

ORIGINAL ARTICLE

Exogenous glucosinolate produced by *Arabidopsis thaliana* has an impact on microbes in the rhizosphere and plant roots

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A specificity of Brassicaceous plants is the production of sulphur secondary metabolites called glucosinolates that can be hydrolysed into glucose and biocidal products. Among them, isothiocyanates are toxic to a wide range of microorganisms and particularly soil-borne pathogens. The aim of this study was to investigate the role of glucosinolates and their breakdown products as a factor of selection on rhizosphere microbial community associated with living *Brassicaceae*. We used a DNA-stable isotope probing approach to focus on the active microbial populations involved in root exudates degradation in rhizosphere. A transgenic *Arabidopsis thaliana* line producing an exogenous glucosinolate and the associated wild-type plant associated were grown under an enriched ¹³CO₂ atmosphere in natural soil. DNA from the rhizospheric soil was separated by density gradient centrifugation. Bacterial (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Acidobacteria*), *Archaea* and fungal community structures were analysed by DGGE fingerprints of amplified 16S and 18S rRNA gene sequences. Specific populations were characterized by sequencing DGGE fragments. Roots of the transgenic plant line presented an altered profile of glucosinolates and other minor additional modifications. These modifications significantly influenced microbial community on roots and active populations in the rhizosphere. *Alphaproteobacteria*, particularly *Rhizobiaceae*, and fungal communities were mainly impacted by these Brassicaceous metabolites, in both structure and composition. Our results showed that even a minor modification in plant root could have important repercussions for soil microbial communities.

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Introduction

Root exudates are critical in determining the nature of plant–microorganisms interaction in the rhizosphere and shape microbial community structure present in the vicinity of the roots (Butler *et al.*, 2003; Bais *et al.*, 2006; Costa *et al.*, 2006; Paterson

et al., 2007; Broeckling *et al.*, 2008; Haichar *et al.*, 2008). Some studies have focused on the implication of a specific plant defense-signaling pathway as a driving force in structuring native soil microbial communities. Salicylic and jasmonic acids signal molecules reduce natural endophytic and epiphytic bacterial diversity on leaves of *Arabidopsis thaliana* (Kniskern *et al.*, 2007). Systemic acquired resistance can alter the rhizosphere bacterial communities (Hein *et al.*, 2008). The glucosinolate–myrosinase chemical defense system is specific of *Brassicaceae*, *Capparaceae*, *Caricaceae*, *Resedaceae* and *Moringaceae* plants (Fahey *et al.*, 2001). This system influences the active microbial community in the

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rhizosphere of canola (Rumberger and Marschner, 2003) but its real impact on the native microbial community in the rhizosphere of *Brassicaceae* remains unknown.

Glucosinolates are specific secondary metabolites stored in plant cell vacuoles. When plant tissues are damaged, glucosinolates come into contact with the enzyme myrosinase located in the cell wall, cytoplasm or in separate cells and are hydrolysed into glucose, sulphates and biocidal products such as isothiocyanates, nitriles and ionic thiocyanates (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). This defense system is active against aerial herbivores, pests or pathogens (Halkier and Gershenzon, 2006). The incorporation and degradation of Brassicaceous tissues in soil releasing active hydrolysis products is used to tentatively control soil-borne pests (Angus *et al.*, 1994; Brown and Morra, 1997). The effects on fungal and bacterial responses of the glucosinolate-myrosinase defense system have already been described. However, these studies implicated pure strains of pathogen organisms and *in vitro* approaches (Brabban and Edwards, 1995; Kirkegaard *et al.*, 1996; Smith and Kirkegaard, 2002; Souza-Fagundes *et al.*, 2004). The incorporation of crucifer tissues showed a variety of effects on non-target soil microbial populations in studies based on isolation and culture of microorganisms (Scott and Knudsen, 1999; Bending and Lincoln, 2000; Cohen *et al.*, 2005). However, such approaches do not reflect the actual microbial community as 0.1 to 10% of total population in soil can be cultured according to current methods (Keller and Zengler, 2004).

In this study, we focused on the influence of glucosinolates and hydrolysis products on native total microbial populations, through the production of a glucosinolate compound released *in situ* from living *Brassicaceae* roots in natural soil.

We used an original culture-independent stable isotope probing (SIP) approach to describe plant microorganisms fine scale interactions in the rhizosphere. This culture-independent approach allows discriminating root exudate-assimilating microbial populations in plant rhizosphere from those dormant or degrading soil organic matter (Rangel-Castro *et al.*, 2005; Haichar *et al.*, 2008) and to link the identity of microorganisms to their function in soil (Radajewski *et al.*, 2000; Lu *et al.*, 2006).

We chose a transgenic *A. thaliana* plant in which CYP79A1 gene from sorghum (*Sorghum bicolor*) was introduced, which led to the accumulation of up to 3% dry matter of p-hydroxybenzylglucosinolate, an aliphatic glucosinolate derived from tyrosine (Bak *et al.*, 1999; Kristensen *et al.*, 2005). We report that the aliphatic hydroxybenzylglucosinolate is only produced in transgenic *A. thaliana* plant roots and that the modifications of glucosinolate profiles by introduction of CYP79A1 gene leads to specific changes in the active microbial community on the

roots but also in the rhizosphere of *A. thaliana* growing in natural soil.

Materials and methods

Plant growth and ¹³C labelling

Glucosinolates biosynthesis pathways are linked to metabolic pathways of other compounds, like the plant hormone indole-3-acetic acid (Halkier and Gershenzon, 2006) and perturbation of glucosinolate metabolism in plant mutants could cause severe phenotypes (Yan and Chen, 2007). The *A. thaliana* transgenic line CYP79A1 (Bak *et al.*, 1999) was selected for its glucosinolate unusual profile and for its similar phenotype than wild-type plant. It results from the introduction of the CYP79A1 gene from sorghum which led to the production of high levels of the tyrosine-derived glucosinolate p-hydroxybenzylglucosinolate not known to accumulate in wild-type *A. thaliana* ecotype Columbia (Bak *et al.*, 1999). Transgenic CYP79A1 plant seeds were provided by Halkier BA (Royal Veterinary and Agricultural University, Copenhagen, Denmark) and together with wild-type Col, were grown in triplicate in soil under ¹³CO₂ following the protocol described by Haichar *et al.* (2008), to compare their rhizospheric microbial communities. Briefly, after seed sterilization, plants were grown in pots for 18 days before being continuously labelled for 25 days by injection of pure (>99% atom ¹³C) ¹³CO₂ (Purchased from Cortec Net, Paris, France) monitored to maintain an isotope excess at >80% atom ¹³C (Figure 1). Replicates of transgenic CYP79A1 and Columbia plants were grown in a twin growth chamber under the same conditions except unlabelled CO₂ for the analysis of glucosinolate profiles in plants. Triplicate of soil pots without plants were also incubated in the same conditions.

Harvesting procedure and DNA extraction

Roots of each plant replicate were separated from rhizospheric soil, washed with sterile water and both rhizospheric soil and roots were frozen in liquid nitrogen and stored at -80 °C. Total nucleic acids were extracted from 3 × 1.5 g of soil per plant sample and from entire root systems as described by Ranjard *et al.* (2003). Integrity of total DNA was verified on agarose gel and DNA concentrations were assessed using PicoGreen kit staining (Molecular Probes, Paris, France) following the manufacturer's protocol. DNA solutions were then stored at -20 °C. The ¹³C enrichment of total DNA extracted from rhizosphere was checked by a measure of δ¹³C (‰) by isotope ratio mass spectrometry coupled with an elemental analyser (IRMS, Delta+ and Conflo, Thermo-finnigan, Thermo-electron corp, Bremen, Germany). One to 2 µg of each DNA solution were placed into 5 × 9 mm tin capsule (Elemental Microanalysis Limited, UK) and submitted to mass

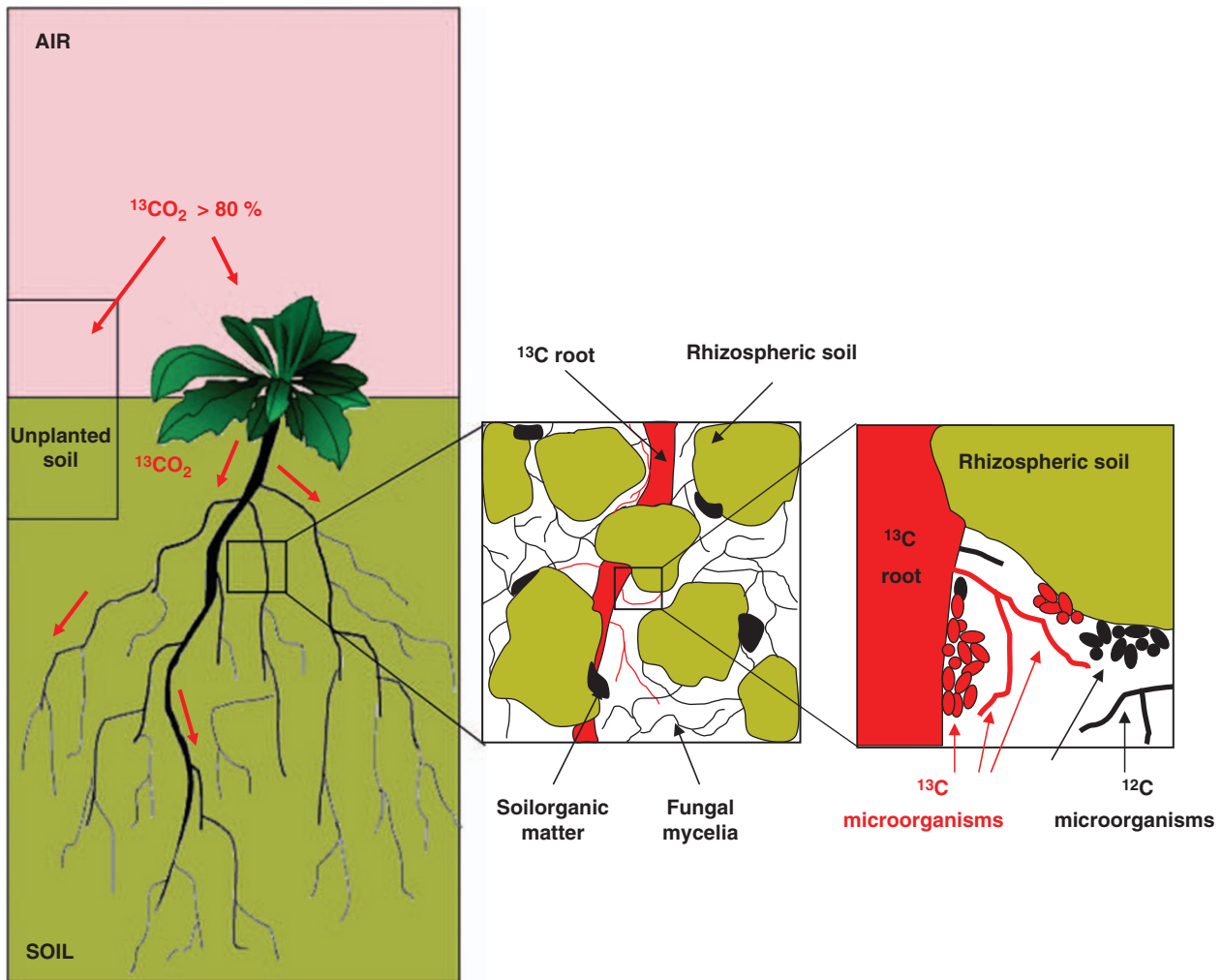


Figure 1 Stable isotope probing of microorganisms living in the vicinity of plant roots. The three fractions (plant roots, rhizospheric and unplanted soil) are presented during ^{13}C enrichment on an idealized plant. The inset shows the schematic representation of probed microorganisms.

spectrometry. $\delta^{13}\text{C}$ (‰) was determined using the equation:

$$\delta^{13}\text{C}(\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}}) - 1] \times 1000$$

where $R = ^{13}\text{C}/^{12}\text{C}$. The R_{standard} was Pee Dee Belemite (Wang and Hsieh, 2002).

Density gradient separation and fractionation

CsCl density gradient centrifugation was performed to separate ^{13}C -labelled and unlabelled nucleic acids extracted from rhizospheric and unplanted soil samples as described by Haichar *et al.* (2007). Approximately $5\ \mu\text{g}$ of DNA were loaded in the density gradient and centrifuged in 4.9 ml polyallomer Optiseal tube in an NVT 90 rotor (Beckman) at $20\ ^\circ\text{C}$, 45 000 r.p.m., for 72 h. Centrifuged gradients were fractionated from the top to the bottom into approximately equal $200\ \mu\text{l}$ by using 1 ml syringes fitted with 21-G needles. Density of each fraction was determined by weighing. DNA concentrations

were fluorometrically quantified using PicoGreen staining kit. For each gradient, one fraction representative of ^{13}C -labelled ('heavy') DNA and one fraction representative of unlabelled ('light') DNA were chosen according to a standardized method based on previous studies in the laboratory (Haichar *et al.*, 2007, 2008) following buoyant density and DNA content of each fraction in comparison with control gradient containing unlabelled soil DNA and ^{13}C labelled DNA from *Escherichia coli*. The heavy fraction was considered to contain DNA representative of populations involved directly or not, in root exudates assimilation and the light one, to contain DNA from populations degrading soil organic matter or inactive (Figure 1) (Rangel-Castro *et al.*, 2005; Haichar *et al.*, 2008).

Microbial community analysis

Nucleic acids were purified from CsCl salts using GeneClean kit (MP Biomedical). PCR amplifications

Table 1 Primers used in this study targeting bacterial, archaeal 16S rRNA and fungal 18S rRNA genes

Primer	Sequence 5'-3'	Specificity	References
F984 (GC-clamp)	CCCCCGCCGCGCGCGGGCGGGCGGGCGGGG	Bacteria	Costa <i>et al.</i> (2006)
R1378	GGCACGGGCCGAACGCGAAGAACCTTAC	Bacteria	Costa <i>et al.</i> (2006)
P3 (GC-clamp)	CGGTGTGTACAAGGCCCGGGAACG CGCCCCCGCGCGCGGGCGGGCGGGCGGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	Bacteria	Haichar <i>et al.</i> (2007)
S10	CCGTCAATTCATTTGAGTTT	Bacteria	Haichar <i>et al.</i> (2008)
F203 α	CCGCATACGCCCTACGGGGGAAAGATTAT	<i>Alphaproteobacteria</i>	Costa <i>et al.</i> (2006)
F948 β	CGCACAAAGCGGTGGATGA	<i>Betaproteobacteria</i>	Costa <i>et al.</i> (2006)
R1494	CTACGGRTACCTTGTTACGAC	Bacteria	Costa <i>et al.</i> (2006)
γ F382	AGGCAGCAGTGGGGAATA	<i>Gammaproteobacteria</i>	Klein <i>et al.</i> (2007)
γ R946	CCCCCGTCAATTCATTT	<i>Gammaproteobacteria</i>	Klein <i>et al.</i> (2007)
γ R1363	ACTAGCGATTCCGACTTCA	<i>Gammaproteobacteria</i>	Klein <i>et al.</i> (2007)
Acid31	GATCCTGGCTCAGAATC	<i>Acidobacteria</i>	Fierer <i>et al.</i> (2005)
Ar3f	TTCCGGTTGATCCTGCCGA	<i>Archaea</i>	Nicol <i>et al.</i> (2003)
Ar9R	CCCGCAAATTCCTTTAAGTTTC	<i>Archaea</i>	Nicol <i>et al.</i> (2003)
PARCH519r	TTACCGCGGCKGCTG	<i>Archaea</i>	Nicol <i>et al.</i> (2003)
SAf (GC-clamp)	CGCCCCCGCGCGCGGGCGGGCGGGGCGGGG GCACGGGGGGCCTAYGGGGCGCAGCAGG	<i>Archaea</i>	Nicol <i>et al.</i> (2003)
NS0	TACCTGGTTGATCCTGCC	Fungi	Costa <i>et al.</i> (2006)
EF3	TCCTCTAAATGACCAAGTTTG	Fungi	Costa <i>et al.</i> (2006)
FF390	CGATAACGAACGAGACCT	Fungi	Vainio and Hantula (2000)
FR1 (GC-clamp)	CCCCCGCGCGCGCGGGCGGGCGGGG CACGGGCCGAKCCATTCAATCGGTAKT	Fungi	Vainio and Hantula (2000)

Primers sets used in the first PCR: F203 α and R1494 (*Alphaproteobacteria*), F948 β and R1494 (*Betaproteobacteria*), γ F382 and γ R946, γ R1363 (*Gammaproteobacteria*), Acid31 and S10 (*Acidobacteria*), Ar3F and Ar9R (*archaea*), NS0 and EF3 (fungi). Primer sets used in the nested PCR: R1378 and F984 (*alpha- and beta-proteobacteria*), S10 and P3 (*Gammaproteobacteria and Acidobacteria*), PARCH519r and SAf (*Archaea*), FF390 and FR1 (fungi).

were performed from root DNA and from light and heavy fractions obtained from the rhizospheric soil of each plant. *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Acidobacteria*, *Archaea* and fungal communities were targeted by amplification of 16S or 18S rRNA gene fragments with adapted primers listed in Table 1, using a nested PCR approach (Muyzer *et al.*, 1993) according to the authors recommendations.

PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) allowing identification of specific populations through band excisions. DGEE was performed with the Dcode Universal Mutation Detection System (BIO-Rad Laboratories, France) on polyacrylamide gels with a denaturant gradient between 28% and 58% (100% denaturant equals 7M urea and 40% (v/v) formamide). Aliquots of PCR products were loaded on the gel and electrophoresis was carried out with 1 \times Tris-acetate-EDTA buffer at 60 °C and at 75 V for 17 h. Gels were silver-stained and scanned. ImageQuant TL one-dimensional gel analysis software (V2003, Amersham Biosciences, France) was used to determine band presence and intensity as described by McCaig *et al.* (2001). Intensities were normalized to avoid background bias and the DGGE matrices obtained were analysed using principal component analysis (PCA) ADE-4 software (Thioulouse *et al.*, 1997). Statistical ellipses representing 90% confidence on PCA plots were used to compare DGGE profiles. If two ellipses representing two different treatments do not overlap, the treatments

have significant different DGGE profiles with an alpha risk of 10%.

After electrophoresis the denaturing gels were stained with a 1:10 000 dilution of SYBR Green I nucleic acid stain (Sigma-Aldrich, USA) and prominent bands were excised from DGGE gels under UV. DNA was eluted in sterile water at 65 °C for 30 min and used as a template for PCR amplification under the conditions mentioned above. In most cases as reported by Mahmood *et al.* (2005), further rounds of band excision, PCR amplification and DGGE analysis were necessary before sequencing the PCR products (Genome express, Meylan, France). A particular attention was paid on ¹³C-labelled populations and those impacted by glucosinolates, specifically in case of minor DGGE bands. The NCBI BLASTN search tool (Altschul *et al.*, 1990) was used to determine the most similar sequences in GenBank database.

Analysis of glucosinolates and derivatives

The roots of six replicates of both Col and CYP79A1 plants grown with unlabelled CO₂ were freeze dried before glucosinolate extractions to avoid activation of 'glucosinolate-myrosinase' system and glucosinolate hydrolysis. Glucosinolates were extracted by 70% v/v methanol following Mellon *et al.* (2002) and Wathelet *et al.* (2004). Analysis of glucosinolates was then carried out using a HPLC/DAD/ESI-MS (HP 1100 series, Agilent Technologies) with a C-18 Nucleodur sphinx reversed-phase column

(250 × 4.6 mm, 5 µm, Macherey Nagel). The system was managed by Chemstation agilent software (Agilent Technologies). Mobile phase was a linear gradient of water with 0.1% v/v trifluoroacetic acid (TFA) (A) and methanol with 0.1% v/v TFA (B) at a flow rate of 1 ml min⁻¹. The linear gradient was 0 to 5 min 0% solvent B; 5 to 25 min 0% to 22% solvent B; 25 to 40 min 22% to 50% solvent B; 40 to 45 min 50% to 100% solvent B. Then, for 10 min the column was washed and equilibrated before the next injection. Spectra were recorded between 200 to 600 nm and the response at 228 nm was used for quantification. Mass spectrometry operating conditions were: gas temperature 340 °C at a flow rate of 11 l min⁻¹, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, capillary voltage 4000 V and fragmentor 150. Full scan spectra from *m/z* 60 to 900 in both positive and negative ion mode were obtained. Identification was based on the molecular weight and previously published information.

Isothiocyanates were tentatively extracted from rhizospheric soil on four replicates of each plant type Col and CYP79A1. Plants were grown in a growth chamber as previously described with some modifications to limit volatilization of isothiocyanates. After 4 weeks, rhizospheric soil of each replicate was carefully collected. Isothiocyanates were immediately extracted with ethyl acetate following a slightly modified procedure of Gimsing and Kirkegaard (2006). They were separated by GC/MS (GC 6890; mass detector HP 5973; Agilent Technologies) using a column HP-INNOWAX (30 m × 0.25 mm with a 0.25 µm film; Agilent Technologies). Helium was used as the carried gaz at 1 ml min⁻¹. One µl of sample was injected in splitless mode at 230 °C. The temperature program was set at 50 °C for 1 min, 50–105 °C at 3 °C min⁻¹, 105–200 °C at 8 °C min⁻¹, 200–240 °C at 30 °C min⁻¹ and 240 °C for 2 min. Mass detection of the samples was performed at 270 °C.

Results

Glucosinolates and their hydrolysis product analysis

The glucosinolate profiles of CYP79A1 transgenic *A. thaliana* plants were compared with the profiles of wild-type plants. We focused on root compartment, as studies on glucosinolate content of CYP79A1 and wild-type plants concerned leaves (Bak *et al.*, 1999; Kristensen *et al.*, 2005). Glucosinolates content of six replicates of seedling root tissues at post-germination stage reveal a different profile for CYP79A1 plants. Besides the exclusive production of *p*-hydroxybenzylglucosinolate by CYP79A1 plants (Figure 2, peak 2), another minor unidentified compound is detected only in CYP79A1 plants (Figure 2, peak 5), and two compounds show significant reduced amplitude in these plants (Figure 2, peaks 1 and 6). Those variations are in agreement with previous results

obtained with CYP79A1 leaves (Bak *et al.*, 1999; Kristensen *et al.*, 2005).

In the rhizosphere soil, isothiocyanates could not be successfully detected because either very low amounts of isothiocyanates were released by plant roots or isothiocyanates turnover was so rapid in the rhizosphere that they were undetectable.

Influence of glucosinolates and derivatives on the structure of root microbial communities

We separated roots and rhizospheric soil because plant root tissues are the source of glucosinolates and derivatives, and the highest effect on soil microflora was expected in this fraction (Figure 1). Actually, PCA analyses show significant differences between Col and CYP79A1 plants for *Alphaproteobacteria*, *Acidobacteria* and fungal root populations (Figure 3). DGGE bands excised were sequenced and affiliated to the closest relatives to identify impacted populations.

One *Acidobacteria*-specific population, closely related to an uncultured clone from a soil sample of radish rich area, is only present in wild type Col plant (Table 2). *Alphaproteobacteria* populations associated with plant roots are all close to members of *Rhizobiales*, underlining the selective effect of the roots on this group (Table 2, Figure 3a). In the *Agrobacterium/Rhizobium* genera, two populations are only associated with CYP79A1 roots and two other populations are located on Col roots (Table 2).

Fungal populations associated with plant roots are close to *Basidiomycota*, *Ascomycota* and *Chytridiomycota* (Table 2). One specific fungal population with a very intense band is only present on CYP79A1 plant roots (Figure 3). This band is assigned to *Syncephalis depressa* (*Zoopagomycotina*), a fungal species known to be a parasite of other fungi.

No significant differences between Col and CYP79A1 plants are detected by PCA on roots for *Betaproteobacteria*, *Gammaproteobacteria* and *Archaea* communities (Supplementary Figure S1). All identified populations of these taxa are detected to be associated with both plant type roots (Table 2).

Influence of glucosinolates and derivatives on the structure of rhizosphere microbial communities

The application of DNA-SIP technique in the rhizosphere allowed distinction between microbial populations actively assimilating root exudates (fresh carbon source) from dormant and/or soil organic matter degrading microorganisms (assimilating ancient carbon source) (Figure 1). *In situ* assimilation of root-derived carbon by soil microorganisms and sufficient incubation time are confirmed by the $\delta^{13}\text{C}$ mean value of total DNA recovered from rhizosphere of ¹³C-labelled plants that reach 261 (±64) ‰ compared to $\delta^{13}\text{C}$ value of 17 (±13) ‰ obtained for DNA recovered from unplanted soil.

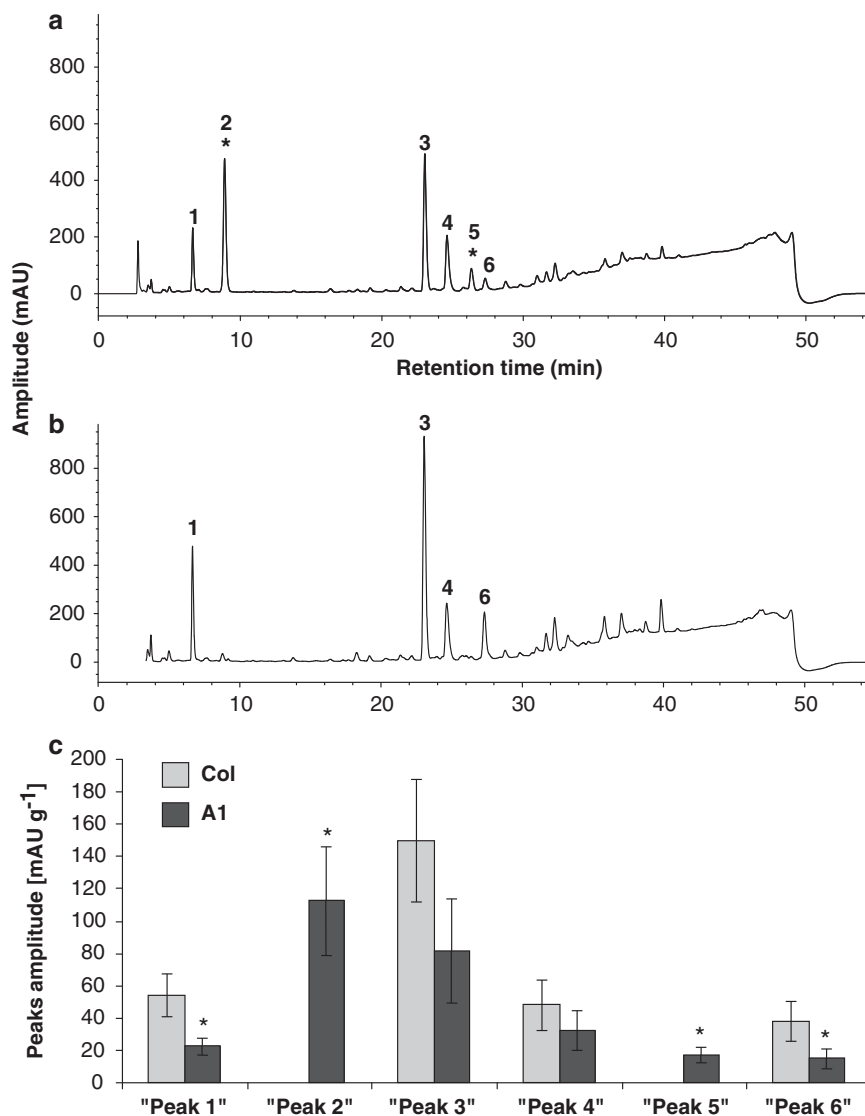


Figure 2 Glucosinolates extracted from *A. thaliana* roots. (a) Representative HPLC chromatogram obtained for CYP79A1 (A1) plant. Stars indicate peaks not detected in root sample of Col plant. (b) Representative HPLC chromatogram for Col plant. (c) Amplitude of each major glucosinolate peak detected by HPLC analyses per gram of Col and A1 plant roots. Means values of six replicates with standard deviations. Stars indicate significant differences in peak amplitude between the two plant types ($P=0.05$).

The DGGE patterns of rhizospheric soil-inhabiting microorganisms are different from those of root-colonizing microorganisms as illustrated for *Alpha-proteobacteria* (Figure 4a). Principal component analyses reveal significant differences between light and heavy DNA fractions (Supplementary Figure S2 and S3). The most important difference based on both PC1 and PC2 axes is observed for *Alpha-proteobacteria* and *Archaea* from transgenic CYP79A1 plant. The ability of some populations to assimilate fresh (¹³C) or ancient (¹²C) sources of soil carbon explains most of the differences in the community structure of these groups 87 and 92%, respectively (Supplementary Figure S3). For all other cases, significant effect becomes slighter, only on the PC2 axis explaining less than 18% of the variability. Visual comparison of corresponding

DGGE profiles reveals, for *Alpha-proteobacteria* and *Beta-proteobacteria*, replicable apparition or clear enrichment of specific bands from heavy DNA fractions corresponding to exudate degrading populations compared with light DNA fractions (Supplementary Figure S4). DNA-SIP allows pointing out a 'rhizosphere effect' of both wild-type Col and transgenic CYP79A1 plant exudates on the structure of the majority of tested taxa. Some specific populations active in the rhizosphere through assimilation of exudates were identified and are presented in Table 3. These populations are not detected in the heavy fractions of unplanted soil showing that ¹³C enrichment does not come from an autotrophic activity (Table 3).

Concerning the effect of the exogenous glucosinolate produced by CYP79A1 transgenic plants, we

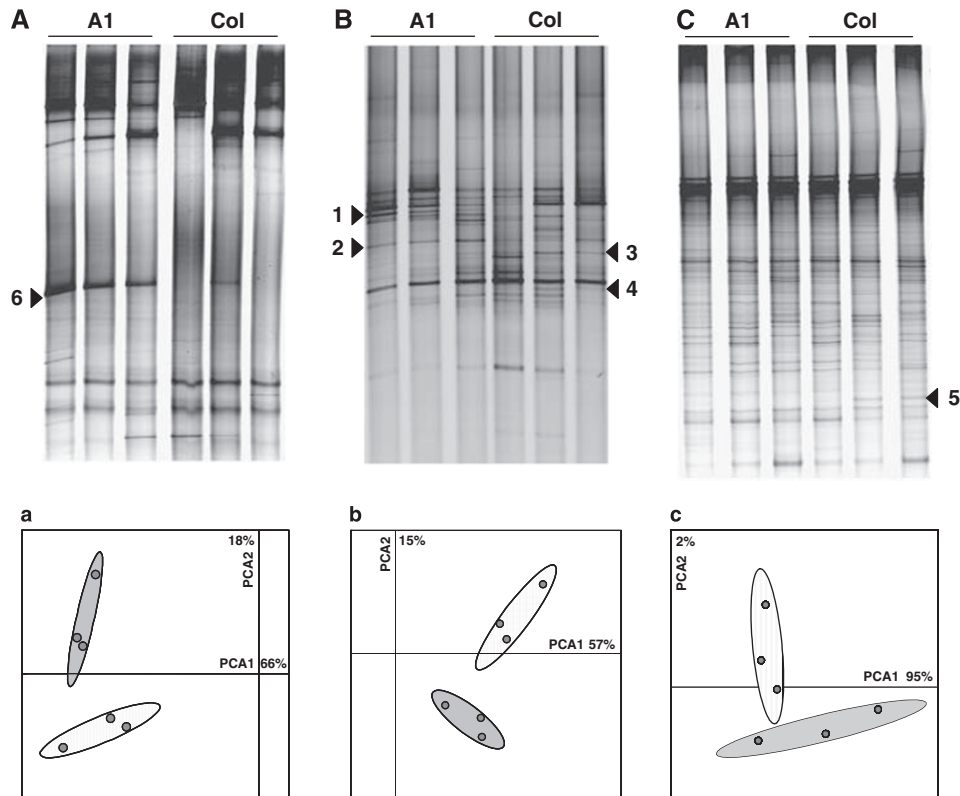


Figure 3 Denaturing gradient gel electrophoresis fingerprint of ribosomal gene fragments from root samples. (A) Fungi, (B) *Alphaproteobacteria*, (C) *Acidobacteria* and their corresponding principal component analyses for (a) fungi, (b) *Alphaproteobacteria*, (c) *Acidobacteria*. Three replicates for each plant type, wild-type Col and transgenic CYP79A1 (A1). Statistical ellipses drawn over the plot replicates represent 90% confidence. Arrowheads indicate examples of DGGE bands specific of one plant type. Legends: ■ Col plants; □ A1 plants. Band numbers are reported in Table 2.

detected significant differences in heavy DNA profiles in rhizospheric soil for *Alphaproteobacteria*, *Gammaproteobacteria* and fungal communities (Supplementary Figure S5). Plant genotype-specific heavy DNA bands are detected on DGGE profiles except for *Gammaproteobacteria* where only variations of band intensities are observed. DGGE profiles obtained with light DNA are similar and no difference was detected.

Alphaproteobacteria are highly influenced in rhizospheric soil by the modification of glucosinolate content. They are more diverse than on roots and affiliated to members of *Rhizobiales*, *Rhodobacterales* and *Caulobacterales* (Table 3). Three populations close to *Mesorhizobium*, *Bosea* and *Rhizobium* are only present in the heavy DNA fraction of CYP79A1 plant rhizosphere. Some other active *Rhizobium* populations are present in rhizospheric soil for both plant types, clearly implicated in the assimilation of root exudates but independently of glucosinolate profiles (Table 3). *Mesorhizobium*, frequently isolated from plant nodules (Wang *et al.*, 2002; Weir *et al.*, 2004), had a plant growth promoting effect on Indian mustard *Brassica campestris* (Chandra *et al.*, 2007) when *Rhizobium* species known for their symbiotic association with members of the *Fabaceae* (*Leguminosae*) or their

ability to form tumours with many plants (Hirsch, 2004) were already isolated from rhizosphere in non-symbiotic context (Yanni *et al.*, 1997; Berge *et al.*, 2009). *Bosea* populations seemed to have active roles in the plant rhizosphere (Martin-Laurent *et al.*, 2006; Dandie *et al.*, 2007) and could grow on specific compounds produced by the root of transgenic *Lotus* Oger *et al.* (2004). DGGE profiles obtained for roots and rhizospheric populations show that one ^{13}C -labelled *Rhizobium* impacted by glucosinolate modifications in roots is also present in rhizosphere but clearly not susceptible to glucosinolate content (Figure 4a).

Fungal community is clearly influenced by glucosinolate profile in rhizospheric soil, with a specific and intense band mainly present in heavy DNA (Figure 4b). As for root compartment, this band is assigned to the fungal parasite *S. depressa* (Table 3). This population is particularly susceptible to plant glucosinolate content and able to use, directly or indirectly through its host, ^{13}C -labelled root exudates. Other populations close to *Basidiomycota*, *Ascomycota* and *Chytridiomycota* are identified in light and heavy DNA fractions of rhizospheric soil for both plant types (Table 3). These populations are not susceptible to glucosinolate profile modifications in CYP79A1 plant.

Table 2 Tentative phylogenetic affiliation of partial 16S and 18S rRNA gene sequences derived from dominant DGGE bands retrieved from root samples (R) of two plant types, Col and CYP79A1 (A1)

BLAST closest relatives (NCBI GenBank database)		Source (NCBI)	Similarity (%)	Compartment		
Bacterial community Alphaproteobacteria Rhizobiales	Rhizobiaceae	<i>Agrobacterium tumefaciens</i>	99	R ^{Col, A1}		
		<i>Agrobacterium</i> sp.	97	R ^{Col, A1}		
		<i>Agrobacterium</i> sp. (Bd 1)	99	R^{A1}		
		<i>Rhizobium</i> sp. (Bd 2)	99	R^{A1}		
		<i>Rhizobium</i> sp. (Bd 3)	99	R^{Col}		
		<i>Rhizobium</i> sp.	99	R^{Col, A1}		
		<i>Rhizobium</i> sp. (Bd 4)	99	R^{Col}		
		Uncultured	99	R^{Col, A1}		
		Betaproteobacteria Burkholderiales	Brucellaceae	<i>Bordetella holmesii</i> .	96	R ^{Col, A1}
				<i>Achromobacter</i> sp.	96	R ^{Col, A1}
<i>Pelomonas puraquae</i>	98			R ^{Col, A1}		
Rhodocyclales	Comamonadaceae Oxalobacteraceae Rhodocyclaceae	<i>Duganella</i> sp.	99	R ^{Col, A1}		
		<i>Herbaspirillum</i> sp.	96	R ^{Col, A1}		
		<i>Zoogloea</i> sp.	98	R ^{Col, A1}		
		Gammaproteobacteria Xanthomonadales	Xanthomonadaceae	<i>Dokdonella</i> sp.	98	R ^{Col, A1}
<i>Dokdonella</i> sp.	97			R ^{Col, A1}		
Acidobacteria		<i>Dokdonella</i> sp.	98	R ^{Col, A1}		
		Uncultured acidobacterium	99	R ^{Col, A1}		
Archaeal community		Uncultured acidobacterium (Bd 5)	98	R^{Col}		
		Uncultured acidobacterium	99	R ^{Col, A1}		
		Uncultured acidobacterium	98	R ^{Col, A1}		
		Uncultured acidobacterium	98	R ^{Col, A1}		
		Uncultured acidobacterium	98	R ^{Col, A1}		
		Uncultured crenarchaeote clone	100	R ^{Col, A1}		
		Uncultured archaeon clone	97	R ^{Col, A1}		
		Uncultured archaeon clone	100	R ^{Col, A1}		
		Uncultured archaeon clone	95	R ^{Col, A1}		
		Uncultured archaeon clone	100	R ^{Col, A1}		
Fungal community Basidiomycota Ascomycota Chytridiomycota		<i>Marchandiobasidium aurantiacum</i>	96	R ^{Col, A1}		
		<i>Nectria</i> sp.	84	R ^{Col, A1}		
		Uncultured clone	97	R ^{Col, A1}		
		Uncultured clone	99	R ^{Col, A1}		
		<i>Olpidium brassicae</i>	98	R ^{Col, A1}		
		<i>Syncephalis depressa</i> (Bd 6)	97	R^{A1}		
Zoopagomycotina						

Each population identified is attributed to all plant types for which the specific DGGE band is detected. Populations impacted by glucosinolates profile are in bold. Band (Bd) numbers in parenthesis are reported in corresponding DGGE gels (Figures 3 and 4).

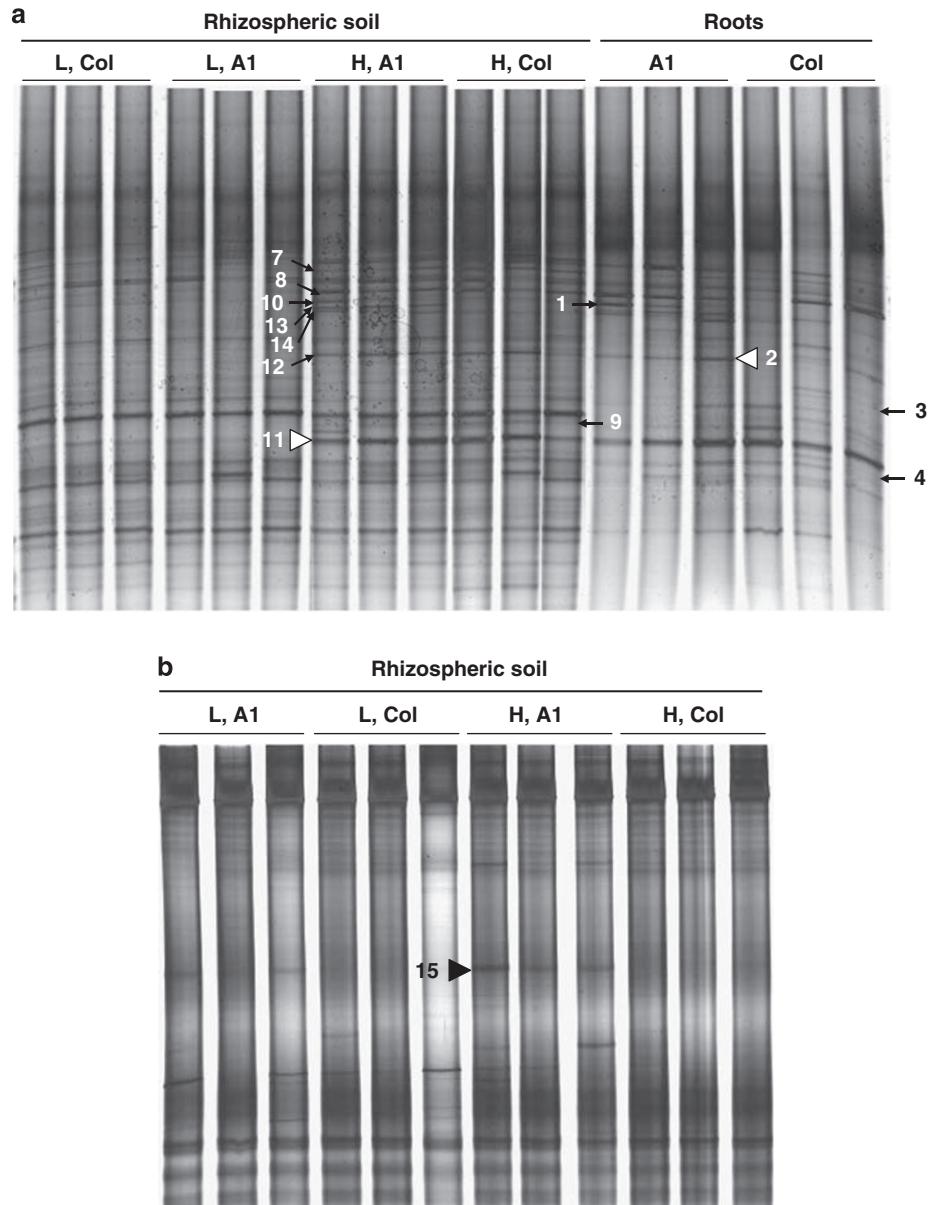


Figure 4 Denaturing gradient gel electrophoresis fingerprints of ribosomal gene fragments from light DNA fraction (L), heavy DNA fraction (H) for wild-type Col plant, transgenic CYP79A1 (A1) plant, three replicates for each treatment, (a) *Alphaproteobacteria*, 16S rRNA gene fragments from rhizosphere soil and root samples. White left-directing arrowhead indicates a ^{13}C -labelled *Rhizobium* population present in both plant type rhizosphere but only on A1 plant roots. White right-directing arrowhead indicates a ^{13}C -labelled *Rhizobium* population present on both plant type rhizosphere and roots. (b) Fungi, 18S rRNA gene fragments from rhizospheric soil. Black arrowhead indicates a population close to *Syncephalis depressa*, specific of A1 plant rhizosphere. Band numbers are reported in Tables 2 and 3.

No significant difference between Col and CYP79A1 plants are detected for *Acidobacteria*, *Betaproteobacteria* and *Archaea* communities by PCA analyses (Supplementary Figure S5). All identified populations for these taxa are detected in both plant types rhizosphere (Table 3).

However, we identify one ^{13}C -labelled archaeal population implicated in root exudates assimilation, only detected in CYP79A1 plant rhizosphere. This population closely related to an uncultured crenarchaeote from soil (Table 3), does not significantly

change global DGGE profile because of high variability for this group. *Crenarchaeota* populations were considered as the most abundant ammonia-oxidizing organisms in soil ecosystems (Leininger *et al.*, 2006).

Discussion

We measure effective production of the exogenous glucosinolate in root tissues of *A. thaliana* CYP79A1

Table 3 Tentative phylogenetic affiliation of partial 16S and 18S rRNA gene sequences derived from dominant DGGE bands retrieved from rhizospheric soil of two plant types, Col and CYP79A1 (A1) and from unplanted soil (uS) for light (L) and heavy (H) DNA fractions

BLAST closely relatives (NCBI GenBank database)		Source (NCBI)	Similarity %	Compartment
Bacterial community				
Alphaproteobacteria				
Rhizobiales	Rhizobiaceae	Pure strain	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Pea	93	H ^{Col, A1}
		Pure strain	97	H ^{Col, A1}
		Pure strain	96	H ^{Col, A1}
		Ground water	95	H ^{A1}
		Nodulating <i>Sesbania exasperate</i>	97	H ^{Col, A1, uS}
		Soybean	94	H ^{Col, A1}
		Ginseng rhizosphere	97	H ^{A1}
		Nodulating <i>Chamaecrista ensiformis</i>	97	H ^{A1}
Bradyrhizobiales				
Phyllobacteriaceae				
		Reservoir water	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Swine wastewater ditch	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Swine wastewater ditch	97	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Agricultural soil	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Decayed wood	95	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Decayed wood	97	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Facultative intracellular bacterium	93	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Alkaline groundwater	94	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Aspen rhizosphere	95	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Distribution system simulator	95	L ^{Col, A1, uS} , H ^{Col, A1, uS}
Uncultured alphaproteobacterium				
		Orchard soil	97	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Orchard soil	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Pure strain	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Type strain	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Type strain	94	H ^{Col, A1} , H ^{Col, A1}
		Ginseng field soil	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Contaminated soil	95	H ^{Col, A1}
		Batch reactor	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Reactor	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Microbial fuel cell	96	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Bioreactor with selenium	97	H ^{Col, A1}
		Termite intestinal tract	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
Betaproteobacteria				
Bulkholderiales	Comamonadaceae			
		Uncultured <i>comamonadaceae</i>		
		Uncultured <i>comamonadaceae</i>		
		<i>Thiomonas cuprina</i>		
		<i>Thiomonas cuprina</i>		
		<i>Thiomonas cuprina</i>		
		<i>Ramlibacter</i> sp.		
		<i>Achromobacter</i> sp.		
		<i>Zoogloea resiniphila</i>		
		<i>Zoogloea</i> sp.		
		<i>Zoogloea</i> sp.		
		<i>Azospira oryzae</i>		
		Uncultured bacterium		
Rhodocyclales	Alcaligenaceae			
	Rhodocyclaceae			
Uncultured betaproteobacterium				
		Drinking water	94	H ^{Col, A1}
		Drinking water	96	H ^{Col, A1}
		Drinking water	93	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Soil		L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Soil	97	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Bulk soil	95	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Cropland	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Pasture	94	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Land soil	98	L ^{Col, A1, uS} , H ^{Col, A1, uS}
		Land soil	99	L ^{Col, A1, uS} , H ^{Col, A1, uS}
Gammaaproteobacteria				
Xanthomonadales	Xanthomonadaceae			
		<i>Dokdonella</i> sp.		
		<i>Dokdonella</i> sp.		
		<i>Dokdonella</i> sp.		
Thiotrichales	Thiotrichaceae			
		<i>Lysobacter</i> sp.		
Acidobacteria				
		Uncultured acidobacterium		
		Uncultured acidobacterium		
		Uncultured acidobacterium		
		Uncultured acidobacterium		

Table 3 Continued

BLAST closely relatives (NCBI GenBank database)

	Source (NCBI)	Similarity %	Compartment	
Archaeal community	Unidentified archaeon	97	L ^{Col} , A1, uS, H ^{Col} , A1, uS	
	Unidentified archaeon	96	L ^{uS} , H ^{uS}	
	Unidentified archaeon	97	L ^{Col} , A1, uS	
	Uncultured crenarchaeote	95	H^{A1}	
	Uncultured archaeon	93	L ^{Col} , A1, uS, H ^{Col} , A1	
	Uncultured crenarchaeote	98	L ^{Col} , A1, uS, H ^{Col} , A1, uS	
	Uncultured archaeon	98	L ^{Col} , A1, uS, H ^{Col} , A1, uS	
	Uncultured archaeon	98	L ^{Col} , A1, uS, H ^{Col} , A1, uS	
	Uncultured archaeon	99	L ^{Col} , A1, uS, H ^{Col} , A1	
	Fungal community	<i>Marchandibasidium aurantiacum</i>	96	L ^{Col} , A1, uS, H ^{Col} , A1, uS
		<i>Sirococcus conigenus</i>	92	L ^{uS}
	Basidiomycota Ascomycota			
<i>Fusarium merismoides</i>		98	L ^{uS} , H ^{uS}	
<i>Stilbocrea macrostoma</i>		99	L ^{Col} , A1, uS, H ^{uS}	
Uncultured chytridiomycota		99	L ^{Col} , A1, uS, H ^{Col} , A1	
Chytridiomycota				
<i>Zoopagomycotina</i>	93	H^{A1}		

Each population identified is attributed to all compartments where the DGGE band is detected. Active populations are in bold. Band (Bd) numbers in parenthesis are reported in corresponding DGGE gels (Figure 4).

transgenic line compared with wild-type plants. Root growth is inevitably accompanied by superficial cell destruction leading to hydrolysis of glucosinolates and release of their degradation products. Living *Brassicaceae* roots could also release these metabolites through root exudation as shown for *Brassica napus* (Choesin and Boerner 1991) and mustard roots (Schreiner and Koide 1993) and directly impact microbe living on roots. Populations closely associated with plant roots directly exposed to these compounds but also rhizospheric populations are impacted by this glucosinolate profile change. We hypothesize that glucosinolates and their hydrolysis products diffuse from roots into the rhizosphere where they are diluted and could be degraded by extracellular myrosinase in soil (Borek *et al.*, 1996) as shown in canola rhizosphere (Rumberger and Marschner, 2003). This gradient of concentrations could explain that some *Acidobacteria* and *Rhizobium* populations are influenced by the modification of glucosinolate content in the root compartment but not in the rhizosphere. *A. thaliana* roots are very small and amounts of glucosinolates and derivatives proportional to roots size could be very low which could preclude their detection by GC. In the rhizosphere, these compounds could act as a signal working at very low concentration and having a short lifespan. In this fraction the modification of glucosinolate content in CYP79A1 plant has an impact mainly on exudate consumer ¹³C-labelled populations. It could indicate that this signal is a part of the plant driving force-selecting populations for its root exudate degradation.

DNA-SIP and continuous labelling of plant allow showing the global impact of glucosinolates and derivatives on active and growing microbial communities in rhizosphere. It could also be interesting to follow the temporal variations of microbial community structure during the plant growth (Petersen *et al.*, 2002; Brown *et al.*, 2003). An RNA-SIP approach combined with pulse labelling could be adequate for detection of punctual changes in active populations at a determined stage during plant culture. RNA is considered as a more sensitive marker for time course experiment because it does not require cell division for isotope incorporation (Whiteley *et al.*, 2006).

Target members of *Alphaproteobacteria* and fungal community appear to be mostly impacted by the change in glucosinolate profile in root and rhizosphere compartments. Fungi modifications are expected according to literature. They were reported to be more sensitive than bacteria to these compounds (Smith and Kirkegaard, 2002). The formation of antimicrobial products from glucosinolates has been also suggested to explain the inability of *Brassicaceae* plants to form arbuscular mycorrhizal (Vierheilig *et al.*, 2000; Roberts and Anderson, 2001). As an exception, *Thlaspi praecox* and *Biscutella laevigata* form arbuscular mycorrhizal symbiosis but only

during the reproductive period that coincided with low level of glucosinolates in roots (Pongrac *et al.*, 2008). However, the development of arbuscular mycorrhizal fungi in the host roots involved a complex cellular relationship both controlled by the plant branching factors strigolactones (Akiyama *et al.* 2005) and the Common Symbiosis Pathway (CSP) plant-signalling pathway. This pathway is common to arbuscular mycorrhizal, rhizobial and actinorhizal root symbioses representing mutualistic relationships in plant root (Markmann *et al.*, 2008). Strigolactone is found in root exudates of many species including *A. thaliana* (Goldwasser *et al.*, 2008) but most CSP gene orthologs were not predicted in the *A. thaliana* genome, whereas a non nodulating mycorrhized plant, *O. sativa* possesses all CSP ortholog candidates (Zhu *et al.*, 2006). Nothing is known about possible a relationship in *A. thaliana* between the presence of the glucosinolate pathway and the absence of CSP, two specificities in *Brassicaceae* plants. Both could have suppressive independent effects on mycorrhizas (Pieterse and Dicke, 2007) and perhaps on some other fungi.

Changes linked to glucosinolates and derivatives in *Alphaproteobacteria* community, particularly *Rhizobiaceae*, were less explored in the literature. Ground root tissue of *Brassica* was toxic to *Bradyrhizobium* pure strains (Trinick and Hadobas, 1995) and *in vitro* colonization of *Brassica napus* roots by the *Alphaproteobacteria Azorhizobium caulinodans* was affected by plant glucosinolate content (O'Callaghan *et al.*, 2000). In our study, we show different susceptibilities of spontaneous *Rhizobiaceae* populations exposed to *Brassicaceae* roots. This unexpected high reactivity could have a role in the selection of populations that are important to explore because rhizobia are of particular interest in soil, according to possible positive or negative effect on plant health and growth.

Differential effects on microbial populations that we observed could result from variable direct susceptibility of populations towards antimicrobial properties of hydrolysis products of glucosinolates (Smith and Kirkegaard, 2002; Kliebenstein *et al.*, 2005; Brader *et al.*, 2006). Beside their antimicrobial effect, glucosinolates and their hydrolysis products could also stimulate microbial growth. In soil, glucosinolates are hydrolysed by microorganisms (Fahey *et al.*, 2001) and microbial degradation is the main mechanism for disappearance of isothiocyanates (Rumberger and Marschner, 2003). Microorganisms could perceive these metabolites as specific signal compounds stimulating growth but also use them as a nutritional substrate (Zeng *et al.*, 2003; Souza-Fagundes *et al.*, 2004).

These compounds could also act indirectly on populations through nutritional or toxic impact on their hosts, competitors, antagonists or predators. Indeed, balance between populations was strictly governed by interaction among community members, each of them might be affected positively or

negatively by each other (Trosvik *et al.*, 2008). It should be the case for the most impacted fungi *S. depressa*, only present in CYP79A1 transgenic plants, that is a parasite of another fungi. Host sensitivity to glucosinolate composition could impact *S. depressa* population development.

We could not exclude that CYP79A1 plants are not only modified in glucosinolates profile because of a known link with other pathways, particularly those implicated in plant defense. Kristensen *et al.* (2005) showed that insertion of CYP79A1 gene and accumulation of the novel glucosinolate had only marginal inadvertent effects on the plant transcriptome and metabolome compared with wild type. However, Brader *et al.* (2006) showed that the accumulation of the novel glucosinolate could reduce the induction of jasmonate-mediated pathway but led to increased levels of salicylic acid. These plant defense pathways could modify the soil bacterial community structure (Kniskern *et al.*, 2007; Hein *et al.*, 2008).

In conclusion, we show that the production of only one exogenous glucosinolate by CYP79A1 plant alters microbial community structure on roots and rhizosphere. The original defense system 'glucosinolate-myrosinase' can be considered as an effective factor of microbial selection in root environment of *Brassicaceae* where inter-strain competition is strong for substrate assimilation. Even small advantages or disadvantages caused by glucosinolates and their hydrolysis products would select the fittest strains. This factor of selection is probably also effective on other organisms such as those of soil microfauna. Further studies are now necessary to draw conclusions about changes in microbial functions because of possible functional redundancy in soil (Wertz *et al.*, 2006).

An interesting area for further study would be to understand the determinants and molecular mechanisms sustaining the differential response of non-targeted microbial populations towards this plant defense system, particularly in a context of living *Brassicaceae* used for soil pest control and disease suppression. *Brassicaceae* species are extensively used as biofumigants towards various soil-borne pests and pathogens (Angus *et al.*, 1994; Brown and Morra, 1997). Our results show that this agricultural practice could also impact non-target microorganisms in soil with possible consequences on following cultures. Recent invasion of eastern North American forests by the non-native garlic mustard well illustrates these ecological issues. Although no single mechanism appears to explain its success, one of the most important is the root release of benzyl isothiocyanate of this *Brassicaceae* (Rodgers *et al.*, 2008) inhibiting the growth of ectomycorrhizal fungi and reducing competitive abilities of native trees (Wolfe *et al.*, 2008). The domination of this new species in forests finally changed the ecology and the function of these systems (Rodgers *et al.*, 2008).

There is also a strong interest to alter levels of specific glucosinolates or generate novel glucosinolates in crop plants that could be an alternative to the use of pesticides. Concerns have been raised about the environmental impact associated with genetically modified organisms particularly GM crop. Variable and transient impacts on non-target microorganisms were already shown (De Vries *et al.*, 1999; Lottmann *et al.*, 2000; Dunfield and Germida, 2003; Castaldini *et al.*, 2005; Rasche *et al.*, 2006). We show here that manipulation of glucosinolate content in *Brassicaceae* could have some repercussions on the microbial community in their close soil environment. Possible consequences on specific microbial functions implicated in ecosystem functioning should be then evaluated.

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