

Minireview

Do transgenic plants affect rhizobacteria populations?

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Summary

Plant genetic manipulation has led to the development of genetically modified plants (GMPs) expressing various traits. Since their first commercial use in 1996, GMPs have been increasingly used, reaching a global cultivating production area of 114.3 million hectares in 2007. The rapid development of agricultural biotechnology and release of GMPs have provided many agronomic and economic benefits, but has also raised concerns over the potential impact these plants might have on the environment. Among these environmental concerns, the unintentional impact that GMPs might have on soil-associated microbes, especially rhizosphere-inhabiting bacteria or rhizobacteria, represents one of the least studied and understood areas. As rhizobacteria are responsible for numerous key functions including nutrient cycling and decomposition, they have been defined as good indicator organisms to assess the general impact that GMPs might have on the soil environment. This minireview summarizes the results of various experiments that have been conducted to date on the impact of GMPs on rhizobacteria. Both biological and technical parameters are discussed and an attempt is made to determine if specific rhizobacterial responses exist for the different categories of GMPs developed to date.

Introduction

In 1996, the first genetically modified seeds were planted in the United States for commercial agricultural production. Eleven years later, more than 12 million farmers in 23 countries are cultivating biotech crops, with a global production area of 114.3 million hectares, steadily increasing for the eleventh consecutive year (James, 2007). Geneti-

cally modified plants (GMPs) with a wide variety of traits have been developed via genetic engineering. While some genetic modifications seek to directly improve the quality of the plant product, many are designed to aid in the cultivation process. Most GMPs developed to date can be grouped into eight main categories: (i) resistance to herbicides, (ii) resistance to pests, (iii) resistance to pathogens, (iv) resistance to environmental stress, (v) plants with altered root exudates, (vi) plants with altered composition, (vii) ability to produce pharmaceutical or industrial compounds and (viii) elimination of pollutants. Although a diversification in modified traits has been seen, the vast majority of GMPs cultivated commercially in 2007 belonged to only two categories: resistance to herbicides and, to a lesser extent, resistance to pests (James, 2007). Biotech soybean continued to be the main commercially produced GMP in 2007, occupying 58.6 million hectares, followed by maize (35.2 million hectares), cotton (11.5 million hectares) and canola (5.5 million hectares) (James, 2007).

The rapid development of agricultural biotechnology and release of GMPs have provided many agronomic and economic benefits, but have also raised concerns over the potential impact these plants might have on human health and the environment. Among the major environmental concerns are the unintended consequences of potential transgene flow to indigenous plants or organisms through pollen transfer (de Vries *et al.*, 2003) or horizontal gene transfer (Nielsen *et al.*, 2001), plant invasiveness (Dale *et al.*, 2002), the development of resistant pests and pathogens (Dale *et al.*, 2002), and non-target effects on flora, fauna and microorganisms (Bruinsma *et al.*, 2003; Liu *et al.*, 2005). Among these environmental concerns, the impact of GMPs on soil-associated microbial communities represents one of the least studied and understood areas. This is possibly due in part to the inherent technical difficulties involved in the study of soil-borne microorganisms.

Plants are known to have a profound effect on the abundance, diversity and activity of soil microorganisms living in close proximity with their roots in a soil zone defined as the rhizosphere (Pinton *et al.*, 2001). Bacteria inhabiting the rhizosphere, also referred to as rhizobacteria, are responsible for numerous functions including nutrient

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cycling and decomposition, which can significantly influence vegetation dynamics (Kent and Triplett, 2002). Among these, plant growth-promoting rhizobacteria represent one of the best-characterized functional group of rhizobacteria known for playing a significant role in plant health and plant development (Siddiqui, 2006). As it can be assumed that any significant impact of plant genetic transformation might alter these fundamental microbial processes, rhizobacteria have been defined as good indicator organisms and have been studied to assess the general impact that GMPs might have on the soil environment (Lynch *et al.*, 2004).

This minireview seeks to: (i) summarize the work done to date on the impact of GMPs on rhizobacteria, (ii) identify the main variables affecting the impact of GMPs on rhizobacteria, (iii) discuss the resolution of the different technical approaches used to assess such impacts, (iv) determine if specific rhizobacterial responses exist for different categories of GMPs and (v) finally suggest a framework for future assessment of GMP effects on rhizobacteria.

Variables affecting the impact of GMPs on rhizobacteria

Nature of the genetic modification

Many factors come into play when trying to address the impact that GMPs might have on rhizobacteria. The nature of the plant genetic modification certainly represents one of the first key elements that naturally come to mind. Different transgenes, allowing the expression of novel plant proteins, can potentially alter rhizobacteria abundance and diversity in a variety of ways. These novel proteins can, for example, be directly released into the rhizosphere through root exudation or their expression can trigger, through alteration of various metabolic pathways, the production and release of additional non-proteinaceous compounds (Raubuch *et al.*, 2007). Although the engineered production of some of these compounds is deliberate, targeting, for example, specific pests or pathogens (Mendelsohn *et al.*, 2003), unintentional effects are susceptible to alter non-targeted rhizobacteria.

The persistence of some of these novel compounds in the rhizosphere, or simply an alteration in the amounts of naturally produced plant compounds due to genetic engineering can also have an impact on rhizobacteria populations (Oger *et al.*, 2000). Even after harvest, decomposition of plant litter can continue releasing novel gene products into the environment (Gebhard and Smalla, 1999), potentially altering rhizobacteria populations over long periods of time. For example, Bt-recombinant DNA and expressed Cry proteins were released in soils through root-exudation and plant tissue

decomposition where they remained intact and chemically active for extended periods of time, up to 240 days (Palm *et al.*, 1996; Tapp and Stotzky, 1998; Zwahlen *et al.*, 2003). However, while evidence for the persistence of transgenic plant DNA exists (Gebhard and Smalla, 1999), transfer of plant DNA to native soil microorganisms has only been shown under optimized laboratory conditions as a rare event (Nielsen *et al.*, 1997; Gebhard and Smalla, 1998) and has not yet been demonstrated to occur naturally under *in situ* conditions.

The recent introduction of transgenic crops expressing multiple traits ('stacked' genes) may further complicate determining the impact of GMPs due to potential interactions among the different transgenes and their respective gene products.

Genetic markers

As it is essential to confirm that a genetic transformation event is successful, selectable genetic markers, usually conferring the ability to survive in the presence of a normally toxic compound, are commonly used to co-transform plants with transgenes of interest. The most widely used selectable genetic marker in plants is the *nptII* gene, which was originally derived from *Escherichia coli* transposon Tn5, and encodes a neomycin phosphotransferase that confers resistance to the aminoglycoside antibiotics neomycin and kanamycin (Gasson, 2000). Such antibiotics are added to growth media when transgenic plants are regenerated from transgenic cells, maintaining selection (Dyer, 1996). It is surprising that the impact of such genetic markers on rhizobacteria has almost been ignored to date. Among the very few studies performed to address this question, Burris and colleagues (2008) showed that overexpression of *Atwbc* in *Arabidopsis thaliana*, which confers kanamycin resistance, had no effect on *E. coli* populations, and Demanèche and colleagues (2008) showed that no significant differences were observed in antibiotic-resistance levels when comparing transgenic and non-transgenic corn fields. However, LeBlanc and colleagues (2007) clearly showed that white spruce transformed with either *nptII*, *cry1Ab* or both genes had a significant impact on the diversity of rhizobacteria, but this impact was different and specific for each transgene. The use of such important antibiotic-resistance gene products in GMPs could also represent additional concerns as they may, for example, inactivate oral doses of antibiotics if the plant is ingested by humans and/or favour antibiotic resistance build-up in microorganisms (Gasson, 2000). According to a study performed in 2003, a DNA construct composed of the *nptII* transgene remained detectable by polymerase chain reaction (PCR) in potato field soil after 4 years of laboratory incubation (de Vries *et al.*, 2003). New generations of transgenic

plants that do not harbour antibiotic-resistance genes of bacterial origin are however in development, which could help address this concern.

Crops

Different crops, and in some cases even different cultivars of the same plant species, are known to favour specific rhizobacteria populations (Mazzola *et al.*, 2004). It has been demonstrated that this impact of different cultivars on rhizobacteria populations can even be more pronounced than plant genetic transformation. For example, Milling and colleagues (2005) have shown that the non-genetically transformed potato cultivar SOLANA displayed a different rhizobacteria population when compared with the non-genetically modified potato cultivar (SIBU) or a transgenic version altered in starch composition (SIBU SI), while rhizobacteria populations associated with SIBU and SIBU SI were not significantly different. This is why appropriate controls, not only including the non-GM parental cultivar, but also additional cultivars of the same plant species, should be included in studies addressing the impact of GMPs on rhizobacteria. Different crop- or cultivar-related factors such as the architecture of the root system, differences in soil nutrient utilization, as well as amounts and composition of root exudates, can be determinant in specific plant–rhizobacteria interactions (Brimecombe *et al.*, 2001).

Production management systems

In general, agricultural, horticultural and forestry practices create a huge perturbation in soil systems, and changes in production management practices can have serious and long-lasting effects on rhizobacteria (Höflich *et al.*, 1999). Soil properties and soil management practices, including tillage, fertilizer use, pesticide applications, herbicide treatments and crop rotation, are of significant importance (Marschner *et al.*, 2004). A 'baseline of normal variation' should be established for each crop under study to assess the effect caused by these practices, allowing to gauge the impact that plant genetic transformation might have as compared with this 'normal variation'. Experimental design for risk assessment studies on GMPs should also take into consideration the changes in management practices associated with cultivation of GMPs. If, for example, the use of a Bt-crop allows avoiding usage of insecticides as compared with the conventional farming approach which relies on their uses, it appears relevant to determine if the effect of genetic transformation on rhizobacteria is still significant as compared with the impact of insecticide application.

Ecosystems parameters

Other biotic and abiotic sources of variation, such as season, weather, plant developmental stage and field location, have been identified as important drivers of microbial community structure alteration in the rhizosphere (Marschner *et al.*, 2004). Proper experimental design is crucial for assessing GMP-induced effects, and should include controls for natural variation within plant–soil systems. Monitoring schemes must relate to ecologically relevant levels of environmental variation, and sampling procedures should take spatial and temporal changes into account. Although time- and resource-consuming, the evaluation of parameters, such as seasonal and year-to-year variations, should be identified and the impact of plant genetic transformation be gauged against them.

Methods used to assess the impact of GMPs on rhizobacteria abundance and diversity

Soil ecosystems are complex, heterogeneous and represent a challenge when it comes to isolate, culture or identify most microorganisms inhabiting this environment. There is strong evidence that most soil bacteria seen under the microscope are viable but fail to be detected by classical culture-dependent microbiological techniques (Ward *et al.*, 1990). Methodological advances, especially the introduction of culture-independent molecular biological techniques (Torsvik, 1980), now enable a thorough examination of GMP-induced effect on rhizobacteria. It is important to keep in mind that different methodological approaches present different resolutions and limitations, which will inevitably affect the conclusions drawn from risk assessment studies investigating the impact of GMPs on rhizobacteria. Although a trend towards molecular and polyphasic approaches is developing, which offer more complete pictures of microbial community dynamics, a significant portion of studies performed to date on the risk assessment of GMPs on rhizobacteria mainly relied on classical culture-dependent methods (Lynch *et al.*, 2004).

Low-resolution techniques

General measures, such as bacterial counts (usually expressed as 'colony-forming units' or 'most probable numbers') or total microbial biomass, can detect changes in terms of microbial abundance only at a rudimentary level. Bacterial counts mainly rely on plating methods which are the most widely used classical culture-dependent methods in microbiology. As counts are usually quite variable, strongly dependent on the incubation conditions used and don't allow the detection of the great majority of soil bacteria because they are not culturable

using standard methods, the information provided is very limited (Liu *et al.*, 2005). Microbial biomass measurements, usually determined using chemical approaches, such as the chloroform fumigation–extraction method, are also quite variable and often not precise (Olf and Scherer, 1996).

Intermediate-resolution techniques

Community-level physiological profiling (CLPP) of microbial populations (e.g. the BiOLOG assay) has been used to characterize microbial diversity based on a pattern of substrate utilization in 96-well microtiter plates. Although these assays can depict community changes between treatments, mainly providing functional ability information, they rely on culture of microorganisms and usually fail at identifying the taxonomical groups associated with functional changes (Garland and Mills, 1991).

Microbial lipid analysis, such as phospholipid fatty acid analysis, fatty acid methyl ester analysis (FAME), ester-link fatty acid analysis and neutral lipid fatty acid analysis, rely on the detection and/or quantification of microbial membrane fatty acid profiles specific to different taxonomical groups of microorganisms. They can provide information on a variety of microbial characteristics, such as abundance, taxonomic and functional identity, and overall community diversity. However, it has been shown that membrane fatty acid components of bacteria may change in response to the availability of nutrients and environmental stress, thereby altering lipid signatures (Kieft *et al.*, 1994). Although the use of lipid-based techniques for microbial community analysis in soil is advantageous because they do not require culturing and can provide quantitative data on entire communities, it has been shown that combined effects of physiological and phylogenetic changes on the lipid composition of a community can confound interpretation of the data (Liu *et al.*, 2005).

Stable isotope probing is a culture-independent method used for linking function with taxonomic identity, which involves using stable isotope-enriched substrates for the identification of metabolically active members within a complex natural environment (Radajewski *et al.*, 2000). This technique is particularly interesting when several uncultivated microorganisms are involved in a process. However, it fails at depicting a portrait of the total community structure present in a sample.

Several other broad-targeted culture-independent molecular methods are now also available for the detection of shifts in total microbial community structure without the need for culturing microorganisms. Most of these methods rely on a three step procedure, an initial DNA extraction step from samples under study (i.e. rhizosphere soil samples), a PCR-amplification step targeting

either taxonomical (e.g. 16S rDNA) or functional relevant genomic DNA sequences, then followed by a third step allowing comparison of the fragments obtained based on either their sizes or their sequences. Depending on the various specificities of the primers used for the PCR amplification step to zoom in on specific microbial lineages, and the nature of the third step to separate PCR amplicons, the resolution of these approaches can vary from intermediate to high.

At the intermediate resolution level, denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), single-stranded conformational polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP) approaches are the most widely used. In DGGE/TGGE analysis, PCR amplicons with the same length but different nucleotide sequences are separated in polyacrylamide gels by a denaturing gradient or a temperature gradient for DGGE and TGGE respectively. DGGE/TGGE bands can then be excised from the gel and sequenced, allowing the identification of the dominant bands. The SSCP, like DGGE/TGGE, detects sequence variations between different PCR amplicons. However, this technical approach relies on using one PCR primer phosphorylated at the 5' end, leading to the degradation of one DNA strand following PCR amplification by selective digestion using a lambda exonuclease. Resulting single strands are separated by electrophoresis under non-denaturing conditions in a polyacrylamide gel, allowing intra-molecular folding of the DNA strand dependent upon DNA variations. The T-RFLP analysis is based on the restriction endonuclease digestion of fluorescent end-labelled PCR amplicons, often 16S rDNA sequences. The amplicons are then separated by gel or capillary electrophoresis. Usually quick to perform as compared with high-resolution techniques, DGGE/TGGE, SSCP and T-RFLP analyses provide a good overview of the microbial diversity present in a sample but generally fail at detecting microorganisms present in low quantities (Lynch *et al.*, 2004). Finally, although being used less frequently since the development of more powerful approaches, culture-independent hybridization techniques, such as slot/dot blots or FISH (fluorescence *in situ* hybridization) rely on the use of oligonucleotidic probes homologous to phylogenetic relevant coding or non-coding regions. In the FISH method, probes are labelled with fluorescent dyes and used for *in situ* detection of cells in environmental samples by whole-cell hybridization (Amann *et al.*, 1990). In slot/dot blots, DNA is directly extracted from the samples under study and hybridized with labelled probes of interest. Probes can target phylogenetic groups at different taxonomical levels, ranging from domain to subspecies and show the relative abundance of major phylotypes in soil microbial communities (Lynch *et al.*, 2004). These approaches are however time-consuming and mainly

used to target specific microbial groups of interest instead of whole community analysis.

High-resolution techniques

Culture-independent molecular based high-resolution techniques available to study microbial diversity mainly include ribosomal intergenic spacer analysis (RISA and ARISA), amplified ribosomal DNA restriction analysis (ARDRA and T-ARDRA) and PCR-cloning sequencing (Lynch *et al.*, 2004).

The RISA or the automated version of RISA called ARISA are based on analysis of length polymorphism of PCR-amplified cloned ribosomal intergenic spacer regions located between 16S and 23S rDNA. This region varies both in length and nucleotide sequence, even between closely related microbial strains. The digested PCR amplicons obtained are then separated by gel electrophoresis (RISA) or first labelled using a forward fluorescent PCR primer and then separated by capillary electrophoresis systems (ARISA). The ARDRA follows basically the same technical approach as RISA with the exception that 16S rDNA is targeted instead of the intergenic spacer region and that this gene only significantly varies in nucleotide composition and not in length. Again, the digested amplicons can either be separated by gel electrophoresis or the approach can be automated (called terminal ARDRA or T-ARDRA) which first involves labelling the amplicons using a forward fluorescent PCR primer and then separation by capillary electrophoresis systems. Both RISA and ARDRA amplicons can be sequenced to identify fragments of interest. If no DNA digestion step is used, the amplicons obtained (either from 16S rDNA or ribosomal intergenic spacer regions) can be cloned, then directly sequenced, an approach generally identified as PCR-cloning-sequencing or referred to as 16S rDNA analysis if the 16S rDNA gene is specifically targeted (Lynch *et al.*, 2004).

Among emerging culture-independent techniques allowing to characterize microbial community dynamics with intermediate to high resolution are metagenomics and taxonomic microarrays. In metagenomics, total genomic DNA (metagenome) derived from microbial communities can be cloned into large vectors, such as bacterial artificial chromosomes, to study microbial diversity and/or functional aspects, as well as the interaction between both (Rondon *et al.*, 2000). In taxonomic microarrays, mainly 16S rRNA-based microarrays, short oligonucleotides targeting phylogenetic groups at different taxonomical levels are hybridized in a high-throughput format with DNA extracted from samples of interest (Sanguin *et al.*, 2006). This allows quick identification of the whole microbial community present, requiring less time and reducing costs as compared with library methods.

Case studies: microbial communities affected by GMPs

Resistance to herbicides

The GMP-resisting or -tolerating herbicides have consistently been predominant since 1996 in biotech agriculture. Mainly two transgenes, the 5-enolpyruvylshikimate-3-phosphate synthase gene and the phosphinothricin-acetyltransferase gene, conferring resistance to the herbicide glyphosate and glufosinate respectively, have been commonly used for plant genetic transformation.

So far, all studies performed on the impact of glyphosate-resistant GMPs on rhizobacteria have shown a clear significant impact on rhizobacterial diversity (Siciliano *et al.*, 1998; Siciliano and Germida, 1999; Dunfield and Germida, 2001; 2003) (Table 1). Different approaches, mainly CLPP and FAME, have revealed clear microbial shifts in the rhizosphere and/or the root interior of different oilseed rape (canola) cultivars, the only glyphosate-resistant plant species tested so far for its impact on rhizobacteria. Interestingly, two studies have addressed this impact under field conditions over a 2 year period (Dunfield and Germida, 2001; 2003). Both showed through PCA analysis that the rhizobacteria population inhabiting the rhizosphere of the genetically modified Quest cultivar had different CLPP and FAME profiles as compared with the population inhabiting the rhizosphere of the closely non-modified Excel cultivar (Dunfield and Germida, 2001; 2003) or the more distantly related non-genetically modified Fairview cultivar (Dunfield and Germida, 2001). This significant effect, although detected at most sampling times, was not persistent the next field season following winter at the pre-seeding stage. This clearly suggests that changes in the rhizobacteria community observed in the rhizosphere of glyphosate-resistant GMPs are temporary and dependent on the presence of the transgenic plant. As only one study has addressed the impact of glyphosate-resistant GMPs on the abundance of rhizobacteria without showing any significant effect (Dunfield and Germida, 2001) (Table 1), it is difficult at this point to clearly conclude on the potential impact that glyphosate-resistant GMPs may have on rhizobacteria abundance.

Contrary to the results indicating that glyphosate-resistant GMPs can alter the diversity of rhizobacteria, studies addressing the impact of glufosinate-resistant GMPs have only shown minor effects (Gyamfi *et al.*, 2002; Sessitsch *et al.*, 2004; Griffiths *et al.*, 2007) or no effects (Schmalenberger and Tebbe, 2002; 2003) on rhizobacterial diversity or abundance (Table 1). Although studies addressing the impact of glufosinate-resistant GMPs have usually addressed impacts under short periods of time and in some cases were conducted under controlled soil conditions (i.e. in pots), at least two studies

Table 1. Studies addressing the impact of GMPs on the abundance and/or diversity of rhizobacteria.

Modified plant	Modified trait	Technique used	Impact on abundance of rhizobacteria ^a	Impact on diversity of rhizobacteria ^a	Reference
Resistance to herbicides					
Canola	Glyphosate resistance	CFU, CLPP, FAME,	No	Yes	Dunfield and Germida (2001)
Canola	Glyphosate resistance	CLPP, FAME, T-ARDRA	–	Yes	Dunfield and Germida (2003)
Canola	Glyphosate resistance	FAME	–	Yes	Siciliano and Germida (1999)
Canola	Glyphosate resistance	CLPP, FAME	–	Yes	Siciliano <i>et al.</i> (1998)
Canola	Glufosinate resistance	DGGE, microbial biomass	Minor effect	Minor effect	Sessitsch <i>et al.</i> (2004)
Canola	Glufosinate resistance	DGGE	–	Minor effect	Gyamfi <i>et al.</i> (2002)
Maize	Glufosinate resistance	SSCP	–	No	Schmalenberger and Tebbe (2002)
Maize	Glufosinate resistance	CLPP, ELFA, PLFA	Minor effect	Minor effect	Griffiths <i>et al.</i> (2007)
Sugar beet	Glufosinate resistance	SSCP	–	No	Schmalenberger and Tebbe (2003)
Resistance to pests					
Cotton	Bt (Cry1Ac)	CFU	Yes	–	Rui <i>et al.</i> (2005)
Cotton	Bt (Cry1Ab)	CLPP	–	No	Shen <i>et al.</i> (2006)
Maize	Bt (Cry1Ab)	CLPP, PLFA	No	No	Griffiths <i>et al.</i> (2006)
Maize	Bt (Cry1Ab)	CLPP, PLFA	No	No	Griffiths <i>et al.</i> (2005)
Maize	Bt (Cry1Ab)	SSCP	–	Minor effect	Baumgarte and Tebbe (2005)
Maize	Bt (Cry1Ab)	CFU	No	–	Saxena and Stotzky (2001)
Maize	Bt (Cry1Ab)	CLPP, PLFA	No	Minor effect	Blackwood and Buyer (2004)
Maize	Bt (Cry1Ab)	DGGE	–	Yes	Castaldini <i>et al.</i> (2005)
Maize	Bt (Cry1Ab)	ARISA, CFU, CLPP	Minor effect	Yes	Brusetti <i>et al.</i> (2004)
Maize	Bt (Cry1F)	CLPP, PLFA	No	Minor effect	Blackwood and Buyer (2004)
Maize	Bt (Cry3Bb)	Microbial biomass, T-RFLP	No	No	Devare <i>et al.</i> (2004)
Maize	Bt (Cry3Bb)	Microbial biomass	Yes	–	Devare <i>et al.</i> (2007)
Potato	Cystatin production	PLFA	Yes	–	Cowgill <i>et al.</i> (2002)
Potato	Concavalin A production	CLPP	–	No	Griffiths <i>et al.</i> (2000)
Potato	<i>Galanthus nivalis</i> agglutinin production	CLPP	–	Minor effect	Griffiths <i>et al.</i> (2000)
Rice	Bt (Cry1Ab)	DGGE, T-RFLP	–	Minor effect	Liu <i>et al.</i> (2008)
Spruce	Bt (Cry1Ab)	ARDRA	–	Yes	LeBlanc <i>et al.</i> (2007)
Resistance to pathogens					
Potato	Cecropin production	ARDRA, ARISA	–	Yes	Sessitsch <i>et al.</i> (2003)
Potato	Attacin and cecropin prod.	T-RFLP	–	Minor effect	Rasche <i>et al.</i> (2006)
Potato	T4 lysozyme	CFU, CLPP, DGGE	Minor effect	Minor effect	Heuer <i>et al.</i> (2002)
Potato	T4 lysozyme	T-RFLP	–	Minor effect	Rasche <i>et al.</i> (2006)
Potato	T4 lysozyme	CFU, CLPP, FAME	No	No	Lottmann <i>et al.</i> (1999)
Potato	T4 lysozyme	CLPP, FAME, rep-PCR	–	No	Lottmann and Berg (2001)
Potato	Magainin II production	CFU	Yes	–	O'Callaghan <i>et al.</i> (2004)
Potato	Barnase/Barstar	T-RFLP	–	Minor effect	Lukow <i>et al.</i> (2000)
Papaya	RP mutant	CFU	Yes	–	Wei <i>et al.</i> (2006)
Plants with altered root exudates					
Alfalfa	neMDH overexpression	CFU, CLPP, 16S rDNA analysis	No	Yes	Testfaye <i>et al.</i> (2003)
Birdsfoot trefoil	Opine production	CFU	Yes	–	Oger <i>et al.</i> (1997)
Birdsfoot trefoil	Opine production	ARDRA, CFU	Yes	Yes	Oger <i>et al.</i> (2004)
Black nightshade	Opine production	CFU	Yes	–	Mansouri <i>et al.</i> (2002)
Tobacco	Ferritin overexpression	CFU	Yes	–	Robin <i>et al.</i> (2006b)
Tobacco	Ferritin overexpression	ARISA, PCR-RFLP	–	Yes	Robin <i>et al.</i> (2006a)
Tobacco	Ferritin overexpression	CFU, RAPD	No	Yes	Robin <i>et al.</i> (2007)
Tobacco	N-AHLS	CFU, DGGE, PLFA	No	No	d'Angelo-Picard <i>et al.</i> (2004)
Plants with altered composition					
Aspen	Altered stem lignin	NLFA, PLFA	Minor effect	Minor effect	Bradley <i>et al.</i> (2007)
Alfalfa	Ovalbumin production	CFU	Yes	Yes	Faragova <i>et al.</i> (2005)
Potato	Altered starch composition	DGGE	–	Minor effect	Milling <i>et al.</i> (2005)
Troyer citrange	Phytohormone balance	CFU, rep-PCR	Minor effect	Minor effect	Cirvilleri <i>et al.</i> (2005)
Troyer citrange	Phytohormone balance	ARDRA, CLPP, DGGE	–	Minor effect	Abbate <i>et al.</i> (2005)
Ability to produce pharmaceutical or industrial compounds					
Alfalfa	Lignin peroxy. production	CLPP, rep-PCR	–	Yes	Di Giovanni <i>et al.</i> (1999)
Alfalfa	Lignin peroxy. production	ARDRA, CFU, CLPP	Yes	Yes	Donegan <i>et al.</i> (1999)
Alfalfa	Alpha-amylase production	CLPP, rep-PCR	–	Yes	Di Giovanni <i>et al.</i> (1999)
Alfalfa	Alpha-amylase production	ARDRA, CFU, CLPP	Yes	Minor effect	(Donegan <i>et al.</i> , 1999)
Tobacco	HSA production	CFU, MPN	No	–	Sabharwal <i>et al.</i> (2007)
Elimination of pollutants					
Tobacco	Nitroreductase overexpression	CFU, CLPP, DGGE	Yes	Yes	Travis <i>et al.</i> (2007)

a. A minor effect indicates that an effect is reported or suggested but that it is either not clearly supported by statistical analyses or that results of statistical analyses are not clearly significant.

CFU, colony-forming unit; CLPP, phospholipid fatty acid analysis; ELFA, ester-link fatty acid analysis; MPN, most probable numbers; NLFA, neutral lipid fatty acid analysis; neMDH, nodule-enhanced malate dehydrogenase; PLFA, phospholipid fatty acid analysis.

reported results obtained under field conditions during two consecutive growth seasons, and their conclusions were not different (Schmalenberger and Tebbe, 2003; Griffiths *et al.*, 2007). Different plant species also did not significantly affect the results as minor effects of glufosinate-resistant canola and maize on rhizobacteria were reported (Gyamfi *et al.*, 2002; Griffiths *et al.*, 2007), while studies reporting no effects used either glufosinate-resistant maize or sugar beet (Schmalenberger and Tebbe, 2002; 2003). The impact of agricultural practices, soil type and seasons was considered more determinant than the incorporation of a glufosinate-resistant gene in the plant's genome for altering rhizobacterial diversity and/or abundance (Griffiths *et al.*, 2007).

Resistance to pests

The GMP engineered for improved resistance against pests, especially insects, have been mainly obtained by the incorporation of *cry* genes, encoding the production of insecticidal crystal proteins (Cry), also known as Bt toxins. Different *cry* genes, encoding various forms of Cry proteins with specific insecticidal effects, were originally isolated from the bacteria *Bacillus subtilis*. Crops engineered with such genes, commonly referred to as Bt-crops, have proved to be effective at controlling a wide variety of insect pests. The GMP producing cysteine proteinase inhibitors (cystatins) conferring resistance to nematodes, as well as GMP producing lectins such as concavalin A and *Galanthus nivalis* agglutinin, improving resistance against invertebrate pests, have also been developed.

Numerous studies have investigated the impact of Bt-crops on rhizobacteria. Plants, such as cotton, maize, rice and even ligneous species such as spruce, have been engineered to produce various Cry proteins, mainly Cry1Ab, Cry1Ac, Cry1Af and Cry3Bb, each active against specific insect groups (Mendelsohn *et al.*, 2003). The impact of such GMPs on rhizobacteria ranged from non-significant to significant effects on rhizobacterial diversity and abundance (Table 1). Neither plant species nor *cry* transgene type used can yet be correlated with specific responses. However, most studies performed under longer periods of time, ranging from 2 (Castaldini *et al.*, 2005; Liu *et al.*, 2008) to 3 years (Baumgarte and Tebbe, 2005; Devare *et al.*, 2007), have shown at least some sort of effects on either rhizobacterial diversity or abundance. This effect was generally reported as being less important than field site, soil and plant growth stage in terms of impact on rhizobacteria. Interestingly, many studies have incorporated non-GMPs as well as non-GMPs treated with insecticides as controls in their experimental set-up (Devare *et al.*, 2004; 2007; Liu *et al.*, 2008). The effect of insecticides application on rhizobacteria was in all cases either minor or absent, again suggesting that other pro-

duction management system or ecosystem parameters were more critical for the alteration of rhizobacterial communities.

Potato plants engineered to produce cystatins (Cowgill *et al.*, 2002) altered rhizobacterial abundance under field conditions, while potato plants producing lectins (Griffiths *et al.*, 2000) had either minor or no effect on rhizobacterial diversity under controlled soil conditions (Table 1). As only very few studies were performed on the impact of cystatin- and lectin-producing plants on rhizobacteria, it is difficult at this point to clearly conclude on the real impact these plants might have.

Resistance to pathogens

Genetic engineering of plants with the aim of achieving better resistance against pathogens has relied on the use of transgenes encoding various lytic peptides and enzymes, mainly with bactericidal and/or fungicidal activities. Such peptides include cercopins, attacins and megainin, while T4 lysozyme is the main enzyme that has been produced so far in GMPs to control pathogens. Risk assessment studies investigating the impact of pathogen-resistant GMPs on rhizobacteria have all used potato, with one exception using papaya engineered with a replicase (RP) mutant gene conferring resistance to the papaya ringspot virus (Wei *et al.*, 2006).

Most studies conducted so far on the impact of pathogen-resistant GMPs have shown at least some sort of effects on rhizobacterial diversity or abundance (Table 1). While all studies investigating the impact of antimicrobial peptide-producing GMPs have shown some effects either on rhizobacterial diversity or abundance (Sessitsch *et al.*, 2003; O'Callaghan *et al.*, 2004; Rasche *et al.*, 2006), studies investigating the impact of T4 lysozyme-producing GMPs have either shown minor or no effects (Lottmann *et al.*, 1999; Lottmann and Berg, 2001; Heuer *et al.*, 2002; Rasche *et al.*, 2006). Only two studies looked at the impact of T4 lysozyme-producing GMPs over more than one growth season under field conditions and showed no effect on rhizobacteria (Lottmann *et al.*, 1999; Lottmann and Berg, 2001), clearly suggesting that T4 lysozyme-producing GMPs have at most only a very minor effect of rhizobacteria. As only one study for each transgene used is available in this category, except for T4 lysozyme-producing GMPs, it is difficult at this point to determine trends or to generalize about the results obtained. The only study performed on GMPs other than potato showed an alteration of rhizobacteria abundance in the rhizosphere of RP papaya.

Plants with altered root exudates

As rhizobacteria populations and microbial gene expression are known for being rapidly altered by root exudation

changes (Matilla *et al.*, 2007; Attila *et al.*, 2008), GMPs have been engineered to produce altered exudation patterns. These GMPs have mainly been developed for risk assessment studies and not for commercial production. The rationale being that if plants with altered root exudation patterns are incapable of altering rhizobacteria populations, other genetic changes, not specifically designed to alter rhizobacteria, should probably cause no significant changes. The GMPs have been developed to produce opines, which are small molecules found in the crown gall tumours induced by the phytopathogenic bacterium *Agrobacterium tumefaciens*, and being used as a growth substrate by the bacteria (Oger *et al.*, 1997; 2004; Mansouri *et al.*, 2002). The GMPs have also been engineered to produce N-acyl homoserine lactones, which regulates quorum sensing in Gram-negative bacteria (d'Angelo-Picard *et al.*, 2004). Finally, plants overexpressing ferritins, which are multimeric proteins that store iron in a soluble and biologically available form, thereby contributing to plant iron homeostasis (Robin *et al.*, 2006a,b), or plants overexpressing a nodule-enhanced malate dehydrogenase, which catalyses the reversible reduction of oxaloacetate to malate, leading to an increased exudation of organic acids in the rhizosphere (Tsfaye *et al.*, 2003), have also been developed.

All studies performed on GMPs with altered root exudates showed a clear impact on rhizobacterial diversity and abundance, with one exception, N-acyl homoserine lactone-producing tobacco (d'Angelo-Picard *et al.*, 2004) (Table 1). These results confirm that plant genetic transformation specifically engineered to alter rhizobacteria can produce the expected effect. This effect appears highly dependent on the transgene used. Clearly, as it is now possible to develop GMPs that will have a significant targeted effect on rhizobacteria, it appears justified to confirm through risk assessment studies that GMP expressing other traits do not exert unintentional impacts on rhizobacteria.

Plants with altered composition

The GMPs with altered stem lignin or starch composition, production of a high-methionine protein ovalbumin or modifications in phytohormone balance have been developed. As traditional breeding has been intensively used to improve plant composition over the years, it appears likely that improving specific plant composition traits through genetic engineering will become very important. This technology has however not yet been significantly used under large-scale commercial field conditions.

All studies performed to date on the impact of GMPs with altered composition have shown at least minor effects on rhizobacterial diversity and abundance (Table 1). As only very few studies have been performed

in this category, usually only one study per transgene, it is difficult to generalize about the results obtained. When looking at the results gathered over more than one growth season, only the impact of Troyer citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] was looked at, showing only minor effects on rhizobacterial diversity and abundance (Abbate *et al.*, 2005; Cirvilleri *et al.*, 2005). Other parameters, such as soil and plant growth stage, were considered more determinant than genetic alteration for their impact on rhizobacteria communities.

Ability to produce pharmaceutical or industrial compounds

The GMPs have been engineered to produce pharmaceutical compounds, such as human serum albumin (HSA) used as a plasma expander, stabilizing agent, drug delivery protein and an adjuvant for novel drugs (Sabharwal *et al.*, 2007). Plants genetically modified to produce industrial relevant compounds, such as alpha-amylase used in starch processing for the production of juice, beer, wine and bread, or lignin peroxidase used for large-scale lignin degradation and as a bleaching agent in biopulping processes, have also been developed (Di Giovanni *et al.*, 1999; Donegan *et al.*, 1999).

All risk assessment studies performed in this category revealed effects on rhizobacterial diversity and abundance, except one study on HSA-producing tobacco (Table 1). Very few studies have however been performed on the impact of GMP producing pharmaceutical or industrial compounds on rhizobacteria. Also, the studies performed to date have tested only two different plant species: alfalfa and tobacco. There is no doubt that plants engineered to produce such commercially important compounds will likely become a significant part of tomorrow's biotech agribusiness.

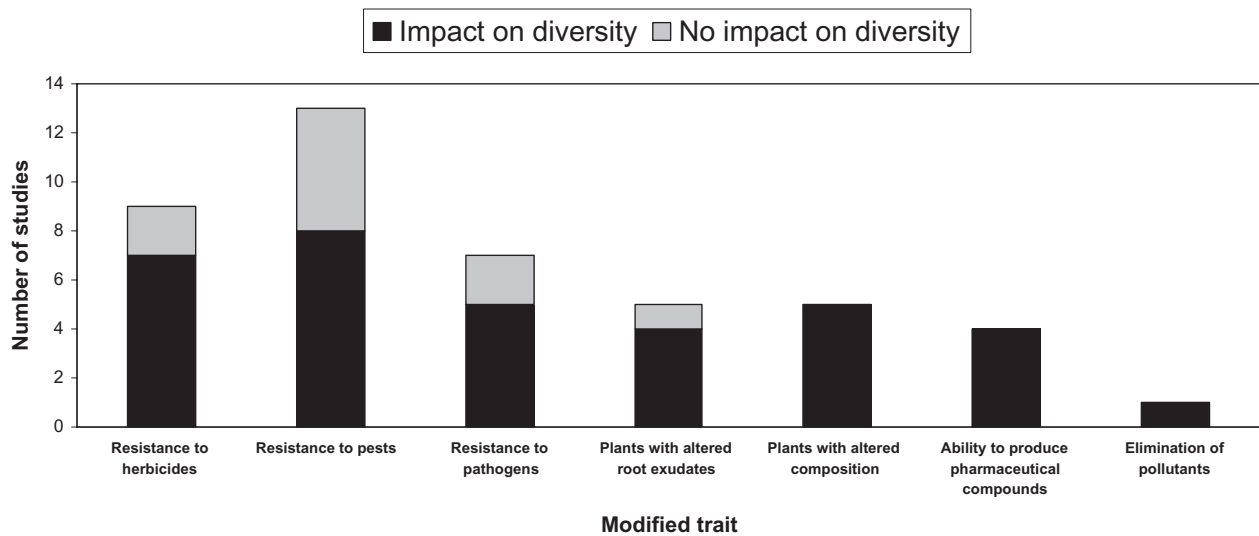
Elimination of pollutants

Only one study reported on the impact of GMPs engineered to eliminate pollutants on rhizobacteria populations. Transgenic tobacco plants overexpressing a bacterial nitroreductase gene were capable of detoxifying soil contaminated with the highly explosive 2,4,6-trinitrotoluene (TNT). These plants significantly altered rhizobacterial diversity and abundance. The effect was however dependent on the concentration of TNT present in soil (Travis *et al.*, 2007).

Concluding remarks

One of the most striking conclusions concerning risk assessment studies performed on the impact of GMPs on rhizobacteria is that so far a wide variety of techniques

A)



B)

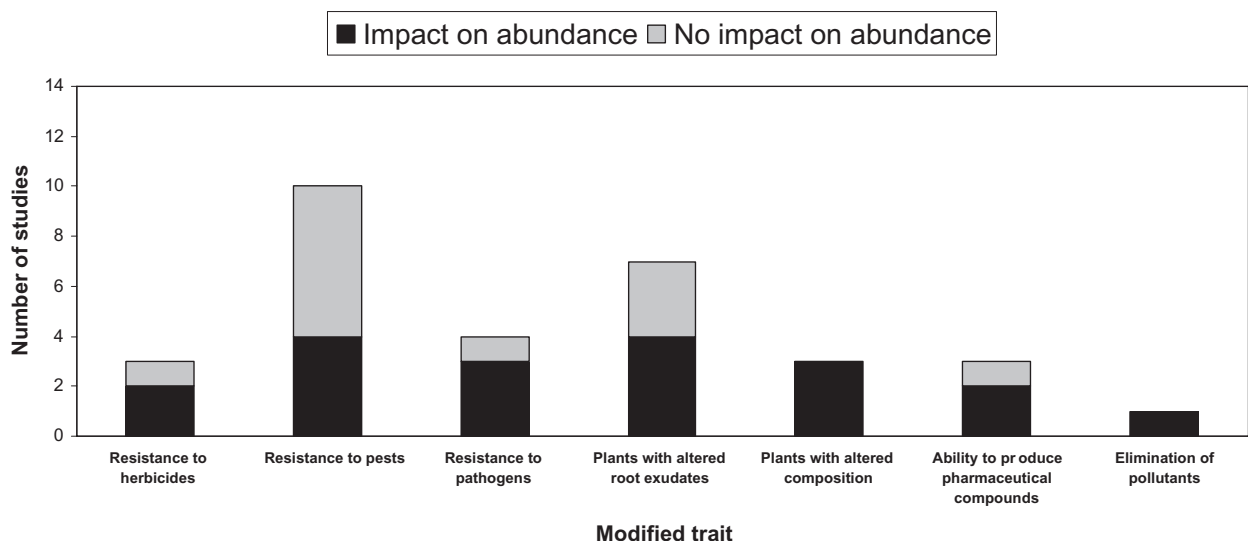


Fig. 1. Studies assessing impacts induced by GMPs on rhizobacterial (A) diversity and (B) abundance. Number of studies showing impact or no impact on rhizobacterial diversity or abundance is presented by categories of plant modification.

have been used to examine numerous parameters and confusion still exists as to which information is really relevant in this context. To date, most studies investigating the impact of GMPs have shown at least minor effects on the abundance and/or the diversity of rhizobacteria (Fig. 1). Despite the limited number of studies performed on this topic, we can now conclude with certainty that the effects observed are not artefacts. Clear rhizobacterial community changes have been shown in the rhizosphere of a variety of plants using different transgenes. The results obtained were reproducible and, in many cases, rhizobacterial responses to different categories of GMPs

appeared more dependent on the nature of the transgene used than any other considerations. However, based on the limited information presently available in the literature, we can suspect that these effects, although not negligible, can be considered 'minor' when compared with other 'normal' sources of variation, such as field site, soil type, seasonal variation, plant growth stage, etc. Agricultural practices, such as irrigation, crop rotation, tillage and use of herbicides and pesticides, have been shown to influence rhizobacteria populations with similar impact and in many cases more importantly than plant genetic transformation. So far, no study has been able to clearly show a

long-term negative effect of plant genetic transformation on rhizobacterial populations. Regardless of the plant species, transgenes and growth conditions used, all rhizobacteria community changes reported during active growth seasons have diminished to undetectable levels in a few weeks or months following plant harvesting.

Framework for future assessment of GMP effects on rhizobacteria

The use of GMPs in a commercial context is in continuous climb. Although it has so far been mainly restricted to agriculture, we can reasonably assume that GMPs will, in the near future, be increasingly used in horticulture and in forestry. Even if no significant long-term negative impact on rhizobacteria has been clearly identified to date, it is imperative that more studies addressing potential non-target effects of GMPs be conducted. One of the main reasons for this is that the technology is recent and only a very limited amount of studies have so far been conducted in this area, limiting the conclusions that can be drawn.

In order for future studies to be as informative as possible, the following framework is proposed. Risk assessment studies on the impact of GMPs on rhizobacteria and microorganisms in general should be conducted under field or 'natural' conditions over long-term periods (i.e. over at least 2 years). These studies should incorporate as many treatments as possible (i.e. using different cultivars, field sites, soil types, sampling at different growth stages etc.) to clearly define a baseline representative of 'natural variation', and should as well incorporate non-genetically transformed control plants and control plants transformed using genetic markers only. The experimental approaches could also follow changes in a rotational cropping context to determine if any effects could still be detected in non-genetically modified intercrops. Finally, from a technical point of view, polyphasic detection approaches should be used to assess microbial abundance and diversity in this context. In order for these recommendations to be realistic in terms of time, work and financial resources, here is a proposed streamlined set of guidelines for risk assessment studies on the impact of GMPs on rhizobacteria, which could also be applied to study the impact of other perturbations (environmental, chemical or physical) on rhizobacteria populations:

- i. At least two different cultivars of the same plant species should be used.
- ii. At least two field sites (each with a different soil type) should be studied.
- iii. Sampling should be performed at least three times per growth season (at planting, mid season and at harvesting).

- iv. GMPs, non-genetically transformed control plants and control plants transformed using genetic markers only should be used.
- v. One culture-dependent and one culture-independent technical approach should be used. BIOLOG assays combined with PCR-cloning sequencing assays represent a powerful combination. As sequencing costs have gone down, it is recommended that 96 clones should be analysed per sample. Alternatively, laboratories on a tight budget could consider other high-resolution techniques, such as RISA, ARISA or ARDRA to cut down on costs.
- vi. At least three replicates per treatment should be used. Based on the experimental set-up proposed here (2 cultivars \times 2 field sites \times 3 harvesting periods \times 3 categories of plant transformation \times 3 replicates), this represents 108 samples to be analysed using two different technical approaches (i.e. BIOLOG and PCR-cloning-sequencing assays).

The conclusion and suggestions on risk assessment mentioned here should not be viewed as static or absolute. They are based on the knowledge presently available. Some key issues concerning the impact of GMPs on rhizobacteria and soil microorganisms might develop or change in light of novel findings.

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