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Exploration of horizontal gene transfer between transplastomic tobacco and plant-associated bacteria

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Abstract

The likelihood of gene transfer from transgenic plants to bacteria is dependent on the transgene copy number and on the presence of homologous sequences for recombination. The large number of chloroplast genomes in a plant cell as well as the prokaryotic origin of the transgene may thus significantly increase the likelihood of gene transfer from transplastomic plants to bacteria. In order to assess the probability of such a transfer, bacterial isolates, screened for their ability to colonize decaying tobacco plant tissue and possessing DNA sequence similarity to the chloroplastic genes *accD* and *rbcl* flanking the transgene (*aadA*), were tested for their ability to take up extracellular DNA (broad host-range pBBR1MCS-3-derived plasmid, transplastomic plant DNA and PCR products containing the genes *accD*–*aadA*–*rbcl*) by natural or electrotransformation. The results showed that among the 16 bacterial isolates tested, six were able to accept foreign DNA and acquire the spectinomycin resistance conferred by the *aadA* gene on plasmid, but none of them managed to integrate transgenic DNA in their chromosome. Our results provide no indication that the theoretical gene transfer-enhancing properties of transplastomic plants cause horizontal gene transfer at rates above those found in other studies with nuclear transgenes.

Introduction

The use of transgenic plants in the field raises questions about the potential transfer of transgenes from these plants to soil bacteria that could lead to the dissemination of unwanted traits among bacterial communities in the environment. The main concern is about the transfer and dissemination of antibiotic resistance genes, usually cloned concomitant with the transgene to allow the selection of transformed plant cells (Dröge *et al.*, 1998). This is due to a higher possibility of recombination-mediated integration of these new plant DNA regions in bacterial genomes facilitated by sequence similarity between the transgene and the DNA of the recipient bacteria (de Vries & Wackernagel, 2002; Meier & Wackernagel, 2003). In addition, studies indicate that several ecosystems in which physiologically active bacteria develop in close physical contact with DNA released by plant tissues constitute 'hot-spots' for horizontal gene transfer (HGT) between transgenic plants and bacteria (Nielsen *et al.*, 1998; Pontiroli *et al.*, 2009). The likelihood of HGT is increased with transplastomic plants in which the

transgene is cloned in the chloroplast genome composed of genes with a typical prokaryotic structure (McFadden, 2001; Chu *et al.*, 2004). The prokaryotic origin of the chloroplast genome suggests that transgene recombination in bacterial genomes could also be initiated based on the chloroplast DNA regions flanking the transgene, including the *accD* and *rbcl* genes routinely used for cloning purposes (Svab & Maliga, 1993; Birch-Machin *et al.*, 2004; Zhou *et al.*, 2008). The prokaryotic *accD* gene encodes the β -carboxyl transferase subunit of acetyl-CoA carboxylase (ACCase) required for fatty acid biosynthesis. Southern hybridization analysis demonstrated that most plants contain this *accD* gene in their genome and have the prokaryotic ACCase in their plastids (Konishi *et al.*, 1996). The chloroplast *rbcl* gene encodes the large subunit of the CO₂-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and is present among *Cyanobacteria* and certain *Proteobacteria* (purple bacteria) (Delwiche & Palmer, 1996). Another factor increasing the likelihood of gene transfer from transplastomic plant to bacteria is the presence of the *aadA* gene. This gene encodes the aminoglycoside adenyl transferase

conferring resistance to both streptomycin and spectinomycin antibiotics and is used to select for chloroplast transformants due to the effective inhibition of plastid protein biosynthesis in wild plants by spectinomycin (Bock, 2001). Moreover, the copy number of a chloroplastic transgene in a plant cell is several orders of magnitude higher than that of a nuclear located DNA insert. A single leaf cell may contain dozens or even hundreds of chloroplasts. Chloroplastic DNA is organized in nucleoids harboring several copies of the plastid genome as typical of a prokaryotic system and several nucleoids are present in each chloroplast. Up to 10 000 (identical) copies of the plastid DNA can be found in a single pea leaf cell and even up to 50 000 copies in a wheat cell (Bendich, 1987).

Studies already performed under conditions favoring HGT included the use of transplastomic plants concomitantly infected by pathogens (Kay *et al.*, 2002a), transgenic plant residuosphere (plant decaying material) (Rizzi *et al.*, 2008) and naturally transformable recipient bacteria specifically constructed to recombine and express transgenes (Nielsen *et al.*, 2000; Kay *et al.*, 2002b; Tepfer *et al.*, 2003; Pontiroli *et al.*, 2009). These conditions simulated those encountered in nature with the use of naturally transformable recipient bacteria commonly found in soil (e.g. *Acinetobacter baylyi*), and molecular constructions specifically engineered to simulate similarity between donor DNA and recipient bacterial genomes. Our study differs from previous ones because we used natural bacterial isolates as recipients.

The objective of this study was to determine the natural occurrence of DNA sequences among bacterial isolates on which recombination with transgene sequences or with the chloroplast DNA regions flanking the transgene could occur. Bacterial isolates able to grow on decaying tobacco plants (Monier *et al.*, 2007) were screened for sequences that share a significant similarity level to three chloroplast-borne genes of transplastomic tobacco plants including *accD*, *rbcL* genes and the *aadA* marker gene. In order to assess the probability of HGT between transplastomic plants and indigenous bacteria, bacterial isolates were tested for their ability to take up extracellular DNA by natural or electrotransformation.

Materials and methods

Bacterial isolates, plants and plasmids

Sixteen environmental bacterial isolates able to grow on decaying plant tissues and presenting sequence similarities to the chloroplastic genes *accD* and/or *rbcL* (Monier *et al.*, 2007) were used in this study. All bacterial strains were grown at 28 °C on a modified Luria–Bertani (LBm) medium (5 g L⁻¹ NaCl). In order to test the resistance of the isolates to streptomycin and spectinomycin (antibiotic resistances encoded by the *aadA* gene), 100 µL of an overnight culture

from the 16 isolates was spread on LBm plates supplemented with both antibiotics (50 µg mL⁻¹ each).

Transplastomic tobacco plants (*Nicotiana tabacum* L. cv. PBD6) were grown in compost potting soil in a greenhouse at 23 °C (± 2 °C), with a daily regimen of 16 h of light and 8 h of darkness. Transplastomic plants harbored the transgenic *aadA* gene cloned between the chloroplastic genes *rbcL* and *accD* (Svab & Maliga, 1993; Kay *et al.*, 2002b) and contained *c.* 7000 copies of the transgene per plant cell.

Escherichia coli strain DH5α(pBHCrec) harbored the pBHCrec plasmid, a pBBR1MCS-3 derivative resistant to tetracycline, which contained the *aadA* gene flanked by part of the *rbcL* and *accD* tobacco plastid sequences (Kovach *et al.*, 1995; Cérémonie *et al.*, 2004). *Escherichia coli* strain DH5α(pLEP01) harbored the pLEP01 plasmid, an ampicillin-resistant cloning vector that contained the *aadA* gene, conferring resistance to spectinomycin and streptomycin, framed with plastid sequences corresponding to the *rbcL* and *accD* regions (Svab & Maliga, 1993; Kay *et al.*, 2002b). These strains were grown at 37 °C on LBm medium supplemented with spectinomycin (50 µg mL⁻¹) and streptomycin (50 µg mL⁻¹). *Pseudomonas* N3, an electrocompetent strain isolated from soil (Cérémonie *et al.*, 2004), was grown at 29 °C on LBm medium and used as a positive control for electroporation experiments. Total transplastomic tobacco DNA, PCR products of the genes *accD*–*aadA*–*rbcL* generated from plasmid pLEP01 and plasmid pBHCrec were used for transformation and electroporation assays as described in the following sections.

DNA extraction

Genomic and plasmid DNA were isolated from bacterial strains using the NucleoSpin[®] Tissue and NucleoSpin[®] Plasmid Kits, respectively (Macherey–Nagel, Düren, Germany), following the manufacturer's instructions. Plant genomic DNA was extracted from plant leaves ground in liquid nitrogen using the DNeasy[®] Plant kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following extraction, nucleic acid purity and concentration were determined by measuring the absorbance of DNA solutions at 260 nm using the NanoPhotometer (Implen GmbH, München, Germany) and by depositing 100 ng on agarose gel for comparison with the 1 kb+DNA ladder (Fermentas, Burlington, Canada).

Genetic amplification by PCR

For isolate identification, the 16S rRNA gene was amplified in a thermocycler (Applied Biosystems Inc., Foster City, CA) by PCR with the primers pA and pH (Table 1) and Titanium Taq 1 × final (Clontech, St-Germain-en-Laye, France) on 70 ng of extracted genomic DNA (Table 1) according to the manufacturer's instructions. The PCR program consisted of

Table 1. Primers used in this study

Target	Primer name	Primer sequence (5'–3')	Amplicon size (bp)	T_h (°C)	T_e	References
16S rRNA gene	pA	AGAGTTTGATCCTGGCTCAG	1534	55	1 min 30 s	Edwards <i>et al.</i> (1989)
	pH	AAGGAGGTGATCCAGCCGCA				
<i>aadA</i>	p415	ATTCCGTGGCGTTATC	382	60	30 s	Ceccherini <i>et al.</i> (2003)
	p416	TGACGGGCTGATACT				
<i>accD–aadA–rbcL</i>	accDF	ACCCACAAATGCCTGTATTTTG	4113	56	4 min 30 s	This study
	rbcLR	TAGCTGCCGAATCTTCTACTGGTA				
Transformants	rbcL455r	GCTTTGTTGATTACTGCGTG	1487	50	1 min 30 s	Monier <i>et al.</i> (2007)
	p416	TGACGGGCTGATACT				

T_h , hybridization temperature; T_e , elongation time.

a hot start at 94 °C for 5 min, followed by 30 cycles consisting of 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 68 °C for 1 min 30 s. A final elongation step at 72 °C for 7 min preceded cooling at 10 °C. Amplified DNA was purified on a 1% agarose gel using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Purified PCR products were sequenced with pA and pH primers (GATC, Konstanz, Germany), assembled with SEQMAN version 7.2.1 (DNASTAR, Madison) and published in GenBank (accession numbers HQ670701–HQ670716).

The screening for the presence of *aadA* gene was performed by PCR with the primers p415 and p416 (Table 1) on 70 ng of purified genomic DNA. Amplifications were performed using the Invitrogen Taq polymerase (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Amplified DNA (5 µL) was then loaded on a 2% w/v agarose gel.

For transformation and electroporation assays, the three genes were amplified by PCR with the primers accDF and rbcLR (Table 1) on 50 ng of plasmid pLEP01. PCR was performed with the Illustra HotStart Mix RTG (GE Healthcare) according to the manufacturer's instructions.

To analyze transformants, integration of the transgene into bacterial isolate's genome either by plasmidic replication or by genomic recombination was monitored by PCR with the primers rbcL455r and p416 (Table 1) on 70 ng of genomic DNA. PCR was performed with the Invitrogen Taq polymerase according to the manufacturer's instructions. Amplified DNA (5 µL) was then loaded on a 1% w/v agarose gel.

Transformation assays

Natural transformation

Natural transformation assays were performed as described previously (Demanèche *et al.*, 2001b), except that overnight cultures were not 100-fold concentrated. Briefly, 100 µL of an overnight culture of the isolates was mixed with 0.1 pmol of plasmid pBHCrec, 0.1 pmol of PCR product or 500 ng of

plant DNA directly on LBm Petri dishes. After drying of the droplets, the plates were incubated at 29 °C for 24 h and bacterial cells were resuspended in 500 µL of LBm medium with a sterile loop. Appropriate dilutions were then plated out on LBm medium supplemented or not with spectinomycin and streptomycin, to enumerate transformant and total cells, respectively. Transformations with plasmid pBHCrec were also plated on LBm medium supplemented with tetracycline (10 µg mL⁻¹) to overcome problems linked to the natural resistance to streptomycin and spectinomycin of soil isolates. Negative controls were conducted without adding DNA. Experiments were conducted in triplicate.

Electroporation

The entry of DNA in bacterial cells due to electric discharges was tested by *in vitro* electroporations using the gene pulserTM apparatus (Bio-Rad Laboratories, Richmond, CA) by mixing 50 µL of electrocompetent cells (Drury, 1996) with 0.1 pmol of plasmid pBHCrec, 0.1 pmol of PCR product or 500 ng of plant DNA. Mixes were incubated for 1 min on ice and electroporated in 0.2-cm cuvette at 12.5 kV cm⁻¹, 200 Ω, 25 µF, for 5 ms and immediately diluted in 450 µL of LBm medium (Drury, 1996). After 2 h at 29 °C, dilutions were plated out on LBm medium supplemented with appropriate antibiotics to determine transformation frequencies. Electroporations with plasmid pBHCrec were also plated on LBm medium supplemented with tetracycline (10 µg mL⁻¹) to overcome problems linked to the natural resistance to streptomycin and spectinomycin of soil isolates. Negative controls were conducted without adding DNA. Positive controls were conducted on *Pseudomonas* N3 electroporated with 0.1 pmol of plasmid pBHCrec. Experiments were conducted in triplicate.

Results and discussion

Similarities of transplastomic genes with sequenced bacterial chromosomes

The transgene *aadA* and both flanking genes *accD* and *rbcL* of the transplastomic tobacco were BLASTed (BLASTN) using

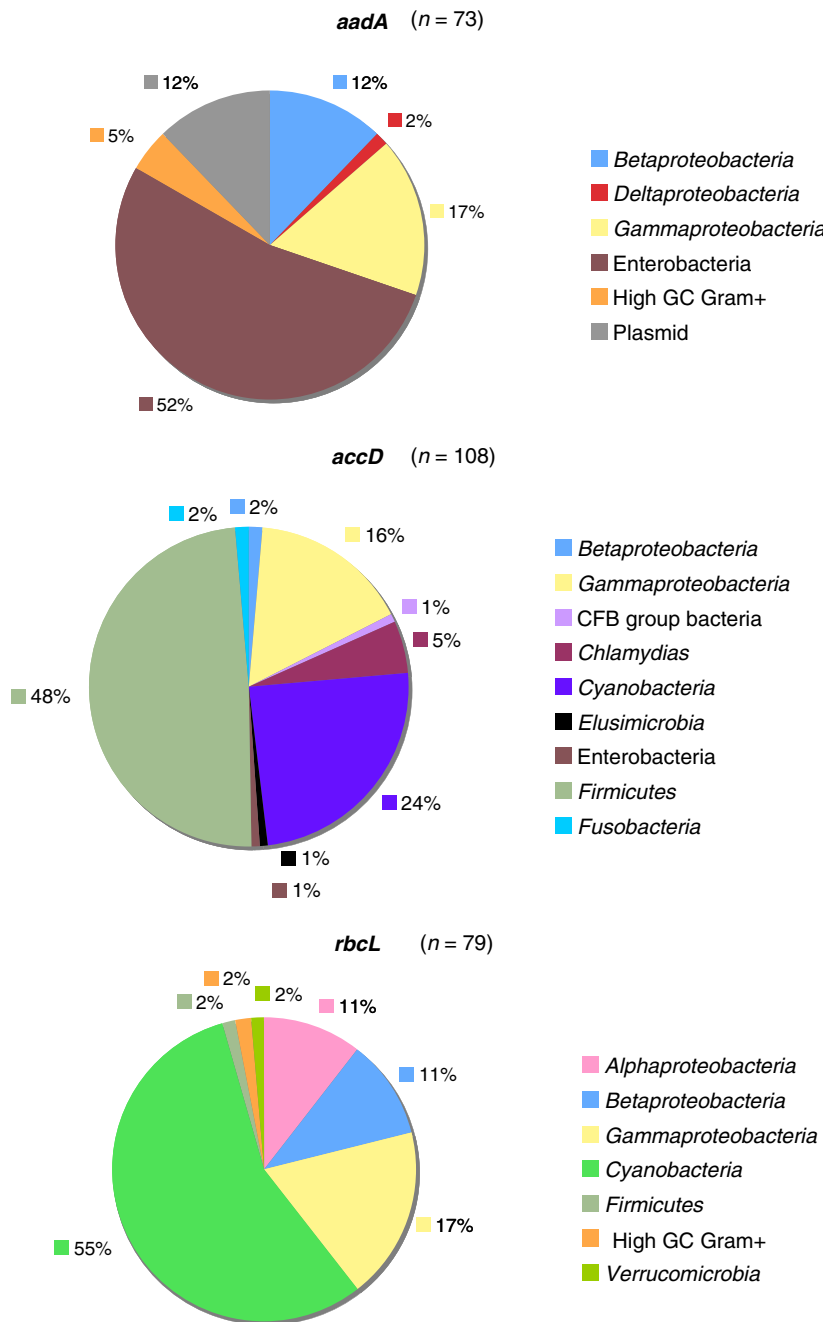


Fig. 1. Phylogenetic composition of prokaryotes possessing significant similarities to *aadA*, *accD* or *rbcL* genes (e -value $> 10^{-5}$) obtained from a BLAST analysis (BLASTN) against the NCBI Genomes database (number of corresponding species are within parentheses).

program BLASTN 2.2.23 (Altschul *et al.*, 1990) against the NCBI chromosome database excluding eukaryotic sequences to detect which prokaryotic organism could be a potential transgene recipient. Seventy-three sequenced chromosomes possessed a high identity (ID) percentage (ID $> 60\%$) to the *aadA* gene, 108 to the *accD* gene and 79 to the *rbcL* gene (Fig. 1). Among BLAST results having an e -value $> 10^{-5}$, 66 sequences presenting 65–100% ID with the *aadA* gene were detected for 52% in enterobacteria, whereas 131 sequences

with 65–87% ID with the *accD* gene were retrieved for 48% in Firmicutes and 66 sequences with 63–87% ID with the *rbcL* gene were rather of cyanobacterial origin for 55% (Fig. 1).

The alignment lengths of the BLASTs ranged from 58 to 1675 nucleotides, thus potentially allowing recombination (Watt *et al.*, 1985; Prudhomme *et al.*, 2002; Monier *et al.*, 2007; Brigulla & Wackernagel, 2010). However, the presence of only one anchor sequence was shown to reduce the transformation frequency up to 10^6 -fold (de Vries &

Table 2. Strains possessing more than 60% identity to *accD* and *aadA* or *rbcl* genes

Strain	Accession number	<i>accD</i>	<i>aadA</i>	<i>rbcl</i>	Lineage	Length (bp)*
<i>Acaryochloris marina</i> MBIC11017	NC_009925.1	+		+	<i>Cyanobacteria</i>	2 158 668
<i>Acinetobacter baumannii</i> AB0057	NC_011586.1	+	+		<i>Gammaproteobacteria</i>	3 163 708
<i>Acinetobacter baumannii</i> AYE	NC_010410.1	+	+		<i>Gammaproteobacteria</i>	2 997 610
<i>Anabaena variabilis</i> ATCC 29413	NC_007413.1	+		+	<i>Cyanobacteria</i>	4 616 865
<i>Cyanothece</i> sp. ATCC 51142	NC_010546.1	+		+	<i>Cyanobacteria</i>	1 160 075
<i>Cyanothece</i> sp. PCC 7424	NC_011729.1	+		+	<i>Cyanobacteria</i>	3 659 837
<i>Cyanothece</i> sp. PCC 7425	NC_011884.1	+		+	<i>Cyanobacteria</i>	506 361
<i>Cyanothece</i> sp. PCC 8801	NC_011726.1	+		+	<i>Cyanobacteria</i>	1 479 116
<i>Cyanothece</i> sp. PCC 8802	NC_013161.1	+		+	<i>Cyanobacteria</i>	1 471 856
<i>Microcystis aeruginosa</i> NIES-843	NC_010296.1	+		+	<i>Cyanobacteria</i>	905 608
<i>Nostoc punctiforme</i> PCC 73102	NC_010628.1	+		+	<i>Cyanobacteria</i>	1 795 936
<i>Nostoc</i> sp. PCC 7120	NC_003272.1	+		+	<i>Cyanobacteria</i>	1 063 322
<i>Prochlorococcus marinus</i> strain AS9601	NC_008816.1	+		+	<i>Cyanobacteria</i>	205 629
<i>Prochlorococcus marinus</i> strain MIT 9211	NC_009976.1	+		+	<i>Cyanobacteria</i>	352 325
<i>Prochlorococcus marinus</i> strain MIT 9215	NC_009840.1	+		+	<i>Cyanobacteria</i>	211 361
<i>Prochlorococcus marinus</i> strain MIT 9301	NC_009091.1	+		+	<i>Cyanobacteria</i>	229 637
<i>Prochlorococcus marinus</i> strain MIT 9303	NC_008820.1	+		+	<i>Cyanobacteria</i>	776 528
<i>Prochlorococcus marinus</i> strain MIT 9312	NC_007577.1	+		+	<i>Cyanobacteria</i>	224 419
<i>Prochlorococcus marinus</i> strain MIT 9313	NC_005071.1	+		+	<i>Cyanobacteria</i>	711 762
<i>Prochlorococcus marinus</i> strain MIT 9515	NC_008817.1	+		+	<i>Cyanobacteria</i>	170 376
<i>Prochlorococcus marinus</i> strain NATL1A	NC_008819.1	+		+	<i>Cyanobacteria</i>	208 055
<i>Prochlorococcus marinus</i> strain NATL2A	NC_007335.2	+		+	<i>Cyanobacteria</i>	208 046
<i>Prochlorococcus marinus</i> ssp. <i>marinus</i>	NC_005042.1	+		+	<i>Cyanobacteria</i>	259 007
<i>Prochlorococcus marinus</i> ssp. <i>pastoris</i>	NC_005072.1	+		+	<i>Cyanobacteria</i>	226 966
<i>Synechococcus</i> sp. CC9902	NC_007513.1	+		+	<i>Cyanobacteria</i>	762 712
<i>Synechococcus</i> sp. JA-3-3Ab	NC_007775.1	+		+	<i>Cyanobacteria</i>	914 137
<i>Synechococcus</i> sp. PCC 7002	NC_010475.1	+		+	<i>Cyanobacteria</i>	1 819 094
<i>Synechococcus</i> sp. RCC307	NC_009482.1	+		+	<i>Cyanobacteria</i>	835 843
<i>Synechococcus</i> sp. WH 8102	NC_005070.1	+		+	<i>Cyanobacteria</i>	885 127
<i>Synechocystis</i> sp. PCC 6803	NC_000911.1	+		+	<i>Cyanobacteria</i>	105 564
<i>Thermosynechococcus elongatus</i> BP-1	NC_004113.1	+		+	<i>Cyanobacteria</i>	143 798
<i>Trichodesmium erythraeum</i> IMS101	NC_008312.1	+		+	<i>Cyanobacteria</i>	2 639 725

*The length corresponds to the number of nucleotides in the chromosome of strains between the two genes presenting more than 60% identity with *accD* and *aadA* or *rbcl*.

Bold, terrestrial strains; underscoring, strains possessing similarities with *aadA* and *accD* genes. +, presence of the gene.

Wackernagel, 2002; Meier & Wackernagel, 2003; Monier *et al.*, 2007). For this reason, the presence of two of the three studied genes was monitored in these strains (Table 2). The presence of similarities to the *aadA* gene concomitant with similarities to the chloroplastic gene *accD* was shown in two strains of *Acinetobacter baumannii*, an aquatic species that can also be isolated from soils and animals, and is phylogenetically related to *Gammaproteobacteria* (Table 2). Both strains are opportunistic pathogens carrying multidrug-resistant genes, insertion sequences, transposons and an ACCase (NCBI accession numbers NC_011586.1 and NC_010410.1). Similarities to both chloroplastic sequences were detected in 30 cyanobacterial strains (Table 2). Most of these *Cyanobacteria* (70%) are found in an aquatic environment, but some genera such as *Cyanothece* and *Nostoc* are also common in terrestrial environments in close contact with plants with which they have developed nitrogen-fixing

symbiotic relationships (NCBI, Genome Project, *Cyanothece* sp. and *Nostoc punctiforme*). The distance-based phylogenetic analysis of complete chloroplastic and bacterial genomes showed that the chloroplast genomes were most closely related to *Cyanobacteria* (Chu *et al.*, 2004) and chloroplast promoters were shown to initiate gene expression in *Cyanobacteria* (Dzelzkalns *et al.*, 1984; Christopher *et al.*, 1999). Consequently, homologous recombination could theoretically occur in these strains. However, considering the fact that the smallest physical distance between both sequences was of 506 361 bp for the terrestrial strain *Cyanothece* sp. PCC 7425 and 105 564 bp for the aquatic strain *Synechocystis* sp. PCC 6803 (Table 1), the likelihood of homologous recombination is considerably reduced. The mechanism to integrate transplastomic DNA could be by homology-facilitated illegitimate recombination (HFIR) or by illegitimate recombination (IR) favored by

Table 3. Identification, antibiotic resistance and transformation frequencies of the isolates studied

Isolate	Strain identification*	Accession numbers	Lineage*	Antibiotic resistance [†]	PCR <i>aadA</i>	Electroporation with pBHCrec [‡]	Transformation with pBHCrec [‡]
AA12	<i>Ochrobactrum</i> sp.	HQ670701	<i>Alphaproteobacteria</i>	I	–	6.5E – 08	7.9E – 09
A013	<i>Acinetobacter</i> sp.	HQ670702	<i>Gammaproteobacteria</i>	I	–	< 10E – 08	< 10E – 09
CA01	<i>Ochrobactrum</i> sp.	HQ670703	<i>Alphaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
CA11	<i>Acinetobacter</i> sp.	HQ670704	<i>Gammaproteobacteria</i>	S	–	1.9E – 08	9.8E – 09
CO03	<i>Pseudomonas</i> sp.	HQ670705	<i>Gammaproteobacteria</i>	S	–	< 10E – 08	< 10E – 09
GA05	<i>Bosea</i> sp.	HQ670706	<i>Alphaproteobacteria</i>	R	–	5.0E – 08	< 10E – 09
MA06	<i>Stenotrophomonas</i> sp.	HQ670707	<i>Gammaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
MC02	<i>Acinetobacter</i> sp.	HQ670708	<i>Gammaproteobacteria</i>	S	–	1.7E – 08	< 10E – 09
MG02	<i>Alcaligenes</i> sp.	HQ670709	<i>Betaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
MG07	<i>Alcaligenes</i> sp.	HQ670710	<i>Betaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
MS01	<i>Stenotrophomonas</i> sp.	HQ670711	<i>Gammaproteobacteria</i>	I	–	< 10E – 08	< 10E – 09
MP07	<i>Stenotrophomonas</i> sp.	HQ670712	<i>Gammaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
PA03	<i>Stenotrophomonas</i> sp.	HQ670713	<i>Gammaproteobacteria</i>	R	–	< 10E – 08	< 10E – 09
PA07	<i>Bacillus</i> sp.	HQ670714	<i>Firmicutes</i>	S	–	< 10E – 08	< 10E – 09
PO01	<i>Acinetobacter</i> sp.	HQ670715	<i>Gammaproteobacteria</i>	S	–	6.4E – 08	< 10E – 09
SO15	<i>Pseudomonas</i> sp.	HQ670716	<i>Gammaproteobacteria</i>	R	–	7.9E – 08	< 10E – 09

*Strain identification and lineage were based on the sequence of their 16S rRNA gene BLASTed (BLASTN) against the nr NCBI database.

[†]Strains were tested for their resistance to spectinomycin and streptomycin; I, intermediate resistant strain; R, resistant strain; S, sensitive strain.

[‡]Electroporation and transformation frequencies were calculated as the mean number of transformant mL⁻¹ divided by the mean total number of cells mL⁻¹; all experiments were conducted in triplicate.

microhomologies. The frequency of IR is very low (2×10^{-10}), but HFIR provide intermediate levels of integration frequencies (3×10^{-3}) compared with the high frequency of homologous integration (Brigulla & Wacker-nagel, 2010). Considering all these data, the probability of gene transfer from plants to bacteria would occur (if any) by HFIR. As a consequence, gene introduction in plants should target chromosomal or plasmid sequences with very low or no similarities to bacterial genomes.

Occurrence of the *aadA* gene in soil isolates

A previous study (Monier *et al.*, 2007) demonstrated that bacterial isolates able to colonize decaying tobacco plant tissue possessed DNA sequence similarities to chloroplastic genes *accD* and/or *rbcl* flanking the transplastomic transgene. We further studied these isolates concerning their natural resistance to spectinomycin and streptomycin, and the presence of the *aadA* gene in their genome.

To test the natural resistance to streptomycin and spectinomycin of soil isolates, overnight cultures were spread on LBm plates supplemented with both antibiotics. Only five isolates (CA11, CO03, MC02, PA07, PO01) were sensitive to streptomycin and spectinomycin, three isolates (AA12, AO13, MS01) presented an intermediate resistance, meaning that one to 100 colonies were able to develop on LBm supplemented with streptomycin and spectinomycin. The eight other isolates were resistant to both antibiotics (Table 3).

The second step included a PCR screening test in order to detect the presence of the *aadA* gene. None of the 16 isolates

yielded a positive PCR signal (Table 3), indicating that the 11 soil isolates that resisted spectinomycin and streptomycin used mechanisms that did not involve products of the *aadA* gene. This is in accordance with the fact that *aadA* is not a gene commonly found in soil, unless it was introduced via manure or wastewater treatment plant spreading (Tennstedt *et al.*, 2005; Srinivasan *et al.*, 2008; Binh *et al.*, 2009). Consequently, this gene should not represent a genomic site of insertion for gene transfer between transplastomic plants and soil bacteria.

In vitro detection of HGT between transplastomic transgene and selected bacteria

The probability of gene transfer from transplastomic plant to soil isolates able to colonize decaying tobacco plant tissue and possessing DNA sequence similarities to chloroplastic genes *accD* and/or *rbcl* flanking the transplastomic transgene *aadA* was then monitored experimentally. Two mechanisms could allow the transfer of genomic DNA into bacteria in the environment: natural transformation or electrotransformation. Natural transformation is restricted to a narrow range of bacteria fitted with adequate genes (Johnsborg *et al.*, 2007). Moreover, different bacteria require different conditions such as a specific growth phase to develop their competence state (Lorenz & Wackernagel, 1994). To cope with these requirements, transformation experiments were carried out on plates so that bacteria were in contact with DNA during all growth phases. The second mechanism, electrotransformation, is based on the

electroporation principle. It was previously shown that gene transfer could occur during lightning discharge and may target the broadest range of recipient bacteria due to the absence of genetic requirements (Demanèche *et al.*, 2001a; Cérémonie *et al.*, 2004).

The broad host-range plasmid pBHCrec (Cérémonie *et al.*, 2004), a pBBR1MCS-3 derivative containing the transplastomic transgene *aadA* with flanking sequences *accD* and *rbcL* and coding resistance to tetracycline, was used for transformation and electroporation assays. Plasmid pBBR1MCS-3 was evidenced to replicate in at least 16 bacterial strains (Kovach *et al.*, 1995). Two isolates, *Ochrobactrum* sp. AA12 and *Acinetobacter* sp. CA11, were able to acquire the plasmid by natural transformation with a respective frequency of 7.9×10^{-9} and 9.8×10^{-9} and six isolates (*Ochrobactrum* sp. AA12, three *Acinetobacter* sp. CA11, MC02 and PO01, *Bosea* sp. GA05 and *Pseudomonas* sp. SO15) were able to acquire this plasmid by electroporation with a frequency of *c.* 1.0×10^{-8} (Table 3). Hybridization experiments on transformant genomic DNA demonstrated that the transgene cloned in pBBR1MCS-3 was never integrated into the chromosome, but was propagated as a plasmid (data not shown). This was in accordance with the absence of transformants when the PCR product and transplastomic plant DNA were used as donor DNA (data not shown). The absence of a sufficient similarity and/or too wide a length between homologous regions in the bacterial genomes for recombination to occur may explain these results.

Conclusion

Transgene transfer from plants to bacteria has been detected under greenhouse conditions with specifically selected donor and recipient organisms, but without anchor sequences, transfer could not be detected (Gebhard & Smalla, 1998; Kay *et al.*, 2002b; de Vries *et al.*, 2004; Pontiroli *et al.*, 2010). In spite of these experiments that simulated an environment under optimized conditions for HGT to occur (transgene construction or genetically modified recipient strains, for example), plant bacteria HGT events remain undetected under field conditions (Badosa *et al.*, 2004; Demanèche *et al.*, 2008). These experimental results are in accordance with theoretical monitoring of the likelihood of gene transfer by examining the similarities between transplastomic plants and associated bacteria and contribute to establish that the probability of gene transfer from plants to bacteria, integration of transferred DNA into bacterial genomes and expression of the carried genes remain very unlikely to occur in nature. Finally, our results provide no indication that the high number of transgene copies in transplastomic plants could cause HGT at rates above those found in other studies with nuclear transgenes.

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