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DEVENIR DE L'ADN TRANSGÉNIQUE DANS LES ENVIRONNEMENTS LIÉS À LA PLANTE ET AU SOL: IMPLICATIONS POTENTIELLES DANS LES TRANSFERTS HORIZONTAUX DE GÈNES ENTRE PLANTES TRANSGÉNIQUES ET BACTÉRIES

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Liste des abréviations

ADN/DNA	Acide désoxyribonucléique
UFC/CFU	Unité Formant Colonie
DO/OD	Densité optique
g	Gramme
mg	Milligramme
µg	Microgramme
ng	Nanogramme
kb	Kilobase
pb	Paire de base
mM	Millimolaire
µM	Micromolaire
PCR	Polymerase Chaine Reaction
rpm	Rotation par minute
Km	Kanamycine
Spe	Spectynomicine
Str	Streptomycine
Rif	Rifampicine
Gm	Gentamycine
A. nal.	Acide nalidixique
LB	Milieu Luria Bertani

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Introduction générale

INTRODUCTION GENERALE

La première trace fossile de vie sur la planète terre date d'environ 3.8×10^9 années et correspond à des cellules d'organismes prokaryotiques (Mojzsis *et al.*, 1996). La présence des Eubactéries mais également des Archaea a contribué énormément à l'évolution de la terre, en premier lieu par une implication dans l'établissement d'une atmosphère stable avec notamment la production d'oxygène permettant l'évolution de formes de vie en aérobiose. Cette très longue évolution des bactéries leur a permis d'atteindre une diversité génétique extraordinaire à la base de leur potentiel pour coloniser tous les biotopes y compris les plus extrêmes, permettant de généraliser que toute forme de vie est nécessairement associée avec des bactéries (où il y a de la vie, il y a des bactéries), l'inverse n'étant pas vrai (Arber, 2000; Torsvik *et al.*, 2002).

L'étendue de cette diversité génétique reste encore à découvrir, le nombre réel d'espèces bactériennes ne pouvant être estimé avec exactitude (Torsvik *et al.*, 2002). Tant du fait de la diversité de leurs fonctions que de leur abondance en terme de biomasse dans les différents environnements de la planète les procaryotes sont des acteurs majeurs de la vie sur terre avec un rôle prépondérant dans le fonctionnement des cycles biogéochimiques (cf. tableaux 1).

Cette extrême diversité génétique s'est toutefois développée en dépit du fait que les procaryotes sont des organismes unicellulaires haploïdes qui, pour la plupart, se reproduisent de façon clonale par divisions binaires successives. Leur évolution est liée à la modification de leur patrimoine génétique causée par des erreurs survenues lors de la réplication de l'ADN et accrue par des interactions avec des facteurs mutagènes de l'environnement.

Ces modifications peuvent être ponctuelles (cas de la substitution d'une base nucléotidique par une autre) ou concerner des régions plus larges du génome comme par exemple lors de la délétion, insertion ou duplication de gènes, phénomènes liés ou non à des mouvements de séquences d'insertions ou de transposons (Arber, 2000).

Environnement	Nombre total de cellules x 10^{28}	Biomasse x 10^{15} g
Sol	26	26
Habitats aquatiques	12	2.2
Fonds sous-marins	355	303
Sous-sol terrestre	25-250	22-215
TOTAL	415-640	353-546

Tableau 1: Nombre et biomasse des procaryotes répartis dans les différents environnements de la planète (d'après (Whitman *et al.*, 1998).

Une fois ces mutations générées et si elles contribuent à accroître la valeur adaptative de la cellule elles seront transmises fidèlement à la descendance selon un transfert dit vertical. Toutefois, le formidable processus évolutif bactérien, caractérisé par la rapidité de la réponse bactérienne à de nouvelles conditions environnementales (stress, nouveaux habitats) ne serait pas uniquement du aux modifications survenues lors de cette reproduction asexuée. L'analyse des données massives de génomique bactérienne aboutit à mettre en évidence le rôle très important des échanges latéraux d'information génétique (Dutta and Pan, 2002; Nakamura *et al.*, 2004b). Il est maintenant parfaitement admis que la structure des génomes bactériens est composite avec des régions mosaïques dont la composition nucléotidique et l'usage des codons sont différents du reste du génome, portant la marque d'une origine exogène (Dutta and Pan, 2002). Les outils bio-informatiques permettent même de déterminer l'identité du ou des microorganismes dont sont issus ces gènes nouvellement acquis (Ochman *et al.*, 2005).

Des opérons entiers peuvent être transférés et on peut penser que leur acquisition et leur fonctionnalité ont conféré à l'hôte bactérien un avantage adaptatif certain. Il a pu être montré que la résistance aux antibiotiques ou la virulence ou une capacité dégradative de molécules chimiques sont des fonctions qui sont régulièrement échangées entre cellules d'une même génération (Davies, 1994; Doolittle, 1998; 2002; Normark and Normark, 2002).

Par analogie, de tels processus bactériens ont été communément affiliés à la reproduction sexuée des Eukaryotes du fait de leur rôle dans le modelage de l'architecture génomique bactérienne et l'analyse et la comparaison de nombreuses données issues du séquençage de plusieurs génomes bactériens ont permis de préciser qualitativement et quantitativement le rôle prépondérant des échanges génétiques. A titre d'exemple, il a été calculé qu' *E. coli* aurait acquis 18% de son chromosome actuel à partir d'autres bactéries et cela depuis la divergence d'avec la lignée qui a conduit à la caractérisation du phylum *Salmonella* (Martin, 1999)

Il est maintenant reconnu que les mécanismes de THG peuvent faire franchir aux gènes de vastes distances phylogénétiques, comme entre bactéries et eukaryotes (Doolittle, 1998), animaux et bactéries (Wolf *et al.*, 1999) et bien entendu entre les formes les plus éloignées de bactéries et jusqu'aux Archées (Nelson *et al.*, 1999). Ces données témoignent de leur importance dans l'évolution des formes vivantes.

L'impact des transferts de gènes interspécifiques est radicalement différent de celui causé par les mutations ponctuelles. Les THG peuvent introduire en une seule étape des traits physiologiques nouveaux modifiant radicalement le potentiel adaptatif tandis que les mutations ponctuelles ne peuvent en revanche que redéfinir et altérer des fonctions métabolique existantes.

Ces mécanismes permettant l'acquisition de nouveaux éléments ont été regroupés sous le terme général de transferts horizontaux de gènes (Majewski, 2001; Ochman *et al.*, 2000). En fait, depuis déjà plusieurs dizaines d'années, antérieurement donc aux données récentes de la génomique et de la post-génomique trois types de mécanismes avaient été répertoriés comme permettant le transfert d'ADN à savoir la transformation, la conjugaison et la transduction (Griffith, 1928; Lederberg and Tatum, 1946; Zinder and Lederberg, 1952).

Le mécanisme de transformation permet aux bactéries naturellement transformables c'est-à-dire équipées des gènes pour développer un stade de compétence d'internaliser l'ADN nu présent dans leur environnement. La conjugaison *strictu senso* permet l'échange d'ADN plasmidique (ou chromosomique dans le cas de transposons) entre une cellule donatrice et une cellule réceptrice après qu'un contact étroit ait été établi entre ces deux cellules. Enfin, la transduction permet, par l'intermédiaire d'un bactériophage qui a incorporé une partie du génome de la souche donatrice de transmettre ces gènes à une cellule réceptrice au cours d'un nouveau cycle d'infection (Lorenz and Wackernagel, 1994).

Depuis une vingtaine d'années des travaux expérimentaux ont été développés afin de déterminer les fréquences aux quelles se réalisent ces échanges de gènes dans différents types d'environnements comme les sols, les eaux, les sédiments mais aussi les environnements de type végétal ou animal dans lesquels se développent les bactéries (De Vries and Wackernagel, 2004). Basées sur des microorganismes modèles, inoculés dans des systèmes de laboratoire de type micro- ou méso-cosmes ces études ont montré que les bactéries échangeaient effectivement leurs gènes en conditions naturelles. Toutefois, les fréquences varient, selon les environnements, les bactéries mais aussi les éléments génétiques étudiés dans de très importantes proportions. Ces travaux, en dépit du fait qu'ils avaient été spécifiquement conçus pour favoriser leur réalisation ont cependant révélé que les échanges d'ADN *in situ* seraient des événements rares (De Vries and Wackernagel, 2004; Nielsen *et al.*, 1998a; Nielsen and Towsend, 2004; Sorensen *et al.*, 2005). Ces résultats semblent très peu concordants avec ceux issus de l'analyse des séquences des génomes complets qui révèlent au contraire une implication à grande échelle des transferts latéraux d'ADN (Nakamura *et al.*, 2004b; Ochman and Moran, 2001). De même des résultats récents obtenus sur l'analyse de nouveaux gènes bactériens codant des enzymes dégradant des composés xénobiotiques confirment ces hypothèses. En effet seuls des échanges d'ADN se réalisant à des fréquences élevées permettent d'expliquer la formation de gènes à partir de plusieurs modules provenant de différentes bactéries (Boubakri *et al.*, 2006).

Ces discordances entre résultats *in silico* et expérimentaux ont conduit à s'interroger plus profondément sur la régulation de ces événements de transfert in situ, régulation par la cellule bactérienne elle-même mais aussi par l'environnement. Par exemple, il a pu être montré que d'autres mécanismes que ceux mentionnés précédemment pouvaient permettre les échanges de gènes. La composition chimique du milieu ou son exposition à des paramètres électriques pouvait conduire à la perméabilisation des enveloppes cellulaires bactériennes et la pénétration de l'ADN (Baur *et al.*, 1996; Cérémonie *et al.*, 2004; Cérémonie *et al.*, 2006).Ces mécanismes passifs, non contrôlés par la bactérie pourraient jouer un rôle fondamental et non suspecté dans les expérimentations réalisées à ce jour.

Schématiquement on peut dire que 3 types de filtres, physiques, physiologiques et génétiques régulent la dissémination horizontale de l'ADN par l'un ou l'autre des mécanismes répertoriés (Thomas and Nielsen, 2005).

Les barrières physiques sont essentiellement abiotiques. Le transfert d'ADN est d'abord dépendant des contacts potentiels entre cellules bactériennes donatrices et réceptrices. Dans les

cas de la transduction ce sont les contacts entre les bactéries et les bactériophages qui sont nécessaires ou dans la transformation active ou passive entre les bactéries et l'ADN extracellulaire dont le pouvoir transformant est lié à son état physique et à sa disponibilité. Par filtres physiologiques s'entend la possibilité ou non pour les bactéries de développer un stade de compétence, ou d'acceptation de la conjugaison ou de l'infection par un bactériophage. Enfin, les filtres génétiques sont principalement (mais pas uniquement) liés aux mécanismes régulant la recombinaison de l'ADN pénétrant avec le génome de la bactérie réceptrice.

Déjà, certaines bactéries ne seront transformables que par un ADN présentant certaines séquences spécifiques nécessaires à l'attachement sur des sites de la paroi, préalable sélectionnant le seul ADN compatible pour les étapes ultérieures de transformation (Lorenz and Wackernagel, 1994).

Après pénétration dans la cellule l'ADN doit échapper aux systèmes de modification et de restriction. Enfin, l'intégration est sous le double et antagoniste contrôle des systèmes SOS et MRS (Mismatch Repair System). A ce titre, les bactéries se classent en 2 catégories selon leur aptitude à intégrer de l'ADN exogène, des plus strictes n'acceptant que l'ADN de leur propre espèce aux plus tolérantes chez lesquelles pratiquement tout type d'ADN pourra être intégré (Dubnau, 1999). Chez ces dernières bactéries, la recombinaison ne nécessite une similarité de séquences que de quelques nucléotides entre ADN donneur et génome récepteur. Mais cette propriété est modulable au sein d'une même bactérie selon que l'on considère une souche sauvage ou son mutant MRS-, mutation qui concerne plus de 1% des individus au sein d'une même population (Denamur *et al.*, 2002). Pour la plupart des bactéries étudiées, les mutants MRS- sont beaucoup plus tolérants pour l'intégration d'ADN hétérologue que ne peuvent l'être les souches sauvages.

Il apparaît cependant que l'étape de recombinaison constitue la principale barrière aux transferts de gènes entre organismes et cette barrière est d'autant plus hermétique qu'organismes donneur et récepteur sont phylogénétiquement éloignés.

A titre indicatif, les différents travaux expérimentaux de transformation bactérienne impliquant l'ADN de plantes transgéniques n'ont conduit à des résultats positifs (présence de transformants) que si les souches bactériennes réceptrices étaient équipées de séquences d'ADN permettant la recombinaison avec les séquences de la plante (de Vries *et al.*, 2001; de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Kay *et al.*, 2002b). De tels événements de

transfert inter-règnes sont encore beaucoup moins probables si une recircularisation d'un fragment d'ADN (cas des plasmides linéarisés et clonés intégralement dans le génome de la plante) est nécessaire pour sa réplication de façon autonome dans la bactérie réceptrice (Schluter *et al.*, 1995).

En fait, les microbiologistes ont rapidement réalisé tout l'intérêt des plantes génétiquement modifiées en vue d'étudier les transferts de gènes chez les bactéries. La transgénèse permet en effet d'introduire dans le génome nucléaire ou du chloroplaste (plantes transplastomiques) des gènes dont les séquences peuvent être spécifiquement identifiées et suivies dans les différents compartiments environnementaux que représentent une plante saine colonisée par des bactéries épiphytes ou endophytes, une plante infectée par un pathogène, une plante en décomposition et enfin la rhizosphère et le sol nu dans lequel l'ADN végétal va se disperser. La plante OGM devient alors un modèle d'étude pour comprendre comment barrières physiques, physiologiques et génétiques peuvent réguler les transferts de gènes dans des environnements qui apparaissent comme les plus favorables à des échanges de gènes entre bactéries (Kay *et al.*, 2002a) mais aussi possiblement entre la plante et ces différents types de bactéries. A ce titre, ces travaux répondront à des questions sociétales sur les potentialités de dissémination des transgènes des organismes génétiquement modifiès (OGM) et plus particulièrement ceux des plantes transgéniques et transplastomiques (Bertolla and Simonet, 1999; Courvalin, 1998; Davies, 1994; Dröge *et al.*, 1999; Nielsen *et al.*, 1998a). La principale question dans ce contexte est de savoir si la dispersion des transgènes introduits dans les OGM vers d'autres organismes de l'environnement est susceptible de modifier l'équilibre des écosystèmes en créant des organismes présentant une compétitivité supérieure à celle de l'espèce sauvage ? La pollinisation entre les plantes transgéniques et les espèces sauvages phylogénétiquement proches constitue le risque le plus souvent évoqué et pour lequel les travaux sont les plus nombreux (Légère, 2005; Rieger *et al.*, 2002; Timmons *et al.*, 1996; Weekes *et al.*, 2005). Cependant la possible dissémination des transgènes vers les microorganismes du sol ou ceux associés aux plantes doit être également prise en considération, tant du fait d'un risque pour la santé humaine avec la dispersion des gènes de résistance aux antibiotiques ou pour l'environnement pour d'autres transgènes d'intérêt agronomique .

Tant pour l'évaluation des risques des plantes OGM que plus fondamentalement pour comprendre l'implication des THG dans l'évolution bactérienne, beaucoup de travaux ont ciblé la transformation qui, du fait qu'elle peut concerner en théorie tout type d'ADN présent dans

l'environnement immédiat de la cellule bactérienne serait le mécanisme-clé des THG. Sa réalisation implique cependant la libération de l'ADN dans l'environnement, sa persistance et le maintien d'une disponibilité jusqu'au contact avec une cellule bactérienne en phase de compétence. Dans une dernière étape, l'ADN transformant doit échapper au contrôle bactérien, s'intégrer dans le génome et potentiellement s'y exprimer.

Chacune de ces étapes a déjà fait l'objet de travaux plus ou moins importants. Récemment, des études ont montré l'implication de pathogènes bactériens dans la lyse des cellules végétales et la libération de l'ADN végétal. Les cinétiques de libération de l'ADN ont pu être comparées à celles résultant d'une décomposition enzymatique du matériel végétal (Ceccherini *et al.*, 2003). Ces travaux ont cependant ouvert de nouvelles interrogations non seulement sur la persistance physique de l'ADN dans le sol déjà abordé précédemment dans de nombreuses études (Gebhard and Smalla, 1999; Romanowski *et al.*, 1993; Widmer *et al.*, 1997; Widmer *et al.*, 1996) mais aussi sur le maintien d'une activité biologique pour cet ADN.
Ensuite, au nombre limité d'espèces bactériennes équipées de la machinerie moléculaire adéquate (De Vries and Wackernagel, 2004) s'ajoutent les difficultés liées au développement *in situ* du stade de compétence (Bertolla et al.1999 ; Nielsen et al., 1997). Enfin, il est certain que les différences de séquences nucléotidiques entre le matériel génétique donneur et le génome récepteur sont les éléments fondamentaux régulant le transfert de gènes qui peut être abordé avec les plantes transgéniques qui possèdent des séquences d'ADN d'origine procaryotique, en particulier les gènes marqueurs de résistance aux antibiotiques.

Considérant ces différents éléments, nos travaux se sont largement basés sur les plantes OGM pour étudier la régulation du transfert de gènes chez les bactéries. Nos objectifs ont été principalement de déterminer le devenir de l'ADN du transgène dans l'environnement et ses implications- interactions dans les transferts horizontaux des gènes avec la microflore indigène du sol mais aussi celle vivant en interaction forte avec la plante. Pour se faire les modèles biologiques que nous avons développés ont été constitués de plantes saines et colonisées par des bactéries épiphytes puis dans un stade de sénescence avant d'aborder le devenir de l'ADN extracellulaire dans le sol nu tant d'un point de vue de la persistance « physique » de la molécule d'ADN que du maintien de son potentiel biologique.
Plantes OGM classiques modifiées au niveau du génome nucléaire par des transgènes dépourvus d'introns et associés à des promoteurs fonctionnels chez les bactéries et plantes transplastomiques contenant un nombre de copies du transgène jusqu'à 10 000 fois plus élevé

que les précédentes (Bendich, 1987; Daniell *et al.*, 1998) ont été développées pour répondre aux différentes questions concernant le devenir de l'ADN dans ces environnements .

Dans un premier chapitre bibliographique rédigé en langue anglaise, nous proposons une synthèse des études expérimentales les plus récentes qui ont cherché à quantifier les conséquences écologiques de la culture des plantes transgéniques dans l'environnement, au niveau de la phytosphère et de la matrice sol et leur impact sur les communautés microbiennes du sol. Un intérêt particulier a été consacré aux études qui visent à déterminer les potentialités du transfert horizontal d'ADN des plantes transgéniques vers les bactéries. Ont été particulièrement pris en compte les facteurs qui peuvent limiter ces transferts interrègnes, à savoir d'un coté la disponibilité dans l'environnement d'ADN biologiquement actif et de l'autre le franchissement des barrières physiologiques et surtout génétiques de la cellule bactérienne.

Les trois chapitres suivants présentent les travaux expérimentaux qui ont été réalisés tout au long de cette thèse et qui reposent sur deux plantes modèles, un tabac transgénique de nouvelle génération (transplastomique) ou une tomate wild type et deux espèces bactériennes, la bactérie phytopathogène *Ralstonia solanacearum* et la bactérie du sol colonisatrice opportuniste *Acinetobacter baylyi*, choisies comme modèles de la microflore indigène et très appropriées pour étudier les transferts horizontaux des gènes *in situ* en vertu de leur aptitude à intégrer l'ADN « étranger » par transformation naturelle.

Dans le second chapitre, nous avons étudié le premier rôle que pourrait avoir l'ADN pénétrant le cytoplasme d'une bactérie à savoir le déclenchement d'un stade mutateur. Cette étude a été conduite *in vitro* et *in planta* sur la bactérie *R. solanacearum* qui, pendant la phase de colonisation de la plante va entraîner une lyse des cellules végétales et une libération d'importantes quantités d'ADN végétal. Les hypothèses formulées incluent une saturation du système de réparation des mésappariements de bases après pénétration de l'ADN hétérologue qui non seulement pourrait accroître le taux de mutations spontanées mais aussi diminuer la barrière génétique vis-à-vis de la recombinaison entre le génome récepteur et l'ADN hétérologue issu du transgène. Nos travaux ont été de développer les expérimentations *in vitro* et *in planta* permettant de valider ou infirmer le rôle de l'ADN homologue ou hétérologue pénétrant le cytoplasme bactérien sur la fréquence de génération de mutants spontanés et la recombinaison avec l'ADN du transgène.

Les objectifs des travaux décrits dans le troisième chapitre ont été de détecter, quantifier et visualiser les transferts horizontaux de gènes entre plantes et bactéries dans la phytosphère. Plus particulièrement, des expérimentations ont été conduites afin de déterminer s'il était possible de caractériser des « hot spots » pour les transferts définis comme un ensemble de conditions très favorables (disponibilité de nutriments et ADN) pour l'accomplissement de ces processus. Pour ce faire, dans une première partie sera décrite la mise au point d'une nouvelle stratégie pour détecter ces transferts *in situ* en dépassant les contraintes liées aux approches traditionnelles basées sur l'isolement et la culture des recombinants sur milieu sélectif (approches destructives); ces travaux ont porté sur la construction d'un nouvel outil moléculaire pour le développement d'une souche *bioreporter* d' *Acinetobacter baylyi*. La mise au point de cet outil et sa validation dans des conditions *in vitro* sont présentées. Dans une deuxième partie, l'étude des transferts d'ADN de la plante à la bactérie a été menée *in situ* par étapes successives. Dans un premier temps, nous avons cherché à identifier quelles niches écologiques dans la phytosphère pouvaient permettre à la fois la croissance de la bactérie modèle *Acinetobacter baylyi* et le développement *in situ* de l'état de compétence, étapes indispensables et préalables à la transformation naturelle. En second lieu et en complément de cette approche éco-physiologique, des travaux ont visé à détecter les transferts de gènes *in planta* par le biais de la visualisation des ces événements par microscopie grâce à la souche *bioreporter* précédemment décrite (approche non destructive).

Enfin, alors que dans les chapitres précédents nos investigations on été conduites au niveau de la plante saine ou en voie de dégradation, dans le quatrième chapitre on s'est intéressé au devenir de l'ADN végétal transgénique pendant la décomposition naturelle de la plante, processus qui va conduire à la libération de l'ADN dans l'environnement sol. Le sol est en effet un environnement incontournable pour les THG, puisqu'il constitue un réservoir inépuisable de bactéries et d'ADN extracellulaire. A l'heure actuelle, certaines études ciblant la persistance des acides nucléiques dans cet environnement ont montré que le turn-over de l'ADN pouvait être relativement lent, conditions d'autant plus favorables à la recapture potentielle par les bactéries telluriques. La persistance des signatures transgéniques dans le sol a également été montrée, toutefois jusqu'à présent aucune information n'était disponible quant à l'activité biologique de cet ADN alors que c'est probablement le plus important indicateur de sa potentielle implication dans les THG. Dans ce chapitre, nos objectifs ont été de quantifier la persistance de l'ADN libéré naturellement lors du processus de dégradation de feuilles de tabac transplastomiques enfouies

dans le sol tant au niveau de la détection « physique » des molécules que de son potentiel biologique, exprimé en terme de pouvoir transformant d' *Acinetobacter baylyi.*

Ce manuscrit est donc composé d'une revue bibliographique et de trois chapitres, en forme d'articles scientifiques, décrivant les études expérimentales effectuées :

-Chapitre 1: Ecology of DNA in the environment (publié sous forme de review dans la revue Environmental Biosafety Research, Pontiroli et al., 2007)

-Chapitre 2: Natural transformation and induction of spontaneous mutants in *Ralstonia solanacearum*

-Chapitre 3: Première Partie: A strategy for *in situ* localization of horizontal gene transfer by natural transformation

Deuxième Partie: Hot spots for horizontal gene transfer from transplastomic plants to *Acinetobacter baylyi* in the phytosphere

-Chapitre 4: Long-term persistence and bacterial transformation potential of transplastomic plant DNA in soil

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Chapitre 1 : Synthèse Bibliographique

Ecology of transgenic DNA in the environment

I-INTRODUCTION

I.1 Genetically engineered plants: history, features and applications

Since the dawn of agriculture, humans have shaped the characteristics of domesticated plants in order to increase yields or get the best adapted varieties, taking advantage of the natural occurrence of mutants. Despite the poor understanding of the process, plant breeding had been a popular activity through centuries until when the in 1865 botanist Gregor Mendel, published his findings on how dominant and recessive alleles could produce the traits we see and could be passed to offspring (http://www.mendelweb.org/Mendel.plain.html). This was the first major insight into the science behind the art, and breeders could soon apply the new understanding of genetics to traditional techniques of self pollination and cross pollination. Later, at the beginning of the last century, scientific advances in disciplines such as physics and chemistry, led to a science based approach to the genetic modification of plants allowing plant breeders to enhance their genetic diversity at a faster pace, recurring to mutagens, irradiation and chemicals.

It was only after the discovery of deoxyribonucleic acid (DNA) in the early 50s as the basis of genetics and, shortly thereafter, of the determination of its structure by Watson and Crick, that the direct manipulation of genetic traits could be envisioned. Techniques for the insertion of foreign genes into bacteria were first developed in the early 1970s, whereas only a decade after, the ability to transfer foreign genes to plants via *transgenesis* was achieved (Comai *et al.*, 1985; Krens *et al.*, 1982)

Thus, plant genetic engineering was added to the long list of methods that aim to broaden the available genetic diversity of a given plant species (Belzile, 2002). The worldwide expectations generated by this technology among scientists and the supporters were so optimistic that the term *Doubly Green Revolution* was introduced to describe the extent of innovation and to sum up the potential benefits for mankind attributed to this technology (Wisniewski *et al.*, 2002).

Early transgenic plants were laboratory specimens, but subsequent research has developed transgenic plants with commercially useful traits, whose first released product appeared on the market in the early 1990s. Actually, genetic engineering introduced single diverse traits such as improved agronomical (plant protection from insects or pests, herbicide resistance, tolerance to stress) and nutritional qualities (such as prolonged shelf life, enhanced nutrient content) into plants. The features of the firstly organisms released were basically of agricultural interest.

For a detailed description the U.S. Food and Drug Administration web site (http://www.cfsan.fda.gov/~lrd/biocon.html) that reports a nearly complete list of currently commercialized genetically engineered plants (GEPs). The potentiality offered by the second generation of transgenic plants currently under development is also important as they are designed to produce vaccines or proteins of pharmaceutical interest. Finally, only by imagination more frivolous applications can be envisioned, such as ornamental bright coloured fluorescent lawn (Marvier and van Acker, 2005). The US patent databases provide a view of the state of the art of wide commercial applications of plant genetic engineering and predict which plant derived products will be available on the market in the near future (Dunwell, 1999).

For the time being, transgenic crops that are cultivated most successfully and widely are herbicide tolerant soybean, oilseed rape, cotton and maize and insect resistant cotton and maize. In 2005, they were grown in a total of 21 countries among which the United States, Argentina, Canada, Brazil, China and South Africa accounted for 99% of the planted surface. Between 1996, when the first transgenic crops were commercialized, and 2005 the global acreage devoted to these plants has increased 50 fold, from 1.7 hectares to 90 million hectares (James, 2005).

I.2 Potential ecological fallouts linked to field release of GEPs

Despite the increasing surface dedicated to these crops, public concerns were raised, particularly in Europe, *in primis* on their safety in relation to human health and the environment, and also on the sustainability of this new agricultural technology and on its impacts on global agro-food production and society at large (Hails and Kinderlerer, 2003).

An argument against approval of transgenic plants involves the dependence upon seeds protected by intellectual property rights and owned by major agrochemical companies, therefore, enriching large corporations and depriving farmers from their rights to reuse the seeds. Other

reasons of dispute concern the elimination of crop and herbicide rotations, the potential for seed dispersal through contamination, cross pollination with wild plants creating “superweeds” and the inability of the public to be adequately informed about the presence of genetically manipulated food (Greenpeace, 2003). During the period up to 2004 a *de facto* moratorium on cultivation and import was in place in Europe; since then resistances to the use of GEPs in Spain, Portugal, France, Germany and Czech Republic are still limiting the acceptable cultivation to a single variety of corn (insect resistant maize expressing the insecticidal pro-toxin of *Bacillus thuringiensis).* In addition, many countries perform risk assessment studies, while in the meantime they wrestle with legal issues concerning their marketing and traceability.

Some researchers suggest ecological theory that planting of transgenic crops can create critical environmental impacts ranging from gene flow to the rapid development of insect resistance and to the impact on non target organisms (Altieri *et al.*, 2004; Marvier and van Acker, 2001; Quist, 2004). A specific concern stems from the fact that commercialized transgenic plants are fitted with prokaryotic derived marker sequences coding for antibiotic resistance. These are central to the controversy due to following arguments: (a) clinical therapy could be compromised due to increased resistance to the antibiotics obtained from consumption of food derived from transgenic crops and (b) there is a possible risk of horizontal gene transfer from plants to their wild relatives with potential contamination of local landraces and (c) to soil and gut microorganisms with uncontrolled spread of antibiotic resistance genes in the ecosystem. The validity of these arguments will be discussed.

Transgenic plants cultivated in the field can be regarded as reservoirs of transgenes, which could be released in the surrounding environment by roots, during the plant decay or by pollen (fig.1). In addition, concern about the release of the gene product for example by roots’ exudation (Saxena *et al.*, 1999) could affect soil health and ecosystem functioning, specifically altering the dynamics of microbial populations. This review assesses current scientific studies that were devoted to understanding the ecological fallouts impact of transgenic plants on microorganisms in the field, at both the plant level (the phytosphere) and the soil matrix. The critical steps involved in the interaction between plant DNA and bacteria and the factors susceptible to condition the gene transfer process will be discussed. In the first section bacteria dependent aspects such as physical-chemical, biological and genetic characteristics will be addressed. In the second part parameters affecting transgenic DNA presence in the environment will be described. In the third section, terrestrial habitats will be evaluated in terms of their

capacity to favour horizontal gene transfer. Finally, studies that assessi possible perturbations of soil bacterial community due to the presence of transgenic plants will be considered.

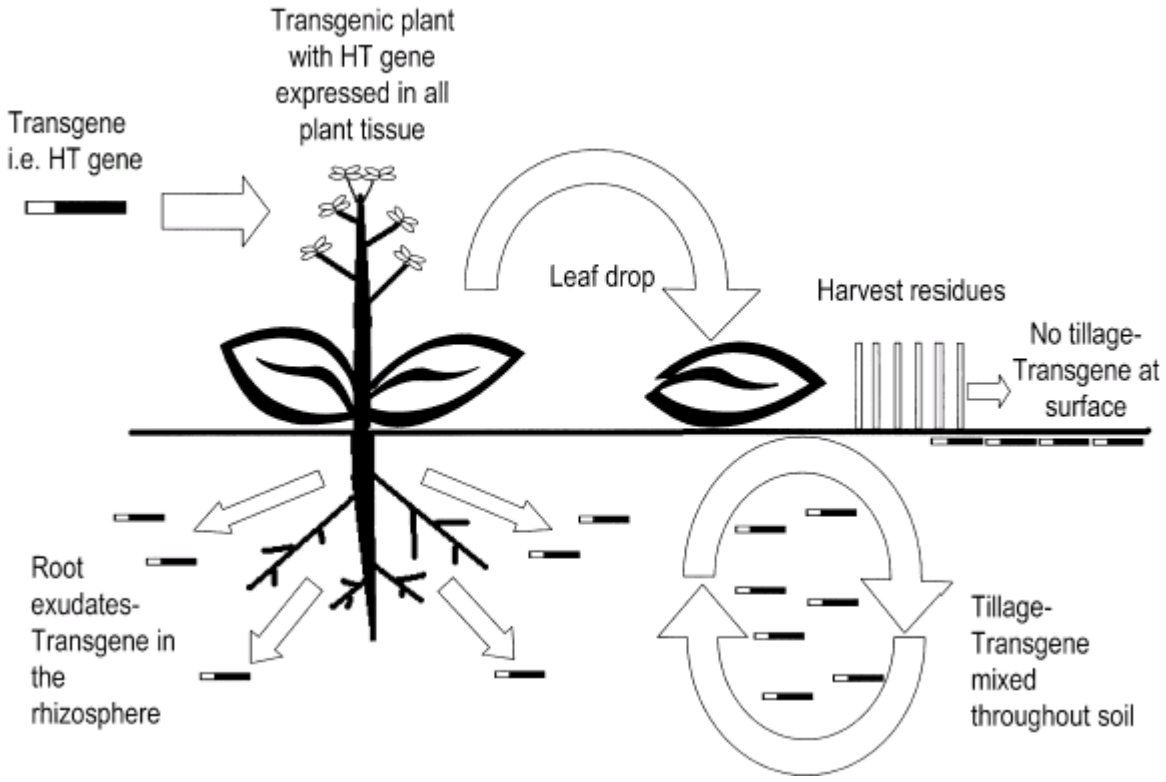

Figure 1. Some possible transgene transport routes from the plant to environmental bacteria (HT= herbicide tolerance gene) (Dunfield and Germida, 2004)

I.3 Current transgenic plants and selectable marker genes

Advances in molecular biology and *in vitro* cell culture led to the development of genetically engineered plants (GEPs). The rapid acquisition of data on the blueprints of life of eukaryotes and prokaryotes and the cloning technology boosted the delivery of novel organisms on the market with traits for wide applications in agriculture. The first GEPs were developed by using *Agrobacterium* mediated transformation, were the new trait was introduced in the nuclear genome after its cloning in an adapted Ti-plasmid, its introduction in the crown-gall disease agent and its inoculation on plant tissues. This technique worked well for most of dycotiledonous species.

Another common approach that was utilized for generating transgenic plants was particle bombardement or “biolistic transformation”: i.e. the mechanical delivery of transgenes into the

nucleus of leaf tissues: this implies that target genes are firstly coated onto a fine mineral particulate (gold or tungsten) and then bombarded into leaf segments with a gun.

Independently of the process used, released transgenic crops first raised concerns about the risk that *de novo* assembled genes could be disseminated in the environment, by pollen, and cross hybridize with native weeds or non transgenic crops, thus creating novel resistant species.

Two recent studies documented the occurrence of cross pollination in herbicide tolerant canola (*Brassica napus* L.) (Hall *et al.*, 2000) and creeping bentgrass (*Agrostis stolonifera*) (Watrud *et al.*, 2004) and also provided evidence that pollen can travel significant distances, up to 21 km in the case of creeping bentgrass.

Meanwhile, researchers have developed other strategies to prevent gene flow from plant to plant. For example, a breakthrough in transformation systems was the delivery of genes, through biolistic transformation, into chloroplast genomes (the plastomes) creating transplastomic plants. Not only does this process yield a higher protein content in the plant due to the elevated copy number of plastomes per cell and of chloroplasts per plant, but , also, it offers a confined placement of transgenes in the plastome: since the latter is maternally inherited, pollen is likely to be transgene free (Daniell *et al.*, 2002).

All transformation systems for creating transgenic plants require separate processes for introducing cloned DNA into living plant cells, for identifying or selecting those cells that have integrated the DNA into the appropriate plant genome (nuclear or plastid) and for regenerating or recovering fully developed plants from the transformed cell. In order to identify transformed cells, which are usually a small percentage of the starting cells, scientists used selectable marker genes. Therefore, genetic constructs in transgenic plants used in agriculture frequently contain antibiotic resistance markers and origins of prokaryotic replication. For the majority of commercial transgenic varieties antibiotic or herbicide resistance selectable marker genes were used such as, most commonly, kanamycin or phosphinotrhicin resistances (Miki and McHugh, 2004). As the source of this genes is mainly bacterial (Tab. 1), the possibility for transgenic plant DNA to by pass the genetic barriers to horizontal gene transfer from plants to bacteria exists and antibiotic resistance determinants might spread among environmental bacteria.

The prokaryotic origin of chloroplasts (and thus potential homology) also potentially increases the likelihood for the horizontal gene transfer from transplastomic plants to environmental bacteria. Indeed, the prokaryotic progenitors of the actual cell organelles were engulfed by a pre-eukaryotic cell in an endosymbiosis-like process. During the gradual integration, the organellar

genomes underwent a dramatic size reduction and gene transfer to the nuclear genome (McFadden, 2001).

Common name	Gene	Gene product and use	Source	Genome	References

Furthermore, genes present in currently commercialized transgenic plants are mostly genes derived from soil bacteria such as the herbicide resistance gene *bar* from *Streptomyces hygroscopicus* or the insect resistance gene *cryA* from *Bacillus thuringiensis.* As soil is an enormous reservoir of bacteria, it is commonsensical to predict that these bacteria could come in contact with DNA released from the live or dead plants. Data has accumulated about some of these bacteria being capable of picking up free DNA and integrating it into their genomes; for example, in the same genus *Streptomyces*, some species were discovered displaying this ability (Lorenz and Wackernagel, 1994).

Are these determinants susceptible of dissemination in their original environments and capable of transfer among the indigenous microbiota? Would hence the equilibrium of existing ecosystems be perturbed by creation of novel organisms? Would these be able to take over other species due to acquisition of an adaptive advantage, therefore becoming more competitive than the wild species? How likely, (e.g. at which frequency) would this happen? And finally, eventual perturbations are detrimental or favourable to soil and plant health? Since the first release of GEPs in the field, these questions have become fundamental issues for scientists, as it was thought that diffusion of drug resistant bacteria in the environment may pose a serious challenge to our means of controlling them and menace the natural biodiversity of species in agroecosystems.

In order to address the likelihood of a gene transfer between plant and bacteria, researcher first performed a certain number of laboratory studies by separating the different steps and stages necessary for the process to happen. *In vitro* studies took into account the different complex environmental parameters that could affect this process and made use of various biological models identified as the most appropriate and likely to occurr *in vivo*. The next step was the design of experimental protocols in which optimal conditions were defined with the aim of obtaining evidence of the transfer *in situ*.

Selectable markers and reporter genes

Antibiotics

Neomycin	*neo, nptII*	Neomycin Phosphotransferases	*Escherichia coli* Tn5	nuclear	(Fraley *et al.*, 1983)
Kanamycin	*(aphA2)*			plastid	(Carrer *et al.*, 1993)
Paramomycin,G418	*nptII (AphAI)*		*Escherichia coli* Tn601		
Aminoglycosides	*aaC3*	Aminoglycosides-N-acetyl transferases	*Serratia marcescens*	nuclear	(Hayford *et al.*, 1988)
	aaC4		*Klebsiella pneumoniae*		
	6'gat		*Shigella sp.*		(Gossele *et al.*, 1994)
Spectinomycin	*aadA*	Aminoglycoside-3-Adenyltransferase	*Shigella sp.*	nuclear plastid	(Svab *et al.*, 1990)
Spectinomycin	*SPT*	Streptomycin	Tn5	nuclear	(Svab and Maliga, 1993)
Streptomycin		Phosphotransferase			(Maliga *et al.*, 1988)
Hygromycin B	*hph (aphIV)*	Hygromycin Phosphotransferase	*Escherichia coli*	nuclear	(Waldron *et al.*, 1985)
Bleomycin	*Ble*	Bleomycin resistance	*Escherichia coli* Tn5	nuclear	(Hille *et al.*, 1986)
Phleomycin				plastid	(Perez *et al.*, 1989)
Sulfonamides	*sul*I	Dihydropteroate synthase	*Escherichia coli* pR46		(Guerineau *et al.*, 1990)
Streptrothricin	*sat3*	Acetyl transferase	*Streptomyces sp.*	nuclear	(Jelenska *et al.*, 2000)
Chloramphenicol	*cat*	Chloramphenicol acetyl transferase	*Escherichia coli* Tn5 Phage p1cm	plastid	(DeBlock *et al.*, 1984) (DeBlock *et al.*, 1985)

Table 1 : common selectable marker genes used for the selection of transgenic and transplastomic plants (Miki and McHugh, 2004).

Common name	Gene	Gene product and use	Source	Genome	References
Herbicides					
Phosphinothricin	*pat, bar*	Phosphinothricin acetyl transferase	*Streptomyces hygroscopicus Streptomyces viridochrimogens* Tu494	nuclear	DeBlock et al., 1989
Glyphosate	EPSP *synthase*	5-Enolpyruvy shikimate-3-phosphate synthase	*Petunia hybrida, Zea mays*	nuclear	(Zhou *et al.*, 1995) (Howe *et al.*, 2002)
	*aro*A		*Salmonella typhimurium Escherichia coli*		(Comai *et al.*, 1988) (della Cioppa *et al.*, 1987)
	Cp4 epsps		*Agrobacterium tumefaciens*		(Barry *et al.*, 1992)
	gox	Glyphosate oxidoreductase	*Ochrobactrum anthropi*		(Barry *et al.*, 1992)
Sulfonylureas	*csr1-1*	Acetolactate synthase	*Arabidopsis thaliana*	nuclear	(Olszewski *et al.*, 1988)
Imidazolinones	*csr1-2*	Acetolactate synthase	*Arabidopsis thaliana*	nuclear	(Aragao *et al.*, 2000)
Oxynils	*bnx*	Bromoxynil nitrilase	*Klebsiella pneumoniae subspecies ozanaenae*	nuclear	(Freyssinet *et al.*, 1996)
Gabaculine	*hem*L	Glutamate-1-semialdehyde aminotransferase	*Synechococcus* PCC6301	nuclear	(Gough *et al.*, 2001)
Cyanamide	*cah*	Cyanamide hydratase	*Myrothecium verrucaria*	nuclear	(Damm, 1998) (Weeks *et al.*, 2000)

Table 1 : common selectable marker genes used for the selection of transgenic and transplastomic plants (Miki and McHugh, 2004).

Common name	Gene	Gene product and use	Source	Genome	References
Pharmaceuticals					
2-Deoxyglucose	*DOG^{R}1*	2-deoxyglucose-6-phosphate phosphatase	*Saccharomyces cerevisiae*	nuclear	(Kunze *et al.*, 2001)
Betaine aldehyde	*BADH*	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	nuclear, plastid	(Daniell *et al.*, 2001a; Ursin, 1996)
S-aminoethyl L-cysteine (AEC)	*DHPS*	Dihydropicolinate synthase	*Escherichia coli*	nuclear	(Perl *et al.*, 1993)
	ocs	Octopine synthase	*Agrobacterium tumefaciens*		(Koziel *et al.*, 1984)
4-Methyl tryptophan (4-mT)	TDC	Tryptophan decarboxylase	*Catharanthus roseus*	nuclear	(Goddijn *et al.*, 1993)
Methotrexate	DHFR	Dihydrofolate reductase	*Escherichia coli mouse*	nuclear	(Herrera-Estrella *et al.*, 1983) (Eichholtz *et al.*, 1987)
			Candida albicans		(Irdani *et al.*, 1998)
Inductors					
D-xylose	*xylA*	Xylose isomerase	*Streptomyces rubignosus*	nuclear	(Haldrup *et al.*, 1998a)
			Thermoanaerobacterium sulfurogenes		(Haldrup *et al.*, 1998b)
D-Mannose	*manA (pmi)*	Phosphomannose isomerase	*Escherichia coli*	nuclear	(Joersbo *et al.*, 1998)
Benzyladenine-N-3-glucuronide	*uidA (gusA)*	β-glucuronidase	*Escherichia coli*	nuclear	(Joersbo and Okkels, 1996)
Reporter genes					
aadA:gfp	*aadA, gfp*	Green fluorescent protein, Aminoglycoside-3-Adenyltransferase	*Aequaria victoria* *Shigella sp.*	plastid	(Khan and Maliga, 1999)

Table 1 : common selectable marker genes used for the selection of transgenic and transplastomic plants (Miki and McHugh, 2004).

II- HORIZONTAL GENE TRANSFER BETWEEN TRANSGENIC PLANTS AND BACTERIA

The occurrence of gene transfer between eukaryotes and prokaryotes depends on the contact between the DNA released by the plant and the indigenous bacteria colonizing the surface of the plant or dwelling into soil. These bacteria are potential recipients of transgenes present in plant residue and root exudates. Bacteria in these habitats are numerous and diverse, and have evolved adapting to specific niches located either on the different parts of plants as epiphytes, endophytes or symbionts. In addition, many bacteria live in proximity to plants as about 20,000 common bacterial species and 500,000 rare species could be present in 30 g of soil (Dykhuizen, 1998).

Some bacteria play key roles in decomposing plant material, either as degraders or as pathogens, potentially leading to the dissemination of nucleic acids in the environment. Clearly, the released DNA may serve as nutritional supply supporting microbial growth in addition to its potential for the incorporation in bacterial genomes. There are many biotic and abiotic factors, which influence the availability of DNA in the environment, which will be reviewed (cf. section 3).

Bacteria generally inherit their genetic material from the previous generation via asexual reproduction (vertical transfer). Hence, the creation of genomic variability allowing rapid adaptation to environmental challenges occurs principally via two major mechanisms: mutation and recombination. Thus, genetic variability is generated by endogenous rearrangements occurring under genetic drift, such as displacement by insertion sequences (IS) or transposons and deletions or duplications of large regions of DNA.

In addition, the bacterial genome diversity stems from a mechanism which drives acquisition of whole new functions from foreign genes: horizontal gene transfer (HGT).

Evidence that a considerable proportion of most bacterial genomes consist of horizontally acquired genes (Nakamura *et al.*, 2004a) has been collected by bioinformatics analysis of sequenced bacterial genomes. Comparative genomics has shown that the high level of similarity between genes from phylogenetically remote organisms could only be result of HGT. Horizontally transferred genes are mostly implicated in environmentally relevant functions, such as pathogenesis, antibiotics or heavy metals resistance and pollutants catabolism.

II.1 HGT mechanisms

More than 50 years ago, the three mechanisms for horizontal gene transfer- conjugation, transduction and transformation - were described for the first time (Avery *et al.*, 1944; Griffith, 1928; Lederberg and Tatum, 1946; Zinder and Lederberg, 1952).

The first mechanism involves an active process of bacterial mating, implying cell to cell contact through specific structures to enable DNA transfer from the donor to the recipient cell. The second mechanism derives from errors in lysogenic phage integration into and/or excision from the chromosome of their host or incorrect packaging of non phage DNA into phage particles in the lytic cycle. The third allows the uptake and stable integration in the chromosome (or autonomous replication) of naked DNA from the environment.

Of the three mechanisms, only natural transformation could play a significant role in the gene transfer between transgenic plants and microorganisms (Bertolla and Simonet, 1999). Transduction has been viewed as largely improbable because of strict host specificity of viruses and until now no virus functioning in both plant and bacteria has been identified. Conjugation has been shown in a specific case: the unidirectional transfer of genes harboured on a conjugative plasmid between the phytopathogenic *Agrobacterium* spp. and the plant. The inverse would require that plant transgenes be carried on a circular plasmid, which is generally not the case for actual GEPs except those in which plasmids have been cloned after linearisation into the genome. In this case, the recircularisation of plasmid would be required before the genetic exchange and so far this option has been viewed as extremely improbable (Schluter *et al.*, 1995).

Since the discovery of natural transformation in *Streptococcus pneumoniae* (Griffith, 1928) about 90 species from water, soil and sediment have been found naturally transformable (De Vries and Wackernagel, 2004). Given the small number of microorganisms investigated compared to the large number of not culturable environmental bacteria and following recent identification *in silico* of competence genetic determinants in several newly sequenced isolates (Bolotin *et al.*, 2001; Claverys and Martin, 2003) , this phenomenon could be more widespread than imagined. The naturally transformable 90 bacterial species currently identified, represent only 2 % of cultured prokaryotic species (De Vries and Wackernagel, 2004), which in turn represent about 1% of the estimated number of existing bacterial species (Kaeberlein *et al.*, 2002). Therefore, studies conducted with well characterized cultured prokaryotic species might severly underestimate the potential for such a transfer. However, there is a considerable variation

in the way bacteria take up DNA, the conditions required, and the specific barriers that limit gene transfer as it will be discussed further.

Genetic transformation has been commonly divided into “natural” and “artificial” transformations. Natural transformation (Lorenz and Wackernagel, 1994) is an active microbial process of DNA uptake requiring specific genes and the development of a competence state in bacteria, while artificial transformation is a passive process requiring chemical (*i.e*.salts) or electrical (*i.e.* electroporation) induction; usually it is performed *in vitro* in order to introduce DNA into bacterial, fungal, plant, or animal cells. By subjecting cells to an electrical current field or a chemical treatment that creates non permanent pores in the lipid bilayer of the cell membranes, DNA is free to move in and out of cells. Evidence is emerging that *in situ* electrical discharges in the form of lightning influence bacterial transformation and gene transfer. Thus, lightening mediated gene transfer (electrotransformation) under natural conditions could be regarded as pivotal as natural transformation in driving bacterial evolution. Indeed, parameters for the *in vitro* process were found to be highly similar to those during lightning of soil (Demaneche *et al.*, 2001a). In addition, indigenous soil bacteria were shown to be electrotransformed by laboratory scale-lightning (Cérémonie *et al.*, 2004; Cérémonie *et al.*, 2006) Similarly, chemical conditions as high salt concentrations in the environment can induce a competent state (Lorenz and Wackernagel, 1994) and in one case provided evidence of *in situ* competence development: calcium dissolved in freshwater induced transformation of *Escherichia coli* in river water (Baur *et al.*, 1996).

II.2 Requisites for natural transformation

Natural transformation involves several steps under natural conditions (Smith *et al.*, 1981): extracellular DNA must be released into the environment, genetically adapted bacterial genotypes must be in close proximity and environmental conditions (biotic and abiotic) favourable for the development of the physiological stage of competence which includes the adsorption of DNA to the bacterial cell surface and the uptake of DNA (Fig. 2). Then, integration in the chromosome via recombination or autonomous replication must be efficient. Finally, the acquired trait has to be expressed by the recipient bacterium. Selective pressure will determine the establishment of the new acquired phenotype and its fixation in the offspring or its disappearance.

All of these steps have been studied as separate events, mostly *in vitro*, thus providing evidence of the bacteria related parameters and limiting factors of each step. The entire gene transfer depends ultimately on fluctuating environmental conditions and correlated cellular intrinsic factors. Hence, assumptions have been made by several authors that the probability that all above mentioned conditions occur for a gene transfer event from plant to bacteria under natural conditions would be extremely low (De Vries and Wackernagel, 2004; Nielsen *et al.*, 1998b).

The successful transformation events are expressed as the ratio of the number of transformed cells to the total pool of cells exposed to DNA, *i.e.* as a frequency of transformation. For most species studied *in vitro*, highest transformation frequencies were obtained when bacteria were exposed to specific DNA sequences harbouring a sufficient degree of homology with the recipient bacteria and under best nutritional conditions, with the exception for instance, of *Azotobacter vinelandii* where highest transformation rates were observed under limited nutritional conditions showing differences imputable to alternative adaptive strategies (Lorenz and Wackernagel, 1994). Although a few of the naturally transformable bacteria are constitutively competent, competence is usually an active physiological state: as a general rule, bacteria become competent in a growth dependent manner. In some gram negative bacteria, such as *Haemophilus influenzae*, *Pseudomonas stutzeri*, *Azotobacter vinelandii* and *Acinetobacter sp.* , competence is internally regulated and its expressed in the late exponential or early stationary phase (Lorenz and Wackernagel, 1994; Paget *et al.*, 1998) while in the gram positive *Streptococcus pneumoniae* and *Bacillus subtilis*, the accumulation of a low molecular mass polypeptide in the growth medium stimulates the expression of the about 30 to 50 genes involved in competence development (Nielsen *et al.*, 1998b). To date only few soil bacteria have been shown to express competence under natural growth conditions. For example, *Acinetobacter baylyi* was transformable in soil and *in planta* (Kay *et al.*, 2002a; Nielsen *et al.*, 1997). Compounds present in roots exudates, such as amino acids, organic acids and sugars, were proven to stimulate competence development in *Acinetobacter baylyi* in soil (Nielsen and van Elsas, 2001). For this bacterium, nutrients accessibility is critical for producing and maintaining its competence in soil. For example, phosphate concentration and humidity were shown to boost transformation frequencies. Furthermore, experiments in soil microcosms demonstrated that *Acinetobacter baylyi* remains competent during several hours, then after reaching stationary phase (after 24 hours) its ability in acquiring host DNA is completely lost (Nielsen *et al.*, 1997). However, the addition of a single carbon source and salt allows stimulates competence which

lasts for up to 6 days in soil.

Demaneche *et al*. (Demaneche *et al.*, 2001c) demonstated that *Agrobacterium tumefaciens* and *Pseudomonas fluorescens* are capable of incorporating plasmid DNA 72 hours after their inoculation in soil microcosms. Similarly, *in planta* nutritional conditions may be favourable for bacterial development and could, thus, constitute an environment which fosters competence. The phytopathogen *Ralstonia solanacearum* became competent during colonisation of plant tissues and its rapid multiplication *in planta* was correlated to an exponential growth phase *in vitro* (Bertolla *et al.*, 1999).

A number of recent studies on bacterial biofilms demonstrate the role of cell signalling (*quorum sensing*) between bacterial aggregates in driving the development of competence and gene transfer (Li *et al.*, 2001; Li *et al.*, 2002). Furthermore, HGT via natural transformation was particularly enhanced in these biofilm associated communities (Molin and Tolker-Nielsen, 2003); hence, several researchers hypothesize that bacteria living aggregately on the different surfaces of the plant could be favourable candidates for HGT (van Elsas *et al.*, 2003a).

Although DNA is degraded in the environment, the probability that sufficient amounts of intact DNA of microbial or eukaryote origin remain available in the environment due to metabolic turnover is not negligible. On the other hand, *in situ*, soil conditions are not always conducive for metabolically active bacteria due to nutrient limited (oligotrophic) conditions. However soil is heterogeneous and composed of a multitude of nutrient rich microhabitats that could foster competence development such as the plant rhizosphere, the phyllosphere, the gut of insects, earthworms and protozoans (van Elsas *et al.*, 2003a).

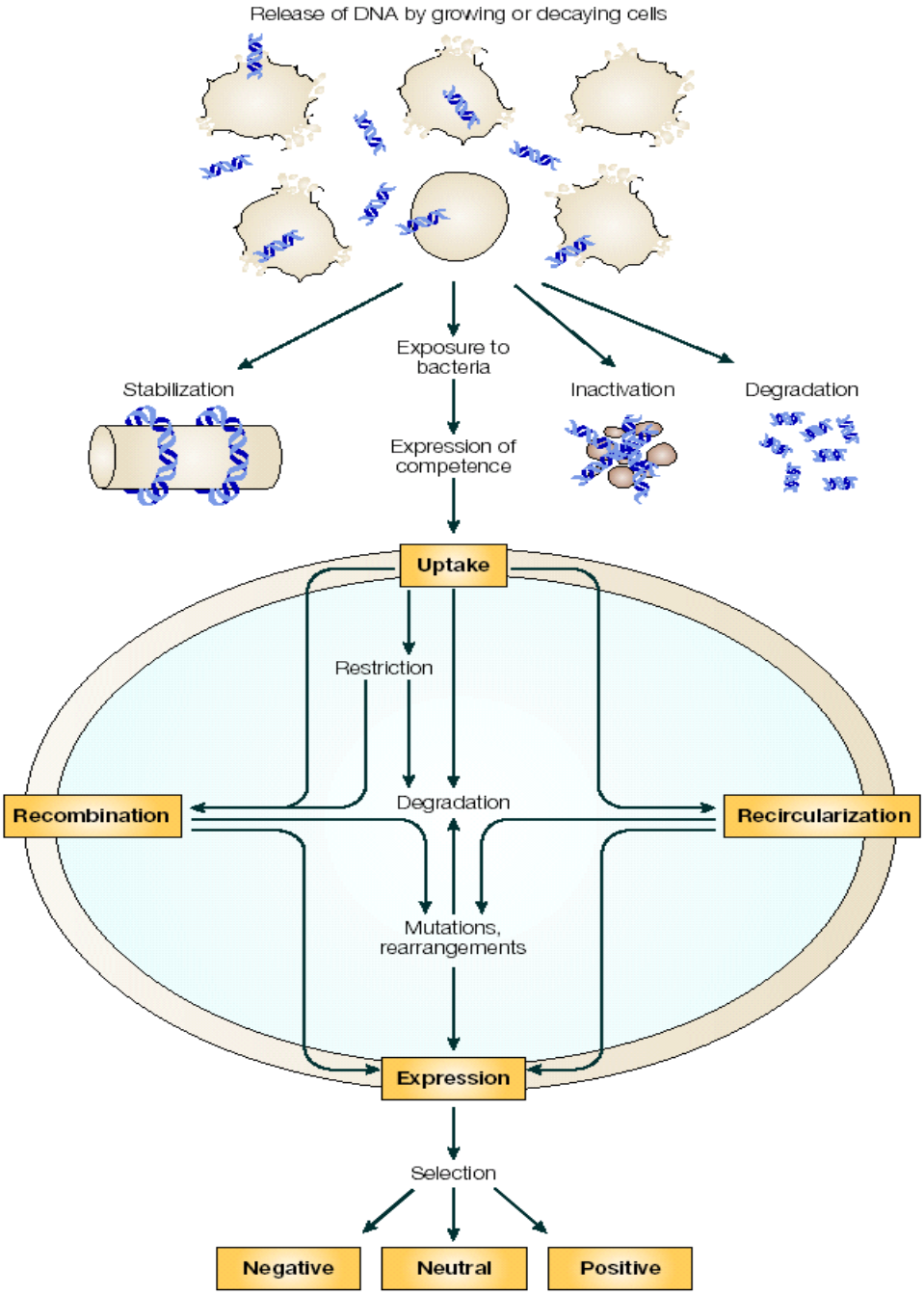

Fig. 2. Natural transformation of bacteria: steps required for a successful transfer of foreign DNA into transformants (Thomas and Nielsen, 2005).

II.3 Barriers to natural transformation

In order to successfully transform a bacterial cell, free DNA must have certain features allowing it to pass a number of stages before its stable integration in the recipient genome (Fig. 2). Assuming that conditions for a physical encounter between the bacterial cell and an intact transgenic molecule are met, we can delineate two further barriers from the bacterial side: the first one concerns the uptake of the DNA into the cell, the following one is then the stable internalisation of the new trait into the genetic content.

II.3.1. Uptake of DNA into the cell

The first step of the transformation process of bacteria depends on the adsorption of extracellular DNA to the cell wall. This process varies among bacterial species. For some bacteria the mechanism requires a short recognition nucleic acid sequence of 10 bp (*Neisseria gonorrhoeae*) or 9 bp (*Haemophilus influenzae)* in the donor DNA and this strategy has been proposed to optimize gene transfer between cells belonging to the same species and to prevent the uptake of foreign DNA (Smith *et al.*, 1995). Other naturally competent bacteria are transformable by any type of DNA, for example *Bacillus subtilis* takes up *E.coli* DNA, phage T7 and various plasmids, whilst *Streptococcus pneumoniae*, *Synechococcus* and *Acinetobacter* acquire DNA from any sources (Lorenz and Wackernagel, 1994).

Once DNA enters the cell, it could be affected by restriction - modification (RM) systems, that degrade some foreign DNA sequences whilst modifying host DNA by methylation, thus protecting it from the RM system. This mechanism reduces the incorporation of new sequences (Bickle and Kruger 1993). RM systems are particularly frequent in the genomes of naturally transformable species (Kobayashi, 2001).

RM systems were certainly involved in the dramatic drop in the transformation efficiency of *Streptomyces avermitil* (Macneil, 1988) and *Pseudomonas stutzeri* (Berndt *et al.*, 2003) when they were exposed to donor DNA that had been propagated in *E.coli* instead of in the same species. However, in three naturally transformable bacteria *(Bacillus subtilis, H. influenzae* and *Streptococcus pneumoniae*), a strong effect of restriction on transformation frequency was not seen (Bron *et al.*, 1980; Lacks, 1984; Stuy, 1976). During natural transformation, only a single strand of the extracellular DNA is transported into the cytoplasm of competent cells (Dubnau, 1999) and restriction enzymes generally recognize and cleave double-stranded DNA (Redaschi

and Bickle, 1996), although now evidence exists that also single stranded DNA can be cleaved (Ando et al., 2000); in addition, conditions such as presence of saturating amounts of extracellular DNA or restriction systems inefficiency could promote natural transformation by overwhelming RM systems (Bickle and Kruger, 1993). Hence, the role of DNA restriction on the integration of foreign DNA is to be seen in the right perspective.

II.3.2. DNA homology: the antagonistic role of mismatch repair system and the SOS system

Probably, the major barrier to the integration of foreign DNA into the genome is the last step, the stable insertion of the incoming DNA either by autonomous replication for plasmid molecules or by genomic integration of linear DNA via homologous recombination (de Vries and Wackernagel, 1998; 2002; Meier and Wackernagel, 2003a).

The size of DNA also influences the success of natural transformation. Before the uptake, because the bigger is its size, the lesser are the chances for it to enter into the cell in a completely intact state. Following uptake, the efficiency of integration of donor DNA in the recipient genome via homologous recombination will depend on the presence, in the incoming strand, of regions of homology with resident DNA. Several studies report that a log-linear decline in the nucleotide sequence identity between donor and recipient leads to a significant decrease in the integration of donor DNA by homologous recombination (Lorenz and Sikorski, 2000; Majewski *et al.*, 2000; Rayssiguier *et al.*, 1989; Roberts and Cohan, 1993; Zahrt *et al.*, 1999; Zahrt and Maloy, 1997).

However, recombination was found to occur *in vitro* even if the degree of homology between donor and recipient DNA was very little, as in homology–facilitated illegitimate recombination (HFIR) where, in paradigmatic cases homologous regions of only 3-8 bp were sufficient to produce integration of longer foreign DNA fragments in *Acinetobacter* BD413 (de Vries and Wackernagel, 2002) and of 3–10 bp in *S. pneumoniae* (Prudhomme *et al.*, 2002). However, the frequencies of transformation *in vitro* were up to 6 log lower than those determined by transforming these strains with entirely homologous donor DNA.

The recombinational process is under the control of two main antagonistic systems, the mismatch repair system (MRS) and the SOS system, which mediate the integration of heterologous DNA. If on the one hand the first one, beside correcting replication errors and base modifications, acts as a strong recombinational barrier by preventing the formation of heteroduplex molecules, in order to maintain a level of genetic stability in the cell, on the other

hand this genetic barrier might be contrasted by the SOS system during stress response (such as to DNA damage). The latter, catalyzed by the RecA protein (Otero and Hsieh, 1995) could promote recombination between divergent stretches of DNA (Matic *et al.*, 1995). Hence, genetic barriers are not inexorably fixed and should be also viewed in a dynamic context as the one in which bacterial cells live in the environment.

II.4 *In situ* transformation of bacteria by plant DNA

Recombination between eukaryotic DNA and prokaryotic DNA was believed impossible due to differences in DNA structure (exon-containing DNA complexed with proteins) and higher methylation rate of eukaryotes DNA, until it was observed in *A. baylyi* under optimized conditions *in vitro* (Gebhard and Smalla, 1998). These conditions included the development of plants in which selectable marker genes were flanked by homologous sequences in order to promote homologous recombination-mediated integration in the recipient host (de Vries *et al.*, 2001). A step further was to research if such transfer could occur *in situ*, e.g. in the phytosphere or in soils cultivated with transgenic plants. Several studies addressed HGT from plant to bacteria *in situ* using different experimental models of recipient bacteria and transgenic plants among which nuclear-modified potatoes, sugar-beets, tomatoes and tobacco fitted with the *nptII* gene (Bertolla *et al.*, 2000; Gebhard and Smalla, 1999; Nielsen *et al.*, 2000b) or transplastomic tobacco plants in which the *aadA* gene was cloned in the plastid DNA (Kay *et al.*, 2002b). However, no gene transfer could be detected when nuclear transgenic tomato and tobacco plants plants where inoculated with *R. solanacearum*, although the agent of bacterial wilt had been found capable of developing a competence state and to exchange genetic material *in planta* (Bertolla *et al.*, 1999; Bertolla *et al.*, 1997). Reasons of this failure were imputed to the low efficiency of transformation of the recipient strain and to the strong dilution of the transgene among the rest of plant DNA in nuclear transgenic plants (Bertolla *et al.*, 2000). Effectively, when a transplastomic tobacco, containing up to 10000 copies of transgene per cell (Daniell *et al.*, 2002) was co-infected with a mix of *R. solanacearum* and *A. baylyi*, the latter was found able to develop competence in infected tissues and to acquire and express the transgene (Kay *et al.*, 2002b). Hence, HGT occurred *in situ*, albeit under optimized conditions of homology between plant and bacterium sequences (Kay *et al.*, 2002b). As regard the soil environment, a microcosm based study revealed that HGT occurred when sterile soil microcosms where inoculated both

with plant DNA and bacteria already in a competent state. No transfer could be detected in similar conditions when non- sterile soil microcosms were used (Nielsen *et al.*, 2000b). To date, the occurrence of HGT in fields planted with GEPs could not be demonstrated (Gebhard and Smalla, 1999). However, crucial aspects in the cited study such as the sampling size and the detection method employed (efficiency of selection) were found to be inadequate to detect those rare transformants that could potentially be present among the high proportion of indigenous soil antibiotic resistant bacteria (Nielsen and Towsend, 2004; Ray and Nielsen, 2005).

III- KEY PARAMETERS AFFECTING TRANSGENIC DNA AVAILABILITY

The earth is entirely covered by nucleic acids which are found in environments such as fresh water and marine, sediment, soil and the terrestrial subsurface (Trevors, 1996). DNA from plants, animals and microorganisms is released into the environment after cellular lysis, but can also be actively excreted by several bacterial species such as *Bacillus subtilis*, *E. coli*, *Acinetobacter sp.* and some species of *Pseudomonas* (Lorenz and Wackernagel, 1991; Paget and Simonet, 1994).

Microbial DNA concentrations in the soil environment can range from 5 to more than 35 µg g of dry soil^{-1} (Ceccherini *et al.*, 1998; Frostegard *et al.*, 1999; Hastings *et al.*, 1997).

During their life cycle, plants also synthesize and replicate DNA, which is released into the soil throughout vegetative growth, by root cap cell lysis and roots turnover, while during anthesis pollen can contribute by adding nucleic acids to soil or water. Moreover, plant tissue decomposition (e.g. following physiological maturity of crops) or enzymatic degradation of cell structures by pathogens (Ceccherini *et al.*, 2003) also release DNA in the environment. Key factor for bacterial transformation frequencies in soil is the availability of not completely degraded and biologically active DNA after its release from dead (or living) cells.

III.1 Persistence of nucleic acids in the environment

Since the release of transgenic crops in the field, interest has grown in the fate and detection of transgenic DNA in the environment after plant removal and has been studied (Gebhard and Smalla, 1999; Romanowski *et al.*, 1993; Widmer *et al.*, 1997; Widmer *et al.*, 1996). A wealth of studies conducted by inoculating microcosms with DNA originating from various sources, like purified genomic (chromosomal or plasmidic) (Gebhard and Smalla, 1999;

Romanowski *et al.*, 1993; Widmer *et al.*, 1996) bacterial lysates (Nielsen *et al.*, 2000b) plant leaves (Widmer *et al.*, 1997) and pollen (Meier and Wackernagel, 2003b) have investigated DNA degradation kinetics in soil.

The persistence of extracellular nucleic acids in the environment seems to be influenced by a number of biotic and abiotic parameters, which favour their protection or which induce their degradation. DNA is altered by physical agents (heat, ultraviolet light), chemical factors (pH, reactive oxygen species, heavy metals, etc.) and by enzymatic hydrolysis by plant or microbial nucleases during senescence or apoptosis. DNase producing bacteria in the soil and water environment account for more than 90% of heterotrophic bacterial (Greaves and Wilson, 1970). However, because nucleic acids are chemically reactive due to their negative electric charge, they can form complexes with equally reactive environmental constituents. Several studies have been done on the interaction of nucleic acids with minerals and are concordant on the protective role that some materials have on this anionic polymer (Demaneche *et al.*, 2001b; Lorenz and Wackernagel, 1994; Romanowski *et al.*, 1991). Thus, the binding of DNA to minerals provides protection against both eukaryotic and prokaryotic DNases (Ahrenholtz *et al.*, 1994; Khanna and Stotzky, 1992; Paget *et al.*, 1992; Pietramellara *et al.*, 2001).

Among these quartz sands, clay minerals, feldspar and heavy minerals were discovered as binding substrates. Nonetheless, also organic compounds such as humic acids were shown to complex DNA (Crecchio and Stotzky, 1998). In addition, proteins and polysaccharides which are present in cellular debris, may also protect DNA from enzymatic degradation (Nielsen *et al.*, 2000a). The protective role offered by these compounds relies on adsorption; namely, the existing electrostatic repulsion between the anionic polymer (DNA) and the negatively charged surfaces of minerals is hampered by the presence of cations. The ubiquitous presence of multivalent cations in the environment and their role as mediators of DNA adsorption to surfaces is probably the reason for the binding of DNA by soils and sediments (Lorenz and Wackernagel, 1994). Several factors influence adsorption in addition to the type of mineral and cations (mono or divalent) present. These factors include pH, soil water content, DNA tertiary structure and size (Franchi *et al.*, 1999; Vettori *et al.*, 1996).

DNA adsorption on clay minerals is more efficient when free water is low and therefore, cation concentration rises, but also when pH is low, because silicates cease being negatively charged below pH 5. Namely electrostatic repulsions between sorbant and adsorbed molecules have to be avoided in order for them to bind. DNA conformation is also important because the higher the number of the degrees of freedom of the molecule, the higher the possible number of

bonds to the sorbant; thus, linear and relaxed circular DNA adsorb better than covalently closed DNA (Melzak *et al.*, 1996; Poly *et al.*, 2000). Both chemical (cation bridging) forces and physical forces (van der Waals) are responsible for the high binding capacity of DNA to minerals (Ogram *et al.*, 1988). The protection from nuclease degradation is not fully understood, but clay particles adsorb DNA molecules and DNAses, thus physically separating the enzyme from its substrate (Demaneche *et al.*, 2001b; Paget *et al.*, 1992). The electrostatic interactions between DNA and clay occur over the entire length of the linear DNA molecule, while for supercoiled molecules, electrostatic attractions seems lower thus leading to bonds higher sensitivity to DNases (Poly *et al.*, 2000) (Fig. 3).

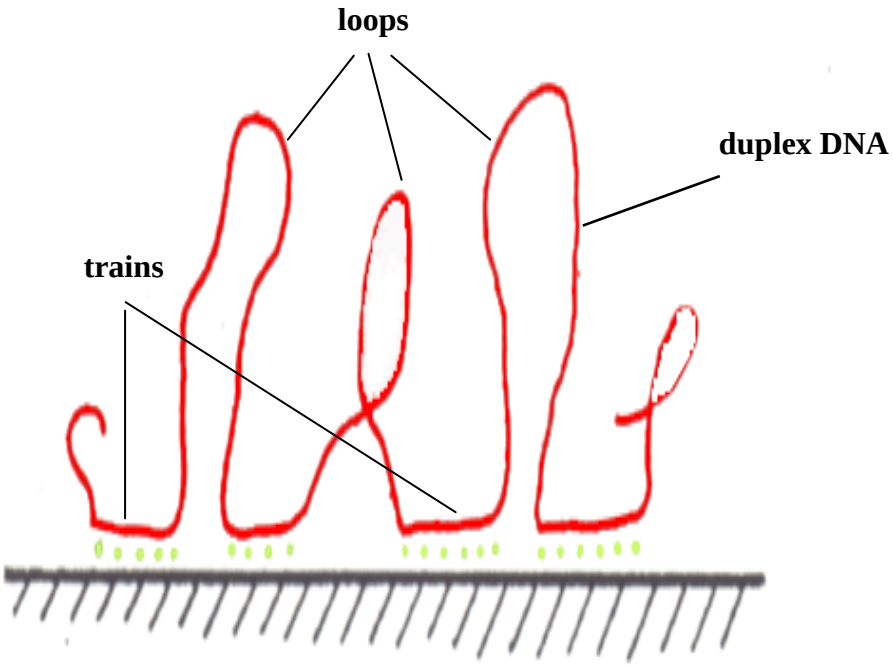

Figure 3. Conceptual adsorption model of a duplex DNA molecule to a solid surface of a mineral particle. The scheme is based on the polyelectrolyte adsorption model of (Hesselink, 1983). Bound segments are termed “trains”, nonadsorbed segments “loops”. There is a dynamic equilibrium between trains and loops. Repulsion forces between the negatively charged surface and DNA are overcome by monovalent cations and divalent cations. The green dots symbolize divalent cations involved in bridging between the negatively charged phosphate groups of DNA and the silicate anions of the mineral (adapted from Wackernagel, 1996).

III.2 Biological potential of DNA

Even if DNA is adsorbed tightly to minerals, it can still display reactivity to enzymes, as it can be amplified by PCR (Vettori *et al.*, 1996) or transform bacteria (Gallori *et al.*, 1994; Paget *et al.*, 1992). Two authors, (Sikorski *et al.*, 1998) and (Demaneche *et al.*, 2001b) demonstrated the biological activity of DNA introduced into soil, and furthermore confirmed previous pioneer results by Graham and colleagues (Graham and Istock, 1978; 1979) which had shown that bacterial transformation can take place in soil.

DNA‘s ability to transform bacteria, that is to integrate into their genome and be expressed in the cell, is dependant on the integrity of the DNA molecules. If the molecule is a plasmid, it has to be intact in order to replicate in the host cell, whereas if it is linear DNA, homologous sequences must not be damaged if homologous or homeologous recombination is to occur. Adsorption and protection of DNA are partial and reversible and when environmental conditions change, DNA is desorbed and becomes accessible to bacteria, but also to DNAses. Extracellular DNA degrades into different environments at rates dependent on viable bacteria. Despite the DNA degrading capacity of microbial habitats, DNA persists and is detected by PCR and hybridization long after it has been seeded in soil. For instance, DNA from transgenic sugarbeet (*Beta vulgaris* L.) was detected up to two years after cropping (Gebhard and Smalla, 1999).

When transgenic tobacco DNA was subjected to decaying process simulating conditions encountered in nature (Ceccherini *et al.*, 2003) within 72 hours of decay most of the intracellular DNA of the plant was degraded by plant nucleases or enzymes. However, the remaining DNA displayed a biological activity by transforming *in vitro* a competent bacterium originating from soil. Even if only a part of plant DNA escapes degradation and reach the soil, its fate will depend on soil microbes present. They could utilize DNA either as a source of nutrients, or internalize it via natural genetic transformation. On the other hand, DNA might bind to soil colloids and be protected from degradation. Because bacteria are heterogeneously distributed in soil, the probability of contact between them and nucleic acids is dependent on DNA mobility. Water might help the dissemination of transgenes from plant debris through soil. Antibiotic resistance genes, whether circular or linear, were shown to move through water saturated soil columns, albeit degradation occurred and was dependent on the time of incubation of nucleic acids in soil (Pote *et al.*, 2003). The DNA remaining in the effluent was biologically active; indicating that

water saturated soil and groundwater could harbour and transport functional DNA. In addition, when roots of transgenic soybean and corn plants were flushed with water, significant amounts of root derived transgenes were found in the leachates (Gulden *et al.*, 2005). These results suggest the possibility that biologically active transgenic DNA can be released from plants into the soil environment, despite a rapid degradation if naked DNA is unprotected.

IV-THE PHYTOSPHERE AND THE "HOTSPOTS" FOR HORIZONTAL GENE TRANSFER

The majority of natural environments, soils and aquatic systems, are often very restricted by the availability of resources for microbial growth, which can severely limit population density and activity. This restricts microbial processes dependent on density and activity, such as all HGT mechanisms. However, a number of specific sites in these natural habitats, mostly related to soil, plant and surfaces in aquatic environments, have been shown to provide favourable conditions for bacterial colonisation, mixing and activity, resulting in the occurrence of locally enhanced densities of active microbial cells. These sites are often conducive to HGT processes and have been considered "hot spots" for bacterial gene transfer activity (van Elsas and Bailey, 2002). Both abiotic (e.g. temperature, pH, moisture content, nutrient availability) and biotic factors (grazing, predation, competing or syntrophic organisms and plants) can affect the microbial environments of these spots. A range of hot spots for HGT in soil and aquatic environments is presented in Table 2.

As natural environments are heterogeneous and dynamic, conditions vary both in time and space. The conditions that reign locally, at each point, control HGT rates.

The presence of large mineral surfaces and organic constituents in soil plays a key role in determining the physiological status of soil-dwelling bacterial cells. Soil is a heterogeneous system (Fig. 4) with gas, liquid and solid phases (Nannipieri *et al.*, 2003). Clay-organic matter complexes are important sites for soil microorganisms, due to their negatively charged surfaces and enhanced nutrient availability (Pietramellara *et al.*, 2001; Smiles, 1988). Water availability in soil is an important factor driving microbial activity. In soil, bacterial cells occur mainly adsorbed to surfaces, and are often present as microcolonies, with restricted movement and contact with other colonisers located elswhere. Hence, most bacterial cells in soil can interact only with partners in their immediate vicinity, unless they are transported by water.

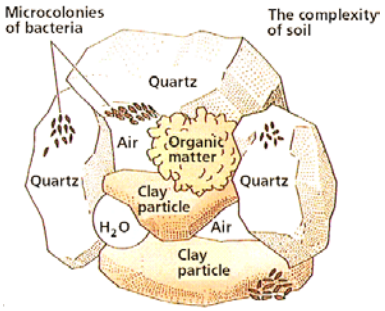

Figure 4. Clay particles, quartz, organic matter, water, air spaces and microbial cells constitute the complexity of soil.

Habitat	*Hot spot*	*HGT Mechanism*
Soil	Rhizosphere and plant tissue	Conjugation Transformation
	Phyllosphere	Conjugation
	Manured soil	Conjugation
	Guts of soil animals	Conjugation
	Epilithon	Conjugation
Aquatic	Sewage/sludge	Conjugation
	Sediment	Conjugation Transformation

Table 2. Environmental hot spots conducive to gene transfers. These hot spots are sites of enhanced gene transfer activity, which is often based on an increase of cell densities, of cell-to-cell contacts, of cellular movement or activity (modified from van Elsas et al., 1992).

In spite of the nutrient-poor status of many soils, nutrients can become concentrated in different locations such as the rhizosphere -the layer of soil influenced by root metabolism- (Hiltner, 1904), the decaying organic material of animal or plant origin (the residuesphere), and the gut of soil animals like earthworms.

The rhizosphere of many plants represents a region with relatively high concentrations of organic carbon and nitrogen, phosphorus and sulphur. The effect of plant roots on soil microorganisms in the rhizosphere has been studied: also known as “rhizosphere effect” it consists in an enhanced biomass and activity of microorganisms tightly linked to the roots as a result of the exudation of compounds.

In addition, water flow in soil induced by plant roots may enhance bacterial movement. Therefore, microbial growth induced by the presence of nutrients and water flow can promote cellular activities and cell-to-cell contacts (De Leij *et al.*, 1995). In addition, *in vitro* transformation was found to be enhanced by a range of different compounds exuded by plant roots into soil (Nielsen and van Elsas, 2001).

The phyllosphere (aerial plant parts) (Normander *et al.*, 1998; Waipara *et al.*, 2002) and the residuesphere (interface soil-plant residues) (de Lipthay *et al.*, 2001; Sengelov *et al.*, 2000) , can also provide nutrient-rich surfaces, resulting in similar high microbial activity. The phyllosphere of plants has been shown to be conducive to conjugative plasmid transfer (Björklöf *et al.*, 1995) and to transduction (Mendum *et al.*, 2001; Stephens *et al.*, 1987). Thus, globally, soil contains physical barriers to cell-to-cell contacts and nutritional limitations, while providing local rich nutrient microenvironments. HGT rates in soil are certainly affected by the combination of these phenomena.

Other plant parts such as the spermosphere, the endosphere and the pathosphere can also sustain consistent densities of bacteria and allow HGT. In this latter microenvironment, in addition, during the co-infection of tobacco plants with the plant pathogen *Ralstonia solanacearum* and *Acinetobacter* BD413 it was shown that *Acinetobacter* was able to capture plant-derived DNA (Kay *et al.*, 2002b).

V-IMPACT OF TRANSGENIC PLANTS ON THE BIODIVERSITY OF THE SOIL MICROBIAL COMMUNITY

Transgenic plants might affect soil microbial communities directly and indirectly.

Among the potential direct effects are changes in soil microbial activity due to differences in the amount and composition of root exudates, changes in microbial functions resulting from gene transfer from transgenic crops and alteration in microbial populations because of the effects of management practices for transgenic crops such as pesticide applications, tillage and application of inorganic and organic fertilizers (Motavalli *et al.*, 2004). Transgenes which enter the soil after the decomposition of plant litter will be concentrated differently in the soil profile based on tillage type (Angle, 1994). Transgenes have been shown to be released directly from the plant roots from sloughed and damaged root cells as well as through root exudation. Transgenic Bt corn (*Zea mays* L.) was found to release the *Bacillus thuringiensis* insecticidal endotoxin from its

roots (Saxena and Stotzky, 2000). Incorporation of transgenic plant products into the soil could alter soil microbial biodiversity due to variable responses by microorganisms to the novel proteins. Decreasing biodiversity is a concern because several authors suggested that the preservation of biodiversity is essential for the maintenance of stable productivity in ecosystems (Tilman and Downing, 1994).

Among indirect effects of transgenic crops on microbially mediated processes, the effects of changes in the amount and composition of transgenic crop residues are the most oftern cited. For example, reductions in corn borer damage or differences in the composition of the residues may increase the amount of undamaged, low N-containing residues remaining on or in soil after harvest due to the expression by the plant tissues of Cry genes coding for the endotoxin. This might reduce the rate of decomposition and nutrient mineralization. Consequentially, the following crop might grow more slowly due to reduced nutrient availability (Boyle *et al.*, 2001). These potential effects are complicated by the possible decrease in soil erosion due to the higher amount of crop residues left in the soil. Furthermore, the higher productivity of the culture conferred by the self protection from the pest also means higher carbon sequestration (Motavalli *et al.*, 2004) which is important in the turnover of this element and would balance its biogeochemical cycle.

V.1 Effects of genetically engineered plants on microbial communities

Several examples demonstrate reduction or increase in the composition of soil microbial communities in the agricultural area cultivated with transgenic crops. Examples targeting transgenic traits affecting plant nutrient acquisition in nutrient limited environments are speculations that the mechanisms mediated by these transgenic crops, such as increased root exudation of organic acids, may reduce the activity of bacterial communities involved in nutrient cycling (*e.g.* the nitrifiers, a keystone indicator of soil fertility) due to a decrease in pH in the rhizosphere (Motavalli *et al.*, 2004). Possible indirect ecological effects of the introduction of transgenic disease-resistant trees on mycorrhizae and other soil biota important in decomposition and nutrient cycling in forest soils were also reported (Boyle *et al.*, 2001). The demonstration that transgenic plants could alter their microbial environment was based on the legume species bird's-foot trefoil (*Lotus corniculatus* L.) engineered to produce opines (low molecular weight amino acid and sugar conjugates that can be used as growth substrates by a few of the root-associated bacteria) (Oger *et al.*, 1997). The population densities of the opine (mannopine and nopaline)-utilizing bacteria were 80 times higher than in non-transformed plants, while the

number of cultivable bacteria was not significantly different. The alterations were both transgene-specific and target population-specific.

A recent study has shown similar findings with different opines, plant and soils, showing that the effect was independent of opine, plant and soil (Mansouri *et al.*, 2002).

Clearly, the assessment study of the introduction of a given transgene into a genetically modified plant will entirely depend on the identification of pertinent target populations.

Siciliano and colleagues (Siciliano *et al.*, 1998) assessed the root-interior and rhizosphere bacterial communities associated with a field–grown genetically engineered canola (*Brassica* spp.) variety and two conventional varieties. The carbon utilisation patterns and fatty acid methyl ester profiles (FAME) of the microbial community associated with the roots of the genetically modified canola variety were different from those of the conventional varieties. Moreover, the cultivable microbial community representative bacteria associated with a genetically modified canola variety was significantly different from two conventional canola varieties tested (Siciliano and Germida, 1999). However, this effects were found to be restricted to a single variety of genetically engineered canola.

Other studies proposed differences in denaturing gel gradient electrophoresis (DGGE) patterns of the eubacterial population associated with transgenic canola herbicide-resistant plants, but they were minor and influenced by seasonal variation, although the *Pseudomonas* populations associated with these plants were different than those found on wild type plants (Gyamfi *et al.*, 2002). Similarly, different populations of *Rhizobium leguminosarum bv.viciae* were hosted by transgenic glufosinate tolerant canola and its non transgenic counterpart (Becker *et al.*, 2001) and differences between the rhizosphere bacterial communities physiological and genetic profiles were detected between parental and transgenic alfalfa (*Medicago sativa* L.) (Di Giovanni *et al.*, 1999; Donegan *et al.*, 1999). On the contrary, microbial communities associated with glufosinate-tolerant transgenic maize were not different based on the SSCP-PCE patterns from the communities linked to wild type maize plants (Schmalenberger and Tebbe, 2002).

Other results from investigation on the effects of GEPs on microbial communities composition seem to indicate that alterations in the microbial diversity would be less significant than environmental factors such as sampling date and field site (Dunfield and Germida, 2001; Dunfield and J.J., 2003). Spatial effects, temporal effects and spatial by temporal interactions were also demonstrated in the terminal restriction fragment length polymorphisms (T-RFLP) patterns associated with the rhizosphere of an herbicide-resistant transgenic potato (Lukow *et al.*, 2000). similarly, a field study conducted with T4 lysozyme expressing transgenic potato

concluded that differences observed between the community structures of rhizobacteria associated with transgenic lines and those of the control isogenic line were negligible if compared to environmental factors (Heuer *et al.*, 2002).

Generally, these studies demonstrated in a variety of plants with different transgenes that these may influence the composition of the plant-associated microbial communities compared to the control plant; however, these effects were often minor if compared with changes associated with cultivar-cultivar comparison or the influence of weather and season.

Community structures are dynamic, as they change through the season and the plant development, and differences observed in many studies were neither fixed nor systematic, raising a further challenge into the monitoring (risk assessment) that, consequently, should be always conducted during long term studies.

To date, only one potentially long-term effect of a GEPs on the soil ecosystem, demonstrating persistent changes, was reported and occurred following the removal of opine producing transgenic *Lotus corniculatus* from the field: the populations of mannopine utilizers, decreased very slowly and after 4 months still produced elevated counts. However, the next fallow bacterial populations isolated from soils that had been planted with transgenic or wild type plants were very similar, indicating that the soil system was somehow resilient (Oger *et al.*, 2000) .

Finally, the importance of comparing the alterations in the microbial diversity associated with GEPs with the non–transgenic counterpart, and with acceptable changes in the agroecosystem (e.g. utilisation of a certain agronomic practice, utilisation of a particular compound) and season should not be forgotten (Dunfield and Germida, 2004; Griffiths *et al.*, 2006).

VI-CONCLUDING REMARKS

Interkingdom transfer from transgenic plants to bacteria was demonstrated to happen *in situ* but under optimized conditions where homologous regions were present in recipient strains. In specific niches where microbial densities as well as conditions are appropriate for development of competence (hot spots) this transfer becomes measurable, albeit occurring at low frequencies (Kay et al., 2002b).

Small fractions of transgenes escape degradation, move into soil and persist in the environment. Further research needs to be done in order to gain better insight into ecological aspects of HGT. Among these, for instance, experimentally or theoretically determined transfer frequencies should be viewed in a context where little is known on growth and the environmental

fitness of bacteria transformed by plant transgenic DNA in the environment. What would be the evolutionary potential of these microorganisms? The transfer event is fixed only if conferring a selective advantage? The answer will necessary be different for each transgene susceptible of the transfer. Generally, successful events occur on an evolutionary time scale (de Vries and Wackernagel , 2004) and require a selective pressure in order to enrich cells transformed by the recombinant DNA. Up to now, quantification of HGT events has been performed with cultivation based approaches (selective plating) which give little information on the survival of transformants in natural settings.

In addition, actual quantitative data obtained from *in situ* studies (transformation frequencies) should be viewed as partial due to the lack of discrimination between original events of transformation and clonal multiplication of these events, hindering their accurate determination. Such questions should be addressed in future investigations.

Discussion of the impact of transgenic plants on environmental bacteria needs to include that antibiotic resistance genes, often located on mobile genetic elements, already exist in the environment and are dispersed in the microbial community due to massive utilisation of antibiotic compounds in animal and human health care all over the last 50 years. Constantly high selective pressure on pathogens ensues in exchange of resistance determinants *via* HGT amid pathogens and is responsible for the loss in efficiency of a growing number of antimicrobial molecules (Davies, 1994). A further element deserving consideration is that also spontaneous mutation could drive the acquisition of antibiotic resistant phenotypes with frequencies close or even higher to natural transformation (Goldstein *et al.*, 2005).

Even if promising technological advances will eliminate selectable marker genes from the plant product, through selectable marker free chloroplast engineering (Daniell *et al.*, 2001a; Daniell *et al.*, 2001b) or recombinase- excision (Ow, 2002), the other functional genes present in the latest generation of transgenic crops such as pharmaceutics will need to be assessed in respect to their potential fallouts on the environment to respond to legitimate societal concerns of biological safety. Nonetheless, the interest in investigating the issue of interkingdom gene transfer remains actual to fundamental research and firstly released GEPs fitted with selectable marker genes still represent the most valuable model available to scientists.

Some evidences of an influence of transgenic plants and plant litter on the composition of the plant-associated microbial communities emerged in a few studies. Effects were found for a variety of plants fitted with different transgenes. However, these effects were shown to depend on field site, seasonal variation and the technique chosen to assess the community. The changes

in microbial communities associated with growing transgenic crops are relatively variable and transient in comparison with some other well-accepted agricultural practices such as crop rotation, tillage, herbicide usage and irrigation. Since minor alterations in the diversity of the microbial community, such as the removal or appearance of specific functional groups of bacteria such as plant- growth promoting rhizobacteria, phytopathogenic organisms, or key organisms responsible for nutrient cycling processes could affect soil health and ecosystem functioning, the impact that plant variety may have on the dynamics of rhizosphere microbial populations and hence plant growth and health, as well as ecosystem sustainability requires further study. The extent of differences observed might be negative or positive and this should be studied in the long term effects of GEPs in rotation (considering each type of GEP as a specific individual with specific properties). Comparison should be made with other acceptable changes in agroecosystems, such as growing a novel non- transgenic plant or the use of a new agronomic practice. Ongoing and future environmental evaluation of the diversity of transgenic crops under development may pose a unique scientific challenge that, in addition, may provide an opportunity for an improved understanding of soil and plant microbial ecology.

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Chapitre 2

Chapitre 2

Avant-propos du chapitre 2

Tant dans un contexte fondamental de compréhension du potentiel évolutif bactérien que plus appliqué d'évaluation des risques de dissémination des transgènes des OGM aux bactéries du sol la recombinaison de l'ADN entrant avec le génome récepteur constitue une étape cruciale conditionnant le succès ou l'échec d'un événement de transfert de gènes en particulier par transformation naturelle. La littérature nous apprend que différents mécanismes sont en charge de préserver l'intégrité du génome en dressant de véritables barrières génétiques qui vont s'opposer à l'intégration de l'ADN hétérologue.

Ces mécanismes agissent directement sur l'ADN qui a réussi à pénétrer l'enveloppe bactérienne lors de la phase de compétence avec un rôle prépondérant pour le mécanisme de réparation des mésappariements de bases (MRS). Ce système intègre l'action de plusieurs enzymes chargées de repérer les appariements entre les deux types d'ADN (donneur et receveur) sur éventuellement de très petites zones d'homologie afin d'hydrolyser ces hétéroduplex préserver ainsi l'intégrité du patrimoine génétique.

Cependant, il a été montré à plusieurs reprises que dans les environnements naturels, des souches bactériennes présentent des défaillances de ce système MRS conduisant la bactérie dans un état qualifié de « mutateur ». Or, pour ces isolats les barrières à la recombinaison interspécifique sont affaiblies ce qui se manifeste par un pouvoir accru à intégrer de l'ADN non homologue mais aussi par des fréquences très élevées d'apparition de mutations ponctuelles. Plusieurs études ont en outre révélé que ces souches mutatrices présentaient des phénotypes de résistance aux antibiotiques.

Un stade mutateur chez une bactérie en contact avec l'ADN d'une plante transgénique est donc susceptible d'accroître la possibilité d'intégrer le gène hétérologue de la plante codant la résistance à l'antibiotique de sélection mais aussi de générer des mutants spontanés résistants au même antibiotique sans qu'il y ait eu transfert d'ADN. De plus dans le cas d'une bactérie naturellement transformable, la pénétration d'ADN végétal hétérologue pourrait saturer le système MRS conduisant un grand nombre de cellules à atteindre un stade mutateur.

Nos objectifs dans ce chapitre ont été de travailler sur cette hypothèse en étudiant le rôle de l'ADN végétal libéré pendant le processus de colonisation de la plante par *Ralstonia solanacearum*, bactérie phytopathogène appartenant au groupe des microorganismes

naturellement transformables. *Ralstonia solanacearum*, agent bactérien responsable de la maladie du flétrissement bactérien chez les Solanacées provoque une libération importante d'ADN lors de l'infection massive des tissus végétaux. Cet écosystème est donc composé d'une importante population bactérienne en phase de compétence se développant au contact d'importantes quantités d'ADN végétal. Nos objectifs ont été de déterminer si la pénétration de l'ADN hétérologue pourrait jouer un rôle dans l'altération du fonctionnement du système MRS par le biais de sa saturation. Les conséquences d'une inactivation du système MRS conduisant à un stade hyper mutateur pourraient être de 2 niveaux, accroissement du taux de mutations spontanées et affaiblissement des barrières s'opposant à la recombinaison hétérologue. Dans ce dernier cas, la possibilité d'une intégration du transgène par la bactérie mutatrice serait accrue. Ces deux hypothèses ont été vérifiées dans ce chapitre à l'aide d'expérimentations conduites in vitro sur des souches bactériennes isolées mais également dans les conditions naturelles produites par l'injection du pathogène dans la plante.

Chapitre 2

Natural transformation and induction of spontaneous mutants in *Ralstonia solanacearum*

I-INTRODUCTION

When evaluating possible implications of horizontal transfer of antibiotic resistance genes from plant to bacteria, considerable attention has been reserved to those bacterial species that establish a strict relationship with the plant and to their potential transformability. Among these for example, *Ralstonia solanacearum*, a naturally transformable bacterium that can develop the competence state both *in vitro* and during infection of its host (Bertolla and Simonet, 1999). Plant tissues, in which some symbiotic or pathogenic bacteria multiply actively, can offer favourable conditions for transformation-mediated gene transfers (Kay *et al.*, 2003). It has been hypothesised that the infection process could lead plant DNA molecules, released by decaying plant cells, to be in close contact with the invading and metabolically active bacterial cells. The traditional and genetic barriers to such interkingdom transfers, due to the molecular mechanisms preventing recombination with foreign DNA in bacteria, could be overcome with transgenic plant DNA because of the prokaryotic origin of the marker genes (Bertolla et al., 2000) and the prokaryotic origin of plastids in the case of transplastomic plants (Belzile, 2002). To this respect, the double status of pathogen and natural transformable bacterium, make of *R. solanacearum* an ideal candidate to study plant – bacteria interactions.

This bacterium is a devastating, soil-borne plant pathogen that naturally infects roots; it has a global distribution and an unusually wide host range. *R. solanacearum* specifically invades and highly multiplies in the xylem vessels. It has been studied both biochemically and genetically, and has long been recognised as a model system for the analysis of pathogenicity (Bertolla *et al.*, 1997; Salanoubat *et al.*, 2002). *R. solanacearum* is responsible for severe damages to many important crops, mainly plants of the *Solanaceae* family and bananas, in tropical and subtropical regions. In Western Europe, where several outbreaks of the disease were recently reported, the disease represents a serious threat (Poussier *et al.*, 2002). *R. solanacearum*

strain GMI1000 is pathogenic on tomato plants, on the model plant *Arabidopsis thaliana*. Strain K60 is pathogenic for *Nicotiana tabacum* plants (Salanoubat *et al.*, 2002). The frequency of natural transformation of *R .solanacearum* GMI1000 depends on the efficiency of DNA uptake following competence development (Bertolla *et al.*, 1997) and DNA sequence divergence between donor and recipient DNA. *R. solanacearum* belongs to the group of bacteria efficiently transformed only with DNA from closely related bacteria (Bertolla et al., 1997).

Figure 1. *R. solanacearum* causes southern wilt, a common and devastating plant disease that affects hundreds of species worldwide (e.g. tobacco in the picture on the right). The bacterium can destroy an entire crop once it infects the root system. It has as many as 100 'effector' proteins that help deliver and spread toxins to the plant (Salanoubat *et al.*, 2002).

Historically, during earlier investigations, the potential of *R. solanacearum* to act as a recipient of plant transgenes *in planta* before these sequences spread among the phytosphere or soil associated microflora was found null, mainly in consideration of the experimental system applied. Plant related aspects such as dilution of transgene in plant genome in model nuclear transgenic plants were called responsible for this negative evidence, since optimal conditions of homology with incoming DNA were provided in the recipient strain (Bertolla *et al.*, 2000). Then, however, further research conducted with transplastomic plants, considered as improved models due to their much greater content in transgenes (up to 10^5-fold copy number) could not allow the rescuing of any recombinant, simply just when the bacterium was exposed *in vitro* to plant DNA, suggesting that the limiting factor of the experience was that transformation frequencies were below the assay detection limit (10^{-9}) in the reason of the relative low transformability of this

bacterium (Mercier, 2003).However, in the course of additional investigations, when the bacterium was directly injected *in planta* to reach complete devastation of transplastomic tobacco plants harbouring the *aadA* transgene coding for streptomycin and spectinomycin resistance, antibiotic resistant colonies could be detected after plating of infected crushed plants suspension on selective solid media. However, following their replication and PCR amplification with specific primers targeting the chimeric regions, no transgenic signatures could be detected in bacterial isolates, suggesting these to be spontaneous mutants (Ceccherini, 2003).

These findings highlight that current assessments of the probability and the biological risk of dissemination of antibiotic resistance determinants from transgenic plants to environmental bacteria and deriving considerations should be made taking into account also other bacterial mechanisms that drive the development of antibiotic resistance phenotypes and their relative importance. For instance, spontaneous mutations could also drive the acquisition of an antibiotic resistant phenotype with frequencies close or even higher than natural transformation (Goldstein *et al.*, 2005).

Two main and antagonistic systems, the Mismatch Repair System (MRS) and the SOS system, regulate genetic bacterial stability by controlling the recombination based integration process. The Mismatch Repair System repairs replication errors such as base-mismatches and insertion–deletions by hydrolysing recombinant strands that contain excess mismatches, thus controlling recombination between divergent sequences and thereby limiting gene exchange amid unrelated microorganisms (Matic *et al.*, 1996). Consequently, inactivation of the *E.coli* MRS by disrupting *mutS, mutL, mutH* genes increases intergenic recombination between divergent DNA sequences which leads to a mutator phenotype. For instance, intergenic recombination beetween *E. coli* and *S. typhimurium* DNA was increased by more than 3 orders magnitude (Rayssiguier *et al.*, 1989). Additionally, non functional *mutS, mutL, mutH* genes make the bacteria incapable of repairing their lesions and generate strong mutator phenotypes displaying 10 to 1000 fold higher spontaneous mutation frequencies as expressed as the development of antibiotic resistant phenotypes (Kurusu *et al.*, 2000). Significant proportion of mutators was evidenced in populations of pathogenic and commensal *E.coli*, *Salmonella spp.*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* isolated from natural environments (Giraud *et al.*, 2001; Humbert *et al.*, 1995; LeClerc *et al.*, 1996; Zahrt and Maloy, 1997). Developing a mutator state is believed to increase the overall fitness of a bacterial population by increasing adaptation rate, the environment selecting the most adapted clones. (Matic *et al.*,

1996). In addition to MRS inactivation, induction of SOS response by stress factors such as UV irradiation also increases potential genetic plasticity by stimulating DNA rearrangements such as transposon excisions and gene transfer to the extent of eliminating genetic barriers between genera in some cases (Matic *et al.*, 2000)

The insurgence of mutators in our model bacterium might be driven by a different modulation of the two antagonistic systems guardians of cell genomic integrity and stability.
One the one hand it can be predicted that during active multiplication *in planta, Ralstonia* might be exposed to various environmental stresses among which, for example, the exposure to compounds released during plant cell decay and tentative defensive response: these might stimulate the bacterial SOS system, typically involved in rapid evolution and adaptability to challenging environmental conditions. On the other hand, the conspicuous amounts of DNA released during the plant decay process that would come in contact with infecting competent *Ralstonia* cells could be involved in driving the acquisition of a mutator phenotype by hampering the functionality of the MRS system. Particularly, this could be viewed as realistic in the light of literature data that report that homeologous DNA penetrating competent cells of *Streptococcus pneumoniae* would saturate the MRS (Humbert *et al.*, 1995), leading in this particular case to an increased interspecific recombination rate. Additionally, as the potentialities of contact between bacteria and DNA of various origins, coding not only for antibiotic resistances, exist in the environments, it is in this sense that studies on spontaneous mutations in strong plant infecting pathogens become important.

The inactivation of MRS could also enhance the rate of spontaneous mutation as expressed as the development of multiple antibiotic resistant phenotypes: it is in this context that we aimed at determining whether heterologous plant released DNA eventually uptaken by competent cells during the transformation process could trigger a mutator state in *R. solanacearum* by saturating the MRS system. Earlier mentioned preliminary experimental data conducted *in planta* and showing development of antibiotic resistant mutants were supportive of our hypothesis. In this study, a first setting of in vitro experiments was performed with the aim of determining if and to which extent the divergence of incoming DNA sequences in respect to recipient genomes could play a role in the generation of spontaneous mutants. Would there be a predictable effect of the type and degree of homology of transforming DNA on the acquisition of drug resistant phenotypes? Secondarily, we aimed at assessing whether the hypothesized generation of spontaneous mutants by saturation of the MRS was effectively dependent on the

dose of penetrating DNA as logically it could be predicted. A second hypothesis of environmental conditions or the bacterium physiological status being at the origin of an increased spontaneous mutations rate was also taken into account and will be discussed.

II-MATERIALS AND METHODS

II.1 Strains and transformation *in vitro*

R. *solanacearum* GMI1000 wt (Boucher *et al.,* 1985) was transformed *in vitro* on plates by the method described previously by Bertolla *et al*. (1997). Overnight cultures grown in 5 ml of B broth were harvested and resuspended in sterile water. An aliquot corresponding to 0.5% of the final culture volume was inoculated into liquid minimal medium (MM) supplemented with glucose and glycerol, and grown with constant shaking at 230 rpm at 28°C up to an OD_{580nm})of 0.8 (corresponding to ca 5.0×10^8 cells ml^{-1}). Fifty microliters of this cell suspension was directly mixed with 100, 250, 500 or 1000 ng of donor DNA of strain GMI1000 (homologue DNA) or DNA of nontransplastomic tobacco and *Burkholderia vietnamiensis* $TVV775^T$ (heterologue DNA) or with water for a negative control. The resulting suspension was used to seed polycarbonate membranes deposited on the surface of solid MM (MMG) and incubated for 48 h at 28°C. Bacterial cells were then harvested from the membrane surface and resuspended in 5 ml of MM, and aliquots were used to inoculate rich medium BG agar plates containing the appropriate antibiotics. Each transformation experiment was replicated three times and included determination of the spontaneous mutation rate by planting water-treated cells on selective media. The antibiotics used for the selection of mutant clones were rifampicin (50 µg ml^{-1}), nalidixic acid (30 µg ml^{-1}), spectinomycin (40 µg ml^{-1}) and gentamycin (10 µg ml^{-1}). Spontaneous mutation frequencies were expressed as the ratio between antibiotic resistant cfu over the number of total cfu exposed to the different DNA.

II.2 Transforming DNA

Genomic DNA used as homologue DNA was extracted from the R. *solanacearum* GMI1000 wt with the DNeasy tissue Kit (Qiagen).

Heterologous DNA were tobacco DNA and *Burkholderia vietnamiensis* genomic DNA**.** Non transplastomic tobacco leaves were chosen to obtain heterologue DNA. The DNeasy plant DNA miniextraction kit (Qiagen) was used to obtain plant DNA, according to the manufacturer's guidelines. Quantification of total DNA was done by spectrophotometric reading at OD_{260nm}. *Burkholderia vietnamiensis* TVV75 DNA was extracted according to the CTAB based DNA extraction procedure (Ausubel *et al.*, 1992). This bacterium DNA was chosen in view of its close relationship with *Ralstonia*, since formerly the *Burkholderia* genus included the *R. solanacearum* species (Yabuuchi *et al.*, 1992). The strain was cultivated in LBm agar or broth media.

II.3 *In planta* transformation

Ralstonia solanacearum GMI1000 (Salanoubat *et al.*, 2002), a pathogen for tomato plants, was cultured in rich BG medium (Boucher *et al.*, 1985), liquid or solid, at 28°C for 48 hours. Cells from an overnight culture (OD_{600} of 1.36) were rinsed with sterile water, centrifuged and resuspended again in sterile water; 200 µl of the bacterial suspension were inoculated into the stems of isogenic tomato plants cv. Marmande using a syringe. The number of cells, in the injected volume, corresponded to ca 1.0×10^6 cfu ml^{-1} for each of the three replicate plants.

The plants were left in a growth chamber with a photoperiod of 16 hours and infection was left to proceed at room temperature in humid conditions. After 6 days from the infection the stems were crushed and homogenised in sterile, distilled water with an Ultra-Turrax T25 homogenizer at 25.000 rpm (IKA-Werke GmbH and Co., Staufen, Germany). The numbers of spontaneous *R. solanacearum* mutants were determined by plating dilutions of the plant tissue suspensions on BG medium supplemented with the antibiotics and containing cycloheximide (200 µg ml^{-1}) to limit fungal growth and 1 % crystal violet (200 µl L^{-1}) to limit eventual contamination of gram positive bacteria. As a control, non infected tomato plants were used.

The antibiotics used for the selection of mutant clones were rifampicin (50 µg ml^{-1}), nalidixic acid (30 µg ml^{-1}), spectinomycin (40 µg ml^{-1}) and kanamycine (25 µg ml^{-1}).

II.4 PCR analysis of spontaneous mutants

Verification of the origin of spontaneous mutants isolated from the *in planta* inoculation experiment was conducted by RFLP analysis on the 16S rDNA amplified from antibiotic resistant clones. Colonies were replicated on antibiotic selective media and isolated with a sterile toothpick, resuspended in 50 µl of sterile ultrapure water and heat denaturated at 95°C for

10 min. Aliquots (1 µl) were amplified by standard PCR with the universal primers pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH' 5'-AAGGAGGTGATCCAGCC
GCA-3' as described in (Edwards *et al.*, 1989). Then the PCR products obtained were digested with *Hae*III or *Msp*I , run on a 1,5 % agarose gel and their electrophoretic profile was compared to the wild type strain GMI1000.

II.5 Assessment of spontaneous mutation rates in different media

In order to assess an eventual effect of the composition of the cultural medium on the development of spontaneous mutants, kinetics of spontaneous mutation were assessed in rich B broth and in minimal MM broth. An overnight culture of GMI1000 was harvested and used to inoculate 100 ml of fresh B broth or MM broth supplemented with glucose. The cultures were grown at 28°C during 48 hours and at different intervals they were harvested to determine in parallel their $OD_{600\ nm}$ and the frequency of spontaneous mutations by plating aliquots on solid media containing the same antibiotics utilized for the in vitro transformation assay. The spontaneous mutation rate was determined as the ratio of antibiotic resistant clones over the total population size.

III-RESULTS AND DISCUSSION

III.1 *In vitro* transformation

In a first series of experiments, the WT control strain R. *solanacearum* GMI1000, transformed with only ultrapure distilled water, showed a frequency of spontaneous mutation equal to ca 4×10^{-10} for each of the four antibiotics tested. Frequencies of mutations between different antibiotics were different during repetition of experiments, as in some cases no spontaneous mutants could be detected and therefore only those providing the most repeatable results have been discussed. According to our hypothesis, natural transformation with homologous DNA without any antibiotic resistance gene from *R. solanacearum*, would not hamper MRS functionality, whilst conversely heterologous DNA would saturate this system thus leading to increased development of mutators.

After natural transformation with homologous DNA the frequency of spontaneous mutation on rifampicin and on gentamycin averaging a mean order of magnitude of 10^{-8} cfu ml^{-1}

was higher than with the other antibiotics, whatever the concentration of DNA used. Similar results were observed when cells were transformed with heterologous DNA, independently of its origin. A slight dose effect could be seen when cells were transformed with 500 ng and 1000 ng DNA amounts in the case of rifampicin resistant mutants, similarly when cells were plated on gentamycin, the highest presence of mutants was induced with 500 ng of DNA, but the general trend suggested that appearance of spontaneous mutants was independent of the dose of DNA applied (table 1).

No significant effect imputable to the degree of homology of donor DNA with the recipient genome could thus be observed on development of spontaneous mutants, and slight differences in frequencies were most probably due to natural biological variability between different replications rather than being significant indicators of an alteration of the repair system. Namely spontaneous mutants were present no matter of which source of DNA cells were exposed to. Furthermore, in some successive experiments, the frequencies of mutation were of the same order of magnitude as those obtained for the control treated with water, indicating that probably other factors than incoming DNA could be involved in triggering the mutator phenotype. To this matter, preliminary investigations were performed in order to determine whether the cultural conditions could be implicated in the development of a mutator phenotype. Assessment of spontaneous mutation on two antibiotics (gentamycin and rifampicin) during the growth of GMI1000 strain in rich or minimal liquid medium revealed that in both cases mutation frequency was maximal during the early stationary phase (OD_{600} = 3.2), and 10 to 300- fold higher spontaneous mutation rates could be detected during the early exponential growth phase up to the begin of the stationary phase when the strain had been grown in minimal medium compared to rich B medium. Under these circumstances, it seems that the growth conditions in minimal medium are more prone to drive a mutational state, which would thus be rather associated with the physiological status of the bacterium.

These preliminary results appear consistent with studies on adaptive mutation that indicate similar trends in the development of spontaneous mutants during *in vitro* growth in other bacterial models, they would consent us to speculate that the enormous potential of adaptation to stressful conditions that this plant pathogen is to likely encounter in its habitat (in soil and *in planta*) might be due to spontaneous mutation. Such conditions were simulated *in vitro* by the minimal medium, whose composition is reported as faithful representative of apoplast conditions (Arlat *et al.*, 1992), and *in vivo* they might be represented by the vascular tissues, during first

stages of infection, when nutrient are probably still limiting. Similarly, expression of *Ralstonia* virulence determinants (*hrp* gene cluster) was driven selectively when the strain was grown in minimal medium (Arlat *et al.*, 1992), attributing to *in planta* conditions a pilot role in the modulation of the ensemble of plant genes.

DNA source	Selection	Quantity			
		100 ng	250 ng	500 ng	1000 ng
R. solanacearum strain GMI1000	Rif+	$< 4.5 \times 10^{-9}$	4.4×10^{-9}	$2.31 \times 10^{-8} \pm 1.6$	2.4×10^{-9}
	Gm+	9.17×10^{-8}	5.89×10^{-8}	$3.86 \times 10^{-7} \pm 2.2$	5.61×10^{-8}
B. vietnamensis strain $TVV75^{T}$	Rif+	8.82×10^{-10}	8.76×10^{-10}	$3.94 \times 10^{-8} \pm 4.4$	6.9×10^{-10}
	Gm+	1.10×10^{-7}	2.76×10^{-8}	$6.26 \times 10^{-7} \pm 4.6$	9.66×10^{-8}
N. tabacum cv. PBD6	Rif+	9.23×10^{-9}	2.04×10^{-9}	$6.61 \times 10^{-9} \pm 2.0$	1.8×10^{-9}
	Gm+	2.04×10^{-7}	1.84×10^{-8}	$1.03 \times 10^{-7} \pm 1$	3.16×10^{-8}
Negative control (water)	Rif+	$2.65 \times 10^{-8} \pm 2.2$			
	Gm+	$1.8 \times 10^{-7} \pm 1.3$			

Table 1. Spontaneous mutation frequencies on two antibiotics as determined after *in vitro* transformation of *R. solanacearum* with homologous and heterologous DNA. (rif+ =rifampicin 50 μg/ml; Gm+ =gentamycine 10 μg/ml)

III.2 *In planta* transformation

The plants infected by R. *solanancearum* GMI1000 showed the characters typical of this kind of pathology: the stem of the leaf became more and more brown, leaves were flaccid, while the control leaves not inoculated remained green and consistent.

At the end of the incubation period, the stems were crushed and homogenised and an aliquot of the homogenate was plated on selective medium in order to observe the presence of spontaneous mutants. The results showed that other opportunistic bacteria replaced the *Ralstonia* cells, maybe taking advantage from the disorganisation of the vegetable tissues caused by the pathogen infection. In particular, the detection based on phenotypic shape and on the 16S rDNA fingerprinting of the single colonies did not show the unique presence of *Ralstonia* clones. However, the bacterial populations on non selective medium isolated from the infected plants were numerous (1.3×10^8 cfu ml^{-1}), more than those present in the control plants (2.4×10^6 cfu ml^{-1}) where the vegetable tissue seemed intact. This might indicate that opportunistic development of endophytes and epiphytes might have been favoured, confirming the results from Kay and colleagues (2002). Namely, although plants had been grown in a growth chamber in cleanliness condition, they were not originally axenic.

Higher numbers of spontaneous mutants were isolated from plates containing the antibiotics rifampicin and gentamycin rather than spectinomycin and kanamycin.

However, the majority of 16S rDNA restriction profiles and in some cases the visual description of the colonies showed that the bacteria present in the infected stems were very different from those in non infected stems; thus two groups of culturable bacterial clones were present. It can be argued that some of the resistant clones could be spontaneous mutants, of some bacterial species which had grown opportunistically, induced by the presence of heterologous plant DNA.

IV- CONCLUSIONS

In this study a correlation between quantity and origin of cell uptaken DNA and the generation of spontaneous mutants could not be shown after *in vitro* exposure of competent *R. solanacearum* cells to nucleic acids. The hypothesis of a saturation of the Mismatch Repair System could, under these circumstances not be validated nor completely excluded since spontaneous mutants were present at detectable and similar frequencies even when the bacterium was cultured in minimal medium, utilized for the induction of competence. Thus, factors

potentially involved in the development of a mutator state in this phytopathogen are the chemical composition of the medium, characterized for its restricted nutritional composition, or intrinsic physiological characteristics of the strain.

However, high mutation rates were observed also following *in planta* inoculation, indicating that factors involved are many and different and that the complexity of spontaneous mutation in this species should be assessed at different levels (e.g. physiological, metabolic and genetic profiles) since it could reveal interesting insights on the development of antibiotic resistances in environmental bacteria. To this extent spontaneous mutation could be regarded as a frequent event, or at least of similar order of magnitude as natural transformation for *R. solanacearum* considering that its potential to be subjected to horizontal gene transfers *in situ* is estimated to be very low.

Indeed, genetic exchanges in bacteria usually involve a very small fraction of the genome, and the frequency of recombination observed in natural populations depends upon both the recombination rate and natural selection (Majewski *et al.*, 2000). Thus, if recombinants are selectively deleterious, they will be lost from the population; if they are selectively neutral, most may be lost but some may be retained; if they are selectively advantageous, they will be retained in the population at a higher frequency than the selectively neutral ones. Similar considerations can be applied to mutators. Most newly arising mutators either have no effect or are harmful, whereas only rare specific mutations are favourable during adaptation under particular selective conditions. In natural environments, the probability that recombinants are advantageous is low, but not abolished. And the probability is greater when organisms are confronted with environmental challenges. In those cases, there is greater opportunity for a new recombinant or spontaneous mutant to survive and to spread in the population.

In natural populations, high mutation rates are favoured during adaptation in spite of the high cost incurred by the generation of numerous deleterious mutations, bacterial mutators somewhat behaving as “genetic altruists” (Radman *et al.*, 2000). That is, when the successful mutator culture grows in nature, two possible scenarios can be envisioned owing to the effective possibility of genetic exchange with members of a similar bacterial culture. In one case, the adaptive mutation may be transferred from the mutator to a nonmutator and enjoy the benefit of a stable genome, thus the original mutator would be overgrown by the adapted nonmutator (which is exempted from mutational load). Alternatively, during genetic exchange with

nonadapted wild type, the mutator could acquire back the functional *mut* gene, becoming an adapted nonmutator which overgrows the adapted mutator and nonadapted wild type.

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Chapitre 2

Chapitre 3

Avant propos du chapitre 3

La deuxième partie expérimentale de ce travail de thèse a consisté à déterminer si la phytosphere pouvait fournir des niches écologiques dans lesquelles les conditions biotiques et abiotiques pourraient être favorables aux transferts horizontaux des gènes entre la plante et la bactérie comme cela a été suggéré par quelques études sur des échanges de gènes entre bactéries (de Lipthay *et al.*, 2001; Lilley *et al.*, 1994; Molbak *et al.*, 2003; Sørensen and Jensen, 1998). Nos travaux se sont focalisés sur différentes sphères de la plante, afin de détecter, quantifier et visualiser les transferts horizontaux des gènes interrègne dans le but de déterminer ceux parmi ces environnements qui pourraient constituer des « hot spots ».

Les échanges de gènes, en particulier par transformation nécessitent la multiplication bactérienne et le développement d'un état de compétence qui sont les pré requis à la transformation naturelle et l'intégration d'ADN étranger. Un tabac transplastomique, possédant un nombre de copies du transgène assez élevé en raison de l'intégration du transgène au niveau du génome des chloroplastes (il y aurait à peu près 7000 chloroplastes par cellule) a été utilisé comme modèle. Le partenaire bactérien est *Acinetobacter baylyi*, une γ-proteobactérie naturellement transformable et isolée du sol et pour laquelle des études antérieures ont précédemment démontré sa capacité à intégrer l'ADN transgénique pendant la croissance « opportuniste » dans une plante de tabac transplastomiques surinfectée par *Ralstonia solanacearum* (Kay *et al.*, 2002b). Dans ce chapitre, notre hypothèse se base sur la disponibilité et sur la présence d'ADN extracellulaire et de nutriments libérés au cours de la dégradation naturelle de la plante, éléments qui pourraient induire la croissance bactérienne, le développement de la compétence et enfin l'intégration de l'ADN dans cette bactérie. Du fait qu'il n'existe pas d'informations concernant l'exacte distribution de ces transferts des gènes *in planta* et que les fréquences détectées jusqu'à présent par les approches traditionnelles sont faibles ou nulles une nouvelle stratégie a été développée visant à détecter les transferts de gènes *in situ* en dépassant les contraintes liées aux approches traditionnelles basées sur l'isolement et la culture des recombinants sur milieu sélectif (approches destructives). La mise au point et les applications potentielles de cette stratégie seront abordées dans la première partie de ce chapitre. Dans la seconde partie, l'étude des transferts d'ADN de la plante à la bactérie a été menée dans des différents compartiments de la phytosphère pour confirmer ou infirmer leur rôle de « hot spots » en étudiant la croissance, le développement de l'état de compétence et la transformation naturelle *in situ*. C'est donc en ce point que la stratégie décrite dans la première partie trouvera

son application directe, permettant d'explorer les transferts des gènes entre plantes et bactéries à l'échelle bactérienne.

Les travaux décrits dans ce chapitre sont issue d'un collaboration, menée dans le cadre du projet européen « TRANSBAC » entre le Laboratoires d'Écologie Microbienne de l'Université de Lyon 1 et le Laboratoire DISTAM de l'Université de Milan . En particulier, la construction des souches bactériennes présentée dans la première partie à été publié sous forme d'article dans la revue Applied and Environmental Microbiology (Rizzi et al., 2008) et les experiences de tranformation naturelle et visualisation dans la residuesphère ont été publies dans la revue Applied and Environmental Microbiology (Pontiroli et al., 2009).

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Première partie:

A strategy for *in situ* localization of horizontal gene transfer by natural transformation

I-ABSTRACT

Bacterial natural transformation in complex environments has been previously studied by plating recipient bacterial reporter cells harboring in the genome recombinogenic sites that can replace or insert a selectable antibiotic resistance gene marker following exposure to the environmental naked DNA. This destructive approach does not allow to spatially localize transformants at the micro-scale level in the environment. Hence we designed improved bioreporter strains that allow for *in situ* localization of transformants, by physically coupling an optical reporter green fluorescent protein gene (*gfp*) with the selectable spectinomycin-resistance marker gene (*aadA*) in the genome of the highly transformable bacterium *Acinetobacter baylyi* strain BD413. A promoter-less *aadA* gene was fused in frame with a *gfp* gene downstream the *rbcL* gene of the tobacco plastid genome and cloned in the *lipBA* operon of the BD413 strain chromosome. The resulting strain BD413(*rbcL-ΔPaadA::gfp*) strain was used in transformation experiments using as donor a transplastomic tobacco DNA containing in the plastid genome the *aadA* gene with the bacterial promoter P*rrn* between the plastid *rbcL* and *aacD* genes. Following recombination in the *rbcL* and *aadA* loci, the replacement of the P*rrn* promoter allowed the expression of the *aadA::gfp* gene fusion in the recipient strain BD413(*rbcL-ΔPaadA::gfp*) and the restoration of the spectinomycin-resistant and fluorescent phenotypes. A 10^{-5} (transformants/recipients) transformation frequency, measured *in vitro* using a plasmid donor DNA, decreased with the increasing dilutions of the donor DNA recombinogenic sites, leading to a frequency of 8.4×10^{-10} using transplastomic leaf tissue homogenates, respectively.

II-INTRODUCTION

Of the three mechanisms involved in horizontal gene transfer (HGT) to bacteria, natural transformation has received the less attention due to the lack of knowledge on the factors regulating transformation and the difficulties to reproduce the conditions of a process that, according to genome sequence comparison, played a major role in shaping prokaryote evolution (Nielsen et al., 1998; Van Elsas et al., 2003; Ochman et al., 2000; Doolittle 1998; Wolf et al, 1999; Bertolla and Simonet, 1999; Koonin et al., 2001). The limited number of species that have been demonstrated to include at least one naturally transformable strain highlights such difficulty (de Vries and Wackernagel, 2004; Thomas and Nielsen, 2005; Gogarten and Townsend, 2005; Smets and Barkay, 2005.

The study of gene transfer by natural transformation received a major impetus by the question of possible transfer of recombinant DNA from genetically modified plants to bacteria (Ceccherini *et al*., 2003; de Vries and Wackernagel, 1998; de Vries *et al*., 2003; de Vries *et al*., 2004; Gebhard and Smalla, 1998; Kay *et al*., 2002; Nielsen *et al*., 1997; Tepfer *et al*., 2003). A main concern has risen about the potential unintended spread to bacteria of antibiotic resistance gene markers present in many transgenic plants (Gasson 2000, Van den Eede, 2004, EFSA report, 2004). Genetically modified plants represent a very interesting model to study HGT by natural transformation between different domains of life (Nielsen et al., 1998; Bertolla and Simonet, 1999).

Most of the studies of HGT by natural transformation in complex environments have been performed using bioreporter bacteria, i.e. naturally transformable strains genetically modified to have on a replicon or their genome sequences homologous to the donor DNA that included a gene coding for a selectable character, typically an antibiotic resistance gene. The antibiotic resistance gene can be either absent (Kay *et al*., 2002) or truncated (Gebhard and Smalla, 1998) but it is replaced by homologous recombination with the same functional gene or its flanking regions in the environmental donor DNA. After exposure of the bacteria to the donor DNA the cells are plated in the presence of a specific antibiotic, and only those cells that acquired a functional antibiotic resistance gene by natural transformation can grow in the selective medium.

A major limitation of this strategy is the culturing step. By plating, samples are destroyed and no information can be obtained about the specific localization of the gene transfer or about the interaction of the recipient cells with the plant material including the donor DNA. Hence it is

not possible to localize micro-hot spots of transformation in a complex environment and analyse the microenvironments conducive for the transformation events.

To overcome such limitation we designed a genetic construct in which a gene coding the resistance to spectinomycin (*aadA*) was fused in frame with an optical reporter gene coding for a green fluorescent protein (*gfp*) so that, when expressed, the recombinant gene produced a single protein with two functional domains, the first determining the antibiotic resistance phenotype and the second conferring fluorescence to the cell. A promoter-less version of the *aadA::gfp* gene fusion was used to construct a bioreporter derivative strain of *Acinetobacter baylyi* BD413 (Vaneechoutte *et al*., 2006), named BD413(*rbcL-ΔPaadA::gfp*), that can reveal natural transformation following the exposure to the DNA of a transplastomic tobacco DNA harboring in the plastid genome a functional *aadA* gene cassette downstream the *rbcL* gene (Kay et al., 2002). In order to mantain two recombination sites that can mediate integration of the *aadA* promoter of the transplastomic tobacco in the genome of strain BD413(*rbcL-ΔPaadA::gfp*), and hence the expression of the protein fusion, the promoter-less *aadA::gfp* cassette was cloned downstream the *rbcL* gene at a chromosomal level in strain BD413 *lipBA* operon (Kok *et al*., 1999). Following exposure to transplastomic tobacco DNA *A.baylyi* strain BD413(*rbcL-ΔPaadA::gfp*) was able to integrate by natural transformation the promoter and express the *aadA::gfp* fusion protein. The functionality of the new bioreporter strain was evaluated with different types of donor DNA *in vitro* and on mechanically damaged tobacco leaf tissues. Potential applications of the construct are discussed.

III-MATERIAL AND METHODS

III.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* strain XL1-Blue MR and *A. baylyi* strainBD413 were grown in Luria Bertani (LB) medium, supplemented with the appropriate antibiotics, at 37°C and 30°C, respectively. For both *E. coli* and *A. baylyi* BD413 the final concentrations of kanamycin and spectinomycin were 50 µg/mL and 100 µg / mL, respectively. Selective media for *A. baylyi* BD413 and derivative strains contained also rifampicin (50 µg / mL) (Sigma, Milan, Italy) .

III.2 Plant material and growthconditions

The plant material used in this study was a transplastomic tobacco (*Nicotiana tabacum*) containing in the plastome the *aadA* marker cassette (1.3 kb) between the genes *rbcL* and *accD* (Kay *et al.,* 2002) and its parental non-transgenic counterpart. Tobacco seeds were germinated in the dark in 1% (w/v) sterile plant agar, melt in Hoagland solution (1:1, supplemented with 4 ppm of $FeCl_3$). In the case of transplastomic seeds, the agar was supplemented with spectinomycin (100 mg / mL). After germination, plants were transferred in the light and grown in hydroponic solution (Hoagland 1:1) for about 15 days (28°C, 12 h photoperiod, 70% relative humidity).

III.3 DNA manipulations techniques

DNA manipulations were performed according to standard protocols (Sambrook *et al*.,1989). Plasmid DNA was prepared from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen, Milan, Italy). Preparative PCR were performed with the high-fidelity enzyme *Pfu* DNA polymerase (Stratagene, Milan, Italy) and DNA fragments generated by PCR were purified using the PCR Purification kit (Qiagen, Milan, Italy) or separated by gel electrophoresis and eluted from agarose gel with a gel extraction kit (Qiagen, Milan, Italy). Agarose gel electrophoresis was performed in Tris-borate-EDTA buffer and gel images were recorded under UV light with the Gel Doc 2000 image system (Biorad, Milan, Italy). Restriction enzymes were commercial preparations and were used as specified by the supplier (Amersham Pharmacia Biotech, Milan, Italy).

III.4 Transforming DNA

Plasmid DNA was purified with the appropriate kit (Qiagen). Total tobacco DNA extraction from leaf or root tissues was carried out by finely grounding in a sterile mortar with liquid nitrogen. Three grams of the resulting powder were transferred into a sterile 50 mL falcon tube, mixed with 5 mL of phenol:chloroform (1:1) and centrifuged at 7000 × g for 10 min at room temperature. After transferring the aqueous phase to a new tube, one volume of isopropanol was added to the samples, mixed and centrifuged at 7000 × g for 20 min at 4°C. The DNA pellet was briefly air dried room temperature, resuspended in one mL of sterile water and 500 µl of 3M sodium acetate were added. DNA from the aqueous phase was precipitated with one volume of isopropanol, centrifuged, washed with 70% ethanol and resuspended in 200 µl of

sterile water. 2 µl RNase A (10 mg / mL) were added to the DNA solution, incubated for 1 h at 37°C and stored at -20°C until use. Extracted DNA was quantified by agarose gel electrophoresis (1.5% agarose) using lambda DNA (Sigma, Milan, Italy) as a molecular weight marker. Fresh tobacco leaf homogenates were prepared as following. Five grams of leaf tobacco were ground in a mortar with liquid nitrogen, transferred into a sterile tube and 10 mL of distilled water were added. After 30 min of incubation on ice, the plant material was centrifuged (10000 × g, 15 min, 4°C) and the resulting supernatant was filtered through a 0.2 µm filter (Millipore, Milan, Italy).

III.5 Construction of *aadA::gfp*-based constructs for cloning in *A. baylyi* BD413

Plasmid pZR80-2(*aadA::gfp*), carrying the *aadA::gfp* gene fusion, was constructed in a triple ligation step from three fragments. A 1292-bp fragment, comprehensive of the *aadA* gene and the upstream P_{rrn} promoter was amplified by PCR from pCEA (cf. page x) as template DNA using primers aadA-F (5-GCAGCAGTGGACGTTTTGGATAAG-3) and aadA-R (5-ACTCATGGCGCCGACTTTAAGACCTTCTACTAGCTCCAATTTTCCTTCAACAAGTTCTTTGCCCACTACCTTGGTGAT-3') which includes an amino acid linker sequence (underlined) and a *Nar*I restriction site (double underlined) with 5'- overhang. The 930-bp fragment, containing the red-shifted *gfp* gene and the transcription terminator T_{rrn} was obtained by standard PCR using p*PnptII::gfp* (Stiner and Halverson, 2002) as template, using primers GFP-F (5'-TATTATAGGCGCCATGAGTAAAGGAGAAGAACTTTTCACTGGA
GTTGTCCC-3'), carrying a *Nar*I restriction site (underlined), and Tend-R (5'-CCAATTCCTGGCAGTTTATG-3'). The two fragments, digested with *Nar*I, were ligated into pZR80-2 digested with *Stu*I, yielding plasmid pZR80-2 (*aadA::gfp*) that carries the *aadA::gfp* fusion into the *A. baylyi lipBA* operon and a functional *aphA3* marker (Kok *et al*., 1999). The *aadA*::*gfp* fusion protein contains the two functional domains of antibiotic resistance and GFP joined together by a 17 amino acid residues linker (ELVEGKLELVEGLKVGA).

The expression of the *aadA::gfp* gene fusion in the pZR80-2(*aadA::gfp*) was inactivated by deleting the *Prrn* promoter. A P*rrn*-containing 461 bp fragment was eliminated from pZR80-2(*aadA::gfp*) through *Eco*RI and *Bam*HI restriction digestions. The entire plastidic *rbcL* gene was amplified with standard PCR by using plasmid pCEA as template and primers ChloroEcoF (5'-ATTATTGAATTCGGTAGAGCCGTTTATGAA-3') and RbclBamR (5'-CTTTCTATTGTTGTCTTGGATCGGATCCAATAAT-3'), carrying a *Eco*RI and *Bam*HI restriction site (underlined), respectively. After digestion with *Eco*RI and *Bam*HI, the *rbcL*-

containing 1148-bp fragment was cloned into the *Eco*RI-*Bam*HI-digested pZR80-2(*aadA::gfp*), yielding plasmid pZR80-2(*rbcL*-ΔP*aadA::gfp*).

III.6 Construction of *aadA::gfp*-tagged *A. baylyi* BD413 strains

In order to introduce inactive *rbcl-aadA::gfp* cassettes in the chromosome of *A. baylyi* BD413 in the *lipBA* operon, DNA fragments, containing the different *lipB-rbcL-aadA::gfp-aphA3-lipA* regions prepared as explained in the previous paragraph, were amplified from pZR80-2(*rbcL*-ΔP*aadA::gfp*) clones, with primers LipB1 (5'-TGCAGGGCTGTTCGGCTCAG-3') and LipA (5'-TTATAGATTTTGACCTTTGAGACG-3'). The amplified DNA fragments were introduced in *A. baylyi* BD413 strain by natural transformation and selection on kanamycin (conferred by the *aph3A* cassette). *A. baylyi* BD413 transformants that were sensitive to spectinomycin and did not show GFP fluorescence were obtained and named strain BD413(*rbcL*-Δ*PaadA::gfp*). The presence of the desired cassettes in the *A. baylyi* BD413 recombinants was confirmed by PCR, standard restriction analysis and by sequencing. The constructed strains were transformed with linearized plasmid pCEA to test the capability of restoring the *aadA::gfp* gene cassette functionality.

III.7 *In vitro* marker rescue experiments

Marker rescue transformation experiments were basically carried out by filter transformation (Nielsen *et al*., 1997, Nielsen *et al*., 2004). Competent cells of the reporter strain BD413(*rbcL*-Δ*PaadA::gfp*) strain were obtained by harvesting late-log phase cells (O.D. 0.8-0.9) grown in LB medium supplemented with rifampicin and kanamycin. The cells were washed once, resuspended in saline added of 15% (w/v) glycerol and stored at -80°C. One hundred µl of frozen competent cells (ca 3.4×10^7 CFU) were mixed with 1 µg of pCEA or 5 µg of purified plant DNA or 100 µl of fresh crushed leaf homogenate. The mixture was placed on a GSWP nitrocellulose membrane filter (Millipore, Milan, Italy) placed on top of a LB agar plate and grown for 24 h at 28 °C. The bacterial layer was resuspended in 4 mL of saline and various serial dilutions plated on LB agar plates supplemented with rifampicin (50 µg / mL) and kanamycin (50 µg / mL) for total count or rifampicin (50 µg / mL), kanamycin (50 µg / mL) and spectinomycin (100 µg / mL) for enumeration of transformants. The number of colonies was determined after 72 h of incubation. The transformation frequency was determined as the ratio of transformants to the total count. Each assay was performed at least in triplicate. Controls were

made with water and non transgenic tobacco DNA. The detection limit of transformation was the reciprocal value of the total recipient cells in the transformation assay. The restoration of *aadA::gfp* cassette promoter in antibiotic-resistant transformants was assessed by PCR analysis with primers Promo F (5'-ATCTTTCTATTGTTGTCTTGGAT-3') and Promo R (5'-GGTCACCGTAACCAGCAAATCAA-3'), designed upstream and downstream of the deleted promoter. In the presence of the promoter, this PCR product gives a 333 bp fragment, while its size is 190 bp in the recipient strain. Transformants were also checked for restoration of fluorescence by examining cell suspension under a fluorescence microscope.

IV-RESULTS

IV.1 Construction of a *A. baylyi* BD413-based marker rescue system

In order to develop a non-destructive system for the *in situ* detection of horizontal gene transfer by natural transformation, a marker rescue cassette was designed (Fig. 1) and inserted in the chromosome of *A. baylyi* BD413 as recipient bacterium. The construct was designed using as a donor DNA model the *aadA*-tagged plastid genome of a transplastomic tobacco (Fig. 1). The developed reporter strain carries into the *lipBA* operon an inactive *aadA*::*gfp* gene fusion downstream the chloroplast gene *rbcL* that in the donor tobacco is just upstream the *aadA* cassette. The expression of the *aadA*::*gfp* gene fusions in BD413 rwas inactivated by silencing the expression of the fused protein through a deletion of the P_{rrn} promoter that is present in the tobacco chloroplast genome. Homologous recombination by a double crossing over with plant DNA in the *rbcL* and the *aadA* loci gives rise to insertion of the P_{rrn} promoter that allows the transcription of both *aadA* and *gfp* domains of the *aadA::gfp* gene fusion and the restoration of spectinomycin resistance and fluorescence phenotypes. Detection of transformants was accomplished by plating in selective-medium as well as by direct cell fluorescence observation *in situ*. As depicted in Fig. 1, the recipient bacterium was provided with two regions homologous to the chloroplast DNA, respectively of 0.8-1.1 kb and 0.5-0.8 kb in the left (*rbcL*) and the right hand side (*aadA*) of the construct. The donor DNA docking site is at chromosomal level to maximize genetic stability in environmental applications and to reduce the risk of transfer to other microorganisms. To ensure single cell detection of cells containing a single copy of the *gfp* gene, we used a red-shifted *gfp* mutant with enhanced green fluorescent intensity and the constitutive ribosomal promoter P_{rrn} (Errampalli *et al*., 1999).

The pZR80 plasmid (Kok *et al*., 1999), modified by deleting the ampicillin resistance gene *bla*, was used for targeting the *aadA*::*gfp* gene fusion in the *A. baylyi* BD413 chromosome. The functionality of the reporter strain was tested using pCEA plasmid as donor DNA. This plasmid is not replicative in *A. baylyi* BD413 and contains tobacco transplastomic sequences and the *aadA* gene inserted between *rbcL* and *accD* plastid genes. Transformants grown on selective medium, showed in all cases a fluorescent phenotype when donor plasmid was linearizedeven if most of them exhibited a reduced fluorescence, thought clearly distinguishable from the control.

IV.2 Transformation frequencies of marker rescue experiments *in vitro*

The evaluation of the transformation frequencies of strain BD413(*rbcL*-Δ*PaadA::gfp*) was performed by using plasmid pCEA as donor DNA, total DNA isolated from transplastomic tobacco leaf or root tissue and transplastomic tobacco leaf homogenate (Table 2). With pCEA plasmid as donor DNA transformation frequencies per recipient cell were in the order of 10^{-5} (transformants/recipients), whereas with transplastomic tobacco DNA extracted from leaf tissue frequencies decreased by 3 orders of magnitude, as expected by the decrease of available donor sites (10^{9} with plasmid and 10^{11} with plant DNA) and by the effect of competing non-target DNA (Kay *et al*., 2002). Plasmid linearization by *Sca*I did not appreciably change transformation frequencies that were of 1.8 (± 0.4) × 10^{-5} and 1.4 (± 0.4) × 10^{-5}, with circular and linearized plasmid, respectively.

When the reporter strains were exposed to DNA extracted from root tissue transformation in the presence of LBm medium frequencies were of 3.5 (± 2.9) × 10^{-9}, about one-tenth of the value obtained with leaf DNA. Transformation frequencies using as donor DNA aqueous extracts from transplastomic tobacco leaf homogenate, was 10^{-10}.

V-DISCUSSION

Horizontal gene transfer of naked DNA to bacteria has been investigated by marker rescue transformation using suitable recipient bacterial strains specifically prepared to detect DNA transfer event (Gebhard and Smalla, 1998; De Vries and Wackernagel, 1998; Kay et al., 2002). Marker rescue transformation is based on a recipient bacterium containing the same antibiotic resistance gene marker present in the donor DNA, but inactivated (e.g. by an internal deletion). Following exposure of the recipient bacterium to external naked DNA, plating in presence of the antibiotic easily allows to detect transformants resulting from two crossover

events (Nielsen *et al*, 1997; Gebhard and Smalla, 1998; de Vries and Wackernagel 1998; Kay *et al*., 2002). This approach is useful and, despite it uses an artificial reporter strain specifically predisposed to highlight the transformation events, it allowed to assess transformation frequencies and efficiencies in different conditions simulating natural environments (Gebhard and Smalla, 1998; Nielsen *et al.,* 2000; Kay *et al*., 2002; Tepfer *et al*., 2003; de Vries *et al*., 2003; de Vries *et al*., 2004), and hence allowed to define values of these important parameters to be used in risk assessment models for example of the gene flow from transgenic plants to bacteria (Nielsen and Townsend, 2004). However this approach relies on transformants that could be cultured, whereas it is not known if the entire recipient cell population can grow in plates following transformation. Moreover, plating provides limited information on the localization of the transformation events in the environment at a microscale level.

Here, the traditional marker rescued transformaiton (MRT) assay takes advantage from the use of the *gfp* as a reporter gene physically linked with the selectable gene marker *aadA*. The expression of the fusion protein is inactivated in the reporter strain by silencing transcription. When transcription is restored following homologous recombination with donor DNA and integration of the upstream promoter the antibiotic resistance and the fluorescence phenotypes are activated allowing selective plating as well as *in situ* detection of the transformants.

By schematizing, in the developed reporter strain different functional domains can be recognized: i) a recombinogenic domain (*rbcL* and *aadA* genes); ii) a selection domain (*aadA*), permitting the enrichment of transformants by subculturing in the presence of the antibiotic; iii) a reporter domain (*gfp*), allowing for the detection and localization of transfer event directly *in situ*. The developed system differentiates from all MRT assays developed by now, since it is not destructive, *i.e*. does not require transformant cell extraction from the environmental sample and culturing, therefore provides additional information about the occurrence of HGT in natural habitats *in situ* and at a microscale level. Another advantage of such a approach is the visualization of all the transformants independently from the assumption that all transformants can be cultured in a selective medium. Another advantage of this strategy is the highly specific DNA monitoring excluding false positives due to natural diffusion of antibiotic resistance genes in the environment or the phenotypic resistances not mediated by a specific antibiotic resistance protein.

Regarding transformation frequencies of the reporter strains BD413(*rbcL*-Δ*PaadA::gfp*), *in vitro* experiments indicated lower values compared to determinations previously reported by some authors for two-side homologous transformation with the *aadA* gene inserted in transplastomic tobacco (Kay *et al.,* 2002; de Vries *et al.,* 2004). In the presence of homologous

regions with length similar to those used in our constructs, observed transformation frequencies two orders of magnitude higher than us. The frequencies we measured are in the same order of those measured by Ghebard and Smalla (1998) by using the same filter protocol. Factors that could influence transformation efficiency are the location of homologous recombination sites or a possible slight toxic effect of GFP protein (Dandie et al., 2001). Concerning recombination site location, it has been shown that recipient strains with chromosomal docking sites are as efficient as strains with the docking sites on plasmid replicons (data not shown; De Vries et al., 2003). When the reporter strains were exposed to DNA extracted from root tissue transformation in the presence of LBm medium frequencies were of 3.5 ($\pm$ 2.9) $\times$ 10^{-9}, about one-tenth of the value obtained with leaf DNA. This is in accordance with previous results (de Vries et al., 2004) and with the concept that plastids are more numerous in green tissues than in roots. Transformation frequencies using as donor DNA aqueous extracts from transplastomic tobacco leaf homogenate, was 10^{-10}, confirming the capacity of the strain to transform in presence of cellular components like cell debris, proteins and polysaccharides (Gebhard and Smalla 1998; Kay *et al.*, 2002).

The developed reporter strain could provide a suitable tool to study in more detail sites potentially conducive for bacterial gene transfer activity, such as rhizospherethe phytosphere, soil, or the gut of animals directly *in situ*. The use of such model reporter could offer through microcosm and field studies a deepest insight into factors that affect HGT by natural transformation, e.g. availability of DNA, physiological state of bacterial cells and their ability to develop competence, effects of competing or promoting organisms, etc. For example, in the case of the study of HGT from transgenic plant tissue to bacteria, direct microscopic observations of transformants could evidence particular sites where sufficient amounts of donor DNA for transformation are released. For example in the rhizosphere, this could be the case of the sloughing off of root cap cells that accompanies root growth (Tepfer *et al*., 2003), or root wounds, where DNA released, coupled with a relatively high concentration of nutrients, like root exudates or released plant cell content might favor DNA uptake and integration and induction of bacterial competence. Tepfer *et al* (2003) recently showed that the root physiological state can influence HGT to bacteria, since root pre-treatment in sterile water or in medium deficient in micronutrients determined high frequency of transfer, though no root damage was visible upon microscopic examination.

The presence of plant pathogenic bacteria, such as *Ralstonia solanacearum*, has been shown to favor the colonization and competence development of *A. baylyi* BD413 as a co-infecting opportunistic organisms *in planta* (Kay *et al*., 2002). However, plating technique gave

no information about possible preferential microsites of HGT event, and moreover, was not able to discriminate between isolated bacteria resulting from independent transfer events or from a clonal multiplication of rare transformants (Kay *et al*., 2002).

The phyllosphere that has been revealed as a potential hot spot for conjugal transfer between bacteria (Normander *et al*.,1998 ; Bjorklof *et al*., 1995), it is thought to be conductive also to transformation transfer, even though, up to now, it has never been investigated (Nielsen *et al*., 2001). Similarly to rhizosphere, the phyllosphere is colonized by a large number of bacteria (10^6-10^7 cells/cm^2, 10^5 CFU/g), thus constitutes a very large microbial reservoir for HGT from genetically modified plants to bacteria, considering the 90 millions hectares cultivated with genetically modified plants worldwide (Clive 2005). Complexity of microbial community in the phyllosphere has been evidenced in a pioneering study (Yang *et al*., 2001), but the role of epiphytes and endophytes on transgene transfer potential needs to be still evaluated. Although the phyllosphere is not considered the primary environmental niche of *A. baylyi* BD413, different *Acinetobacter* sp. strains have been isolated as epiphytic bacteria (Yang *et al*., 2001) and moreover, colonization on leaves of non-epiphytic bacteria under humid conditions was also demonstrated (Brandl *et al.*, 2002; O'Brien *et al.*, 1989). The reporter strains presented here could be useful as model for the detection of gene transfer event in conditions of release of plant DNA, like wounds determined by plant-feeding mesofauna.

Soil is also an important potential environment for natural transformation. DNA adsorbed on clay mineral surfaces is protected against DNases and available for transformation of naturally competent bacteria (de Vries and Wackernagel, 2004). The use of the GFP-tagged reporter strains presented here could allow to non-invasively observe the occurrence and rate of transformants on clay or soil particles.

In addition, the present system could be improved in order to provide also quantitative information. By tagging the recipient strain with a dual-labelling approach, as described for *in situ* monitoring of conjugation (Nancharaiah *et al.*, 2003), the differentiation of donors and trasformants is allowed. By chromosomally labeling the recipient strain also with dsRed fluorescent protein, transformed cells, expressing both green and red fluorescent proteins, should appear visualized in yellow, whereas non-transformed cells remain red, thus permitting to microscopically estimate *in situ* transfer frequencies without cultivation.

A weak point in this approach is the detection limit since transfer rates from genetically engineered plants to bacteria are rather low. In this case it may be necessary to examine by fluorescence microscope a discrete number of samples in order to detect transfer event. However, it should be straightforward when studying HGT between bacteria where relatively

high amount of free DNA becomes available as a result of cell death or autolysis. In this context, Nielsen *et al.* (2000a) showed in microcosm studies that cell lysates of kanamycin-resistant donor bacteria were capable of transforming the kanamycin-sensitive recipient *A. baylyi* BD413(pFG4) restoring the deleted marker gene, and concluded that natural transformation might provide populations of *Acinetobacter* cells of a mechanism for generating genetic variability (*e.g.* mosaic genes) by taking up chromosomal DNA released from soil bacterial donors. On the other hand, in a number of species gene-exchange by natural transformation is provided by a well-controlled mechanism of autolysis. For example, in *Streptococcus pneumoniae* DNA is actively released from cells during competence development by competence-induced lysis of a subfraction of the cell population (Steinmoen *et al*., 2002). Localization of transformation events by GFP activation could enable to elucidate the participation of streptococci in gene exchange when located in biofilms in sites such as the oral cavity and the nasopharynx. To this aim, similar constructs, providing sequence homology between donor and recipient and GFP expression upon transformation, should be designed.

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TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Bacterial strains		
E. coli XL1-Blue	*RecA1, endA1, hsdR,* Tc^r	Stratagene
A. baylyi BD413 (ADP1)	Rif^r mutant of BD4	Juni, 1972 (ATCC 33305)
A. baylyi BD413 *aadA::gfp*	BD413 containing chromosomally into the *lipBA* operon the fusion *aadA::gfp;* Rif^r, Km^r	This study
A. baylyi BD413(*rbcL-ΔPaadA::gfp*)	BD413 containing chromosomally into the *lipBA* operon the cassette *rbcL-ΔP-ΦaadA::gfp* with *aadA::gfp* fusion inactivated by P_{rrn} deletion; Rif^r, Km^r	This study
Plasmids		
pCEA (named also pLEP01)	Cloning vector, containing *aadA* gene between *rbcL* and *accD,* Amp^r ,7.0 kb	Kay *et al*., 2002
pZR80-2	pZR80 derivative, integrative vector at *lipBA* operon site, Km^r, 4.9 kb	DISTAM
pZR80-2 *aadA::gfp*	pZR80-2 containing the fusion *aadA::gfp,* Km^r, Spc^r, 7.2 kb	This study
pZR80-2 (*rbcL-ΔPaadA::gfp*)	pZR80-2 deleted of P_{rrn} upstream of *aadA::gfp* and with a 1.1-kb fragment containing *rbcL,* Km^r; Spc^s, gfp negative, 7.8 kb	This study

[a] Tc^r, tetracycline resistance; Rif^r, rifampicin resistance; Amp^r, ampicillin resistance; Spc^r, spectinomycin resistance; Km^r, kanamycin resistance.

TABLE 2. Transformation experiments of *A. baylyi* BD413(*rbcL-ΔPaadA::gfp*)[a] exposed to plasmid pCEA and to different sources of transplastomic donor DNA *in vitro*

Donor DNA	Amount of material used	No. of donor genes per assay	No. of transformants per assay[b]	Transformants per target gene[b]	Transformation frequency[b]
Circular pCEA plasmid	1μg DNA	1.3×10^{11}	$(1.0 \pm 0.4) \times 10^{5}$	$(0.7 \pm 0.3) \times 10^{-6}$	$(1.8 \pm 0.4) \times 10^{-5}$
Linear pCEA plasmid	1μg DNA	1.3×10^{11}	$(1.3 \pm 0.6) \times 10^{5}$	$(1.0 \pm 0.4) \times 10^{-6}$	$(1.4 \pm 0.4) \times 10^{-5}$
Transplastomic total DNA from leaf tissue	5μg DNA	5.5×10^{9}	$(1.3 \pm 0.2) \times 10^{2}$	$(0.2 \pm 0.03) \times 10^{-7}$	$(2.4 \pm 0.4) \times 10^{-8}$
Transplastomic total DNA from root tissue	5μg DNA	n.d.[c]	$(1.8 \pm 1.3) \times 10$	n.d.[c]	$(3.5 \pm 2.9) \times 10^{-9}$
Transplastomic leaf homogenate	500 mg[b]	n.d.[c]	$(3.2 \pm 1.9) \times 10$	n.d.[c]	$(8.4 \pm 2.2) \times 10^{-10}$

[a] Recipients were 3.4×10^{7} CFU. In the case of homogenate reported data comes from ten pooled assays.
[b] Data are means ± standard deviations of three independent experiments.
[c] n.d., not determinable.

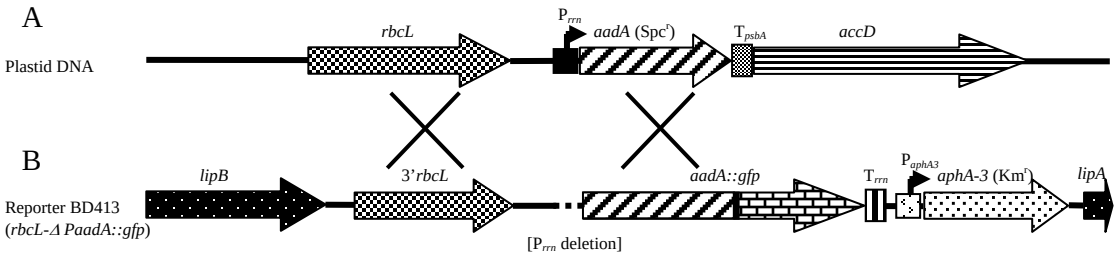

Figure 1. Schematic representation of the marker rescue system for biomonitoring DNA transfer from transplastomic tobacco to the developed reporter strains *A. baylyi* BD413(*rbcL-ΔPaadA::gfp*). (A) Transgene cassette in tobacco plastome. The transgene cassette inserted in the tobacco plastome contains the marker gene *aadA* under control of a modified plastid ribosomal RNA operon promoter, P_{rrn} and the 3' region of the plastid *psbA* gene (psbA3'). The cassette is flanked by plastid genes *rbcL* and *accD*. (B) Recipient cassette in BD413(*rbcL-ΔPaadA::gfp*). The recipient strain contains the cassette *rbcl-ΔPaadA::gfp-aphA3,* introduced by double crossing-over at the level of *lipA* and *lipB* genes into the BD413 strain to generate the BD413(*rbcL-ΔPaadA::gfp*) strain, kanamycin resistant (*aphA3*). The *aadA* gene, which is both recombinogenic and selective domain is fused by an amminoacidic linker to the reporter domain gfp. The restoration of *aadA::gfp* gene fusion requires a double cross-over event of homologous recombination (indicated by X) followed by the integration of promoter P_{rrn} in strain BD413(*rbcL-ΔPaadA::gfp*).

The interruption of the line in the recipient strains indicates the position of the deletion.

Deuxième partie:
Hot spots for horizontal gene transfer from transplastomic plants to *Acinetobacter baylyi* in the phytosphere

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I-ABSTRACT

The different plant compartments, including aerial surfaces, roots and internal tissues, are colonized by numerous and diverse bacterial species. We have recently reported that plant DNA released during the degradation process of plant tissues can persist and remain biologically active for significant periods of time, suggesting that soil or plant-associated bacteria could be in direct contact with plant DNA. In addition, nutrients released during the decaying process may provide a copiotrophic environment conducive for opportunistic microbial growth. Our objectives were to determine whether the natural or pathogen-induced degradation of plant tissues could promote i) opportunistic bacterial growth, ii) development of a competence state and iii) possible acquisition of exogenous plant DNA by bacteria through natural transformation. Transplastomic tobacco plants harboring the *aadA* gene (conferring resistance to Streptomycin and Spectynomycin) were used as the plant model. *Acinetobacter baylyi* BD413 carrying the chloroplastic DNA sequences flanking the transgene (pBAB2) or a promoter-less *aadA::gfp* fusion were used as recipient strains, to quantify and visualize transformants, respectively. We report here that opportunistic growth occurs on decaying plant tissues and that a significant proportion of the bacteria developed a competence state. Quantification of transformants clearly suggests that the phytosphere could constitute a hot spot for horizontal gene transfer between plant and bacteria in the environment. Furthermore, the non disruptive approach developed allowed for the visualization of transformants *in planta*, and provided new insights on environmental factors influencing HGT on plant tissues.

II-INTRODUCTION

Despite the yearly growing number of acreage planted with genetically modified plants worldwide, the ongoing debate on their ecological safety is controversial and gave impetus to various investigations, of which the putative horizontal gene transfer of recombinant DNA from plant to bacteria (HGT) constitutes a main issue. Such interest arose either to answer to practical societal concerns especially related to the potential dissemination of antibiotic resistance determinants in the environment but also questions of fundamental order (evolution-related). The different parts of a plant (globally intended as the phytosphere) can support growth of numerous and diverse bacteria, which colonize surfaces or internal tissues and display advantageous, neutral or pathogenic functions towards the plant (Andrews and Harris, 2000; Hirano *et al.*, 1982; Hirano and Upper, 2000). However, the plant as a whole is exposed to many environmental challenges and would not entirely provide the same favorable conditions for bacterial growth. The latter depends on several factors such as the presence of nutrients, moisture, shelter from desiccation and UV rays, shelter from grazing and predation, which are rapidly fluctuating parameters and heterogeneously distributed. Hence, growth seems to occur mostly in nutrient rich but few and localized microhabitats on plant surfaces were bacteria would form aggregates (Lindow and Brandl, 2003). The presence of large clusters of bacteria developing at sites of relative nutrient abundance on plant surfaces might also increase the potential for metabolic and genetic exchange. Namely, bacterial growth and remarkably high rates of transfer of conjugative plasmid were reported taking place on plants (Bailey *et al.*, 1996). Similarly, availability of growth substrates, a high bacterial density, and the presence of solid surfaces on the leaf were imputed to induce gene transfer by conjugation in the phyllosphere at significant high rates (Normander *et al.*, 1998).

Of the three mechanisms of horizontal gene transfer in bacteria, natural transformation is thought to be the only one that could be effectively implied in the passage of DNA from transgenic plants to bacteria (Bertolla and Simonet, 1999). Although as stated earlier, plants seem supportive for bacterial growth, to this day only speculations were made that DNA released by naturally degrading plant tissues could be involved in a natural transformation process. Namely, recent data (Ceccherini *et al.*, 2003; Pontiroli *et al.*, submitted) showed that during the decaying process most of the plant DNA was degraded within a few weeks by plant nucleases *in planta*, yet a significant proportion escaped degradation and was still able to transform a recipient soil isolate *in vitro*.

In order to assess plant to bacteria gene transfer some studies have been conducted investigating separately the different plant parts . For example, the possibility for *Acinetobacter baylyi* BD413 to grow opportunistically into *Ralstonia solanacearum* infected plant tissues revealed a new niche for this soil bacterium: the pathosphere (Kay *et al.*, 2002b). Furthermore, this bacterium could be naturally transformed therein by artificially added or indigenous transgenic DNA (Kay *et al.*, 2002a; Kay *et al.*, 2002b). Yet, other plant parts could be as propitious to HGT; for instance, the residuesphere (*i.e.* the naturally degrading plant material at the interface with soil) has been shown to provide conditions for growth and conjugal gene transfer between indigenous soil bacteria (de Lipthay *et al.*, 2001; Sengelov *et al.*, 2000). The litter and residues of annual crops, especially, represent an important amount of final plant production, which, according to common agricultural management practices is often left in the field after harvest, and in most cases make up for up to 60 % of the world plant biomass (Lal, 2005). Decomposition of residues in field conditions is mainly microbial and influenced by factors such as microbial biomass, litter source, amount of litter input, its chemical composition and by soil texture, temperature and humidity (Berg *et al.*, 1984; Howard and Howard, 1974; Pote *et al.*, 2005).

Up to now, the assessment of natural transformation events *in situ* has, however, revealed several methodological challenges and biases since quantification of transformation events has always been conducted with a cultivation based approach requiring the plating of recipient bacteria on selective media supplemented with antibiotics. Antibiotic resistance determinants, though, are widespread into soil environments, providing a technical intricacy in the discrimination between the newly acquired trait bearing recipients and indigenous antibiotic resistant flora, naturally fitted with analogous genes (Courvalin, 1998; Davies, 1994). In addition, discrepancy in transformation frequencies determined by cultivating on selective media and those determined by cell densitometry, revealed that the latter were usually higher by a two order log of magnitude (Sørensen *et al.*, 2004) leading to an underestimation of the phenomenon in natural settings, which so far has been viewed as quite rare. In addition, the uncertainty that each colony enumerated on plate belongs to a single event of transformation rather than being a clonal multiplication of a unique event adds an element of complicatedness to the study. Another drawback of the plating step is the disruptive sampling of material to rescue transformants, which implies a deficiency in information on the spatial distribution and pattern of HGT, at the microscale level, where effectively bacteria interact on plant and soil surface, while. it seems more probable that rare transfer events would occur into localized spots most conducive for HGT, where optimal conditions would be gathered both for nutrients and DNA bioavailability.

Hence, evidences and knowledge on the effective topology and ecology of HGT in the phytosphere are lacking.

The rationale of this study was to determine whether during the natural or pathogen-induced decay of plant tissues new niches able to foster growth, development of a competence state and possible acquisition of exogenous plant DNA by the *Acinetobacter baylyi* strain BD413 *via* natural transformation could be revealed. A step further was to understand the spatial bacterial colonisation pattern both of the naturally degrading plant material and of intact leaves and to detect HGT events *in situ* at the microscale level. To this aim, a novel approach allowing visualisation of HGT *in situ* based on a cultivation–independent assay that relies upon a bioreporter tool has been developed. Microcosm-based experiments revealed that bacterial growth and competence development occur in the different compartments of the plant. Isolation and direct visualization of transformants *in situ* suggests that some compartments of the phytosphere may provide conditions conducive for HGR between plant and bacteria and hence be regarded as environmental hot spots for HGT.

III-MATERIAL AND METHODS

III.1 Plant material

Wild-type (WT) and transplastomic tobacco plants (*Nicotiana tabacum* L. cv. PBD6) were grown in potting compost soil in a greenhouse at 23 (± 2) °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 72% at night. Transplastomic tobacco plants harbored the transgenic *aadA* gene (conferring resistance to spectinomycin and streptomycin) cloned between the chloroplastic genes *rbcL* and *accD* (Kay *et al.*, 2002b) and contained an estimated 7000 copies of the transgene per plant cell (Pontiroli *et al.*, submitted). Entire leaves were sampled at the 5 leaves stage, when plants were 9 weeks old.

III.2 Bacterial strains, plasmids, and culture media

Escherichia coli strain DH5α(pCEA) strain harbored the pCEA plasmid, a pBluescript®II SK+ derived cloning vector that had been used to transform tobacco plants (Kay *et al.*, 2002b) containing the *aadA* gene flanked with part of the *rbcL* and *accD* plastid sequences (Fig. 1). The strain was grown at 37 °C on LBm medium (LBm: Bacto Tryptone extract 10 g/L;

NaCl 5 g/L, yeast extract 5 g/L in 1 L distilled water) supplemented with ampicillin (50 µg/mL) and spectinomycin (50 µg/mL) (Sigma, St.Louis, California).

The naturally transformable *Acinetobacter baylyi* strain BD413 was chosen as the model bacterium. A first strain harbored the recombinant plasmid pBAB2 that contained a recombinogenic site with tobacco plastidic sequences *rbcL* and *accD* (Kay *et al.*, 2002b) to favor homologous recombination (Fig. 2A). The strain was cultured at 28 °C on Luria Bertani modified medium supplemented with ampicillin (50 µg/mL) and nalidixic acid (20 µg/mL) (Sigma, St.Louis, California) and for solid medium, Bacto™ agar (15 g/L) (Beckton Dickinson, Franklin Lakes, USA). Transformants were selected on LBm medium containing ampicillin (50 µg/mL), nalidixic acid (20 µg/mL) and spectinomycin (50 µg/mL) (Sigma, St.Louis, California) after 2 days of incubation at 28° C.

To quantify and visualize total bacterial populations and transformants *in situ*, a marker rescue transformation system relying on the bioreporter *A. baylyi* strain BD413(*rbcL-aadA::gfp)* and its non functional counterpart *A. baylyi* strain BD413(ΔP*aadA::gfp*) were used (Fig. 2B). Respectively, these two *Acinetobacter* strains carry an *aadA::gfp* fusion protein containing the two functional domains of antibiotic resistance and green fluorescence protein, joined together by a 17 amino acid residues linker (ELVEGKLELVEGLKVGA) next to a functional *aphA3* marker cloned into their *lipBA* operon. The second strain, BD413(ΔP*aadA::gfp*) carries a 143 bp deletion in the *aadA* promoter sequence resulting in sensibility to spectinomycin, and a 1148 bp fragment of the *rbcL* plastid gene, to allow homologous recombination. The construction of *aadA::gfp*-based constructs for cloning in *A. baylyi* BD413 was isreported in the first part of Chapter Three. The two strains were routinely grown at 28°C on LBm medium supplemented with kanamycin (50 µg/mL), rifampicin (50 µg/mL) and, in the case of the *A. baylyi* strain BD413(*aadA::gfp)* or, to rescue transformants, with spectinomycin (50 µg/mL) (Sigma, St.Louis, California).

Ralstonia solanacearum strain K60, a plant pathogen responsible for bacterial wilt in solanaceous crops, was used to perform tobacco leaf co-inoculation experience was cultured in B solid medium (Boucher *et al.*, 1985) supplemented with 12 µg/mL gentamycin, at 28 °C for 48 h or in B broth medium until reaching an $OD_{600\ nm}$ of 0.8

III.4 DNA extraction

Plant genomic DNA was extracted from tobacco leaves. Leaves were sampled and dismissed of the central vein with a sterile scalpel and individually ground in liquid nitrogen.

Plant DNA was extracted from 100 mg of the resulting leaf powder using the DNeasy® Plant kit (Qiagen, Mannheim, Germany) following manufacturer's instructions.

Plasmid pCEA was isolated from *E. coli* DH5α(pCEA) using the QIAfilter™ Plasmid Midi Kit (Qiagen, Mannheim, 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, DNA purity and concentration were determined by measuring the absorbance of DNA solutions at 260 nm (OD_{260}) with the Eppendorf® Biophotometer (Eppendorf, Westbury, USA) spectrophotometer and by depositing 1 µl of extract on 1% agarose gel for comparison with the Massruler ™ DNA Ladder (Fermentas, Burlington, Canada).

III.5 Plant inoculation and incubation

Intact and ground tobacco leaves were inoculated with *A. baylyi* strain BD413(pBAB2). Discs of 2 cm diameter were cut from WT tobacco leaves and soaked twice in a 5% (w/v) bleach solution for 10 min, then rinsed twice in sterile distilled water and dried on sterile Whatman™ paper (Whatman, Maidstone, UK). Half of the discs were then ground in distilled sterile water with an Ultra-Turrax T25 homogenizer (IKA-Werke GmbH and Co., Staufen, Germany) operating at 25,000 rpm in a proportion of 1 g/ 5 mL for 5 min. Intact leaf disc (ca 0.1 g) and ground leaf tissue (ca 0.2 g) were placed on 2.5 cm diameter Isopore ™ 0,2 µm membrane filters (Millipore, Billerica, USA) laying in Petri dishes containing a thin layer of agar supplemented with cycloheximide (200 µg/mL).

A. baylyi strain BD413(pBAB2) was cultured overnight in LBm broth medium and then diluted ten-fold in fresh medium and left growing until the culture reached an optical density corresponding to 2.3 x 10^8 CFU/mL. Then, cells were harvested, centrifuged at 5000 rpm, washed and resuspended in sterile saline solution (NaCl 0.8 %).

One hundred µl of a 1000-fold dilution of the bacterial suspension corresponding to a final inoculum of ca. 10^4 CFU were spotted in droplets of ca 10 µl on ground or intact leaves placed on filters and left to incubate at 28°C for up to 15 days with three replicates per leaf. In parallel, as a negative control, 100 µL of sterile saline solution were also spotted on leaves.

A similar experimental set up was used to quantify and visualize transformants *in situ*, with the exception that in this case crushed leaf discs of transplastomic tobacco were used and inoculated with either *A. baylyi* strains BD413(pBAB2) or BD413(*rbcL*-ΔP*aadA::gfp*),

respectively. Visualization of the colonization pattern of plant tissues was performed after inoculating ground or intact wild type tobacco leaf discs with *A. baylyi* strain BD413(*aadA::gfp*). Incubation was left to proceed for up to 15 days.

In order to detect gene transfer following pathogen induced plant decay, individual transplastomic leaves were inoculated with a mixture of *R. solanacearum* strain K60 and *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*). *R. solanacearum* cells were centrifuged and resuspended in 1 mL of saline solution and the concentration adjusted to an OD_{600} value of 0.8 corresponding to ca 2 x 10^8 CFU/mL. A suspension of the strain BD413 (*rbcL*-ΔP*aadA::gfp*) was prepared by harvesting an early exponential phase growing culture and resuspending it in 2 mL of saline solution, corresponding to a final density of ca 2.5 x 10^8 CFU/mL (OD_{600} =0.8). The final inoculum was prepared by mixing 1 mL of the *R. solanacearum* K60 cell suspension with 2 mL of the *A. baylyi* cell suspension. Three hundreds µl of the final suspension were injected into each tobacco leaf with a syringe, by piercing the central vein of the leaf at intervals of 2 cm. Leaves were incubated at 28°C into Petri dishes containing a thin layer of water agar supplemented with cycloheximide for up to 11 days or until appearance of severe symptoms of wilting.

III.6 Growth and competence development of *A. baylyi* strain BD413

Growth and competence development of *A. baylyi* strain BD413(pBAB2) in the phyllosphere (*i.e.* intact leaves) and the residuesphere (*i.e.* ground leaves) was estimated by determining population sizes and transformation frequencies *in vitro*, respectively. Three leaf discs were sampled every 24 hours for up to 7 days. Filters were removed with sterile tweezers under a laminar flow hood, and individually placed in 50 mL Falcon™ polypropylene tubes (Becton Dickinson, Franklin Lakes, USA), washed in 5 mL of saline solution (NaCl, 0.8%) by vortexing the tubes for 2 min at maximum speed. Total and revertant population sizes were determined by serially diluting and plating suspensions on LBm media supplemented with the appropriate antibiotics.

Assessment of competence development was performed by exposing 500 µl of the above described bacterial suspensions obtained by leaf tissue washing to 1 µg of the pCEA plasmid in 50 µl of ultrapure water for 2 hours at 28°C. Cells were serially diluted in saline solution and then plated on LBm agar medium supplemented with appropriate antibiotics and incubated at 28°C. Total population sizes and transformants were determined by colony counts after 2 days incubation.

Growth and competence development were also determined for *A. baylyi* strain BD413(pBAB2). Cells were grown overnight in LBm broth medium, diluted 25-fold with fresh LBm medium supplemented with the appropriate antibiotics and cultured for 54 hours. During this time interval, 350 µL of the culture were periodically sampled and exposed to 1 µg of the pCEA plasmid in 50 µl of ultrapure water, incubated for 2 hours at 28°C, diluted and plated on LBm medium supplemented with appropriate antibiotics.

III.7 Natural transformation of *A. baylyi* strain BD413 *in vitro* and in the phytosphere

The transformation ability of the different strains was initially determined *in vitro* to assess their respective transformation frequencies under optimal growth conditions. Aliquots of exponentially growing cultures (500 µL; OD_{600}= 0.8) of *A. baylyi* strain BD413(pBAB2) and *A. baylyi* strain BD413(*rbcL*-Δ*aadA::gfp*) were exposed to pCEA plasmid DNA, purified transplastomic DNA and leaf homogenate and incubated with constant shaking (300 rpm) at

28 °C for two hours. Then bacterial cultures were harvested, serially diluted and plated on selective LBm medium. Each experiment was done in three replicates for each assay.

A. baylyi strain BD413(pBAB2) and *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) cells were recovered from intact or ground transplastomic leaf discs at 0, 24 48, 72 h by washing each disc in a Falcon tube with 5 mL of sterile saline solution and vortexing the tubes for 5 min at maximum speed. Total, transformant, revertant population sizes were determined by serially diluting and plating suspensions on solid agar media supplemented with the appropriate antibiotics. In order to avoid *ex planta* transformants, suspensions were treated with DNAse I (0.1 mg/mL) (Roche Diagnostics GmbH, Mannhein, Germany) at 37°C for 1 hour before plating all the remaining volume of suspension on selective LBm solid media for the enumeration of transformant cells.

Three *R. solanacearum* infected leaf segments (1 by 2 cm) were randomly cut per leaf, weighed and utilized to quantify and visualize transformants. Quantification was performed after crushing infected segments in 5 mL sterile saline solution with the Ultra-Turrax T25 homogenizer. Then, the suspension was treated with DNAse I (0.1 mg/mL) at 37°C for 1 h before plating onto selective LBm media supplemented with the appropriate antibiotics.

Stability of the different constructs of the bioreporter strains of *A. baylyi* was assessed *in vitro* and *in planta* by determining the number of revertants (spontaneous mutants resistant to spectinomycin). Cells of the two strains were grown in LBm broth for up to 7 days and total and revertant population sizes were determined every 24 hours by plating on LBm and spectinomycin - supplemented LBm solid medium. Cells of the two strains were inoculated on ground leaf tissues of wild type tobaccos for up to 15 days and total population size and revertant population size were determined every 24 hours by washing and plating on LBm and LBm supplemented with spectinomycin (as described above). Reversion frequencies were determined as the number of revertants (*i.e.* spontaneous mutants resistant to spectinomycin) observed divided by the total number of bacterial.

III.8 Analysis of transformants

The presence of the transgene in spectinomycin resistant clones of *A. baylyi* strain BD413(pBAB2) resulting from transformation with plasmid or plant DNA was confirmed by PCR using specific primers p1351cpl2up (5'-TTTCTATTGTTGTCTTGGAT-3') and p416 (5'-TGACGGGCTGATACT-3') (Ceccherini *et al.*, 2003). These primers targeted an 853-bp

sequence and were complementary to a part of the *rbcL* and the *aadA* gene. PCR reactions were conducted with 1 µl of template DNA extracted from putative recombinants and using a previously published thermal protocol (Ceccherini *et al.*, 2003).

Confirmation of the rescue of the marker system in the *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) spectinomycin resistant clones was performed by PCR on a template of 1µl of genomic DNA. Primers Promo F (5' ATCTTTCTATTGTTGTCTTGGAT-3') and Promo R 5'-GGTCACCGTAACCAGCAAATCAA-3' were used to discriminate between the recombinant clones containing a functional promoter (size of the amplicon = 333 bp) and the recipients bearing a deletion in the promoter (size of the amplicon = 190 bp). The PCR reaction consisted in a denaturation step at 95° C for 4 min followed by 35 cycles consisting in 40 s at 95° C, 40 s at 56° C, and 40 s at 72° C, and a final extension step at 72° C for 5 min. PCR products were run on an agarose gel by depositing 10 µL of PCR product on 1.5 % agarose gel for comparison with the Fastruler ™ DNA Ladder (Fermentas, Burlington, Canada) or the 1 Kb Plus™ DNA Ladder (Invitrogen, Carlsbad, California).

III.9 Visualisation of bacterial cells on plant tissues

The spatial localization of total and transformed bacterial cells was determined directly *in situ* by epifluorescence microscopy using *A. baylyi* strain BD413(*aadA::gfp*) constitutively expressing GFP and *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) respectively . Processed sampled (Cf. Plant inoculation section) consisted of intact or ground transplastomic tobacco leaves that had been inoculated with the *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) and *A. baylyi* strain BD413(*aadA::gfp*) or of symptomatic leaves infected with *R. solanacearum* K60 and coinoculated with *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*).Three samples consisting of either intact or ground leaf tissues were selected for each treatment. Four segments of approximately 1 cm² were cut from each intact leaf and placed on a glass slide. Ground leaf tissues incubated on filter membrane were observed undisturbed under the microscope. A drop of Aqua-Poly/Mount "mounting" semipermanent medium (Polysciences Inc., Warrington, PA) was placed on the center of a coverslip which was then gently pressed down onto the leaf material. To visualize total bacterial cells, a solution of ethidium bromide (1 µg/mL) was mixed to Aqua-Poly/Mount (Polysciences Inc., Warrington, PA) before mounting. Samples were then immediately observed using an AxioSkop Zeiss microscope equipped with a 10x/0.30, 20x/0.50,

40x/0.75, or 100x/1.30-numerical-aperture Plan Neofluar objectives (Zeiss Inc., Oberkochen, Germany). Zeiss filter set 10 (excitation, BP 450/490 nm; beamsplitter, FT 510 nm; emission, BP 515/565 nm) was used to visualize green fluorescence emitted by cells expressing the GFP. Total cells stained with ethidium bromide were visualized using Zeiss filter set 15 (excitation, BP 534/558 nm; beamsplitter, FT 580 nm; emission, LP 590 nm). Images were captured with an AxioCam MRc5 digital camera and the AxioVision V4.3 software (Zeiss Inc., Oberkochen, Germany).

IV-RESULTS

IV.1Growth of *A. baylyi* BD413 in the phytosphere

A. baylyi strain BD413(pBAB2) cells grow on intact leaves referred to as the phyllosphere. Population sizes determined at different intervals indicated effective and consistent growth of the strain from the time of inoculation (Fig. 3). The number of recovered cells had increased from 6.00 x 10^5 (± 1.16) CFU/g (fresh weight) at sampling time 0 (approximately 4 hours after inoculation) to 1.08 x 10^8 (± 1.51) CFU/g (fresh weight) after one week, in the stationary phase. The bacterial cell generation time during the exponential growth phase on intact leaf surfaces was ca. 3.4 hours. Likewise, when the same number of cells of *A. baylyi* was spotted on crushed leaves, the number of recovered cells indicated that consistent and rapid growth had occurred. The generation time during the exponential growth phase on ground leaf tissues was estimated at 1.8 hours. After 24 hours they had increased by 4 log-orders of magnitude, reaching a density of 3.41 x 10^8 CFU/g (± 1.08) of fresh tissue (Fig. 4). After seven days incubation discs were supporting a population size of 1.31 x 10^{10} (± 2.35) CFU/g (fresh weight). Growth kinetic of the same strain determined *in vitro* (Fig. 5) showed that the population size incremented exponentially during the first 8 hours, with a generation time estimated at 1.3 hours, and entered the stationary phase thereafter.

IV.2 Competence development of *A. baylyi* BD413 *in vitro* and in the phytosphere

Development of a competence state in the *A. baylyi* strain BD413(pBAB2) was assessed in the phyllosphere and in the residuesphere of tobacco. After *in vitro* transformation,

spectinomycin resistant clones could be detected from suspensions recovered from intact leaf discs after 48 hours incubation. The transformation frequency was on average 1.47 x 10^{-6} (± 3.01) for samples taken after 2 days and decreased to 1.21 x 10^{-8} (± 1.51) after 7 days incubation on intact leaves (Fig. 3). Transformants failed to be detected either when bacteria grown on leaves were sampled before 48 hours, or on leaves that had been inoculated only with sterile saline solution. When suspensions of the *A. baylyi* strain BD413(pBAB2) were recovered from tobacco leaf residues after 8 hours inoculation, 5.70 x 10^{-7} (± 2.21) transformants / recipients were detected. After 16 hours competence had reached to a maximum 4.57 x 10^{-6} (± 3.04) corresponding to the late exponential growth phase, while shortly thereafter a decline in transformation frequencies was observed.

As for growth kinetics, the transformation ability of the strain was also determined *in vitro* for up to 40 hours. Transformants were detected at a frequency of 2.69 x 10^{-4} when aliquots of ca 10^{7} CFU/mL were sampled at time 0, while a peak in transformation frequencies was reached after 4 hours and a decrease followed thereafter and up to 40 hours with a global decline of 2 logs of magnitude.

IV.3 Natural transformation of *A. baylyi* BD413 by transgenic DNA *in vitro* and in the phytosphere

The strains of *A. baylyi* BD413 used in this study rely on different marker rescue systems: to verify whether the strains could be comparable in terms of their ability of being transformed, we first determined *in vitro* their transformation potential with different forms of transgenic DNA (plasmid, purified plant DNA, leaf homogenate). Frequencies of transformation for *A. baylyi* strain BD413(pBAB2) were on average higher than those of strain BD413(*rbcL*-Δ*P*a*adA::gfp*) by two log-orders independently of the type of transforming DNA used (Table 1). For both strains they decreased according to the number of donor genes present in the transformation mixture, as it appears logical if we consider that even if cells where exposed to equal amounts of DNA, in the case of plant DNA and plant leaf homogenate transgenic signatures were diluted among total DNA compared to pure plasmid DNA. A step further in the investigation was to determine if *A. baylyi* that proved to grow and develop competence in the phytosphere could be naturally transformed *in situ* by the DNA released from the plant. Experiments conducted with the *A. baylyi* strain BD413(pBAB2) on intact and crushed leaf discs revealed that no transformants could be detected from bacterial cells recovered from intact leaf

tissues. However, when bacteria recovered after incubation on crushed leaf discs for 8, 24 and 96 hours were plated on spectinomycin containing medium, transformants were detected, at frequency of 2.0 x 10^{-10}. PCR amplifications of the transgene signature in spectinomycin resistant clones confirmed their recombinant nature (Fig. 4).

Transformants isolated from washings of ground leaf tissues that had been inoculated with *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) could be detected on plates only after an incubation of 48 hours, with a frequency averaging 10^{-11}. Fluorescence could be detected in all colonies recovered. When analyzed by PCR spectinomycin–resistant clones were discriminated from their amplification product on agarose gel, with the appearance of a band of 300 bp characteristic of the restored promoter (Fig. 7). No revertants were observed after sampling inoculated ground leaves and broth culture during 15 days and 7 days, respectively.

Transformants were also detected after plating suspensions derived from co-inoculation experiments. Total population size of *A. baylyi* BD413(*rbcL*-ΔP*aadA::gfp*) reached 2.85 x 10^{11} (± 1.65) CFU/g (fresh weight) after 7 days and transformant frequencies averaged 1 x 10^{-7} (± 5.4).

IV.4 Visualization of total and transformant bacteria *in planta*

The approach developed allowed to determine the bacterial colonization patterns of the phytosphere and *in situ* detection of HGT. Using epifluorescence microscopy, total populations were visualized in the different spheres either by tracking the constitutively GFP expressing *A. baylyi* strain BD413, or by staining the cells with ethidium bromide when the bioreporter bearing the non-functional GFP was used. After 2 days incubation of *A. baylyi* strain BD413(*aadA::gfp)* on intact plant tissues, cells constitutively expressing GFP could be observed by epifluorescence microscopy using GFP filter set: this enabled visualization of individual cells and small aggregates that would otherwise be masked by the strong autofluorescence of the leaf. After 2 days incubation, cells in the phyllosphere were localized around and at the base of glandular trichomes, with a scattered uneven distribution all over their surface. Cluster of cells were mostly found near trichomes (Fig. 8).

TABLE 1. Transformation assays of *A. baylyi* BD413 (*rbcL*-ΔP*aadA::gfp*) and *A. baylyi* BD413(pBAB2) exposed to plasmid pCEA and to different sources of transplastomic (TP) and wild type (WT) donor DNA[a].

Strain	Donor DNA	Amount of material used	No. of donor genes per assay	No. of transformants per assay[b]	Transformants per target gene[b]	Transformation frequency[b]
A. baylyi BD413 (*rbcL*-ΔP*aadA::gfp*)	Linear pCEA plasmid	1μg DNA	1.3×10^{11}	$(1.3 \pm 0.6) \times 10^{5}$	$(1.0 \pm 0.4) \times 10^{-6}$	$(1.4 \pm 0.4) \times 10^{-5}$
	Total DNA from TP leaf tissue	5μg DNA	5.5×10^{9}	$(1.3 \pm 0.2) \times 10^{2}$	$(0.2 \pm 0.03) \times 10^{-7}$	$(2.4 \pm 0.4) \times 10^{-8}$
	TP leaf homogenate	500 mg[b]	n.d.[c]	$(3.2 \pm 1.9) \times 10$	n.d.[c]	$(8.4 \pm 2.2) \times 10^{-10}$
	WT leaf homogenate	500 mg[b]	0	0	n.a.[d]	$<10^{-11}$
A. Baylyi BD413 (pBAB2)	Linear pCEA plasmid	1μg DNA	1.3×10^{11}	$(5 \pm 0.05) \times 10^{5}$	$(3.85 \pm 0.05) \times 10^{-6}$	$(1{,}69 \pm 0{,}8) \times 10^{-3}$
	TP total DNA from leaf tissue	5μg DNA	5.5×10^{9}	$(6.0 \pm 0.8) \times 10^{2}$	$(1.09 \pm 0.05) \times 10^{-7}$	$(4.1 \pm 2.3) \times 10^{-6}$
	TP leaf homogenate	500 mg[b]	n.d.[c]	$(1.0 \pm 0.1) \times 10$	n.d.[c]	$(1{,}45 \pm 6.0) \times 10^{-8}$
	WT leaf homogenate	500 mg[b]	0	0	n.a.[d]	$<10^{-11}$

[a] Recipients for BD413(*rbcL*-ΔP*aadA::gfp*) were 3.4×10^{7} CFU/mL, and for BD413(pBAB2) were 7×10^{8} CFU/mL

[b] Data are means ± standard deviations of three independent assays.

[c] n.d., not determined.

[d] n.a., not applicable.

Microscopic observations of ground leaf tissues revealed a heterogeneous distribution of anatomical structures of the plant such as leaf nervures, trichomes and vacuoles (Fig. 9): chloroplasts and veins were clearly identifiable when samples were observed with a long pass DAPI filter (Fig.9). Total cells were spread on the surface of the mashed plant material, and it appeared that the denser the amount of debris, the higher the amount of cells residing on it as it could be revealed by ethidium bromide staining and utilisation of an appropriate filter set (Fig. 10). *In situ* observation of fluorescence revealed that cells preferentially colonized the margins (Fig. 11 C, D) of leaf residues or formed aggregates on the leaf debris surface (Fig 11 A, B). Alternatively, when observing samples at a smaller scale, it appeared that groups of cells aggregated just in close proximity of damaged plant cells (Fig.11 E, F).

When the reporter strain BD413(*rbcL*-ΔP*aadA::gfp*) was inoculated on leaf residues, ethidium bromide staining of recipient cells and the fluorescence emitted by recombinant cells enabled their discrimination during microscopic observation. The utilization of the marker rescued transformation system coupled to epifluorescence microscopy allowed for identification of HGT events at single cell level (Fig. 12) without need of selective pressure: recombinants could be visualized directly out of the total population of recipients by using different filters. Combining images captured with identical fields of view but different filters allowed localization of single recombinants (Fig. 12) as well as microcolonies (Fig. 13). Observations of microcolonies (ca. 60 cells on figure 13), enabling a rough estimation of the number of transformants *in situ* which appeared to be higher than that determined on solid media *ex situ* for the same treatment.

When leaves infected by *R. solanacearum* were sampled, tissues showed the typical symptoms of bacterial wilt such as chlorosis and necrosis, the leaf appeared softer and flaccid (Fig. 14 A) and the distribution of the phytopathogen could be inferred by observing the central and secondary veins, which leaked bacterial mucilage (Fig. 14). Leaf segments observed by microscopy revealed a heterogeneous disintegration of plant cellular components. Transformants of the *A. baylyi* strain BD413(*rbc*L-ΔP*aad*A*::gfp*) were observed in destroyed tissues, mostly veins (Fig. 14 B, C, D) as well as microcolonies form on the tissues nearby veins (Fig. 14 E, F).

V-DISCUSSION

The present study revealed that *A. baylyi* strain BD413 can develop significant populations on tobacco leaf tissues. Population sizes reached on intact leaves were, however, 100-fold smaller than on leaf homogenates simulating plant tissue degradation, suggesting that conditions for microbial growth are more favourable on the latter due, most likely, to increased availability of nutrients and moisture. An additional element in support of this consideration is the different growth rate of the strain in the two habitats, which was more rapid on crushed leaves. Likewise, growth was assessed and occurred also on intact leaf discs that had been only slightly abraded on the surface to favor release of cellular content; in this case, the population sizes were of the same order of magnitude of those obtained on ground leaves (data not shown). A previous study reports that cells densities of *A. baylyi* BD413 when inoculated alone in healthy stems decreased over a ten days incubation period (Kay *et al.*, 2002a) and only when it was co-inoculated with a vascular plant pathogen would the strain take advantage of plant tissue disintegration, indicating that the major factor allowing growth would be the release of nutrients from sloughed and damaged cells. However, we observed that growth occurred also when the strain was inoculated on healthy plant tissues; examination of leaf surface colonisation patterns revealed that cells were preferentially associated to trichomes and interstices supporting the general finding that these microhabitats offer a preferred habitat for bacterial survival (Lindow, 1991; Normander *et al.*, 1998).

We might easily speculate that growth of *Acinetobacter* in the phyllosphere occurred due to the consumption of those leaf exudates such as carbohydrates, amino acids and organic acids known to be emitted on intact leaf surface and reported as supporting consistent epiphytic bacterial densities, up to 5×10^7 CFU/g (fresh weight) under humid conditions (Bramwell *et al.*, 1995; Brandl and Mandrell, 2002; Kidambi *et al.*, 1994; Mercier and Lindow, 2000). Moreover, similar conditions were effectively reported stimulating gene transfer by conjugation in the phytosphere (Lilley *et al.*, 1994; Sørensen and Jensen, 1998). To the best of our knowledge this is the first study reporting active growth and multiplication of this strain in a different habitat than soil from which it was isolated (Heidelberger *et al.*, 1969; Juni and Janik, 1969) with the exception of vascular tissues of tomato plants that had been deliberately co-infected with a phytopathogen (Kay *et al.*, 2002a). Likeliness for this event to occur in natural systems is not

negligible, since litter and naturally decaying plant tissues are tightly associated with soil in the residuesphere.

Furthermore, we found that a significant proportion of *Acinetobacter baylyi* BD413 cells was able to develop a competent state *in situ*, as assessed by exposure of bacterial cells recovered from leaves to a selectable marker bearing plasmid. Competence development kinetics were in accord with those observed *in vitro* and found in the literature (Palmen *et al.*, 1993) and clearly related to the exponential growth phase, although the fraction of cells that became competent in the phyllosphere and the residuesphere was lower by two log order when compared to *in vitro* results. Not casually, these plant compartments have, among others, been indicated as conducive to bacterial growth and potentially to horizontal gene transfer among plant and soil associated bacteria and are referred to as "hot spots" (Fig. 15) (Sørensen *et al.*, 2005; van Elsas and Bailey, 2002; van Elsas *et al.*, 2000). These spots, were bacteria can cluster and form microcolonies or even biofilms (e.g. on roots, aerial plant surfaces decaying organic matter, and at the interfaces between organic phases and mineral particles) are characterized by their capacity to enhance bacterial metabolic activity and hence, rates of genetic exchange (and mutational change) processes (Fig. 15) (van Elsas *et al.*, 2000).

As we have pointed out earlier, microscopic observations revealed that bacteria were not uniformly distributed on leaf tissues, revealing a heterogeneous spatial distribution; this was especially true in the case of residual tissues: bacterial aggregates seemed to privilege margins and extracellular interstices. We hypothesize that in these spots nutrients and, potentially, transgenic signatures are more concentrated. Our approach allowed isolation and quantification of bacterial transformants out of this copiotrophic environment, and, most significantly, permitted their direct visualisation *in situ* without the need of cultivation. To our knowledge this is an original finding and the first report of *in situ* plant to bacteria gene transfer visualisation by natural transformation.

In the phyllosphere, characterized by a higher degree of oligotrophy than residuesphere, as already mentioned, the probability of a physical encounter between DNA molecules and competent bacteria would be less favored due to the fact that *a priori* not a significant amount of DNA would be released. This in turns could explain why no transformants could be detected in our microcosms simulating phyllosphere conditions.

Assessement of the potential of natural transformation in the pathosphere confirmed the ability of *A. baylyi* to grow as an opportunistic bacterium (Kay *et al.*, 2002a), and was helpful in validating the experimental approach in a further compartment of the phytosphere considered as

hot spot. There, bacterial densities were extremely high and so were transformation frequencies if compared with those we obtained in the residuesphere or those reported in the literature (Kay *et al.*, 2002a; Kay *et al.*, 2002b), suggesting that of the three compartments investigated in this study, the pathosphere would be the most conducive spot for plant to bacteria gene transfer probably due to higher availability of nutrients by increased plant cell leakiness consequent to *Ralstonia* infection. Although considerations can be made about the possible competition for resources due to simultaneous growth of the two bacterial species *in planta*, our findings would show that the carrying capacity of leaf tissues could allow coexistence of conspicuous bacterial populations. In respect to previous studies that found transformation frequencies near to detection limit in the pathosphere and on the basis of low frequencies of transfer detected for the same reporter strain in the residuesphere, conditions were optimized in our system as denser inocula of the recipient strain were injected in plant veins during coinoculation. This resulted in a higher colonisation of wilting tissues that might have supported a higher number of transformation events. However, it could be also questioned if the important number of transformants detected after plating suspensions recovered from infected leaf segments was representative of individual recombinants or the result of a clonal multiplication of few original single transformation events, thus leading to an overestimation of HGT frequencies.

Previous field studies attempting to detect or quantify transfer of engineered plant transgene by natural transformation into resident field bacterial population (Gebhard and Smalla, 1999; Paget *et al.*, 1998) relied on detection strategies based on the isolation of cultivable bacteria from soils, their cultivation on selective media and the screening, *via* various biomolecular techniques, for the presence of transgenic DNA. Although they resulted in isolation of high number of antibiotic resistant colonies on transformant selective media, neither of them was able to confirm incorporation of transgenic plant DNA into the bacterial isolates. Published results on this issue are, however, scarce and limited and when critically reviewed were blamed for relying on detection methods and sampled sizes inadequate to identify rare bacterial transformants among the 10^8 bacteria ideally found in every gram of soil (Nielsen and Towsend, 2004; Ray and Nielsen, 2005). Furthermore, these cells occupy only 0.02 % of the colonisable volume which is available in such amount of soil (Pallud *et al.*, 2004) and one of the more important stochastic factors influencing horizontal gene transfer in the environment is the proximity of donor DNA and recipient cells (van Elsas *et al.*, 2003b). These arguments backed our rationale of investigating gene transfer by natural transformation at the microscale level, during the initial steps of plant tissue degradation.

This study is the first attempt to describe the spatial distribution and quantification of natural transformation events at the microscale level; although visualisation of transformants *in situ* in various hot spots was possible with our detection system, technical issues resulted from our experiments, deserving thorough consideration for future investigations. Namely, in some cases the number of transformants that were detected by epifluorescence microscopy observation *in situ* seems to exceed the number of transformants recovered after plating. This could be principally due to the presence of bacterial cells in the so called viable but not culturable state (vbnc) or of dying or dead cells still positive for GFP expression that might have led to an overestimation of transformation events. Alternatively, biases could be due to mechanical reasons, because removal of bacterial cells from vegetal samples by washing might have been only partly efficient, leading to an underestimation of potential cultivable transformants. In addition, during direct visualisation of leaf samples transformants were often found developing in clusters such as microcolonies, suggesting that such aggregate formation most probably might result from the clonal multiplication of single transformation events (Fig. 13); however, overestimation of gene transfer events might occur also with cultivation based methods, since recombinants isolated on solid media have no additional guarantees of deriving from independent transfer occurrences. Uncertainty on transformation frequencies could be resolved by coupling the reporter gene approach to new analytical methods not requiring cultivation like flow cytometry (FACS) as recently proposed by Sørensen and colleagues, which allows fine enumeration of recombinants (Sørensen *et al.*, 2005; Sørensen *et al.*, 2003). Hence, the use of the bioreporter tool could be envisioned in natural environments with new experiments relying on flow cytometry and differential genetic labelling of recombinant and recipient cells. Under an ecological perspective, however, focusing strictly on determination of precise transformation frequencies *in situ* might be limiting (if we omit risk assessment purposes), since in theory even single extremely rare transformation events may hinder a chain of ecological consequences.

Finally, our experiments were conducted in a nearly sterile environment; hence, the transformation frequencies we observed are likely to be different to those that would be observed in the presence of the natural microflora of plant leaf surfaces in reason of the different types of possible interactions (*e.g.* competition or synergy).

If our approach allowed the detection of rare transformation events in the course of preliminary laboratory experiences with microcosms simulating natural conditions, the application of this tool to field studies could provide deeper insights into factors that affect HGT by natural transformation between prokaryotes (with appropriate modifications) or interkingdom.

Particularly, in the latter case (*e.g.* selectable antibiotic marker bearing transgenic plants), background antibiotic resistances already intrinsic to soil bacteria may be partially circumvented thanks to fluorescent reporter genes. In this preliminary study we have assessed and reported bacterial growth, competence development and visualisation of plant to bacterium gene transfer by natural transformation in the phytosphere, revealing potential new habitats for a naturally transformable soil bacterium. Further than allowing to confirm that the phytosphere constitute a hot spot for microbial growth and horizontal gene transfer, this study provides new insights on the complexity of factors affecting HGT *in situ* and hints for future lines of investigation.

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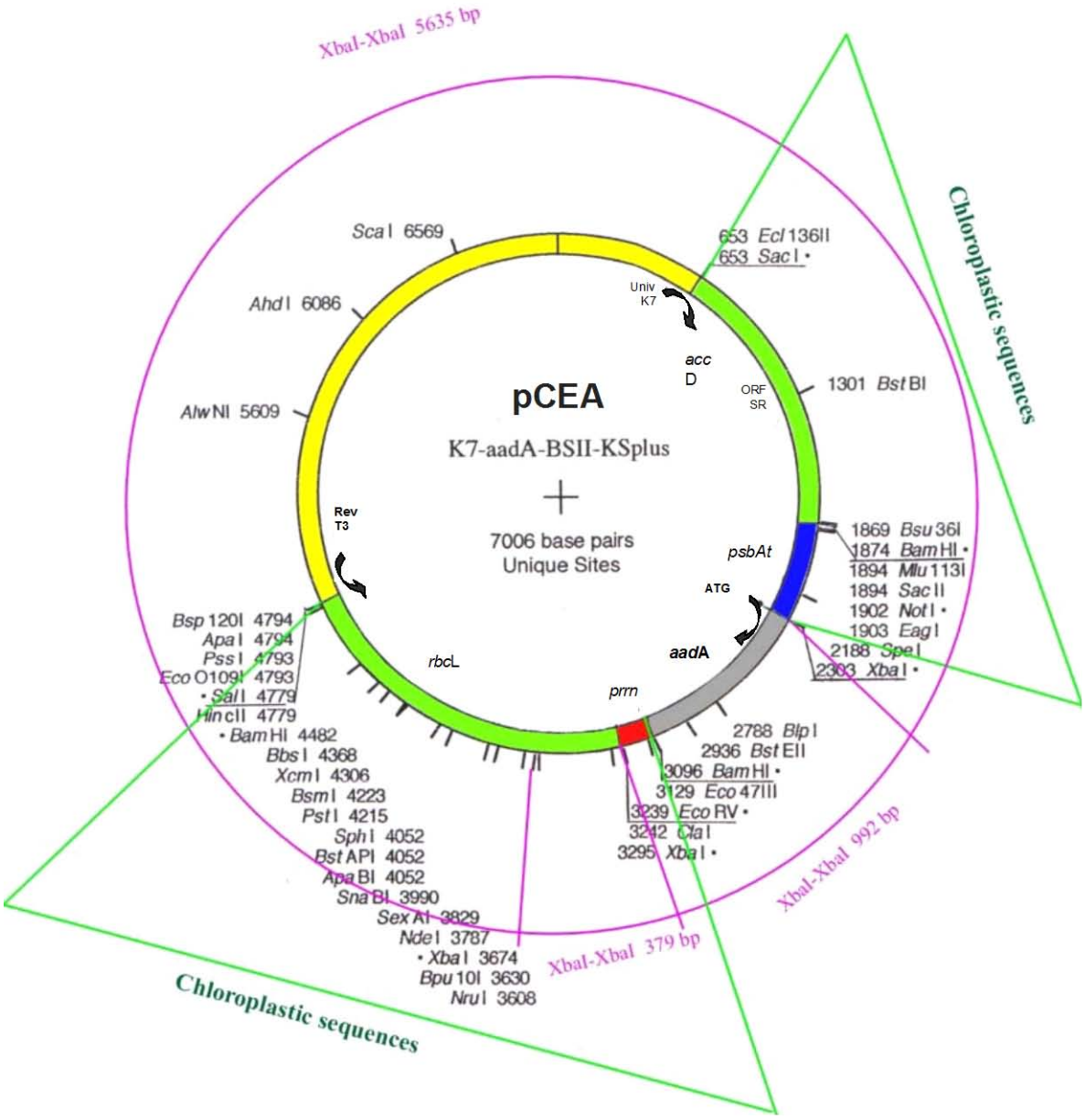

Figure 1. Schematic representation of pCEA. The plasmid harbors the *aad*A selectable marker gene (grey box) coding for spectinomycin and streptomycin resistance flanked by the two chloroplastic sequences *rbc*L and *acc*D (green boxes).

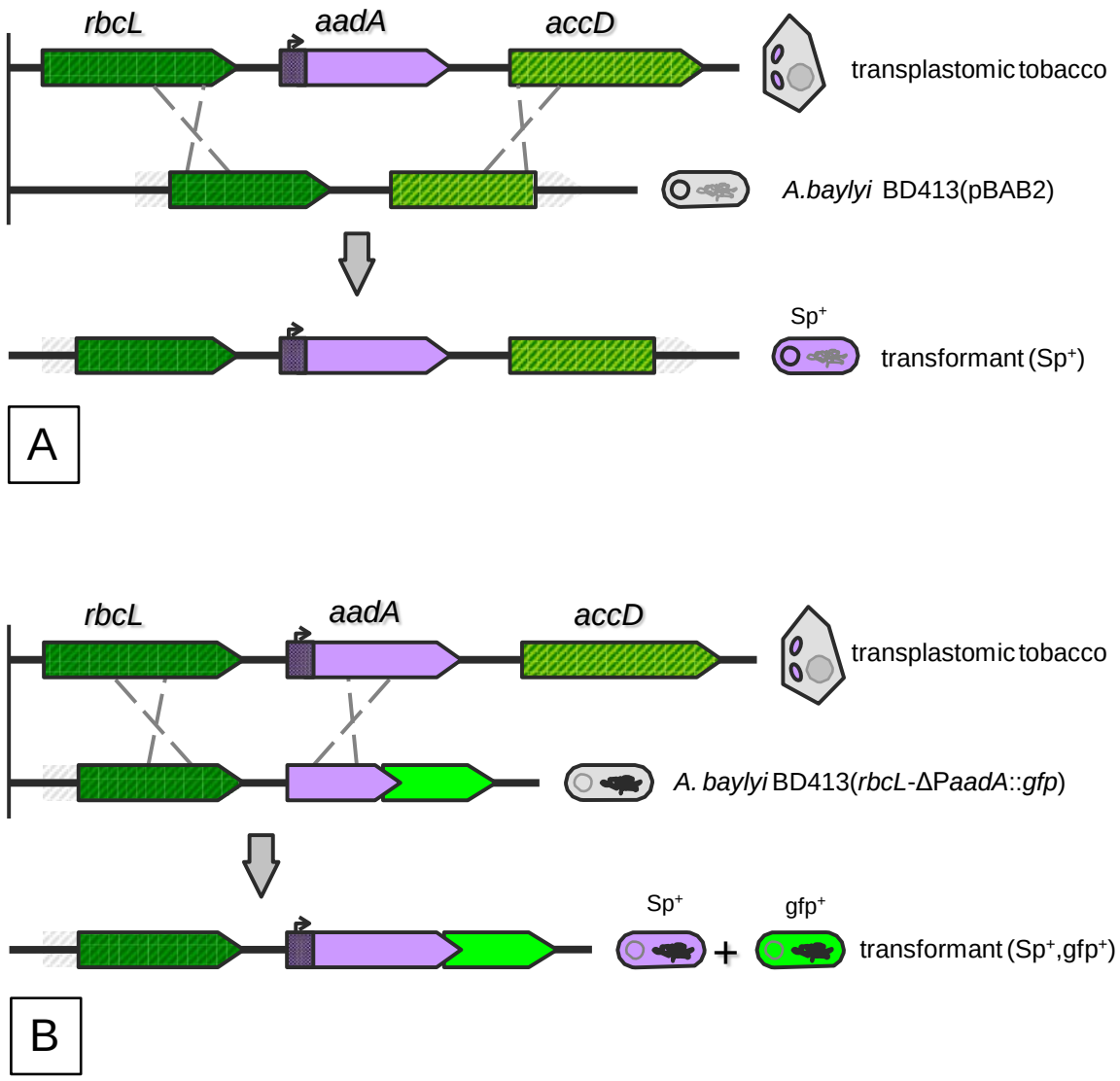

Figure 2. Schematic representation of homologous recombination events in *A. baylyi* reporter strains used in this study. (A) *A. baylyi* strain BD413(pBAB2) harbors the pBAB2 plasmid (Kay *et al*., 2002b) which contains two neighboring chloroplastic gene sequences (*accD* and *rbcL*) favoring recombination with homologous sequences of the transplastomic tobacco DNA flanking the *aadA* transgene. After homologous recombination with plant DNA the transgene is inserted in the plasmid and confers a spectinomycin resistant phenotype to the transformed cell. (B) *A. baylyi* strain BD413(*rbc*L-Δ*PaadA*::*gfp*) carries a promoterless *aadA::gfp* fusion downstream of *rbcL* cloned in the chromosome between the *lipB* and the *lipA* genes. The functionality of the *aadA::gfp* fusion is restored after homologous recombination with the transgenic DNA, conferring both antibiotic resistant and green fluorescent phenotypes to the recombinant cells.

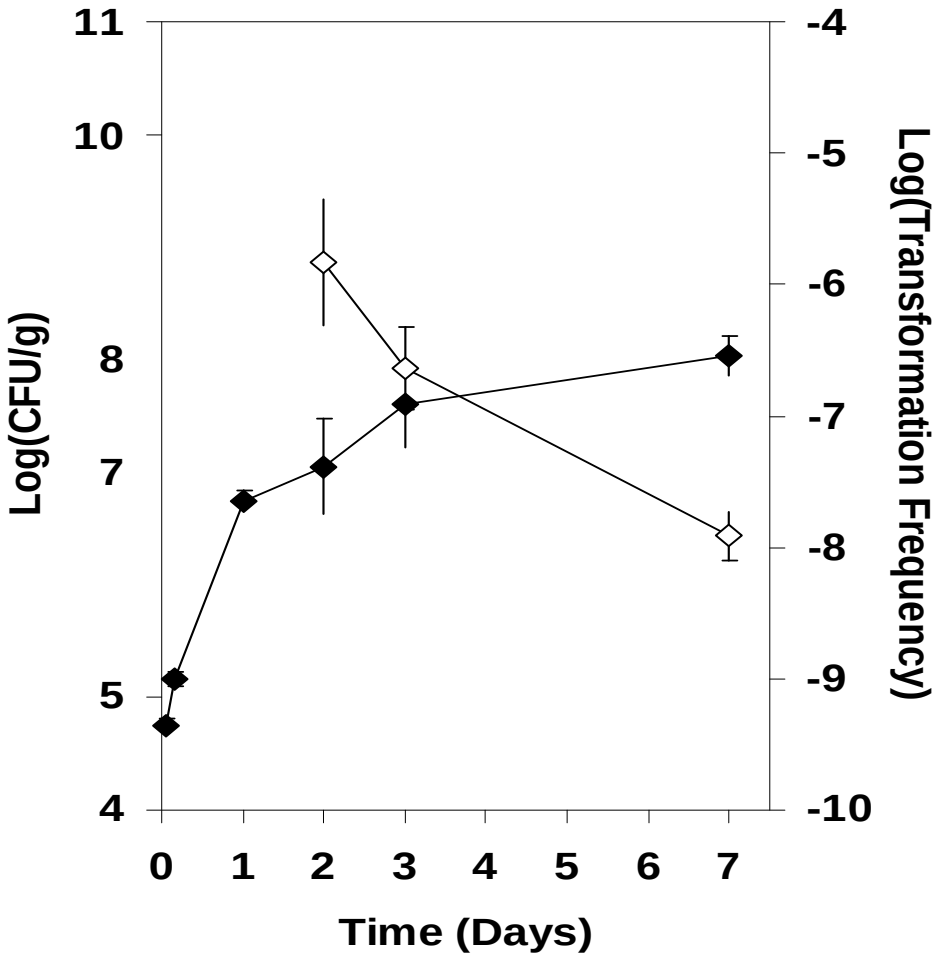

Figure 3. Growth (black diamonds) and competence development (white diamonds) kinetics of *A. baylyi* strain BD413(pBAB2) inoculated on leaf surfaces of WT tobacco plants and incubated for 7 days. Competence development is expressed as the log number of transformation frequencies (transformants/recipients) determined after exposure of the bacterial suspensions recovered from leaf surfaces to plasmid pCEA *in vitro*.

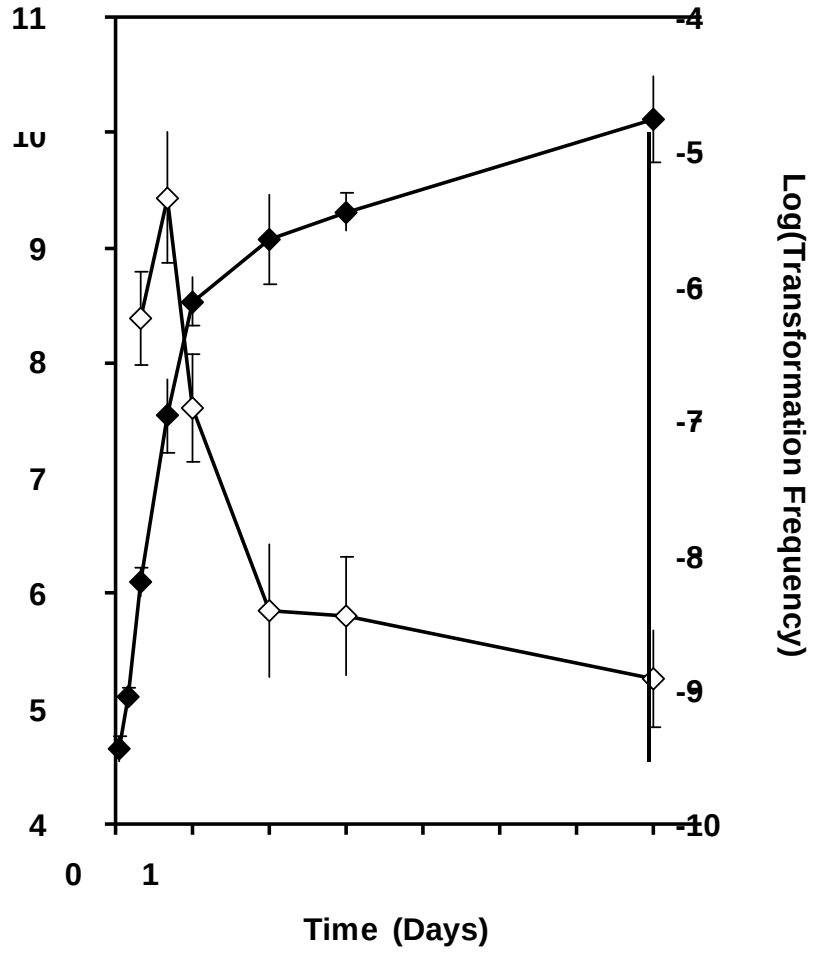

Figure 4. Growth (black diamonds) and competence development (white diamonds) kinetics of *A. baylyi* strain BD413(pBAB2) inoculated on ground leaf discs of WT tobacco plants and incubated for 7 days. Competence development is expressed as the log number of transformation frequencies (transformants/recipients) determined after exposure of the bacterial suspensions recovered from leaf tissues to plasmid pCEA *in vitro*.

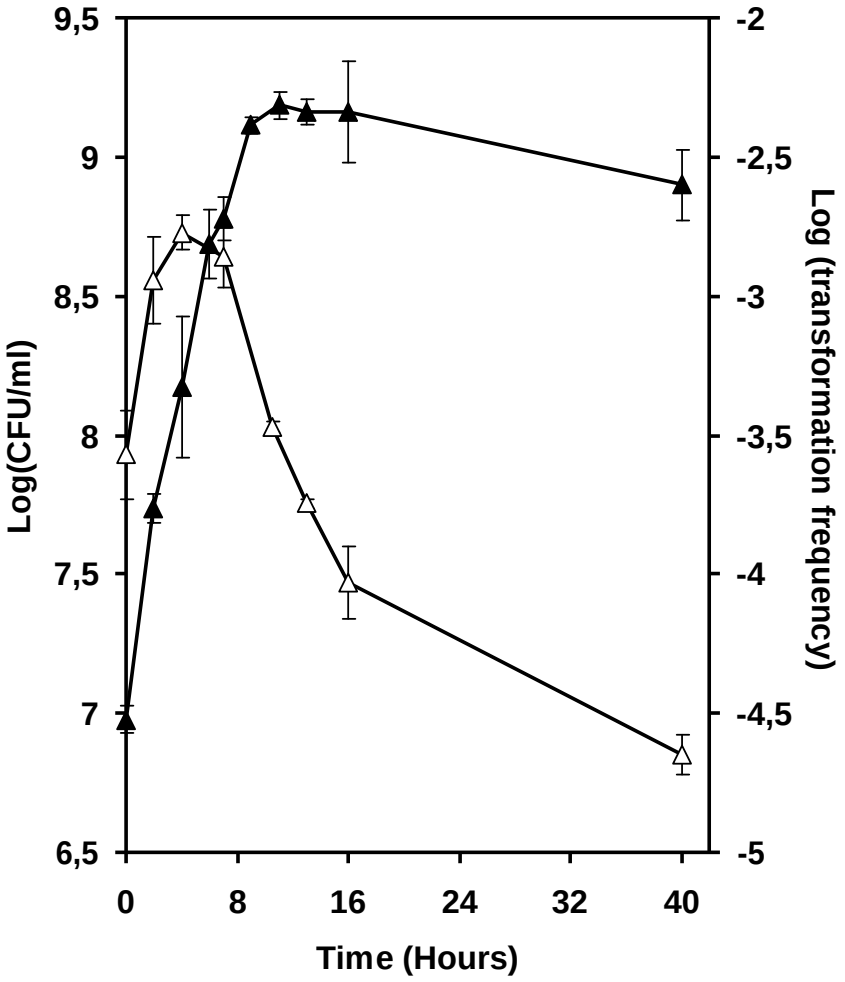

Figure 5. Growth (black diamonds) and competence development (white diamonds) kinetics of *A. baylyi* strain BD413(pBAB2) inoculated in LBm broth and grown for 40 hours. Competence development is expressed as the log number of transformation frequencies (transformants/recipients) determined after exposure of the bacterial suspension to plasmid pCEA.

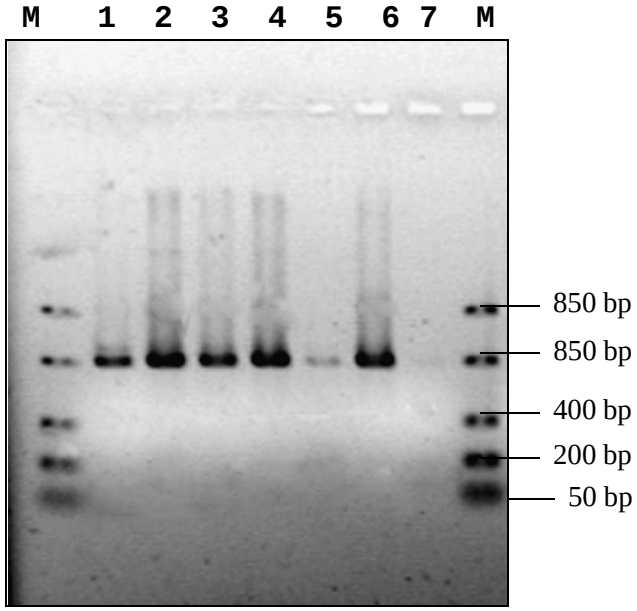

Figure 6. Amplification of transplastomic plant DNA sequences in the genome of transformants isolated from decaying leaf discs. Detection of the transplastomic sequence was performed by PCR using primers p1351cpl2up and p416 targeting a region upstream the gene and a region within the gene, leading to amplification of a 853-bp fragment. Lane 1 to 6: PCR products obtained from six transformant clones of *A. baylyi* strain BD413(pBAB2) isolated from plants. Lane 7: negative control (non transformed recipient strain). M: size marker (Fastruler, low range).

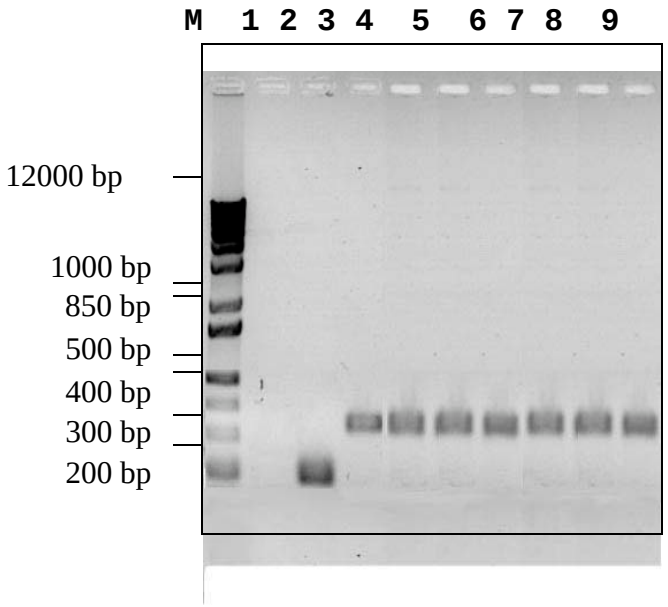

Figure 7. Amplification of the P_{rrn} promoter region located upstream of the *aadA::gfp* fusion on genomic DNA extracted from *A. baylyi* strain BD413(*rbc*L-ΔP*aad*A*::gfp*) clones recovered from ground leaves. PCR was conducted with primers Promo -F and Promo-R targeting the P_{rrn} promoter region. A 333-bp band is characteristic of the restored promoter, while the disrupted promoter size is 190 bp. Lane 1: negative control (water); Lane 2: negative control (non transformed recipient strain BD413(*rbc*L-ΔP*aad*A*::gfp*); Lanes 3-10: PCR products obtained from seven transformant clones of BD413(*rbcL-ΔPaadA::gfp*); M: 1 Kb plus size marker.

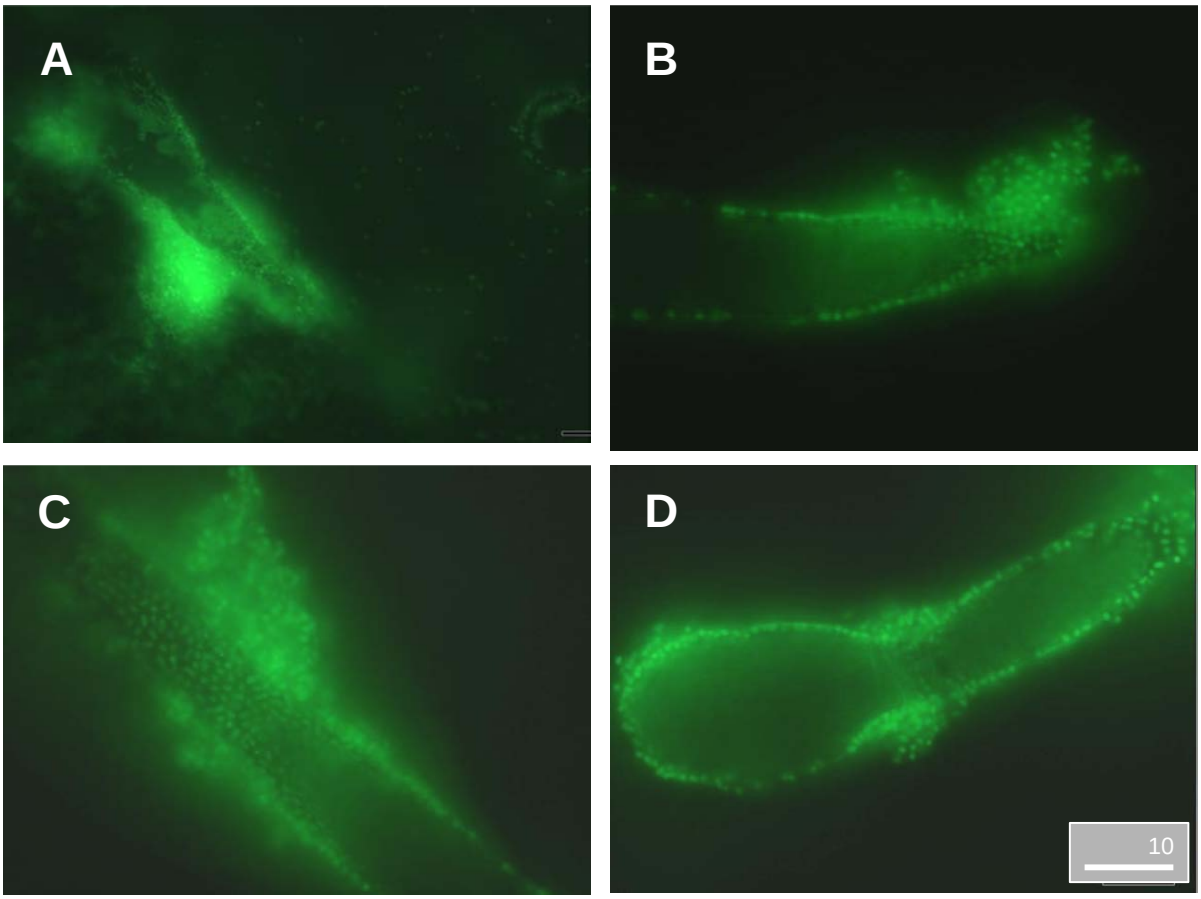

Figure 8. Epifluorescence micrographs of *A. baylyi* strain BD413(*rbcL-aad*A::*gfp*) inoculated on intact leaves. Bacterial cells constitutively express the green fluorescent protein (GFP). The spatial distribution of the cells revealed that growth on leaf surfaces had preferentially occurred near trichomes. (A, B): hooked trichomes; (C, D): glandular trichomes. (Bar =10µm).

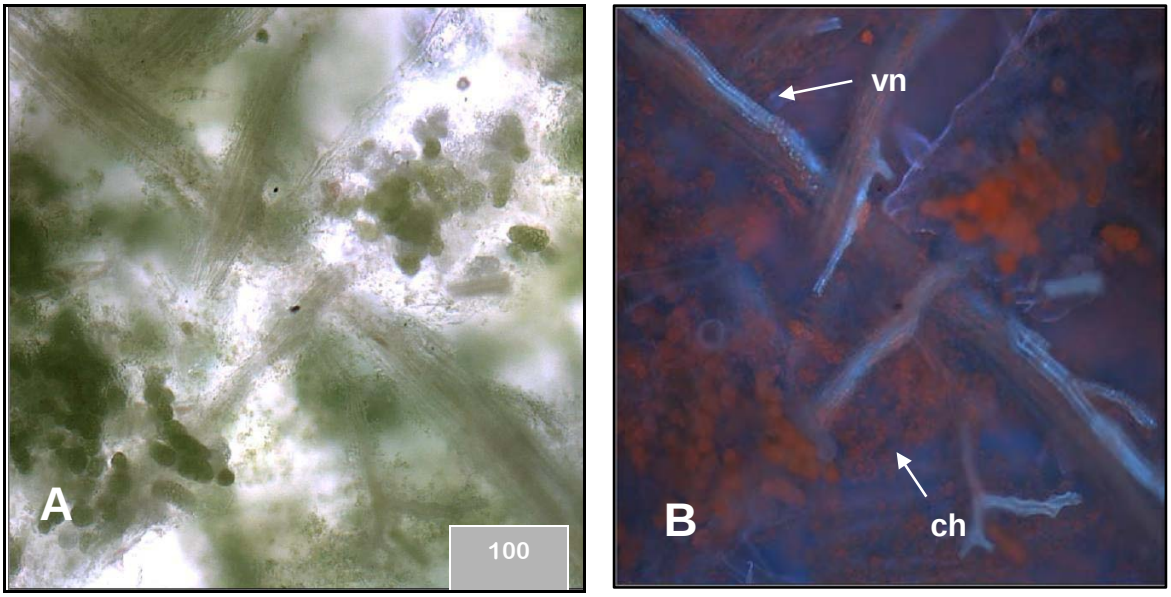

Figure 9. Micrographs of ground leaf tissues. Images of the same field of view were captured with the bright field (A) and epifluorescence using a long pass DAPI filter (B) revealing veins (vn) in blue and chloroplasts (ch) in red. (Bar = 100 µm)

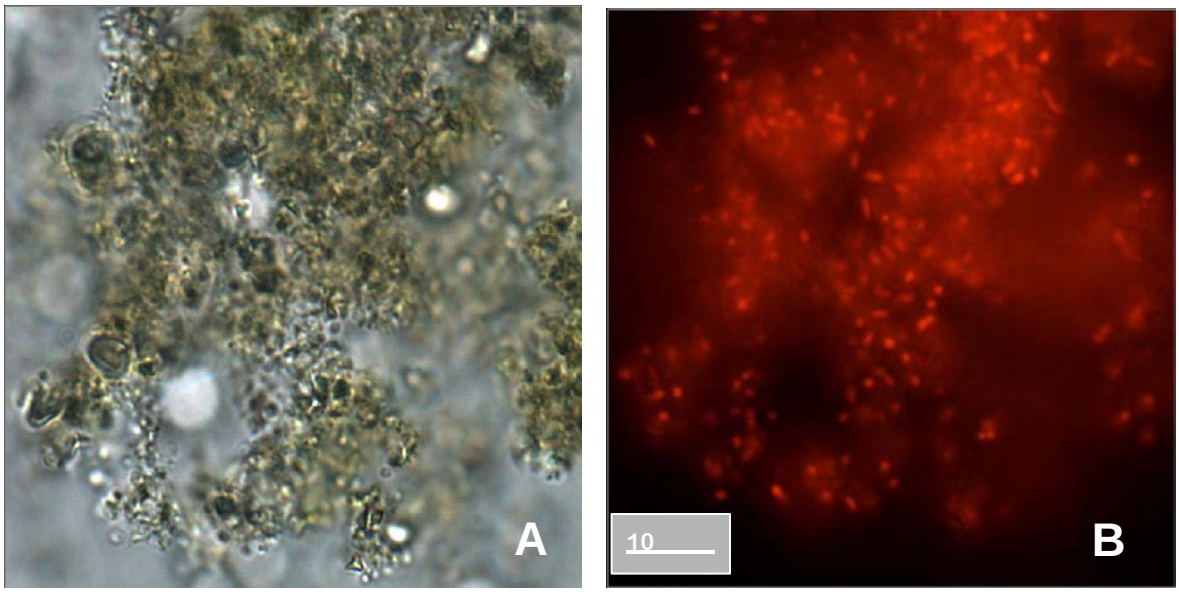

Figure 10. Micrographs of leaf debris inoculated with *A. baylyi* strain BD413(pBAB2). Cells were observed after two days of incubation. Bright field image showing leaf tissues (A) and the same field of view observed by epifluorecence where bacterial cells stained with ethidium bromide appear in red (B).

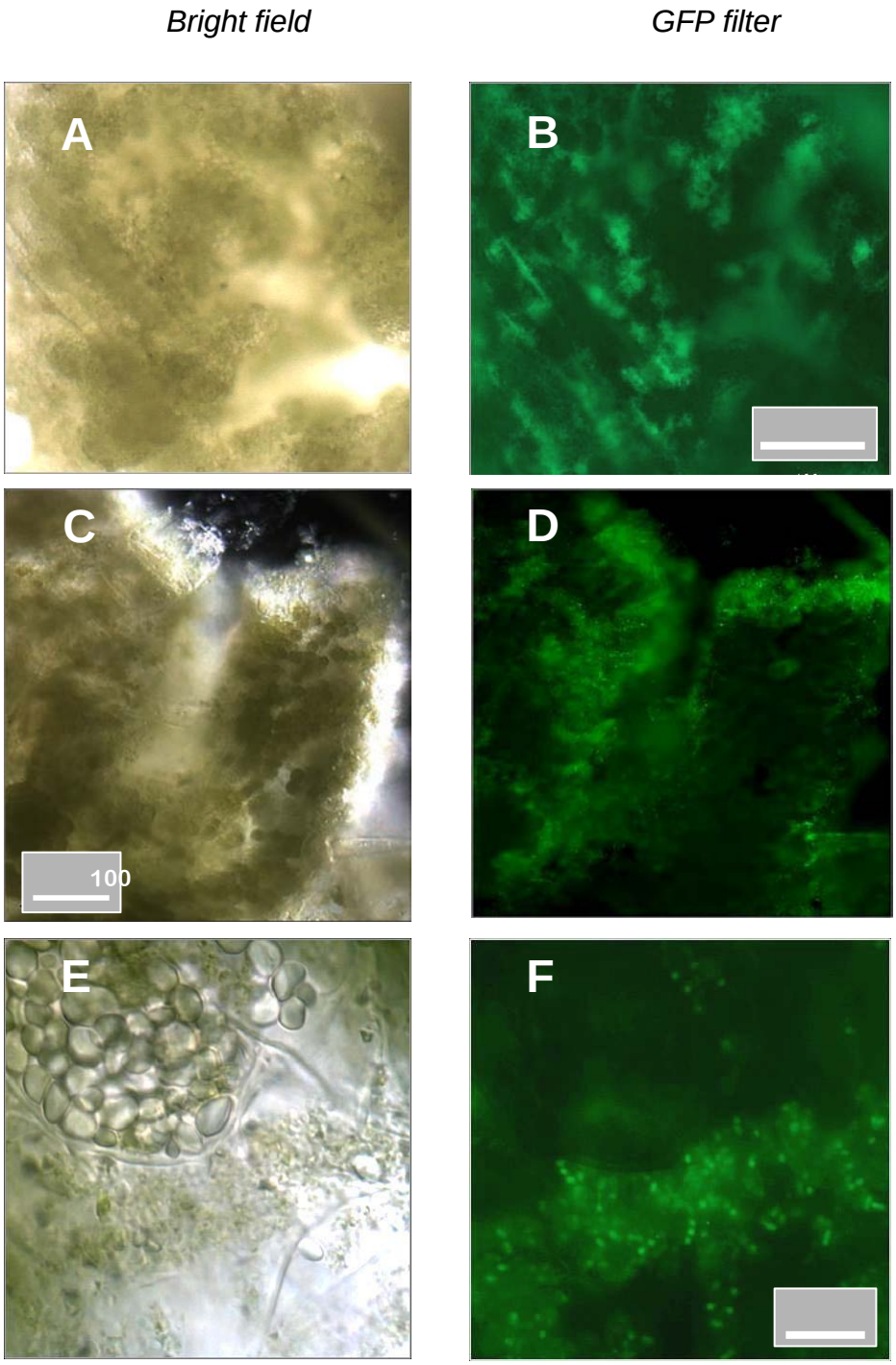

Figure 11. Micrographs of leaf debris inoculated with *A. baylyi* strain BD413(*rbc*L-*aad*A::*gfp*) constitutively expressing the GFP. Samples were observed after two days incubation. Aggregates formed on the surface of the ground leaves (A, B). Aggregates were however preferentially observed (C, D). Details of a cell cluster formed on the edge of ground plant cells. (Bars = µm).

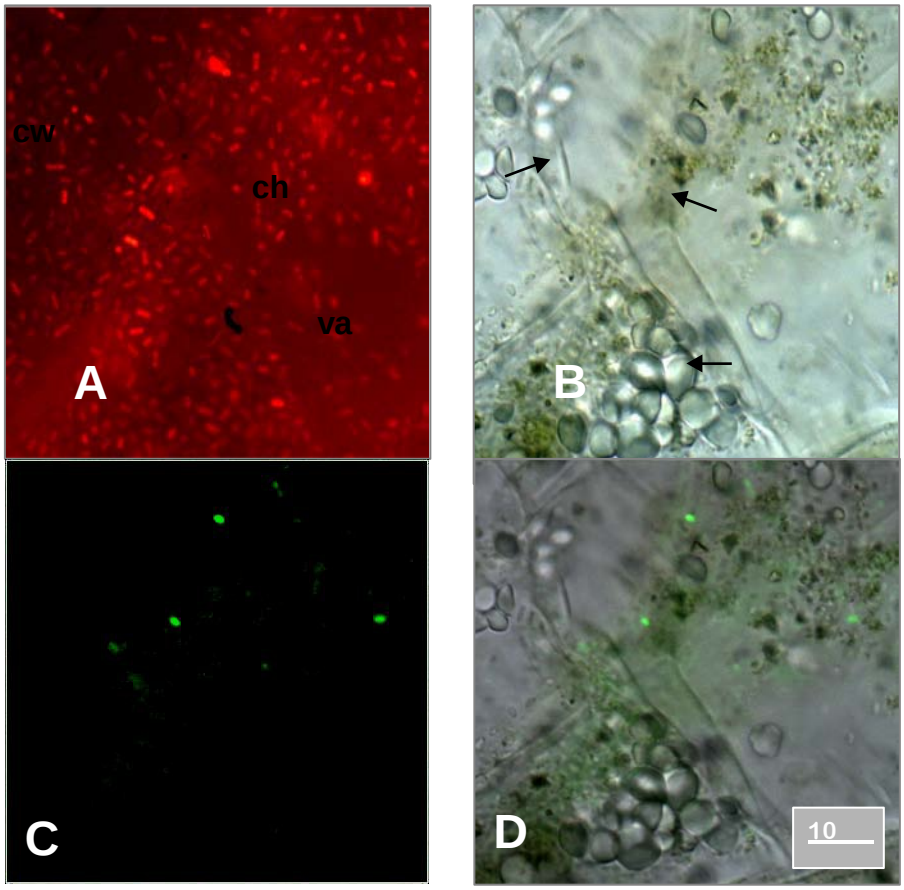

Figure 12. *In situ* visualization of plant to bacterium gene transfer in the residuesphere using *A. baylyi* strain BD413(*rbcL*-ΔP*aadA::gfp*) as a bioreporter strain. The same sample stained with ethidium bromide was observed (A) under bright field, (B) by epifluorescence with the rhodamine filter to visualize total bacterial cells and (C) with the GFP filter to visualize transformant cells. (D) Superposition of image A and C brings additional insight on the localization of the recombinants on plant residues. cw: cell wall; va: vacuoles; ch: chloroplasts. (Bar = 10 µm).

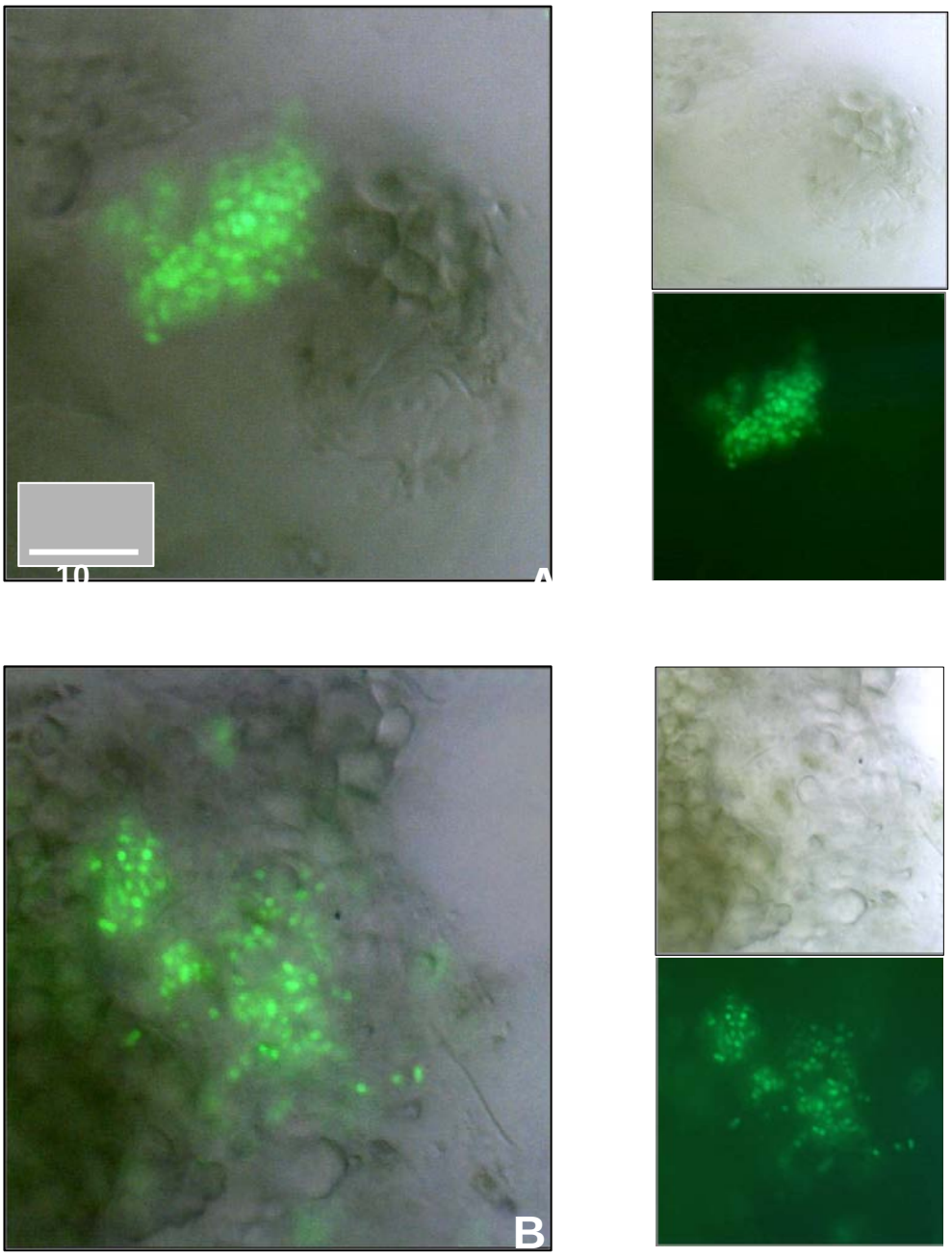

Figure 13. Cluster of recombinant cells of *A. baylyi* strain BD413(*rbc*L-*aad*A::*gfp*) observed in the residuesphere after 5 days of incubation. The aggregated nature of recombinants is especially evident as a microcolony in (A) and opened a methodological question on actual frequencies. Processed images were obtained by combining images obtained with bright field and with GFP filters shown on the right hand side. (Bar = 10 µm).

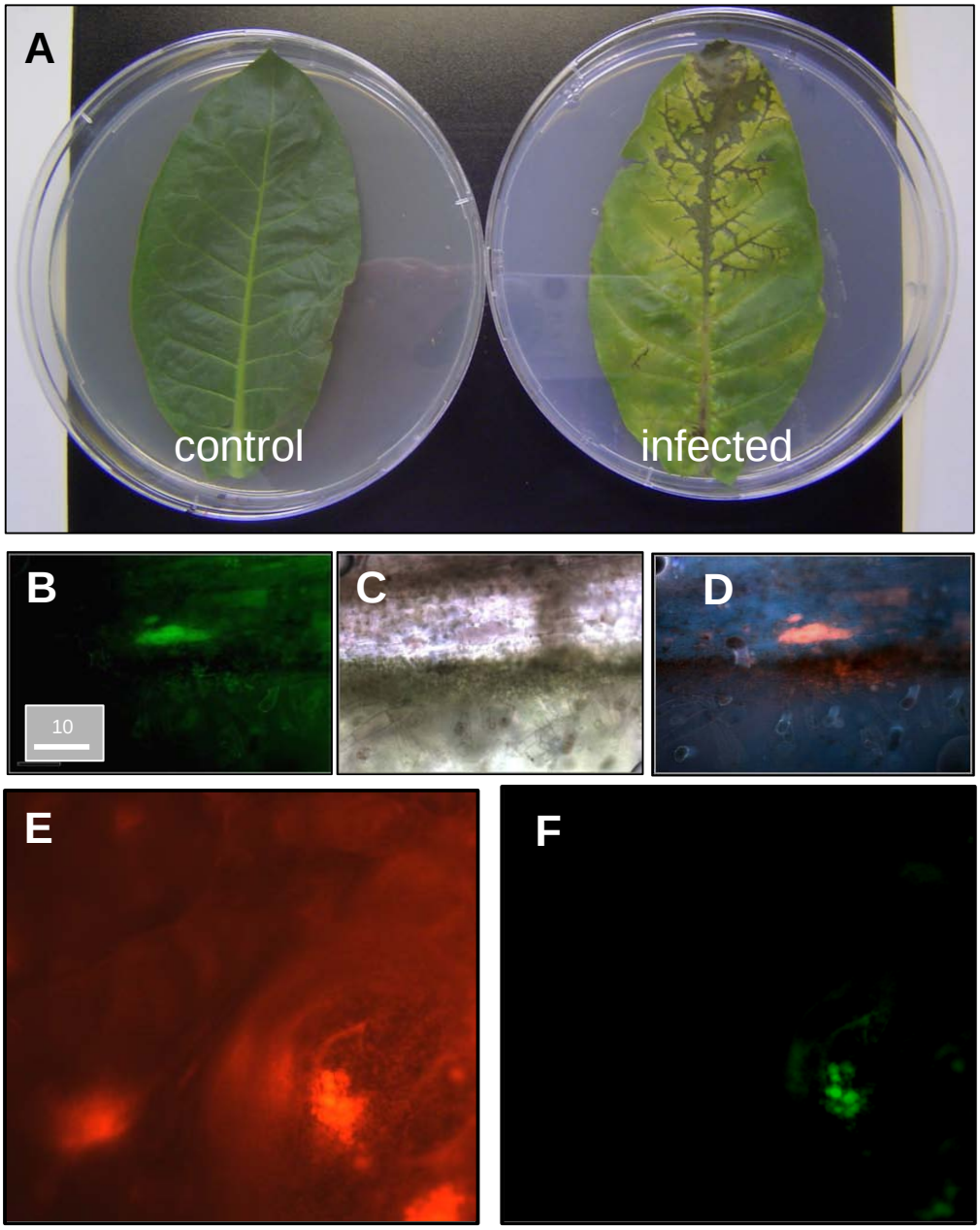

Figure 14. *In situ* detection of plant to bacterium gene transfer in tobacco leaves infected by *R. solanacearum* strain K60. Leaves co-infected with a mixture of *R. solanacearum* K60 and *A. baylyi* BD413(*rbcL*-Δ*aadA::gfp*) incubated for 11 days (A); section of a leaf vein observed with the GFP filter (B), with the brigth field (C) and with the rhodamine filter to visualize total cells stained with ethidium bromide (D). Total bacterial cells (E) and recombinant clones of *A. baylyi* BD413(*rbcL*-Δ*aadA::gfp*) (F) clustered near a stomatal chamber (Bar = 10 µm).

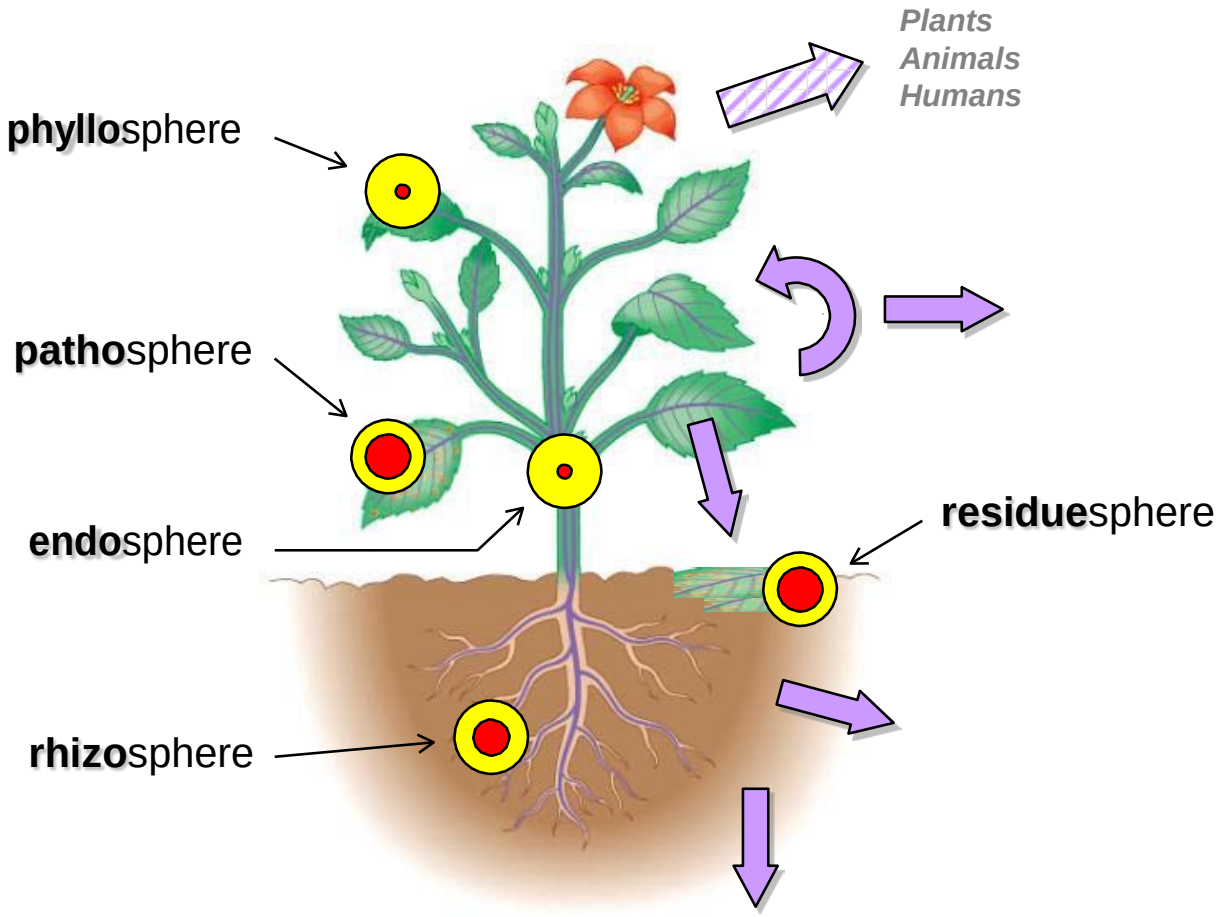

Figure 15. Compartments of the plant that could foster bacterial growth and plant to bacterium gene transfer due to localized conducive conditions such as enhanced presence of nutrients and extracellular DNA. Yellow circles represent bacterial populations, red circles represent the putative proportion of the cells exposed to plant DNA as a measure of probability of HGT *in situ* based on the literature. Arrows indicate potential transgene flow from the transgenic plant to the environment.

Chapitre 4

Avant propos du chapitre 4

Dans le chapitre précédent nous nous sommes intéressés aux interactions potentielles entre l'ADN transgénique et la bactérie colonisant la phytosphère. Ces travaux nous ont permis de détecter des transferts de gènes quand la plante est sujette à un processus de dégradation. Si l'ADN libéré par les cellules de la plante est majoritairement dégradé (Ceccherini *et al*., 2003) quelques molécules peuvent être incorporées dans les bactéries colonisant cet environnement mais on ne peut négliger que le dernier récepteur des débris de la plante sénescente, y compris l'ADN soit le sol.

Si la « residuesphere » caractérisée par une étroite interaction entre les tissus végétaux et la matrice tellurique peut être considérée comme un « hot spot » pour le transfert de gènes en permettant la croissance bactérienne, le développement de la compétence et la transformation naturelle *in situ* par l'ADN extracellulaire des questions demeurent quant à l'implication de l'environnement « sol » pour permettre l'acquisition d'ADN de la plante par les bactéries indigènes. Une première étape dans ce processus de transformation concerne la persistance dans le sol de l'ADN de la plante transgénique à l'état extracellulaire.

Plusieurs études ont déjà abordé cette question tant au niveau de microcosmes de sol étudiés au laboratoire que de situations de terrains notamment en utilisant la technique PCR pour détecter la présence des séquences d'ADN de la plante transgénique. Ces travaux ont montré la survie de l'ADN dans le sol, ces molécules pouvant persister jusqu'à plusieurs années dans le sol et demeurer détectables par PCR après extraction directe. Cette survie de l'ADN serait due à son adsorption sur les minéraux du sol et en particulier les argiles qui assurent une protection contre une dégradation enzymatique.

Toutefois des informations manquent sur le maintien du pouvoir biologique de cet ADN après incubation dans le sol. Dans ce chapitre ces deux volets ont été abordés en basant nos études sur les modèles biologiques précédemment décrits, le tabac transplastomique et la bactérie *Acinetobacter baylyi*. En utilisant une approche PCR le temps de persistance des signatures transgéniques dans le sol a été déterminé après que de l'ADN extracellulaire ou des tissus végétaux aient été inoculés dans les échantillons de sol. Cette étude a cependant été complétée en parallèle par la recherche du rôle biologique que pouvait avoir cet ADN, exprimé par sa capacité à transformer *in vitro* la bactérie réceptrice *Acinetobacter baylyi*. Nos travaux ont pour objectif

de déterminer si l'ADN qui persiste dans le sol maintient son potentiel biologique et pourrait être impliqué dans un mécanisme de transformation des bactéries colonisant naturellement les microstructures, en dépit du fait que le sol a longtemps été considéré comme étant un milieu oligotrophe. En effet, d'un coté la matrice tellurique est hétérogène et composée de micro-habitats dans lesquels l'état de compétence des bactéries du sol pourrait être atteint, ou induit par les chocs physico-chimiques auxquels elles sont plus ou moins constamment soumises.

Ces travaux ont été publies dans la revue Research in Microbiology (Pontiroli et al., 2010)

Long-term persistence and bacterial transformation potential of transplastomic plant DNA in soil

I- ABSTRACT

The long-term physical persistence and biological activity of transplastomic plant DNA (i.e. of plants genetically engineered in the chloroplast genome) either purified and added to soil or naturally released by decaying tobacco leaves in soil was determined. Soil microcosms were amended with transplastomic tobacco leaves or purified plant DNA and incubated for up to 4 years. Total DNA was extracted from soil and the number of transgenes (*aadA*) was quantified by Real Time PCR. The biological activity of these transgenes was assessed by transformation in the bacterial strain *Acinetobacter* sp. BD413(pBAB2) *in vitro*. While the proportion of transgenes recovered increased with the increasing amount of transplastomic DNA added, plant DNA was rapidly degraded over time. The number of transgenes recovered decreased about 10,000 fold within 2 weeks. The data reveal, however, that a small fraction of the plant DNA escaped degradation. Transgene sequences were still detected after 4 years and transformation assays showed that extracted DNA remained biologically active and could still transform competent cells of *Acinetobacter* sp. BD413(pBAB2). The approach presented here rapidly quantified the number of transgenes released and persisting in the environment over time and provided new insights on the fate of transgenic plant DNA in soil.

II-INTRODUCTION

Horizontal gene transfer (HGT) is a fundamental mechanism of bacterial evolution and adaptation (Garcia-Vallve *et al.*, 2000; Rensing *et al.*, 2002) but becomes a source of concern if bacteria in the environment integrate and express specific non-indigenous genes from transgenic plants. Questions rise about the possible impact of such transformation-mediated gene transfer if the newly acquired traits lead to an increased fitness of these genetically-transformed bacteria. Bacterial population shifts might occur with the specific risk that the number of bacteria containing the newly acquired genes increases significantly.

While no such transfer has been determined under natural field conditions, the spatial organization of soil and plant-associated bacterial communities and the presence of plant tissues and plant DNA in soil could enhance HGT between plants and bacteria. Soil is a heterogeneous matrix structured into microenvironments in which numerous and diverse soil bacteria develop micro-colonies (Grundmann, 2004). Some soil bacteria possess a genetically encoded natural transformation potential (Demaneche *et al.*, 2001c; Paget and Simonet, 1994) that can be expressed *in situ* (Demaneche *et al.*, 2001c). Moreover, indigenous bacteria might be passively transformed when subjected to lightning discharges *in situ* (Cérémonie *et al.*, 2004; Cérémonie *et al.*, 2006; Demaneche *et al.*, 2001a). In addition, other chemical and physical conditions similar to those that caused the bacterium *Escherichia coli* to be genetically transformed in river water (Baur *et al.*, 1996) might occur. The probability of transgene transfer to bacteria increases for transgenes containing prokaryotic sequences (Bertolla *et al.*, 2000; de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Kay *et al.*, 2002b) This suggests that the probability of HGT from plants to bacteria depends in part on the presence of homologous sequences in soil bacteria, which would favor homologous or homeologous (i.e.between similar but not identical sequences) recombination.

Based on gene transfer regulation, parameters such as the origin, transgene sequence, and copy number of the transgene in the plant, have a significant role in HGT. A significant percentage of DNA from decaying tobacco leaves escapes degradation and maintains its biological activity as shown by its ability to produce bacterial transformants *in vitro* (Ceccherini *et al.*, 2003). Although several reports have shown that DNA released by plant material could persist in soil for months or years as extracellular material (Gebhard and Smalla, 1999; Paget *et*

al., 1998; Widmer *et al.*, 1997; Widmer *et al.*, 1996), little is known about the biological potential of this long lasting DNA. Given that the greater the quantity of transgenic DNA released by the plant, the greater the potential for a soil bacterium to incorporate these sequences, transplastomic plants, which contain up to 10,000 copies of the transgene per cell (Bendich, 1987; Daniell *et al.*, 1998), are particularly well suited models for studying the fate of transgenes in soil and potential HGT between plants and bacteria.

The objective of this study was to investigate the physical persistence and biological activity (transformation frequency) of extracellular DNA either purified and added to soil or naturally released by decaying transplastomic tobacco leaves in soil. Soil microcosms were amended with purified plant DNA and stored at room temperature for up to 4 weeks before total DNA was extracted and quantified in Real Time PCR assays and transformed in to *Acinetobacter* sp. BD413 *in vitro*. The fate of plant DNA naturally released by transplastomic tobacco leaves in soil microcosms was similarly monitored for up to 210 weeks.

III-MATERIALS AND METHODS

III.1 Plant material

All 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. Description of the construction of the transplastomic plants has been reported (Kay *et al.*, 2002b).

III.2 Bacterial strains, plasmids, and culture media

The naturally transformable bacterium *Acinetobacter* sp. strain BD413 harbors the recombinant plasmid pBAB2 (Kay *et al.*, 2002b) in which plastid sequences corresponding to the *rbcL* and *accD* regions flanking the transgene have been cloned to facilitate homologous recombination with the transplastomic tobacco sequences. *Acinetobacter* sp. strain BD413(pBAB2)was used as the model strain for evaluation of the natural transformation

potential of plant DNA. It was routinely grown at 28°C on Luria-Bertani modified medium (Bacto Tryptone, 10 g/l; yeast extract, 5 g/l; NaCl 5 g/l) (LB*m*) supplemented with ampicillin (50 µg/ml) and nalidixic acid (20 µg/ml) (Sigma, St. Louis, USA). Transformants were selected on LB*m* medium containing ampicillin (50 µg/ml), nalidixic acid (20 µg/ml), and spectinomycin (50 µg/ml). The population sizes of recipient and transformant cells were estimated from colony counts after 2 days of incubation on plates at 28°C. *Escherichia coli* DH5α harboring the plasmid pCEA was grown at 37°C on LB*m* medium supplemented with ampicillin (50 µg/ml) and spectinomycin (50 µg/ml) (Sigma, St. Louis, USA). Plasmid pCEA (pLEP01) which had been used to transform tobacco plants (Kay *et al.*, 2002b), is a pBluescript® II SK+ derived, ampicillin-resistant cloning vector of a size of 7006 bp containing the *aadA* gene flanked with plastid sequences corresponding to part of the *rbcL* and *accD* regions (2.5 Kb) (Kay *et al.*, 2002b).

III.3 Soil microcosms

Microcosms consisted of non-sterile, sandy loam soil (50% sand; 41% clay; organic matter; 40.6 g/Kg of dry soil; pH 5.6) from La Côte Saint-André (Isère, France) in which intact or ground tobacco leaf tissue or purified plant DNA were added. Transplastomic and wild-type tobacco plant leaves were either cut into disks or ground in liquid nitrogen. A total amount of either 0, 0.05 g or 0.5 g of plant material, corresponding to approximately a total number of 0, 8 x 10^9 and 8 x 10^{10} *aadA* genes, respectively, was added to 10 g of soil. Soil-plant mixtures were placed into 50 ml Falcon™ polypropylene tubes (Becton Dickinson, Franklin Lakes, USA) and maintained at room temperature for up to 210 weeks. Similar experiments were performed by adding 1 ml of a purified plant DNA solution. Transplastomic plant DNA was added to 10 g of soil in amounts ranging from 0 to 10^6 ng (0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 ng) in 1 ml of ultrapure water. Mixtures were kept as described above for up to 4 weeks. Each experiment was performed in triplicate.

III.4 DNA extraction

Plant genomic DNA extraction from tobacco leaves was performed using the DNeasy® Plant Kit (Qiagen, Mannhein, Germany) according to the manufacturer's instructions. Plasmid DNA was isolated from *E. coli* DH5α(pCEA) using the QIAfilter™ Plasmid Midi Kit (Qiagen,

Mannhein, Germany) following manufacturer's instructions. Total DNA was extracted from microcosms using UltraClean™ Soil DNA Kit Mega Prep (Mo Bio Labs, Solano Beach, USA) following manufacturer's instructions, 0, 2, 3, 4, and 210 weeks after addition of DNA to microcosms. DNA degradation was assessed by electrophoresis on 0.8% agarose gels. Purity of extracted DNA was assessed by PCR using primers pA and pH complementary to part of the 16S ribosomal DNA gene (Edwards *et al.*, 1989). DNA concentrations were determined by measuring the absorbance of the solution at 260 nm (OD_{260}) with the Eppendorf® Biophotometer (Eppendorf, Westbury, USA).

III.5 Detection and quantification of transplastomic sequences

Detection and quantification of the transplastomic sequence signature was determined by PCR and Real Time PCR, respectively. Detection of transplastomic sequences in soil microcosms was performed by touch-down PCR using published specific primers p1531cpl2up (5'-TTTCTATTGTTGTCTTGGAT-3') and p416 (5'-TGACGGGCTGATACT-3') targeting a 853-bp sequence and with primers p415 (5'-ATTCCGTGGCGTTAT-3') and p416 (5'-TGACGGGCTGATACT-3') complementary to part of the *aadA* gene and targeting a 382-bp fragment (Ceccherini *et al.*, 2003). Denaturing and elongation steps were done at 95°C and 72°C, and the annealing temperature decreased by 2°C during 10 cycles starting from 60°C to 50°C, followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The last cycle was done at 72°C with a 7-min extension before storage at 4°C. Transgene DNA sequences were quantified by Real Time PCR using the LightCycler® 1.5 system (Roche, Meylan, France). Real Time PCR assays were carried out with 50 ng of template DNA using primers p415 and p416 to amplify a 382-bp fragment. The PCR reaction was adapted from Ceccherini et al. (Ceccherini *et al.*, 2003) and consisted of an initial denaturation at 95°C for 480 s followed with 45 cycles of 95°C for 10 s, 55°C for 8 s, and 72°C for 16 s. Plasmid pCEA was used as a template to establish calibration curves and was serially diluted in distilled sterilized water at concentrations ranging from 3.2 ng to 3.2 x 10^5 ng/µl. Data were analyzed with LightCycler® software 3.5.3 (Roche, Meylan, France). The number of *aadA* fragments in each sample was quantified by plotting the respective crossing-point value against standard concentrations. All samples were tested in triplicate, and the average value was used for quantification.

III.6 Natural Transformation of *Acinetobacter* sp. BD413

The transformation potential of the extracted DNA was determined by transforming *Acinetobacter* sp. BD413(pBAB2) with each DNA extraction *in vitro*. An overnight culture of *Acinetobacter* sp. BD413(pBAB2) was diluted 25-fold with fresh LB*m* medium supplemented with the appropriate antibiotics and cultured for 2 h at 28°C (OD_{600} = 0.9) to reach the competent state as described by Palmen et al., (Palmen *et al.*, 1993). Transformations were carried out by adding 12.6 µg, 1.4 µg, or 1 µg of total DNA extracted from microcosms in 50 µl of ultrapure water or 50 µl of serial dilutions of purified plant DNA (4000, 400, 40, and 4 ng) to 250 µl of 2,6 x 10^8 cfu/ml competent bacterial cells. The resulting mixtures were incubated for 2 h at 28°C. Appropriate dilutions were plated on LB*m* medium supplemented with the appropriate antibiotics. Population sizes of recipient and transformant cells were estimated from colony counts after 2 days of incubation at 28°C. Three replicates were used for each sample. PCR assays using primers p1531cpl2up and p416 were performed to confirm the presence of the transgene resulting from transformation by plant DNA in newly spectinomycin resistant clones of *Acinetobacter* sp. BD413(pBAB2). To assess the effect of soil-derived DNA on the transformation potential of *Acinetobacter* sp. BD413(pBAB2) by transplastomic plant DNA, different amounts of purified soil - and plant-derived DNA were mixed and used to transform *Acinetobacter* sp. BD413(pBAB2) as described above. DNA mixtures consisted of 800 ng of total DNA,where plant-derived DNA represented 0.05%, 0.5%, 5%, 50%, and 100% of the total amount.

III.7 Modeling the fate of plant DNA in soil

A simplified model assessing the fate of plant DNA in soil as a function of time was used based on the total amount of plant DNA recovered in different experiments. The model equation is $n_o = n_t - n_d - n_a$, where n_o, n_t, n_d, and n_a represent the number of *aadA* gene fragments observed, added, degraded, and adsorbed, respectively, where

$$n_t = \alpha.A$$
$$n_d = d.n_t$$
$$n_a = a.n_t / A$$

and *A* and *a* represent the amounts (µg) of plant DNA added and adsorbed, respectively; *d*,the percentage of degraded plant DNA; and α is the number of *aadA* genes/ µg of transplastomic plant DNA.

$$n_o = n_t \cdot \left(1 - d - \frac{a}{A}\right) \quad and\ if \quad \left(d + \frac{a}{A}\right) \geq 1 \ \ then \ \ n_o = 0$$

The number of *aadA* genes/ µg of transplastomic plant DNA (α) was determined experimentally by quantifying the number of *aadA* genes in 1 ng of purified transplastomic plant DNA by Real Time PCR. This DNA was supplemented with increasing amounts of plasmid DNA (pCEA, 7006 bp) ranging from 2 x 10^{-5} to 2 ng for which the exact number of *aadA* genes/ng plasmid DNA was determined based on its sequence and on the size of the transgene (resulting in x aadA genes/ng). The average number of copies of the transgene per plant cell in the transplastomic tobacco plants was calculated based on *Nicotiana tabacum* chloroplast genome size (plastome has 155939 bp; GenBank accession no.Z00044) and the size of the transgene, multiplied for the average number of plastomes in a tobacco cell.

III.8 Data transformation and statistical analysis

Data transformation, estimations of the total number of *aadA* genes/µg of total DNA, descriptive statistics, statistical analyses, and modeling of the fate of DNA in soil were performed using Microsoft Excel software (Microsoft Co., Redmond, USA).

IV- RESULTS

IV.1 Release of DNA in soil from decaying plant material

Deterioration of plant tissue became visible within the first weeks after burial in soil. After 3 weeks, leaf disks appeared greenish, flaccid, and water-soaked. Leaf disk degradation was more pronounced after 4 weeks in soil and, although while the 0.5-g leaf disks remained intact, 0.05-g leaf disks were fragmented into small pieces. After 210 weeks, plant material incorporated into soil could not be detected visually. The quantity of total DNA extracted from soil microcosms where plant material was added increased significantly as a function of time.

The average quantity of total DNA extracted from microcosms doubled, on average, after 4 weeks. The amount of total DNA extracted after 210 weeks (5.5 ± 0.7 µg/g of soil) was higher than that extracted after 4 weeks (4.8 ± 0.6 µg/g of soil), but not significantly different (P=0.34). During the first 4 weeks, the amount of total DNA extracted from microcosms where ground leaf disks were added was more than 2 fold greater than that of microcosms where intact leaf disks of the same weight were added. This difference was less pronounced after 210 weeks: 5.9 ± 2.0 µg/g and 4.9 ± 1.0 µg/g were extracted from soil microcosms where 0.5 g of ground and intact leaf discs were added, respectively.

IV.2 DNA extraction from microcosms amended with purified DNA

The initial amount of total DNA extracted from control soil was 6.0 ± 5.7 µg/g of soil and did not differ significantly between experiments. The amount of total DNA extracted at time 0 from microcosms amended with 10 µg or less of purified plant DNA/10 g of soil did not differ significantly from control soil and averaged 6.1 ± 1.2 µg/g of soil (Figure 1). The quantity of DNA extracted at time 0 from microcosms amended with greater quantities of plant DNA were significantly greater than control microcosms and were 15.0 ± 1.4 µg/g of soil and 19.5 ± 3.0 µg/g of soil for microcosms amended with 100 µg and 1 mg of purified plant DNA/10 g of soil, respectively (Figure 1). Although the average amount of total DNA extracted from microcosms increased over time, no significant differences were observed between microcosms with different amounts of plant DNA added initially. On average, 10.9 ± 1.3 and 9.3 ± 1.9 µg of DNA/g of soil were extracted from microcosms amended with purified plant DNA after 2 weeks and 4 weeks of incubation, respectively.

IV.3 PCR detection of transgene sequences in soil

Transplastomic sequence signatures were successfully amplified by PCR performed with DNA solutions extracted from soil microcosms that had been amended with purified transplastomic plant DNA or plant tissue. Aliquots of DNA solutions extracted from each soil microcosms were successfully amplified using primers complementary to highly conserved regions of the 16S rDNA attesting their purity (data not shown). Touch-down PCR assays with primers p415 and p416 and primers p1531cpl2up and p416 successfully amplified part of the *aadA* gene (382 bp) and a sequence containing part of the *aadA* gene and the adjacent plastid

DNA region specific of the transgene (853 bp), respectively. Transplastomic plant DNA sequences were not amplified from total DNA solutions extracted from control microcosms.
Transplastomic sequences were successfully amplified from total DNA solutions extracted immediately after addition of purified plant DNA to soil microcosms, regardless of the amount of purified plant DNA added, which ranged from 10^{-3} to 10^{3} µg/10 g of soil (Figure 2). After 4 weeks, transplastomic DNA sequences were only amplified from microcosms having been amended with 1 µg or more of plant DNA and only with the primer set p415 and p416 targeting the 382-bp fragment. The presence of the transgene was also detected in microcosms amended with transplastomic leaf tissue. The expected 853-bp DNA fragment was amplified in all DNA extractions from soils amended with the transplastomic plant leaves and was absent from all soil microcosms where wild type leaf tissue was added (Figure 3). After 3 weeks, DNA extracted from microcosms, that initially contained 0.5 g of intact or ground plant tissue yielded stronger PCR-fragment intensities than from microcosms amended with 0.05 g of leaf disks (Figure 3A). The same trend was observed after 4 years for microcosms in which transplastomic DNA sequences were still amplified by PCR (Figure 3B).

IV.4 Real Time PCR quantification of transgene sequences in soil

Real Time PCR results confirmed those obtained with touch-down PCR with the detection of transgene sequences after up to 210 weeks and only in soil samples amended with transplastomic leaves or purified transplastomic purified DNA. The number of *aadA* genes/µg of plant DNA (α) added to soil microcosms was $1.3 \pm 0.18 \times 10^{9}$ *aadA* genes / µg of plant DNA, which corresponded to about 7000 copies of the transgene/plant cell. Regardless of the initial amount or form of transplastomic DNA added to microcosms (*i.e.*,purified, intact, or ground tissue), the kinetics of degradation of plant DNA in microcosms showed that the number of *aadA* gene fragments decreased dramatically over time (Figure 4). The total number of *aadA* gene fragments decreased by over 10,000 fold within the first 2 weeks and by only 10 fold during the following 207 weeks of the experiment. No significant differences in the number of *aadA* gene fragments were observed between microcosms amended with the same amount of plant DNA as either purified DNA or intact and ground leaf tissue at any time point. The number of *aadA* gene fragments amplified from soil amended with 0.5 g of leaf disks was about 10-fold larger ($3.1 \pm 0.2 \times 10^{6}$) than that of soil microcosms amended with 0.05 g ofleaf disks ($3.3 \pm 0.1 \times 10^{5}$) after 4

weeks. After 210 weeks, *aadA* fragment sequences were amplified from microcosms amended with 0.5 g of leaf disks but not from those amended with 0.05 g of leaf disks (Figure 4).

IV.5 Fate of transplastomic plant DNA released in soil

The fate of transplastomic plant DNA added to soil microcosms was determined as a function of time. The presence of the transgene was quantified by Real Time PCR. Data were normalized and expressed as the number of *aadA* gene fragments divided by the total amount of DNA. Regardless of the amount of transplastomic plant DNA added to soil microcosms, the number of transgenes recovered decreased rapidly over time, thus confirming the data presented previously. The presence of the transgene could not be detected in microcosms amended with 0.1 µg or less of purified plant DNA after 0 and 2 weeks and in microcosms amended with 1 µg or less after 4 weeks (Figure 5). The percentage of transgenes recovered immediately after addition of purified plant DNA to microcosms was on average about 2 % of the number of transgenes added. The number of transgenes recovered after 2 and 4 weeks from microcosms amended with 10 µg or more of purified plant DNA did not differ significantly. No significant differences were observed in the amount of transgenes recovered after 4 weeks from microcosms amended with plant tissue or with an equivalent amount of purified plant DNA. Nonetheless, the proportion of transgenes recovered was roughly proportional to the number of transgenes added to the soil microcosms and increased with the increasing amount of transplastomic DNA added (Figure 5). For example, immediately after the addition of plant DNA (and also after 2 weeks), the percentage of transgenes recovered from microcosms amended with 10^3 µg of purified plant DNA was about 10-fold greater than that of microcosms amended with 1 µg of purified plant DNA (the addition of 1 µg of plant DNA corresponded to approximately 10^7 *aadA* / µg of total DNA).

To assess the amount of plant DNA that was degraded, or adsorbed as a function of time, a model, defined as $n_o = n_t - n_d - n_a$ (where n_o, n_t, n_d and n_a represent the number of *aadA* gene fragments observed, added, degraded and adsorbed, respectively) was applied to the data set (Figure 6). Degradation of plant DNA increased rapidly with time and only 2.3 %, 0.01 %, 0.01 % and 0.002 % of added DNA were recovered after 0, 2, 4 and 210 weeks incubation, respectively. While DNA extraction yield remained an unknown variable, most likely resulting in an overestimation of the amount of DNA degraded or adsorbed, the amount of plant DNA

recovered after 2 weeks and 210 weeks was, on average, 200 fold and 1,200 fold smaller, respectively, than that recovered immediately after addition of plant DNA to soil microcosms. Adsorption of plant DNA to soil organic and inorganic constituents also increased with time (Figure 6).

IV.6 Biological potential of plant DNA extracted from soil

Transplastomic plant DNA present in soil for up to 210 weeks still transformed competent cells of *Acinetobacter* sp. strain BD413 (pBAB2) *in vitro*. Transformants were obtained only with DNA extracted from soil samples at time 0 (*i.e.,* immediately after addition of purified DNA to soil microcosms) and not after 2 and 4 weeks. Alternatively, transformants were obtained using total soil DNA extracted from soil samples amended with 0.5 g ground leaf disks after 3 and 210 weeks, but not with any soil from of the microcosms amended with only 0.05 g leaf disks as either ground or intact tissue. PCR amplifications and sequence analysis confirmed that the spectinomycin- resistant clones obtained resulted from transformation by plant DNA extracted from microcosms. Transformation frequencies obtained *in vitro* with transplastomic plant DNA extracted from soil microcosms were similar to those obtained with purified plant DNA and were proportional to the number of transgenes present in the DNA extracts used to transform *Acinetobacter* sp. (Figure 7). The slopes of the linear regressions were 0.57 and 0.55 for transformation assays performed with purified DNA and extracted DNA, respectively, confirming the similar trend of the DNA biological activity (transformability) for both samples. Transformation frequencies obtained with DNA extracted from soil were slightly lower (by about 2.8 fold) than those obtained with purified plant DNA (Figure 7). To assess the effect of soil DNA on the transformation potential of *Acinetobacter* sp. by transplastomic plant DNA, different amounts of purified soil DNA and plant DNA were mixed together and used to transform *Acinetobacter* sp. BD413(pBAB2). Transformation efficiencies were weakly affected by the presence of nontarget DNA or soil impurities and were a function of the number of transgenes. For example, when 400 ng of purified plant DNA was mixed with 400 ng of purified soil DNA, transformation frequencies were, on average, 2.6-fold lower than those obtained with purified plant DNA only (-5.3 ± 0.5 and -5.7 ± 0. for plant DNA and plant DNA mixed with soil DNA, respectively).

V-DISCUSSION

Investigation of the physical persistence and biological activity of extracellular plant DNA added or naturally released by decaying transplastomic tobacco leaves in soil revealed that DNA sequences persist in soil and remain biologically active for several months. Here the fate of plant DNA in soil was addressed by investigating the main factors that prevent DNA to be recovered qualitatively and quantitatively, including adsorption and enzymatic degradation. The approach used to assess the amount of specific DNA sequences persisting in soil included direct extraction of total DNA, its purification, and the use of specific primers to amplify target DNA by PCR. Although the Real Time PCR method used could detect as few as 10^2 copies of the transgene/µg of soil DNA, methodological biases could, however, lead to an underestimation of the actual number of DNA sequences present in soil. For example, precise DNA extraction yields, which remained undetermined, or the dilution of target DNA sequences among the total genomic DNA (the metagenome) could have biased the PCR amplification process leading to an underestimation of the actual number of specific targets. Despite these potential methodological biases, the number of the transgenes amplified was systematically lower after 2 and 4 weeks than at time 0. The decrease was greater within the first 2 weeks (up to 3 orders of magnitude) than between weeks 2 and 4 for which the number of transgene and the amount of DNA recovered were similar. DNA remaining after 2 and 4 weeks most likely belonged to the fraction that was neither adsorbed initially onto soil components nor subjected to a rapid enzymatic degradation by nucleases naturally present in soil. The reduced rate of DNA degradation with time may have resulted from an increasing spatial segregation of DNA and the microorganisms responsible for its degradation in the soil matrix. Perturbation of the soil by mixing or adding water would most likely result in an increased degradation rate of the remaining plant DNA.

DNA adsorbs tightly to soil components such as clay particles, humic acids (Crecchio and Stotzky, 1998) or sand (Lorenz and Wackernagel, 1987), and therefore, DNA recovery rates can be low and even less than 1% for clay-rich soils (Frostegard *et al.*, 1999). Quantities of DNA recovered were probably underestimated for microcosms amended with the lowest amount of DNA. The theoretical value leading to saturation of binding sites as well as the amount of DNA that was adsorbed to soil components was assessed based on microcosms amended with different amounts of purified DNA (Figure 6). The amount of plant DNA that was adsorbed increased as a function of time. According to previous reports in which DNA adsorption potential was tested

with clay particles, most of the adsorbed DNA remained accessible to both nucleases and bacteria while the rest was protected against degradation and unavailable for transformation of competent bacteria (Demaneche *et al.*, 2001b). In our study, a fraction of the adsorbed DNA might have remained accessible to degradation, but the adsorption of DNA onto soil components may also increase with decreasing DNA fragment size, which results from the degradation of free DNA, as previously reported (Ogram *et al.*, 1994; Ogram *et al.*, 1988) .

Plant DNA is probably released gradually from decaying tissue (Ceccherini *et al.*, 2003), resulting in adsorption and degradation of DNA that can not be satisfactorily simulated by amending soil with pure plant genomic DNA. Developing and using soil microcosms in which plant material was allowed to decay was another step to simulate the release of DNA under natural conditions, assess the persistence of transforming DNA. The number of transgenes amplified after 2 weeks or more of incubation did not differ from that amplified from microcosms amended with a comparable amount of purified plant DNA. While purified plant DNA might have been degraded more rapidly in soil than the plant DNA contained in leaf tissue, no differences in the number of *aadA* gene fragments were observed after 2 weeks, suggesting that degradation of DNA in soil, regardless of its state (purified or within plant tissues), occurs rapidly. Although purified DNA may have been degraded more rapidly, extractions of DNA performed after only 2 weeks may have been too late to observe a difference in the degradation kinetics of purified plant DNA and DNA inside plant tissues. However, most of the DNA released from decaying tobacco leaves appears to be degraded within 72 hours (Ceccherini *et al.*, 2003). Both total DNA and numbers of *aadA* gene decreased remarkably within 72 hours, suggesting that plant nucleases were active and that the shearing process, which decreases the average size of plant DNA fragments, may prevent amplification of *aadA* sequences. It has been suggested that bacterial cell debris may protect DNA from inactivation in soil (Nielsen *et al.*, 2000a). Although this beneficial effect might apply to plant material, plant nucleases may counteract the protective effect of plant cell debris and account for the rapid degradation of plant DNA observed in soil.

In addition to the quantification of persistence of transgenes in soil, the transformation potential of extracellular DNA was considered. Extracellular DNA in soil may remain undetected, yet, some could be involved in a genetic transformation with indigenous soil bacteria. Assessment of DNA availability and transformation potential by inoculating soil with recipient bacteria to trap

DNA before isolating transformants suffers major flaws. Inoculated bacteria colonize a small proportion of the soil, often limited to the outer soil compartments without penetrating soil microaggregates (Recorbet *et al.*, 1995), thus avoiding most of the indigenous extracellular DNA. Moreover, naturally-transformable bacteria are transformed at very low frequency *in situ* due to a rapid loss of competence ability in soil (Nielsen *et al.*, 1997).As testing transformation directly in soil seems unsatisfactory for many reasons, tests were developed to determine transformation efficiency of a recipient strain exposed *in vitro* to DNA extracted from soil. Controls werecarried out with pure transplastomic plant DNA in the presence, or absence of soil contaminants traces and indigenous DNA. Transformation efficiency of extracted DNA was related to the Real Time PCR determined-number of target genes in the DNA and was slightly lower than that of pure plant DNA (Figure 7). Purified DNA added to soil and immediately extracted without any incubation exhibited transformation efficiency lower than that ofpure DNA for the same number of PCR target genes, suggesting a discrepancy between the presence of the transgene as detected by Real Time PCR amplification and the biological potential of these DNA molecules to transform bacteria. The addition of DNA to soil for no more than a few seconds might have been too brief for soil nucleases to degrade DNA, but long enough for chemical alteration or adsorption to reduce transformation efficiency. DNA extracted from soil samples amended with leaf disks for 2, 4, or 210 weeks had the same discrepancy between thecalculated number of target genes and those determined by Real Time PCR and the transformation efficiency. Such differences may have resulted from the presence of inhibitory compounds on extraction and PCR amplification yields. On the other hand, quantification of *aadA* genes by Real Time PCR using primers targeting only part of the *aadA* gene (382 bp) may have resulted in an overestimation of the actual number of functional *aadA* gene fragments that could transform *Acinetobacter* sp. strain BD413. Amplification of a sub-fragment of the transgene does not necessarily imply that the entire transgene and flanking regions long enough to allow recombination in the recipient strain were present,which could explain why transformants were not obtained with some of the DNA extracts containing an *a priori* significant number of transgenes (Figure 7). Such hypotheses could also explain the difference observed in the transformation potential of DNA extracted from microcosms amended with purified DNA and those amended with leaf disks. Transformants were obtained with soil samples amended with purified plant DNA at the sampling time 0 only, whereas transformants were obtained with DNA extracted after 3 and 210 weeks from microcosms amended with leaf tissue. PCR amplification of the transgene, as shown in Figs. 2 and 3 confirmed this hypothesis:

after 4 weeks, only 382-bp fragments were amplified with DNA extracted from microcosms amended with purified DNA, whereas 853-bp fragments were still present after 210 weeks in microcosms amended with leaf tissue. We hypothesize that plant DNA gradually released from decaying plant tissue was not as degraded as purified DNA, and thus, for the same number of *aadA* gene fragments amplified by Real Time PCR, the number of functional *aadA* genes (i.e., larger DNA fragments) was more abundant in microcosms amended with leaf tissue. Although plant DNA is rapidly degraded by plant nucleases during the decaying process, cell debris may have a protective role and allow plant DNA to persist and remain biologically active in soils for long periods of time. The presence of cell debris may be important for the protection of DNA against enzymatic hydrolysis and interaction with soil particles (Nielsen *et al.*, 2000a). During the first two weeks of the experiment, the half-life of plant DNA added to soil was estimated to be 23 h, which implies that after about 40 weeks, no transgene should be amplified from soil. However, the transgene was detected for up to 210 weeks, with a half-life of 83 days when long-term data (longer than 2 weeks) was used. Such observations suggest that although DNA is rapidly degraded in soil, a small proportion of plant DNA escaped degradation by soil nucleases. Although cell debris and adsorption of DNA to soil particles may have a protective role, it is also possible that under the conditions tested, some plant DNA was spatially isolated from soil nucleases or microorganisms and, therefore, escaped degradation. However, under natural conditions, soil is exposed to fluctuating environmental conditions (e.g., temperature, moisture…) that might affect the persistence of DNA in soil differently than those reported in this study.

While determination of copies of the transgene in extracted DNA may not provide exact data about DNA availability for transformation of bacteria *in situ*, such an approach most likely underestimates the gene pool that could be involved in a transformation process. The physical persistence and biological activity of extracellular plant DNA released by decaying transplastomic tobacco leaves in soil lasted for up to 4 years. The low number of transgenes amplified after two weeks revealed that plant DNA was rapidly degraded. Although persistence and biological activity of plant DNA in soils was long-term, the direct contact between soil bacteria and plant DNA probably occurs during the first days of plant tissue degradation. The presence of degrading plant tissue in soil may i) promote the growth of soil bacteria or bacteria naturally occurring on plant tissue by providing a peculiar and abundant source of nutrients, ii) lead to a localized copiotrophic environment potentially fostering cell competence, and iii) favor

direct contact between bacterial cells and plant DNA. Therefore, while plant DNA may persist in soils for long periods, horizontal gene transfer and acquisition of the transgene by soil bacteria is most likely to occur during the initial degradation of plant tissue in soils, when plant DNA is still abundant and bacterial cells are actively growing.

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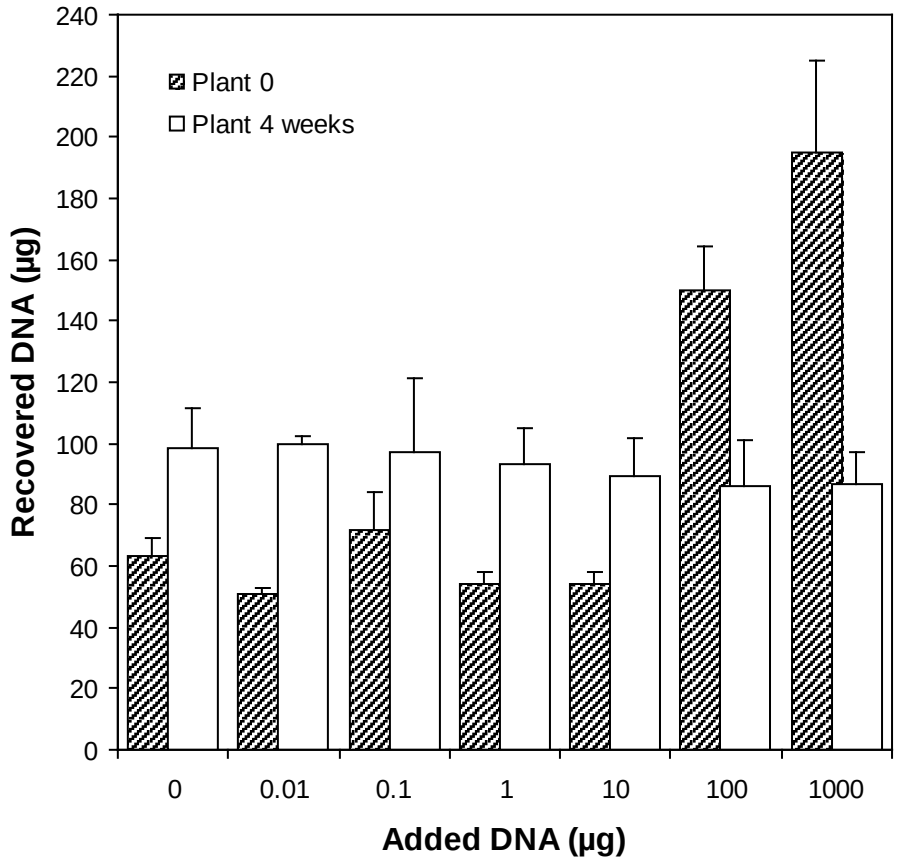

Figure 1. Total amount of DNA extracted as a function of the amount of purified plant DNA added to soil (10 g) microcosms immediately after addition (*shaded bars*) and after 4 weeks in soil (*white bars*). Error bars represent the standard error of the mean amount of DNA recovered.

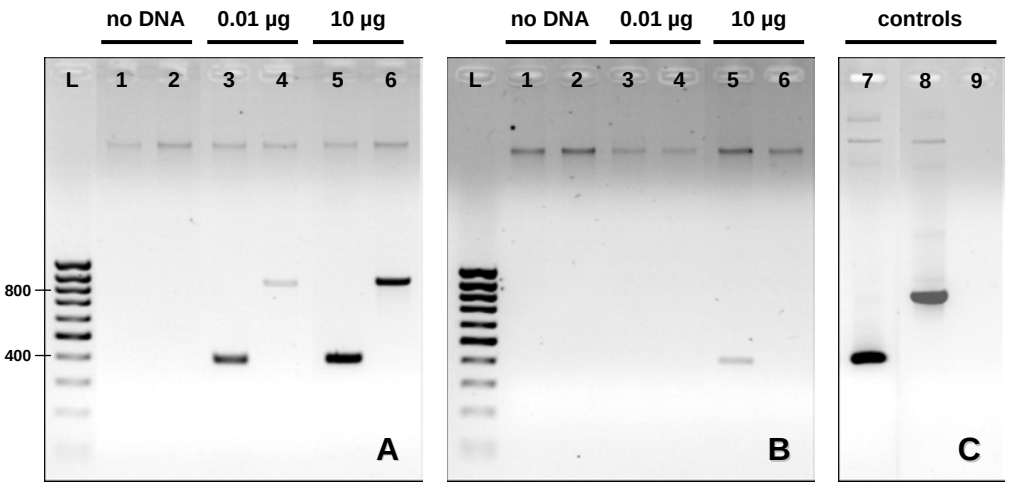

Figure 2. Amplification of transplastomic plant DNA sequences extracted from soil microcosms immediately after addition of purified plant DNA (A) and after 4 weeks in soil (B) visualized by agarose gel electrophoresis. Detection of transplastomic sequences in soil microcosms was performed by PCR using 2 primer sets targeting 382-bp (left) and 853-bp (right) fragments, respectively. DNA extracted from nonamended microcosms (1, 2), from microcosms amended with 0.01 µg of purified plant DNA (3, 4), or with 10 µg of purified plant DNA/10 g of soil (5, 6) are shown. (C) Positive controls performed using purified plasmid DNA as a matrix (7, 8) and negative control, H_2O (9).

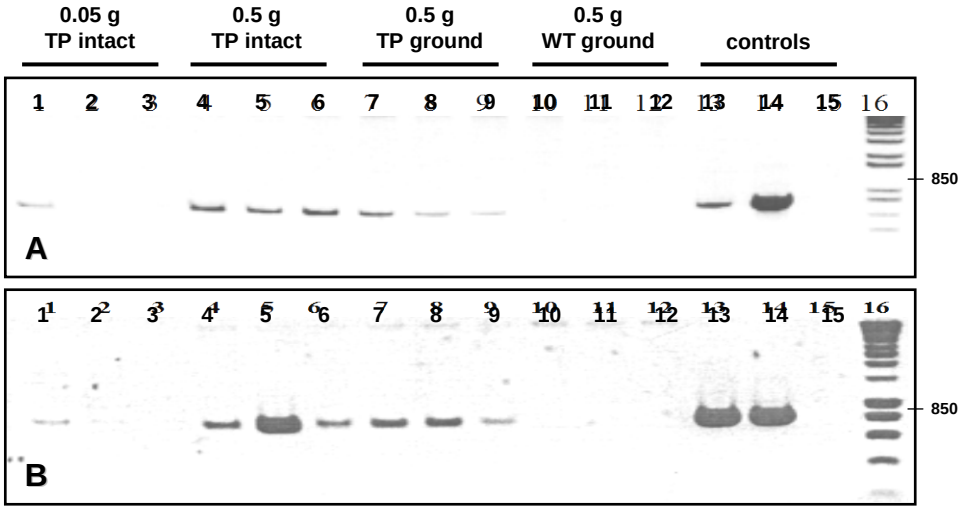

Figure 3. Amplification of transplastomic plant DNA sequences extracted from soil microcosms amended with leaf disks after 3 weeks (A) and 210 weeks (B) visualized by agarose gel electrophoresis. Detection of transplastomic sequences was performed by PCR using primers p1531cpl2up and p416 targeting a 853-bp fragment. DNA extracted from microcosms amended with 0.05 g intact leaf disks (1-3), with 0.5 g intact leaf disk (4-6), with 0.5 g ground leaf disks (7-9) of transplastomic tobacco plants (TP), and with 0.5 g ground leaf disks of non transplastomic tobacco plants (WT); Purified plasmid DNA (pCEA, 16 ng) (13); Purified transplastomic plant DNA (30ng) (14); Negative control (H2O) (15).

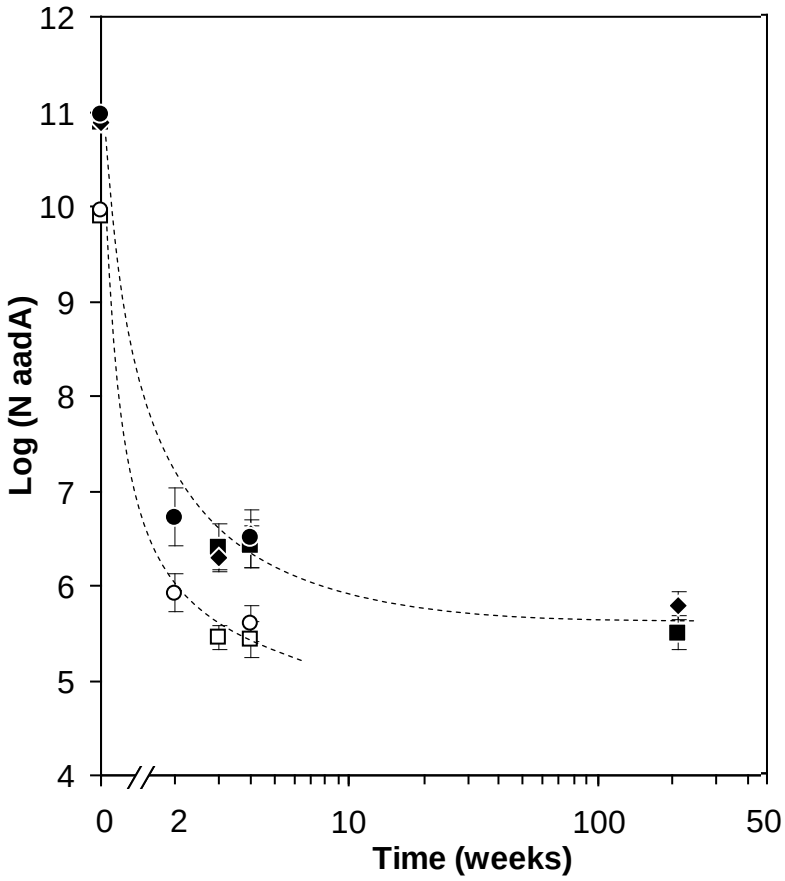

Figure 4. Kinetics of plant DNA degradation in soil microcosms as estimated by the number of *aadA* gene fragments amplified by Real Time PCR. Different amounts of purified plant DNA were added to soil microcosms (10 µg, *white circle*; 100 µg, *black circles*) and degradation was monitored for up to 4 weeks. The number of *aadA* gene fragments in soil microcosms where intact 0.05 g (*white squares*) and 0.5 g (*black squares*) of leaf disks and 0.5 g of ground leaf disks (*black diamonds*) were added was quantified for up to 210 weeks. Dotted lines represent best fit of the equations. Vertical bars represent the standard error of the mean number of *aadA* gene fragments amplified.

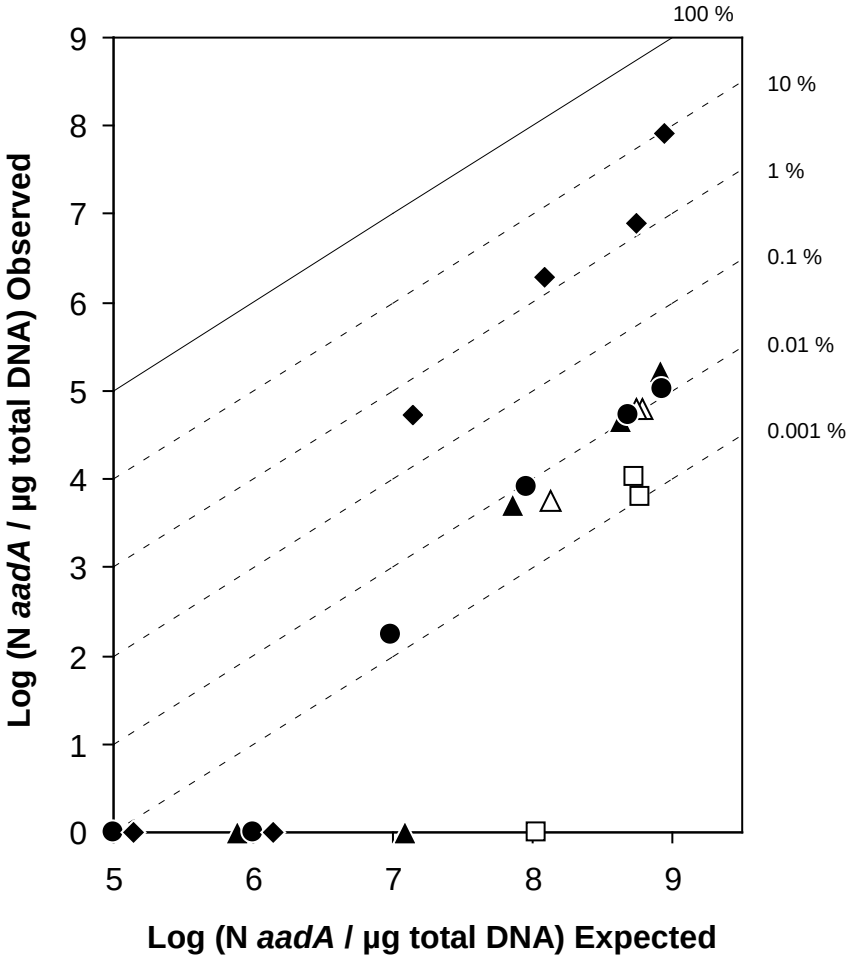

Figure 5. Number of *aadA* gene fragments recovered as a function of the number of *aadA* genes added to soil microcosms. Data were normalized and expressed as the number of *aadA* gene fragments divided by the total amount of DNA extracted. Quantification was performed after 0 (*diamonds*), 2 (*circles*), 4 (*triangles*), and 210 weeks (*squares*) for purified plant DNA (*black*) or leaf disks (*white*) in the soil microcosms. The addition of 1 µg of plant DNA corresponded to approximately 10^7 *aadA* / µg of total DNA. Dotted lines correspond to the relative percentage of *aadA* gene fragments recovered and amplified by Real Time PCR. Standard error bars were removed for clarity but averaged 0.25 ± 0.08 Log (N *aadA* / µg total DNA).

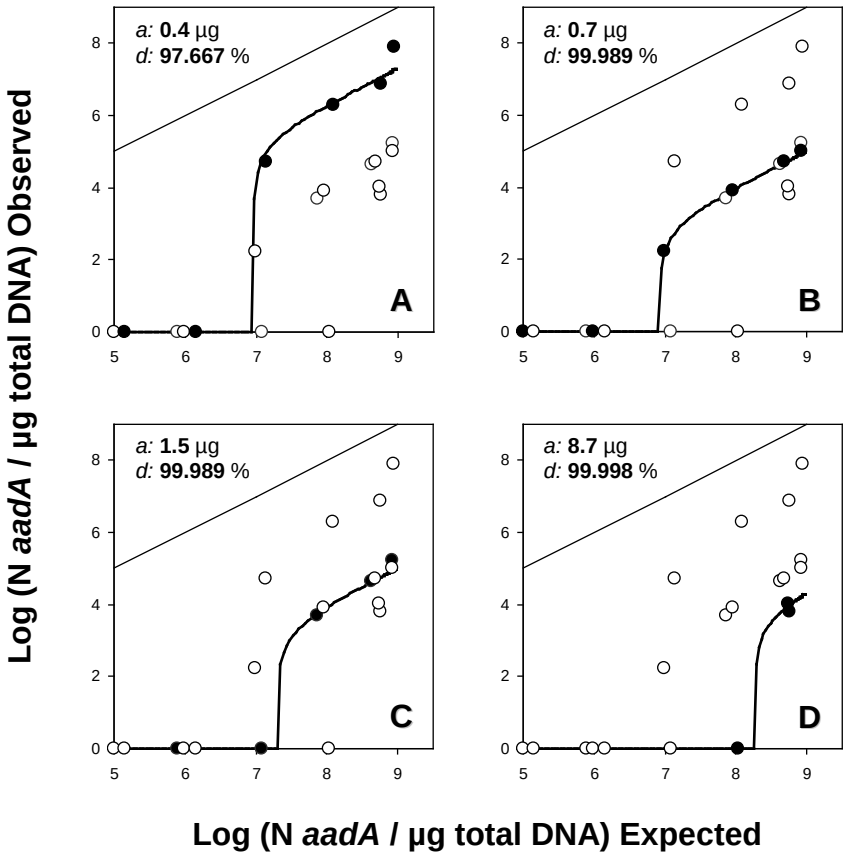

Figure 6. Assessment of the amount of plant DNA that was adsorbed (*a*) and degraded (*d*) following its addition to soil microcosms for 0 (A), 2 (B), 4 (C), and 210 weeks (D), as a function of the amount of DNA added to microcosms. The model was defined as $no = nt \cdot (1 - d - a/A)$, where *no* and *nt* represent the number of *aadA* gene fragments observed and added, respectively, *A* and *a* represent the amounts (µg) of plant DNA added and adsorbed, respectively, and *d* the percentage of degraded plant DNA.

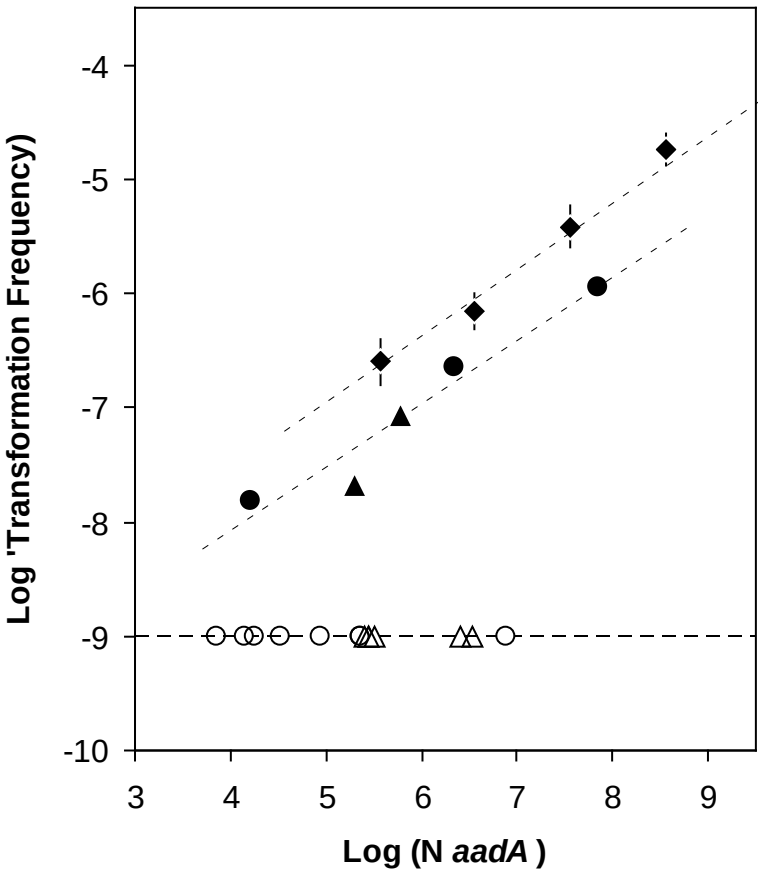

Figure 7. *In vitro* transformation of *Acinetobacter* sp. BD413(pBAB2) with purified plant DNA (*diamonds*), and with DNA extracted from soil microcosms amended with purified plant DNA (*circles*) or leaf disks (*triangles*). Open symbols correspond to transformation frequencies below the detection level (~10^{-9}). Dashed lines represent the linear regression of the transformation frequency of *Acinetobacter* sp. BD413(pBAB2) as a function of the number of *aadA* genes present in the solution for purified plant DNA ($y = 0.57x - 9.85$, $R2 = 0.986$) and for DNA extracted from soil ($y = 0.55x - 10.29$, $R2 = 0.942$).

Conclusion Générale

"Science is as much about uncertainty as certainty. To acknowledge uncertainty and recognize its origins is scientific strength, not weakness; to insist on certainty is arrogance, not strength"

Alan RAYNER

CONCLUSION GÉNÉRALE

Il est maintenant parfaitement reconnu que le transfert horizontal d'ADN constitue un processus évolutif majeur au sein du monde procaryotique. C'est très certainement la possibilité d'échanger de l'information génétique qui a permis aux bactéries de coloniser les différents écosystèmes de notre planète. Ce sont encore ces mécanismes qui aujourd'hui permettent aux bactéries de s'adapter très rapidement aux variations de leur environnement, que ce soit pour résister aux antibiotiques ou pour dégrader des molécules xénobiotiques synthétisées par l'homme. Depuis plus d'une trentaine d'années le transfert latéral d'ADN est étudié à partir de quelques bactéries modèles, d'une part pour élucider au niveau moléculaire les différentes étapes de la conjugaison, de la transformation ou de la conjugaison mais aussi pour déterminer leur occurrence dans différents environnements. C'est cependant grâce aux progrès de la génomique et la comparaison des séquences des génomes complets de plus de 200 bactéries que l'ampleur du transfert horizontal dans la structuration des génomes bactériens a pu être évaluée. Ces analyses *in silico* ont toutefois révélé que le taux de gènes d'origine exogène est très variable selon les bactéries, de quelques pour cent à plus de 40% (Nakamura *et al.*, 2004b). Il convient toutefois de signaler les limites de ces techniques bio-informatiques, qui ne détecteront pas les événements de transfert les plus anciens, ou ceux impliquant de très courts fragments d'ADN. De même ces outils ne peuvent révéler que les portions d'ADN présentant des caractéristiques suffisamment différentes du reste du génome, impliquant que les échanges de gènes entre bactéries phylétiquement très proches ne pourront pas être détectés. Enfin, l'analyse des génomes ne va révéler que le très faible pourcentage de gènes dont l'expression après pénétration et intégration ont accru la valeur adaptative de la bactérie réceptrice entraînant leur fixation dans la population. On peut donc en conclure que les données issues de l'analyse *in silico* non seulement sous estiment le nombre de gènes acquis par transfert horizontal et fixés dans la population mais aussi ne prennent pas en compte tous les événements de transfert « avortés », soit que l'ADN n'ait pu s'intégrer au génome soit qu'il en ait été ultérieurement éliminé (délétion) ou encore qu'il ait diminué la valeur adaptative de la bactérie réceptrice. Ces données sont à mettre en parallèle avec les résultats des travaux expérimentaux concernant l'occurrence des transferts de gènes dans l'environnement. S'il est acquis que les bactéries peuvent échanger du matériel génétique

par conjugaison, transduction et transformation dans de nombreux environnements, les fréquences auxquelles ces échanges de gènes se réalisent demeurent très faibles. En particulier, le principal environnement dans lequel vivent les bactéries, le sol, présente des résultats contradictoires quant à sa capacité à permettre les échanges de gènes entre bactéries. Les bactéries y vivent la plupart du temps dans un état physiologique très ralenti peu propice aux échanges de gènes. Par exemple, les conditions telluriques ne sembleraient pas propices au développement du stade de compétence (Nielsen *et al.*, 1997) nécessaires à l'intégration de l'ADN par transformation. Cependant, ces résultats négatifs peuvent s'expliquer par l'hétérogénéité de la matrice tellurique et la présence de micro-environnements dans lesquels les conditions physiologiques pour les bactéries pourraient être très différentes du reste du sol ne peut être exclue. Or ces micro-niches demeurent difficilement colonisables par des bactéries nouvellement introduites soulevant ainsi le problème du réalisme des expérimentations à simuler les conditions naturelles. D'autres éléments militent en faveur de fréquences élevées du transfert de gènes dans l'environnement, y compris dans le sol. La densité bactérienne est généralement très élevée favorisant ainsi les probabilités de contact entre bactéries ou entre ADN et bactéries. De nombreux travaux ont montré que le sol était un environnement dans lequel l'ADN libéré par les organismes qui le colonisent pouvait persister sur des temps très longs. Cette persistance pourrait être un élément clé pour permettre l'acquisition par les bactéries de l'information génétique d'origine bactérienne ou autre. Ces conditions de densité cellulaire élevée, d'état physiologique plus ou moins actif des bactéries et de présence d'ADN à l'état libre sont aussi susceptibles de se rencontrer dans d'autres situations environnementales que le sol. Les différents environnements de la planète peuvent alors être considérés selon leur capacité à permettre les transferts de gènes chez les bactéries. La notion de « hot spot » est alors proposée quand les fréquences auxquelles les transferts de gènes par l'un ou l'autre des mécanismes répertoriés sont significativement plus importantes que dans les autres environnements.

A ces filtres physiques et physiologiques s'ajoute la barrière génétique ou moléculaire exercée par la bactérie réceptrice de façon à réguler l'intégration de l'ADN transformant dans son génome. De nombreux travaux ont montré le rôle antagoniste des systèmes SOS et MRS qui selon leur niveau d'expression vont réguler l'évolution du génome entre maintien d'une stabilité et génération d'une diversité nécessaire.

Nos travaux ont pris en compte ces différents aspects de la régulation des transferts de gènes. En utilisant des plantes transgéniques comme modèles biologiques nous avons abordé la recherche

d'environnements qui pourraient constituer des hot-spots pour les transferts de gènes, en particulier par le mécanisme de transformation. Nous avons ainsi montré que la plante en décomposition (residuesphere) réunissait les différents critères nécessaires à la réalisation des transferts de gènes, à savoir une colonisation bactérienne importante, le développement d'un stade de compétence traduisant un état physiologique actif et la présence d'importantes quantités d'ADN extracellulaire résultant de la lyse cellulaire. Grâce au développement d'outils moléculaires adaptés nous avons montré que le transfert d'ADN pouvait se réaliser dans ces conditions y compris avec l'ADN du transgène libéré naturellement par la plante. Ces outils devraient nous permettre à terme de déterminer les fréquences exactes des événements de transfert en particulier en séparant ce qui résulte d'événements de transfer indépendants de ce qui est du à une multiplication des transformants. La capacité de pouvoir localiser les cellules transformantes permettra également d'appréhender plus précisément la régulation des transferts par les paramètres physico-chimiques du milieu.

Mais nos travaux ont également été de prendre en compte l'environnement « sol nu » et de considérer l'élément le plus important pour la réalisation des transferts de gènes par transformation à savoir la persistance de l'ADN à l'état extracellulaire. Si nos travaux ont d'abord confirmé des études préliminaires sur la capacité de détecter par PCR des fragments d'ADN plusieurs mois après leur libération dans le sol ils ont surtout apporté des informations sur le maintien du pouvoir transformant de ces molécules même après plusieurs années. Ces travaux ouvrent de nouvelles perspectives quant à l'implication de cet ADN dans le mécanisme de transformation des bactéries colonisant naturellement les microstructures du sol. Les outils sont maintenant disponibles pour prendre en compte les concepts liés à l'hétérogénéité de la matrice tellurique grâce à la microspatialisation des échantillons mais aussi pour étudier le rôle de la transformation naturelle codée génétiquement comme celui lié à une perméabilisation des enveloppes cellulaires suite à des chocs physico-chimiques auxquels sont plus ou moins constamment soumises les bactéries du sol. Enfin, nos travaux sur la possible saturation du système de réparation des mésappariements de base en particulier par une entrée massive d'ADN végétal nous ont permis d'aborder la régulation des transferts de gènes au niveau moléculaire. Ces résultats nous ont permis de constater la solidité de cette barrière génétique qui semble jouer un rôle prépondérant dans la régulation de l'acquisition de l'ADN hétérologue. A l'avenir, les plantes transgéniques, dans lesquelles les séquences des transgènes peuvent être adaptées en fonction des objectifs recherchés constitueront un modèle d'étude inestimable pour déterminer in

situ l'implication des facteurs physiques, physiologiques et génétiques dans la régulation du transfert d'information génétique. En plus d'une avancée d'ordre fondamental vers une meilleure compréhension des mécanismes évolutifs et adaptatifs bactériens de tels travaux répondent à une demande sociétale forte concernant l'évaluation du risque lié à ces nouvelles technologies.

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DEVENIR DE L'ADN TRANSGENIQUE DANS LES ENVIRONNEMENTS LIES A LA PLANTE ET AU SOL: IMPLICATIONS POTENTIELLES DANS LES TRANSFERTS HORIZONTAUX DE GENES ENTRE PLANTES TRANSGENIQUES ET BACTERIES

Les transferts latéraux d'information génétique sont reconnus comme étant la force majeure de l'évolution des bactéries leur ayant permis de coloniser la grande majorité des écosystèmes de la biosphère. Les mécanismes impliqués dans ces transferts de gènes sont la transduction, la conjugaison et la transformation. Aujourd'hui, ce sont ces mécanismes qui assurent aux bactéries un potentiel adaptatif conséquent vis à vis de différents stress liés aux pollutions chimiques par des composés xénobiotiques ou à l'usage massif des antibiotiques. Les transferts latéraux de gènes sont aussi responsables de la défiance généralisée vis à vis de nouvelles technologies comme la bio-ingénierie végétale, pouvant être à l'origine de la dispersion par transformation naturelle, des transgènes des plantes transgéniques vers les bactéries du sol. Ce dernier processus se caractérise par une certaine universalité, les bactéries pouvant acquérir des gènes d'autres microorganismes plus ou moins proches phylogénétiquement ou même d'eucaryotes comme des plantes. Le projet de recherche a spécifiquement porté sur l'étude du devenir de l'ADN végétal transgénique dans l'environnement et de ses interactions avec le microbiote. A cette fin ont été utilisés des plants de tabac transplastomique caractérisés par leur nombre très élevé de copies du transgène par cellule et plusieurs approches complémentaires de microbiologie et de biologie moléculaire afin de retracer les différents niveaux d'interaction entre l' ADN végétal et les bactéries de la phytosphère et du sol.

Nos travaux ont permis de déterminer le niveau de persistance des molécules d'ADN libérées dans le sol par la plante en décomposition et le maintien du potentiel biologique de cet ADN vis à vis de la microflore tellurique. Différentes niches écologiques particulièrement favorables aux transferts horizontaux des gènes entre la plante et les procaryotes ont été identifiées. C'est notamment le cas des tissus végétaux en décomposition (la residusphère) qui offrent les conditions nécessaires pour la croissance bactérienne et le développement physiologique d'un état de compétence génétique. Ces travaux confirment que certaines conditions environnementales peuvent être très favorables aux échanges de gènes entre bactéries et permettre, le cas échéant, une dissémination des transgènes végétaux vers la microflore environnementale.

FATE OF TRANSGENIC DNA IN PLANT AND SOIL ENVIRONMENTS: POTENTIAL IMPLICATIONS IN HORIZONTAL GENE TRANSFER BETWEEN TRANSGENIC PLANTS AND BACTERIA

Horizontal gene transfers (HGT) are considered as the major force of bacterial evolution, which allowed for the colonisation of most ecosystems of the biosphere. Mechanisms implied in such gene transfers are transduction, conjugation and natural transformation. Today, these mechanisms endow bacteria with an adaptive potential ensuing from different stress linked to chemical pollution by xenobiotics or massive antibiotic utilisation. HGT are also responsible for societal concerns about new technologies such as plant bioengineering, being held responsible of the dissemination of transgenes via natural transformation from transgenic plants to soil bacteria. This last HGT process is characterized by some universality, since bacteria may acquire genes of other microorganisms more or less phylogenetically related or even from eukaryotes such plants. This thesis project focused on the study of the fate of plant transgenic DNA in the environment and of its interactions with the microbiota. To this aim transplastomic tobacco plants, characterized for an extremely high transgene copy number per cell, and several complementary microbiological and molecular biological approaches have been used, to track the different levels of interaction between plant DNA and bacteria dwelling in the phytosphere or in soil.

Our work allowed to determine the degree of persistence of DNA molecules released into soil by the decaying plant and of the maintenance of the DNA biological potential *vis- à -vis* the telluric microflora. Several ecological niches particularly favourable to horizontal gene transfer between plant and prokaryotes have been identified. This is paradigmatic in the case of decaying plant tissues (the residuesphere) which provided conditions conducive to bacterial growth and competence development. This work confirms that certains environmental conditions might be highly favourable for genetic exchange between bacteria and potentially allow the dispersion of plant transgenes towards the environmental microflora.

DISCIPLINE : Écologie Microbienne
MOTS-CLÉS : Transfert de gènes, OGM, transformation naturelle, compétence

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