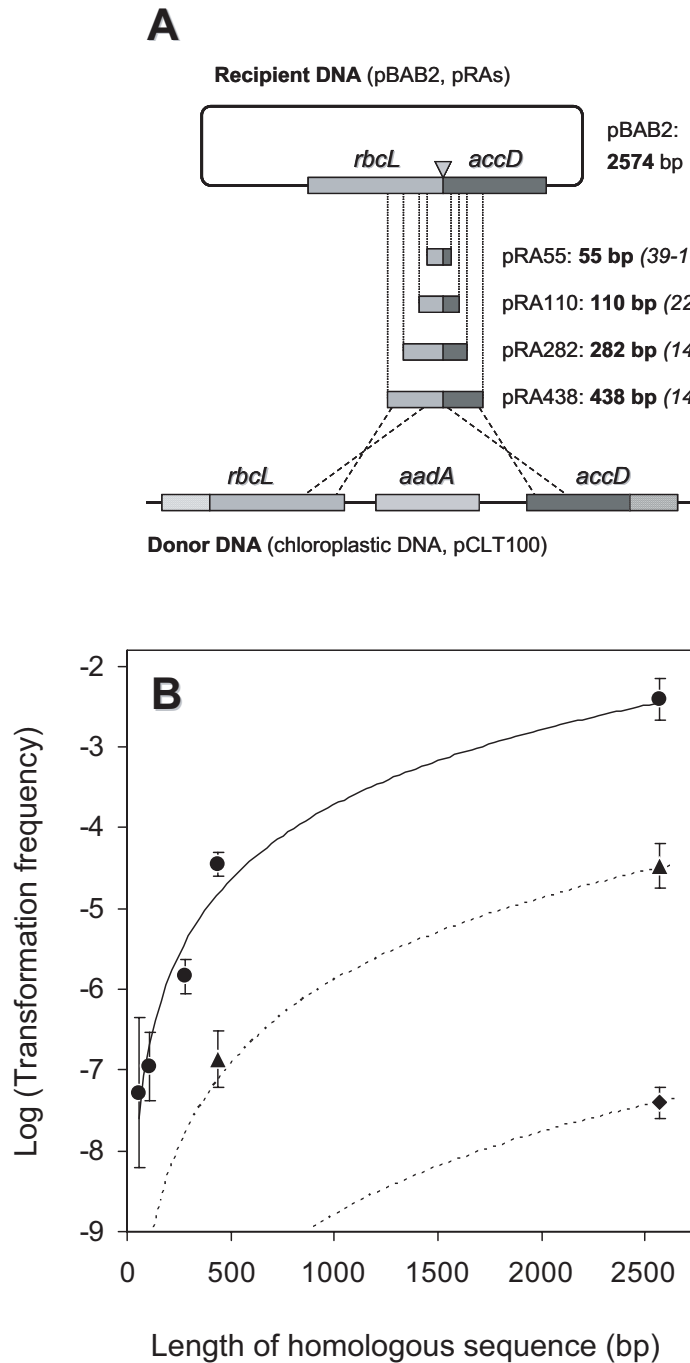
A

Recipient DNA (pBAB2, pRAs)

rbcL *accD*

pBAB2:
2574 bp *(1149-1425)*

pRA55: **55 bp** *(39-16)*

pRA110: **110 bp** *(22-88)*

pRA282: **282 bp** *(147-135)*

pRA438: **438 bp** *(140-298)*

rbcL *aadA* *accD*

Donor DNA (chloroplastic DNA, pCLT100)

B

Log (Transformation frequency) vs *Length of homologous sequence (bp)*

Figure 1. Effect of homologous sequence length on transformation frequency. (A) Plasmids harboring fragments, varying in size, of the *rbcL* and *accD* genes flanking the transgene *aadA* (1.35 kb in size) and schematic representation of homologous recombination between donor DNA (chloroplastic DNA, pCLT100) and recipient DNA harbored in *A. baylyi* strain BD413 on plasmids pBAB2 or pRAs. (B) Transformation frequencies obtained with *A. baylyi* strain BD413 harboring the different constructs and plasmid pCLT100 (circles), purified plant DNA (triangles) and crushed leaves (diamonds) as donor DNA. Each experiment was performed in triplicate. Vertical bars represent the standard error of the mean. Hypothetical tendency curves drawn for transformation assays performed with purified plant DNA and crushed leaves correspond to the curve obtained for plasmid DNA (y = 1.34 ln(x) − 12.96; R^2 = 0.969).

Acinetobacter cells showed that the two populations increased by the same order of magnitude (Kay et al., 2003), suggesting that in our study, bacterial population extracted from soil and injected into plants may have increased by up to 1000-fold. In addition, differences observed in the proportions of soil bacteria resistant to different antibiotics before injection into and after recovery from plant tissues suggested that not all bacteria were able to grow on infected plant tissues. For example, the percentage of bacteria resistant to kanamycin or tetracycline recovered from soil was *ca* 0.1% before injection, and reached almost 10% after cells had grown on plant tissue. While such changes may reflect selection of antibiotic resistant phenotypes, it may also suggest that horizontal transfer of resistance elements are fostered while bacteria are growing in plant tissues, which is often considered as a hot spot for HGT between bacteria (van Elsas et al., 2003).

Out of the 305 bacterial isolates collected from infected plant tissue, 17 could not be grown again on synthetic media after storage at –80 °C and, therefore, a total of 288 isolates were analyzed in this study. The antibiotic resistance profile of each bacterial isolate was assessed in order to determine which plasmid harboring which specific antibiotic resistance gene could be used for transformation experiments. The different antibiotics tested revealed that for the different samples and among the collection of isolates obtained (Fig. 2A) resistance to tetracycline was the least common and resistance to ampicillin was the most common. As a consequence, all transformation experiments were performed with plasmid pBBR1MCS3 (tetracycline resistance) or in the case of isolates naturally resistant to tetracycline with plasmid pBBR1MCS2 (kanamycin resistance). As expected, several isolates exhibited multiple antibiotic resistance, and over 70% of isolates were resistant to at least two of the antibiotics tested with five isolates (2.1%) resistant to all five antibiotics tested (Fig. 2B). A more extensive analysis of the antibiotic resistance potential of soil microorganisms was recently reported and showed what the authors referred to as the underappreciated density and concentration of environmental antibiotic resistance in soil (D'Costa et al., 2006). Such reports would tend to minimize the concern about the spread of antibiotic genes used as markers in transgenic plants to environmental bacteria (Miki and McHugh, 2004). However, our data and that from D'Costa et al. (2006) only measure phenotypes and not the actual resistance genes.

Screening for sequence similarity with DNA sequences flanking the transgene

Two sets of radioactive labeled DNA probes were used to screen the collection of 288 bacterial isolates for sequences homologous to the *rbcL* and *accD* genes flanking the transgene. After an initial screening, a total of 22 bacterial isolates (7.6%) exhibited a positive hybridization signal with one or more probes. No hybridization signals were observed for other isolates, as well as negative controls (water and total DNA of *A. baylyi* strain BD413). DNA from these 22 isolates was hybridized a second time with all probes, and positive hybridization signals were only confirmed for 16 of them. Hybridization signal intensities of positive isolates were slightly weaker than, but comparable to, signal intensity obtained for plant DNA (Fig. 3). The estimated copy numbers of targeted region per spot were *ca* 9×10^7 and 2.6×10^8 for plant and plasmid DNA controls, respectively. Assuming the presence of at least one sequence homologous to either flanking regions of the transgene in the genome of bacterial isolates, their estimated number per spot would range from 3.0×10^7 and 1.8×10^8 under the conditions tested (values calculated for genome sizes of 1 Mb and 6 Mb, respectively). Signal intensity of isolates being weaker than positive controls suggested that complete similarity was not detected. Therefore it is most likely that similarity to the probes was only partial. Among the 16 isolates exhibiting similarity, one isolate hybridized with probes targeting both regions and a total of five (1.7%) and ten (3.5%) isolates exhibited a positive signal when hybridized with probes targeting the *rbcL* and *accD* regions, respectively (Tab. 1). While the DNA sequence of the bacterial isolates presenting similarity with the chloroplastic genes was not characterized, the relatively high percentage of confirmed isolates presenting sequence similarity may not be surprising, since both plastid genes, which are of prokaryotic origin, are likely to be present in soil and may exhibit significant similarity. The initial purpose of this work was not to characterize the nature and identity of sequences similar to the *rbcL* and *accD* genes, yet further investigations might be to determine the nature and numbers of DNA sequences that could serve as recombinational anchors for integration of exogenous transgenic plant DNA. As reported for several bacterial species, transformation events may lead to the incorporation of non-homologous DNA into resident DNA when the DNA contains a single small region of homology (de Vries and Wackernagel, 2002; Meier and Wackernagel, 2003; Prudhomme et al., 2002). Currently, the transgenes in transgenic plants are mainly derived from soil bacteria (Miki and McHugh, 2004; Pontiroli et al., 2007) and could serve as recombinational anchors favoring insertion of flanking genes. While our study focused on the *accD* and *rbcL* genes, our results suggest that, when designing transgenic or transplastomic plants, the potential of flanking regions to serve as recombinational anchors (*i.e.*, sequence similarity to bacterial genomic DNA) should be taken into consideration.
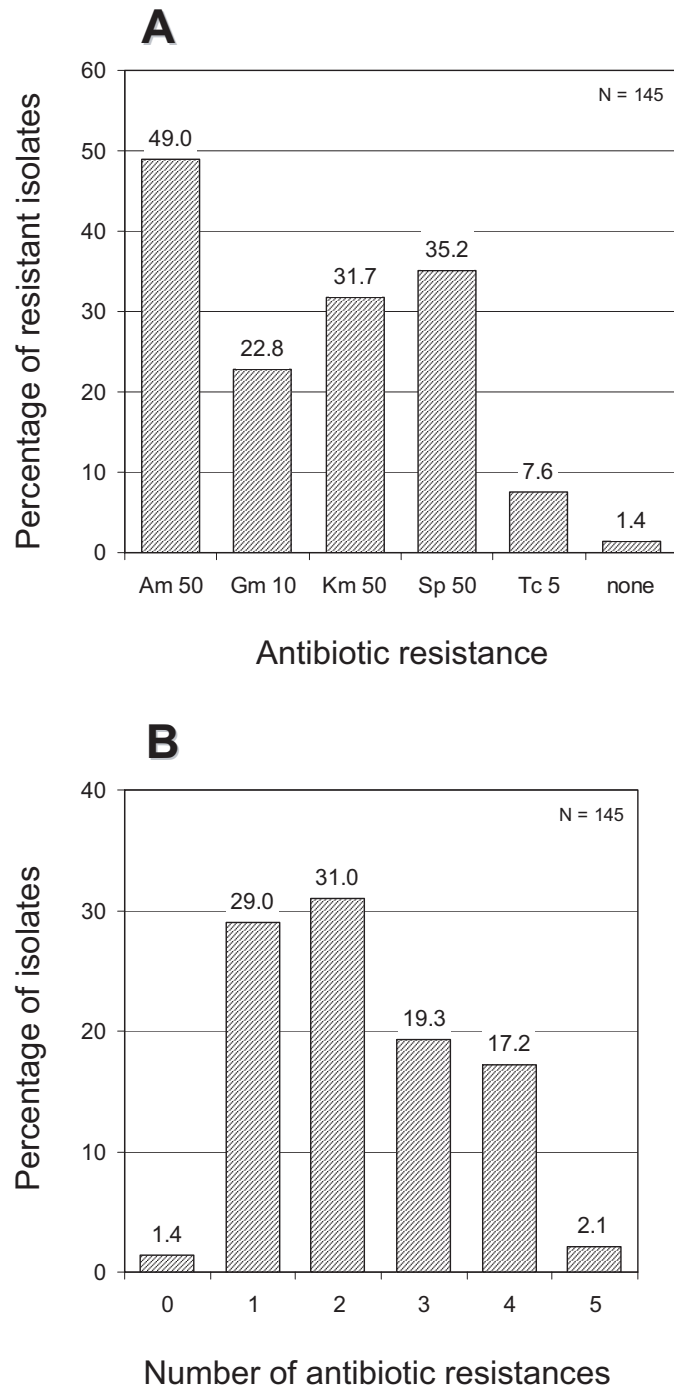
A

B

Figure 2. Antibiotic resistance profile of bacterial isolates. Antibiotic resistance profile of bacterial isolates recovered from infected tobacco plant tissues that were able to grow on TSA/10 medium supplemented with either ampicillin (50 µg.mL^{-1}), gentamicin (10 µg.mL^{-1}), kanamycin (50 µg.mL^{-1}), spectinomycin (50 µg.mL^{-1}) or tetracycline (10 µg.mL^{-1}) or exhibiting no resistance to any of the five antibiotic tested (none). (A) Percentage of isolates resistant to a given antibiotic. (B) Percentage of isolates resistant to 0, 1, 2, 3, 4 or 5 of the antibiotic tested. Data presented in this figure correspond to the antibiotic resistance profile of 145 isolates randomly selected from the 305 collected.

Table 1. Hybridization and transformability results for isolates exhibiting a positive signal after hybridization with probes targeting the *rbcL* and *accD* regions flanking the transgene.

Isolate	Hybridization signal		Transformability[1]			Soil[2]
	rbcL flank	*accD* flank	Broth	Filter	Electro	
AA12	-	+	-	-	-	SA
AO13	+	-	-	-	-	M
CA01	-	+	-	-	-	SA
CA11	-	+	$2.2 \ (\pm 0.9) \times 10^{-6}$	$4.5 \ (\pm 1.6) \times 10^{-6}$	-	SA
CO03	+	-	-	-	$3.9 \ (\pm 0.6) \times 10^{-5}$	M
GA05	-	+	-	-	-	SA
MA06	+	+	-	-	-	M
MC02	+	-	-	-	-	M
MG02	-	+	-	-	-	M
MG07	-	+	-	-	-	M
MP07	-	+	-	-	-	M
MS01	-	+	-	-	-	M
PA03	-	+	-	-	-	SA
PA07	-	+	-	-	-	SA
PO01	+	-	-	-	-	M
SO15	+	-	-	-	-	M

[1] Transformability is expressed as the number of transformants per recipient cells (mean ± SD).

[2] M: Montrond; SA: La Côte Saint-André.

Transformability of bacterial isolates

All 16 bacterial isolates that exhibited positive hybridization signals with probes targeting the *rbcL* and *accD* plastid regions were tested for their ability to incorporate the broad-host-range plasmids pBBR1MCS by natural transformation in suspension, on filter membrane, and by electro-transformation. All isolates but three were sensitive to tetracycline and transformation experiments were conducted with plasmid pBBR1MCS3 (Tc^R). The remaining three isolates, which were resistant to tetracycline, were kanamycin-sensitive and transformation experiments were conducted with the plasmid pBBR1MCS2 (Km^R). Under the conditions tested, two isolates were shown to be transformable and able to express the resistance gene (Tab. 1). Isolate CA11 was naturally transformable, and exhibited transformation frequencies (expressed as transformants per recipients cells) of 2.2×10^{-6} and 4.5×10^{-6} in suspension and on filter membrane, respectively. Isolate CO03 was naturally electro-transformable under the conditions tested, and transformation frequencies observed were on average 3.9×10^{-5} transformants per recipient cells (Tab. 1). Isolates CA11 and CO03 were identified based on a partial 16S ribosomal DNA sequence as *Acinetobacter* sp. and *Pseudomonas* sp., respectively. Transformation frequencies observed for isolate CA11 were similar than those of our control strain *A. baylyi* strain BD413 which exhibited transformation frequencies ranging 1.2 to 2.3×10^{-6} transformants per recipients cells under the same experimental conditions. While for isolate CO03, transformation frequencies observed were less than those reported for the electro-transformable *Pseudomonas* sp. isolated from soil, electro-transformation protocols used in this study were different and not optimized for *Pseudomonas* strains (Cérémonie et al., 2004). The ability of isolate CO03 to integrate the transgene by electro-transformation was tested with donor DNA plasmid pCLT100 and purified transplastomic tobacco plant DNA carrying the *aadA* gene. However, isolate CO03 is naturally resistant to spectinomycin and streptomycin, and while integration of the transgene might have occurred, such event could not be detected. In order to confirm or invalidate the ability of both isolates, CA11 and CO03, to integrate transgenic plant DNA, plasmid-based transgenic constructs as well as transplastomic tobacco plants carrying different marker genes between the *rbcL* and *accD* regions are currently being investigated in the laboratory.

While HGT between transgenic plant DNA and bacteria isolated from soil has not been assessed, the approach developed in this study revealed that potential
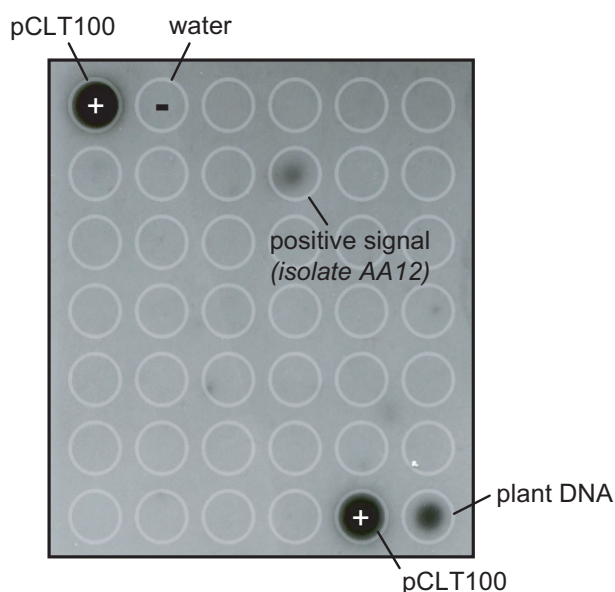
Figure 3. Dot blot hybridization. Example of dot blot hybridization obtained with the probe *accD*1064 (1064 bp in length) with one isolate (AA12), out of 38 spotted on that membrane, showing a positive signal after exposure of the membrane to X-ray films for 24 h. Positive controls: plasmid pCLT100 (1 ng) and purified plant DNA (100 ng); negative control: water.

transgenic DNA recipients might be present among soil bacteria. Two isolates exhibited all the required characteristics to be a potential transgenic plant DNA recipient (*i.e.*, colonization of infected plant tissues, sequence similarity with regions flanking the transgene and transformability). Additional characterization of the homologous sequences, optimization of transformation protocols and generation of different transgenic constructs harboring different marker genes will influence the ability of these isolates to integrate transplastomic plant DNA in their genome. The ability of the two isolates to grow and develop a competent state *in planta* will also be assessed.

While none of the 16 other isolates were identified as naturally transformable, a limited number of experimental conditions were tested to assess their transformability. The low or undetected frequencies of plasmid transformation may have been cause by the lack of easy entry or replication of plasmid DNA into the cell, or the lack of expression of the resistance gene. Further testing using different protocols or donor DNA might reveal other transformable bacteria among our collection of isolates (Ray and Nielsen, 2005). Identification of the 16 bacterial isolates (based on their 16S rRNA gene sequence) should help us refine transformation protocols adapted to the species and may lead to identification of other

transformable isolates. On the other hand, the conditions tested for *in vitro* transformation may not be encountered by bacteria in the environment, and further investigations of natural and electro-transformability of the different isolates under natural conditions will be required.

MATERIAL AND METHODS

Bacterial strains and plasmids

The plant pathogenic bacteria, *Ralstonia solanacearum* strain K60 (Kelman, 1954), the causal agent of bacterial wilt on a wide range of species including tobacco (Buddenhagen and Kelman, 1964), was used to perform plants co-inoculation experiments. Cells were grown on B agar medium (Boucher et al., 1985) at 28 °C for 48 h or in B broth until reaching an optical density of 1.0 at 600 nm (OD_{600}). The naturally transformable bacteria *Acinetobacter baylyi* strain BD413 (Juni and Janick, 1969) was chosen as the model bacterium for transformation assays. *A. baylyi* strain BD413 (pBAB2) harbored plasmid pBAB2 that contained a recombinogenic site with tobacco plastidic sequences *rbcL* and *accD* (Kay et al., 2002b) to favor homologous recombination. Plasmids containing fragments of different sizes of the *rbcL-accD* regions were constructed. Plastid sequences ranging from 55 bp to 438 bp were amplified by PCR using primer sets listed in Table 2 and cloned into pGEM®-T Easy vector (Promega, Madison, Wisconsin) according to manufacturer's instructions. Plasmids were then digested with *Eco*RI and the resulting fragments containing plastid sequences were purified with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Each of these fragments was then subcloned into the broad host spectrum vector pMMB190 (Morales et al., 1991) previously digested with *Eco*RI. Plasmids containing *rbcL-accD* fragments of different sizes were purified using QIAfilter™ Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturer's instructions and introduced into *A. baylyi* strain BD413 by natural transformation. *A. baylyi* cells were grown at 28 °C on modified Luria Bertani (LBm) medium (5 g.L^{-1} NaCl) supplemented with ampicillin (50 μg.mL^{-1}) and nalidixic acid (20 μg.mL^{-1}) (Sigma, St. Louis, California). Transformants were selected on LBm medium containing ampicillin (50 μg.mL^{-1}), nalidixic acid (20 μg.mL^{-1}) and spectinomycin (50 μg.mL^{-1}) (Sigma, St. Louis, California) after 2 days of incubation at 28 °C. Plasmid pCLT100, which has been used to generate transplastomic tobacco plants (Kay et al., 2002b), and antibiotic-resistant derivatives of the broad-host-range plasmid pBBR1MCS (Kovach et al., 1995), were used to perform transformation assays with *A. baylyi* strains and bacterial isolates, respectively. As shown by incompatibility testing, pBBR1 does not belong to the broad host range IncP, IncQ or IncW groups (Antoine and Locht, 1992).

Table 2. Primers used to amplify and clone plastid sequences and the corresponding number of nucleotides in identical sequences with plant DNA on each side of the transgene.

| Plasmid | Size (bp) of the fragment cloned | | | Primer's sequence (5'-3') |
	Total	*rbcL* flank	*accD* flank	
pRA55	55	39	16	*f:* AAATCTAAGACTCAAATC *r:* CGTAGGATTAATTGTGGA
pRA110	110	22	88	*f:* TTTCTATTGTTGTCTTGGAT *r:* GGTGTGATACTTGGCTTG
pRA282	282	147	135	*f:* AAAGGATTGAGCCGAATACA *r:* TTTCAACACGGAACAAAGG
pRA438	438	140	298	*f:* GAGCCGAATACAACAAAGAT *r:* GGCTTCTCCTATGTCGTG
pBAB2	2574	1149	1425	(Kay et al., 2002b)

Plant materials

Experiments were performed using wild-type tobacco plants (*Nicotiana tabacum* cv. PBD6) and transplastomic plants of the same cultivar harboring a chimeric *aadA* gene, which confers resistance to both spectinomycin and streptomycin, inserted between the *rbcL* and *accD* plastid genes (Kay et al., 2002b). Plants were grown in potting compost (Grassot, Brignais, France) in a greenhouse at 22 °C with a daily light regimen of 16 h and 8 h of darkness. The relative humidity rate was on average 55% during the day and 70% at night. *Ralstonia*-infected tobacco plants were transferred to a growth chamber and grown at 28 °C with a light regimen consisting of 16 h of light and 8 h of darkness at 70% relative humidity.

DNA extractions

Plant genomic DNA was extracted from entire tobacco leaves. Leaves were sampled and the central vein was removed with a sterile scalpel and individually ground in liquid nitrogen. Plant DNA was extracted from 100 mg of the resulting leaf powder by using with the DNeasy® Plant kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Plasmids were isolated from bacterial cells using the QIAfilter™ Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Total bacterial and plasmid DNA extraction from putative recombinants and recipients were performed using the NucleoSpin® Tissue and NucleoSpin® Plasmid kits, respectively, (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Following extraction, nucleic acid purity and concentration were determined by measuring the absorbance of DNA solutions at 260 nm (OD$_{260}$) with the Eppendorf® Biophotometer (Eppendorf, Westbury, USA) or by depositing 1 μL on agarose gel for comparison with the Massruler™ DNA Ladder (Fermentas, Burlington, Canada).

Acinetobacter baylyi transformation assays

In vitro transformation assays of *A. baylyi* strain BD413 were performed using plasmid and plant DNAs purified with the appropriate kits as well as with crushed plant material. Two grams of fresh leaves were crushed in 10 mL of distilled sterile water with an Ultra-Turrax T25 homogenizer operating at 25 000 rpm (IKA-Werke GmbH and Co., Staufen, Germany). Aliquots of purified DNAs and fresh plant material were used for *in vitro* transformation experiments. *A. baylyi* strain BD413(pBAB2), BD413(pRA55), BD413(pRA110), BD413(pRA282), BD413(pRA438) were grown overnight, before an aliquot was diluted 25-fold into fresh LBm broth medium and cultured for an additional 2 h to reach the competent state as described previously (Palmen et al., 1993). A final amount of 1 μg of plasmid pCLT100 DNA or purified transplastomic plant DNA in 40 μL of sterile distilled water, or 40 μL of crushed leaves, were used to transform 360 μL of competent *A. baylyi* cells. Mixtures were then incubated for an additional 90 min at 28 °C for DNA uptake before plating on LBm medium supplemented with the appropriate antibiotics. The transplastomic sequence signature was detected by PCR using previously described primers

p415 and p416 complementary to part of the *aadA* gene and targeting a 382-bp fragment (Ceccherini et al., 2003).

Soil samples and recovery of bacteria

Extraction of soil bacteria was performed on fresh soil samples using a Nycodenz® gradient separation method (Bertrand et al., 2005; Courtois et al., 2001). Soil samples from La Côte Saint-André (48% sand, 41% silt, 9% clay, organic matter 40 g.kg^{-1} of dry soil, pH 5.6) and Montrond (44% sand, 40% silt, 18% clay, organic matter 47 g.kg^{-1} of dry soil, pH 5.9) were collected from the upper 5–25 cm and sieved through 2 mm mesh before recovery of bacteria. Two hundred g of each soil sample were mixed with 300 mL sodium hexametaphosphate solution 0.2% and homogenized in a Waring blender for 1 min. Coarse soil particles were eliminated through a first centrifugation step at low speed (700× *g*, 15 min at 10 °C) using a Beckman J2-21M/E centrifuge (Beckman Coulter, Fullerton, California). The supernatant was collected and centrifuged at 7500× *g* during 20 min at 10 °C. The microbial cell fraction contained in the pellet was resuspended in 40 mL of sterile 0.8% sodium chloride solution by vortexing to obtain a homogeneous suspension. Twenty-five mL aliquots of the suspension were transferred to an ultracentrifuge tube containing 10 mL of Nycodenz® solution (Nycomed, Oslo, Norway) at 1.3 g.mL^{-1} density (8 g of Nycodenz® to 10 mL of sterile ultra pure water). Cells and soil particles were separated by centrifugation at high speed (14 600× *g*, 40 min at 10 °C) with a Kontron TGA-50 ultracentrifuge equipped with a Kontron TST 2838 swing-out rotor (Kontron, Eching, Germany). The white ring containing bacterial cells formed at the interface between the Nycodenz® solution and the overlying aqueous layer was carefully recovered using a pipette. Recovered bacterial cells were centrifuged at 7500× *g* for 10 min at 10 °C and rinsed twice in sterile ultrapure water.

Plant inoculation and recovery of bacterial isolates

Tobacco plants were co-infected with a mixture the bacterial cells recovered from soil (Nycodenz® ring) and *R. solanacearum* strain K60 cells. One milliliter of an overnight culture of *R. solanacearum* strain K60 was harvested, rinsed twice and resuspended in sterile distilled water to adjust the concentration to *ca* 10^7 cells.mL^{-1}. The numbers of bacterial cells recovered from the different soil samples were assessed by microscopy using a Malassez counting chamber and the suspensions were diluted in sterile distilled water to adjust the concentration to *ca* 10^7 cells.mL^{-1}. Population sizes of the different suspensions used to inoculate the plants were determined

from colony counts after plating serial dilutions onto agar media and incubation at 28 °C for up to 5 days. The final inoculum was prepared by mixing 1 volume of the *R. solanacearum* strain K60 stock suspension with 2 volumes of a suspension of bacteria extracted from each soil. Two hundred and 500 μL of the different inocula were injected into the central veins of leaves of 8-week-old tobacco plants and stems of 4 weeks-old tobacco plants, respectively. As control experiments for disease development, sterile distilled water and *R. solanacearum* strain K60 cells were also injected into plant tissues. Between eight and ten injections were performed for each different mixture. After 5 days incubation, or until development of severe wilting symptoms, infected plant tissues were sampled and their weight determined. One gram of infected plant tissue was then mixed with 10 mL of sterile 0.8% sodium chloride solution and homogenized using an Ultra-Turrax T25 operating at 25 000 rpm (IKA-Werke GmbH and Co., Staufen, Germany). Serial dilutions of the plant tissue suspensions were plated on Trypticase Soy Agar medium diluted one-tenth (TSA/10) supplemented with cycloheximide (200 μg.mL^{-1}) and nystatin (50 μg.mL^{-1}) (ICN Biomedicals Inc., Aurora, Ohio) to limit fungal growth, without antibiotics, or with one of the following antibiotics ampicillin (50 μg.mL^{-1}), gentamicin (10 μg.mL^{-1}), kanamycin (50 μg.mL^{-1}), spectinomycin (50 μg.mL^{-1}) or tetracycline (10 μg.mL^{-1}) (Sigma, St. Louis, California). Plates were incubated at 28 °C for up to 5 days and population sizes were estimated from colony counts. A collection of bacterial isolates able to colonize *Ralstonia*-infected tobacco plant tissues was obtained. Individual colonies were picked from each plate using sterile toothpicks and purified by streaking them on fresh TSA/10 plates supplemented with the appropriate antibiotics. The antibiotic resistance profile of all bacterial isolates was also assessed by determining the ability of each isolate to grow on TSA/10 medium supplemented with either ampicillin (50 μg.mL^{-1}), gentamicin (10 μg.mL^{-1}), kanamycin (50 μg.mL^{-1}), spectinomycin (50 μg.mL^{-1}) or tetracycline (10 μg.mL^{-1}) (Sigma, St. Louis, California). Stock suspensions of each isolate (*ca* 300) were prepared by mixing cells grown overnight in Trypticase Soy Broth (TSB) medium to glycerol (1:1 vol:vol) and stored at –80 °C until use.

Screening for similarity by dot-blot hybridization

In order to screen for DNA sequence similarity between the genome of bacterial isolates and tobacco plant chloroplastic sequences, probes of different lengths targeting the *rbcL* and *accD* regions neighboring the transgene were defined (Tab. 3). Amplifications of 1064-bp, 516-bp and 215-bp fragments of the accD region were

Table 3. Primers used to amplify portions of the *rbcL* and *accD* genes flanking the transgene. Amplified products of different lengths were used as probes to screen for the presence of DNA sequence similarity between the genome of bacterial isolates and tobacco plant chloroplastic sequences.

Target	Product size (bp)	Primer's sequence (5'-3')
accD	1064	*f:* TCGTCTACTGGAACCCTCGT *r:* TCACACCTCTTTCTACCCATCC
	516	*f:* GCCCATTGATTTATTTAGCCC *r:* TATTGTCCCCTTTGTTCCGT
	215	*f:* CGGCTTCTCCTATGTCGTG *r:* CACCTCTTTCTACCCATCCTGT
rbcL	1408	*f:* TGTTGTATTCGGCTCAATCCT *r:* GCTGCCGAATCTTCTACTGG
	1071	*f:* TCCTTATCCTTCTTTATTTCCTGCT *r:* CGAATCCCTCCTGCTTATGT
	455	*f:* TCCTTATCCTTCTTTATTTCCTGCT *r:* GCTTTGTTGATTTACTGCGTG

performed by PCR using primer sets accD-1064F/accD-1064R, accD-516F/accD-516R and accD-215F/accD-215R, respectively. Amplifications of 1408-bp, 1071-bp and 455-bp fragments of the rbcL region were performed by PCR using primer sets rbcL-1408F/rbcL-1408R, rbcL-1071F/rbcL-1071R and rbcL-1071F/rbcL-455R, respectively. All primers were defined with a Tm of 60 ($\pm$ 1) °C. Radioactive labeling of DNA probes was performed by random oligonucleotide-primed synthesis using HexaLabel^{TM} DNA Labeling Kit (Fermentas, Burlington, Canada) using [α–^{32}P]-dCTP and following manufacturer's instructions. Unincorporated label was removed by chromatography on Sephadex® G-50 columns (Amersham Biosciences, Piscataway, New Jersey). In order to avoid variability due to differences in bacterial cell lysis efficiency, total bacterial DNAs of the collected 288 isolates were extracted as described previously (*DNA extractions*) before blotting on positively charged nylon membranes using a 96-well Bio-Rad dot blot (Bio-Rad, Hercules, California). Total DNA from *A. baylyi* strain BD413 and water were used as negative controls, whereas total DNA extracts from *A. baylyi* strain BD413(pBAB2), tobacco plants and pCLT100 plasmid DNA were used as positive controls. One hundred µL of denatured DNA (95 °C for 10 min) containing a total amount of 100 ng of total bacterial DNA or 1 ng of plasmid DNA were added to each well. Four membranes harboring *ca* 80 different DNA extracts of the different isolates and controls were prepared, placed in individual tubes and hybridized overnight at 65 °C with each sets

of probes. Membranes were exposed to X-Ray films for 24 h and 72 h.

Screening for naturally and electro-transformable bacterial isolates

Bacterial isolates that exhibited a positive hybridization signal with one or more probes targeting the *rbcL* and/or *accD* regions flanking the transgene were screened for their ability to incorporate plasmid DNA by natural transformation in suspension or on filter and by electro-transformation. Based on the antibiotic resistance profiles of the selected bacterial isolates, transformation assays were performed with either plasmid pBBR1MCS3 or plasmid pBBR1MCS2, tetracycline- and kanamycin-resistant derivatives of the broad-host-range plasmid pBBR1MCS (Kovach et al., 1995), respectively. For all transformation assays, bacterial isolates were grown overnight in TSB medium before an aliquot was diluted 25-fold into fresh TSB medium and grown until an OD_{600} of *ca* 1.0 was reached. Natural transformation assays in broth media were performed by adding 1 µg of plasmid DNA in 10 µL of distilled water to 390 µL of the bacterial suspensions. Mixtures were then incubated for 90 min at 28 °C before plating on TSA medium supplemented with the appropriate antibiotics. Natural transformation assays on filter membranes were performed by depositing 250 ng of plasmid DNA in 2.5 µL of distilled water mixed to 87.5 µL of the bacterial suspensions onto GTTP

membrane filters (Millipore, Billerica, USA) placed on TSA plates. Plates were incubated for 2 to 5 days at 28 °C. Membrane filters were individually rinsed in 5 mL sterile distilled water and serial dilutions of the suspension obtained plated on TSA medium supplemented with the appropriate antibiotics. Electro-transformation was performed by placing 40 μL of the bacterial suspensions mixed with 400 ng of plasmid DNA into 2 mm electroporation cuvettes (Equibio Ltd., Middlesex, UK) and applying one pulse using the Gene Pulser® II system (Bio-Rad, Hercules, California) set at 12.5 kV.cm^{-1}, 200 Ω, and 25 μF. In all transformation assays, control experiments were performed by replacing plasmid DNA solutions by an equal volume of sterile distilled water. For natural transformation in broth medium and electro-transformation experiments, prior to plating suspensions on TSA medium, bacterial suspensions were treated with 0.1 mg.mL^{-1} DNase I (Sigma, St. Louis, California) for 15 min at 37 °C to degrade exogenous plasmid DNA added to the suspensions and prevent transformation from occurring on plates. Population sizes of recipient cells and transformant cells were determined from plate counts after 2–5 days incubation at 28 °C. Transformants were selected on TSA medium containing either kanamycin (50 μg.mL^{-1}) or tetracycline (10 μg.mL^{-1}). Each experiment was performed in triplicate. The ability of bacterial isolates exhibiting DNA sequence similarity with *rbcL* and/or *accD* probes and identified as naturally or electro-transformable were also tested for their ability to integrate the transgene. Transformation assays were performed with plasmid pCLT100 and purified transplastomic tobacco plant DNA under the conditions described previously (*Acinetobacter baylyi* transformation assays).

Bacterial isolate identification

Bacterial isolates exhibiting a positive hybridization signal with *rbcL* and/or *accD* probes and identified as naturally or electro-transformable were identified based on their 16S ribosomal DNA sequence. The *rrn* gene of each bacterial isolate was amplified using PA-PH primers and cloned into pGEM®-T Easy vector (Promega, Madison, Wisconsin). Vectors were then isolated from *Escherichia coli* strain DH5α cells with QIAfilter™ Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturer's instructions and sent for sequencing (Genome Express SA, Meylan, France).

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