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Rapid report

DNA-based stable isotope probing enables the identification of active bacterial endophytes in potatoes

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Summary

- A $^{13}CO_2$ (99 atom-%, 350 ppm) incubation experiment was performed to identify active bacterial endophytes in two cultivars of *Solanum tuberosum*, cultivars Desirée and Merkur. We showed that after the assimilation and photosynthetic transformation of $^{13}CO_2$ into ^{13}C-labeled metabolites by the plant, the most directly active, cultivar-specific heterotrophic endophytic bacteria that consume these labeled metabolites can be identified by DNA stable isotope probing (DNA-SIP).
- Density-resolved DNA fractions obtained from SIP were subjected to 16S rRNA gene-based community analysis using terminal restriction fragment length polymorphism analysis and sequencing of generated gene libraries.
- Community profiling revealed community compositions that were dominated by plant chloroplast and mitochondrial 16S rRNA genes for the 'light' fractions of $^{13}CO_2$-incubated potato cultivars and of potato cultivars not incubated with $^{13}CO_2$. In the 'heavy' fractions of the $^{13}CO_2$-incubated endophyte DNA, a bacterial 492-bp terminal restriction fragment became abundant, which could be clearly identified as *Acinetobacter* and *Acidovorax* spp. in cultivars Merkur and Desirée, respectively, indicating cultivar-dependent distinctions in ^{13}C-label flow. These two species represent two common potato endophytes with known plant-beneficial activities.
- The approach demonstrated the successful detection of active bacterial endophytes in potato. DNA-SIP therefore offers new opportunities for exploring the complex nature of plant–microbe interactions and plant-dependent microbial metabolisms within the endosphere.

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Introduction

Endophytes are microorganisms that colonize the intercellular space and vascular tissues of plants without exhibiting pathogenicity (Wilson, 1995). Many bacterial endophytes inhabiting plant shoots offer plant growth-promoting and pathogen antagonistic activities and thereby can have beneficial effects on crop yield and health (Sturz *et al.*, 2000). Most bacterial endophytes identified so far that colonize roots as well as above-ground tissues are heterotrophs. Therefore, it is assumed that endophytes depend on assimilates from the plant as growth substrates to be able to colonize this ecological niche. Similarly, the quantity and the composition of these plant species-specific assimilates may control the structure of endophytic communities and even regulate their activity. Previous cultivation-independent analyses have identified a high diversity of plant shoot-associated endophytic communities and revealed that individual plants, or even cultivars of the same species, are colonized by bacteria belonging to different phylogenetic groups (Araújo *et al.*, 2002; Zinniel *et al.*, 2002; Idris *et al.*, 2004; Berg *et al.*, 2005; Rasche *et al.*, 2006a). Furthermore, bacterial endophyte communities have been shown to respond to abiotic factors as well as to biotic plant stress, indicating again that these populations respond to altered plant physiology (Dalmastri, 1999; Kinkel *et al.*, 2000; Araújo *et al.*, 2002; Rasche *et al.*, 2006a).

Such DNA-based community analyses generally allow a cultivation-independent analysis of bacterial populations; yet no direct information on the respective metabolic properties of the detected community members has been obtained. However, it is possible to identify directly specific microbial populations actively involved in a given metabolic process by nucleic acid-based stable isotope probing (SIP, Radajewski *et al.*, 2000). Stable isotope probing is based on the incorporation of ^{13}C from a labeled substrate into cellular biomarkers such as nucleic acids, the subsequent separation of labeled and unlabeled nucleic acids by density-gradient centrifugation and, finally, the molecular identification of specifically labeled population members within the 'heavy' nucleic acids (Radajewski *et al.*, 2000). Recently, SIP has been successfully applied to identify key plant assimilate-dependent microbes in rhizospheric environments (e.g. methanogenic populations on rice roots (Lu *et al.*, 2005), rhizosphere bacteria in a grassland soil (Rangel-Castro *et al.*, 2005), or microbes inhabiting roots of upland and peatland grasses (Vandenkoornhuyse *et al.*, 2007)). However, SIP has never been applied to endophytic bacteria of plant shoots.

The objective of this study was to verify whether DNA-SIP can be applied to identify shoot-associated endophytes that most directly utilize plant-assimilated photosynthates. We furthermore aimed to test whether different plant genotypes host different endophytic populations directly consuming the photosynthates. Therefore, a glasshouse experiment was performed with two potato genotypes that were either treated or not treated with $^{13}CO_2$ followed by the subsequent detection of labeled bacterial DNA isolated from plant shoots using SIP.

Materials and Methods

A glasshouse experiment was performed with two potato (*Solanum tuberosum* L.) cultivars, Desirée and Merkur: six tubers from each cultivar were cultivated in pots (size: 9 × 9 × 9 cm, one tuber per pot) filled with a chernozem (sand, 43%; silt, 37%; clay, 21%; organic carbon, 2.7%, pH 7.0; NO_3-N, 81.9 mg kg^{-1}; P_2O_5, 227 mg kg^{-1}; K_2O, 338 mg kg^{-1}; Mg, 120 mg kg^{-1}) taken from the top layer of a potato field close to Seibersdorf (Austria). Plants were grown under controlled conditions (average air temperature, 22.5°C; average daylight, 7.000 lux; relative air humidity, 60.1%; daily irrigation, 100 ml of tap water). After plants had reached the stem-elongation stage (Hack *et al.*, 1993), three pots per cultivar were transferred into an airtight incubation tent (4000 l total tent volume) built of transparent, ultraviolet-radiation-permeable plastic foil to separate plants from the ambient glasshouse atmosphere. Repeated pulse labeling (three pulses per day) with $^{13}CO_2$ (99 atom-%, 350 ppm) was performed over a 4-d period, resulting in an average concentration of 41.47 atom-% $^{13}CO_2$ in the tent, as measured by gas chromatography combined with isotope ratio mass spectrometry (delta plus; Finnigan MAT, Bremen, Germany) over the incubation period. During night-times, however, evolving $^{12}CO_2$ was exhausted from the system using a pump. The incubation time was defined after a pre-experiment using the same cultivars and conditions described above in which plants showed the highest incorporation rates of $^{13}CO_2$ after 4 d of labeling (data not shown). Control plants were treated accordingly, but using nonlabeled CO_2. After incubation, plant shoots of 15 cm length of three replicate plants of both treatments (control plants and $^{13}CO_2$-treated plants) were surface-sterilized and macerated (Rasche *et al.*, 2006a). Fluid obtained from macerated material was used for analysis of the isotopic signature (atom-%) by an elemental analyzer coupled to an isotope ratio mass spectrometer (EA/IRMS) (Euro EA (Eurovector Milan, Italy) and MAT 253 (Thermo Finnigan, Bremen, Germany), respectively).

The fluid obtained from both potato cultivars from $^{13}CO_2$-labeled plants as well as from control plants were further used for DNA isolation according to Martin-Laurent *et al.* (2001). The procedure of gradient preparation, isopycnic centrifugation and gradient fractionation was performed mainly as described previously (Lueders *et al.*, 2004). Five micrograms of PicoGreen (Invitrogen, Carlsbad, CA, USA)-quantified DNA extracts were loaded into a gradient medium of 1.71 g ml^{-1} CsCl (Calbiochem, Luzern, Switzerland) dissolved in gradient buffer prepared according to Neufeld *et al.* (2007). Before centrifugation, the average density of centrifugation media was controlled refractometrically (AR200; Reichert Analytical Instruments, Depew, NY, USA) and adjusted, if necessary.

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Centrifugation was performed in 5-ml poly-allomer quick-seal tubes in a VTI 65.2 vertical rotor (both Beckman Coulter, Fullerton, CA, USA) using a Centrikon T-2190 ultracentrifuge (Kontron Instruments, Zurich, Switzerland). Centrifugation runs were carried out at 180 000 ***g*** at 20°C for 65 h. Thirteen fractions of each gradient were collected from 'heavy' to 'light' by displacement with water (Manefield *et al.*, 2002) using a Perfusor V syringe pump (B. Braun Melsungen, Melsungen, Germany) at a flow rate of 1 ml min^{-1}. Refractometric measurement of fraction densities, recovery of DNA from gradient fractions, measurement of total bacterial 16S rRNA gene quantities in gradient fractions and terminal restriction fragment length polymorphism (T-RFLP) fingerprinting were performed as previously described (Kunapuli *et al.*, 2007). The T-RFLP analysis of bacterial 16S rRNA gene amplicons was performed using primers Ba27f-FAM and 907r (Weisburg *et al.*, 1991; Muyzer *et al.*, 1995) and *Msp*I digestion. From each gradient, nine DNA fractions (from fraction #11 to fraction #03) were selected for fingerprinting. Electrophoresis was carried out on an ABI 3730 DNA Analyzer and electropherograms were evaluated using the GENE MAPPER 3.5.1 software (both Applied Biosystems, Foster City, CA, USA). 16S rRNA gene libraries were generated using the same experimental conditions as for T-RFLP analysis, from the fifth 'heavy' fractions (buoyant density = approx. 1.728 g ml^{-1} CsCl) of both $^{13}CO_2$-treated and nonlabeled potato genotypes. Purified amplicons were ligated into the StrataClone™ PCR cloning vector pSC-A (Stratagene, La Jolla, CA, USA); StrataClone™ SoloPack® competent cells (Stratagene) were then transformed with the ligation products. One-hundred clones from each gene library were randomly selected and partially sequenced using the BigDye V3.1 Terminator-Kit (Applied Biosystems) with oligonucleotides 518r (Lane, 1991) and 907r (Lane *et al.*, 1985). Obtained sequence information of approx. 850 bases per sequence were subjected to BLAST analysis in the National Center for Biotechnology Information (NCBI) database and have been deposited in the NCBI database under accession numbers EU450168 to EU450267 and EU450368 to EU450467.

Results and Discussion

Two cultivars of *S. tuberosum* (Desirée and Merkur) were treated with pulses of $^{13}CO_2$ (99 atom-%, 350 ppm) for 4 d at the stem-elongation stage. Analysis of the resulting plant shoot material using EA/IRMS revealed ^{13}C enrichments in biomass of approx. 15 atom-% for both cultivars incubated with $^{13}CO_2$, whereas for the nonincubated control plants, a natural ^{13}C signature of 1.078 atom-% was measured. The incorporation of just 15% ^{13}C into DNA would clearly be at the assumed detection limits of centrifugation-based separation of labeled nucleic acids (Radajewski *et al.*, 2000). However, if specific carbon fluxes from fresh plant assimilate to consuming bacterial endophytes are more directly coupled than average ^{13}C enrichment in total plant biomass, this would still facilitate the detectability of the respective bacteria by DNA-SIP. We were able to substantiate the validity of this assumption using the results obtained from our differential ^{13}C-labeled and unlabeled DNA-centrifugation gradients.

Quantification of bacterial 16S rRNA gene distribution in density-resolved DNA gradients did not indicate increased template detectability in 'heavy' fractions of DNA of $^{13}CO_2$-treated plants (data not shown). Nevertheless, T-RFLP fingerprinting of the 16S rRNA genes amplified using universal bacterial PCR primers revealed distinctions in the bacterial community structure of 'heavy' and 'light' DNA of $^{13}CO_2$-treated plants for both cultivars compared with the nonlabeled control plants (Fig. 1). The T-RFLP patterns were mostly dominated by two specific terminal restriction fragments (T-RFs), namely the 337- and 411-bp peaks. These were affiliated to plant-derived mitochondrial (337 bp) and chloroplast (411 bp) rRNA genes, as shown by cloning and sequencing of the respective amplicons (Table 1). This occurrence of plant-derived T-RFs in generated fingerprints was expected, because the DNA-isolation procedure applied was based on that described by Martin-Laurent *et al.* (2001), which recovers bacterial and plant DNA. Thus, subsequent amplification using universal bacterial 16S rRNA gene primers resulted in a mixed pool of bacterial and plant organelle rRNA gene amplicons, which is known generally to hamper the cultivation-independent analysis of endophyte communities (Sessitsch *et al.*, 2002).

Nevertheless, the comparison of T-RFLP patterns from 'heavy' fractions indicated that the relative abundance of plant-derived sequences, particularly those from mitochondria, was strongly reduced in DNA extracts from $^{13}CO_2$-incubated plants in comparison with control gradients (Fig. 1). In particular, a 492-bp T-RF became abundant in fractions #04 and #05 of labeled plant DNA gradients, which was not detected, or was clearly less abundant, in the respective 'heavy' control gradient fractions from non-$^{13}CO_2$ treatments. This substantiated that the ^{13}C-label was incorporated into non-plant organelle small subunit (SSU) rRNA genes. Cloning and sequencing revealed that the 492-bp T-RF observed in Desirée 'heavy' DNA fractions represented sequences closely related to *Acidovorax* sp., whereas in Merkur this T-RF appeared to be affiliated to relatives of *Acinetobacter* sp. (Table 1).

Furthermore, a considerable diversity of further putative bacterial endophytes was detected in gene libraries, mostly within the alpha-, beta- and gamma-proteobacteria, as well as in the Gram – positive bacteria (Table 1). These were partially also reflected in respective T-RFs observed in different fractions; however, unlike the 492-bp T-RF, their appearance was not consistent or dominant within the 'heavy' fractions of DNA from $^{13}CO_2$-incubated plants. Therefore, specific labeling of SSU rRNA genes other than those represented by the 492-bp T-RFs was not apparent.

The detection of high-G+C Gram-positive bacteria (e.g. *Propionibacterium*, *Micrococcus* and *Microbacterium* spp.) in

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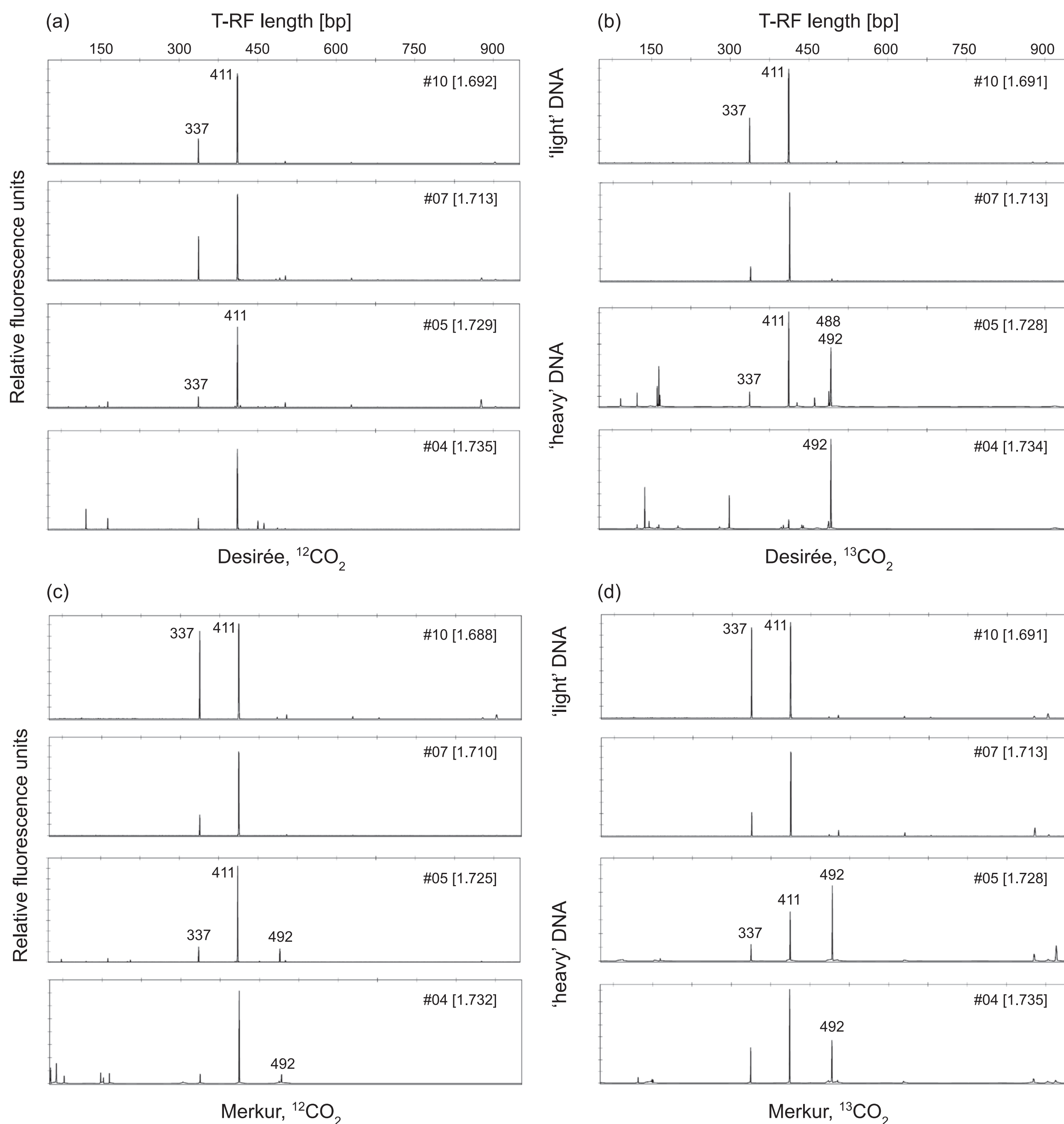

Fig. 1 Representative terminal restriction fragment length polymorphism (T-RFLP) electropherograms of density-resolved fractions #10 ('light') to #04 ('heavy') of nonincubated (a, c) and $^{13}CO_2$-incubated (b, d) plant shoot DNA of *Solanum tubersosum* cultivars Desirée (a, b) and Merkur (c, d). Selected terminal restriction fragments (T-RFs) consistently dominating either 'light' or 'heavy' fractions are indicated via base-pair length. Fraction numbers and CsCl buoyant densities (g ml^{-1}, in parentheses) are also given.

these heavy fractions may also be attributed to their high genomic G+C content, rather than to specific ^{13}C labeling (Manefield *et al.*, 2002; Lueders *et al.*, 2004; Buckley *et al.*, 2007).

The detection of *Acidovorax* and *Acinetobacter* spp. as potato endophytes is not novel. Previous studies have shown that these genera are able to colonize the potato endosphere and also to exhibit plant-beneficial functions (Sturz, 1995; Heuer & Smalla, 1999; Sturz *et al.*, 1999). Nevertheless, our labeling experiment over an incubation time of just 4 d revealed interesting cultivar effects on the SIP-detectable endophytic bacterial community, enabling novel insights into dynamic plant–microbe interactions shaped by cultivar-specific metabolic fluxes. Thus, the ^{13}C-label appeared to be predominately

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Table 1 Phylogenetic affiliation of cloned small subunit rRNA genes amplified from density-resolved 'heavy' $^{13}CO_2$-incubated plant shoot DNA (gradient fractions #05) of *Solanum tuberosum* cultivars Desirée and Merkur

Affiliation predicted T-RF (bp)	No. of clones cultivar Desirée	No. of clones cultivar Merkur	Closest NCBI match (accession number)/% homology
Firmicutes			
554		2	*Listeria grayi* (X56150)/100
High-G+C Gram-positive bacteria			
146	5		*Microbacterium* sp. KVD-unk-64 (DQ490453)/100
163	1	3	*Micrococcus* sp. MACL03 (EF198243)/99
165		7	*Propionibacterium* sp. (AM157438)/99–100
279	8		*Microbacterium* sp. MC3B-10 (AY833570)/99–100
α-Proteobacteria			
150	6		*Sphingomonas melonis* (AB334774)/99–100
152	10	4	*Bradyrhizobium* sp. LAR-20 (EF638789)/99–100
404	1		*Sphingomonadaceae* clone Elev_16S_810 (EF019656)/99
β-Proteobacteria			
141	23	14	*Burkholderia* sp. SAP II (AF052387)/99–100
404		4	Uncultured bacterium clone LF035 (EF417724)/100
489–490	15		*Acidovorax* sp. strain 98-63833 (AY258065)/99–100
498	12		Uncultured *Tepidimonas* sp. clone HB116 (EF648100)/99
γ-Proteobacteria			
321		1	*Pseudomonas citronellolis* (AY147931)/99
490–492	1	33	*Acinetobacter junii* (AB101444)/99–100
494		1	*Pantoea agglomerans* (CP000038)/100
496		2	*Shigella sonnei* (X56150)/99–100
Eukaryota			
338	6	4	*Solanum tuberosum* mitochondrial DNA (AJ252732)/100
413–414	12	25	*Solanum tuberosum* chloroplast DNA (DQ386163)/99–100

NCBI, National Center for Biotechnology Information; T-RF, terminal restriction fragment.

shuttled to *Acidovorax* sp. in Desirée plants and to *Acinetobacter* sp. in Merkur plants. Previous studies based on denaturing gradient gel electrophoresis analysis and on T-RFLP analysis, as well as cultivation of plant-beneficial endophytes, carried out by Van Overbeek & van Elsas (2008) and by Rasche *et al.* (2006a,b), confirmed such distinct cultivar effects on active endophytes by studying the same cultivars. In detail, the authors determined cultivar-specific bacterial antagonists as well as plant growth-promoting endophytes (e.g. *Pseudomonas* sp., *Acinetobacter* sp. and *Sphingomonas* sp.), which were also partially recovered in the present study.

The obtained data support our primary hypothesis that bacterial endophytes found in above-ground plant tissues directly metabolize organic plant assimilates and thus can be resolved by DNA-SIP. It has been previously reported that the different potato cultivars (Desirée and Merkur) used in this study shape cultivar-specific bacterial communities in the corresponding plant shoots (Rasche *et al.*, 2006a,b). However, for a detailed characterization of plant metabolites extracted from $^{13}CO_2$-treated and control plants, it would be necessary to identify the specific composition and quantity of assimilates supporting endophytic bacterial growth (Gleixner *et al.*, 1998).

In summary, we confirmed the specific activity of selected plant shoot-associated bacterial endophytes and showed that these are directly connected to the carbon flow deriving from fresh photosynthates. Therefore, endophyte-SIP, in addition to DNA-SIP of microbes in rhizospheric environments (Lu *et al.*, 2005; Rangel-Castro *et al.*, 2005; Vandenkoornhuyse *et al.*, 2007) may offer a novel perspective for a better understanding of relevant plant–microbe interactions. In the present study, active endophytes were detected at the stem-elongation stage; however, the specifically active community can be expected to change with different vegetation stages and through the influence of biotic or abiotic stress factors (Sessitsch *et al.*, 2002; Rasche *et al.*, 2006a). Such factors lead to a cascade of reactions within the plant, resulting in the production of various enzymes, stress proteins and metabolites. Endophytes might be directly affected by, for example, reactive oxygen species or other antibacterial substances, or they might change as a result of the different availability of plant metabolites. Further investigation of labeled plant metabolites and active endophytes at different vegetation stages or plant stress situations caused by biotic or abiotic stressors will therefore be essential to advance the understanding of dynamic plant–microbe interactions.

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