Structural and functional analysis of the bacterial root microbiota of *Arabidopsis thaliana* and relative species

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`The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not ‘eureka!’ but ‘that’s funny…’

Isaac Asimov

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Abstract

Plants that reside in the soil interact with a plethora of organisms, such as nematodes, fungi, oomycetes or bacteria. Soil bacteria can colonize the rhizosphere and roots of plants and influence plant growth via interference with hormones, mobilization of nutrients or suppression of phytopathogens. With the advances in the last years, we begin to understand the structure of the root and rhizosphere bacterial microbiota and the factors that influence community assembly. However, remarkably little is known about the stability and functional abilities of bacteria in a community context. To address these gaps, two approaches were undertaken that will be discussed in two chapters within this study.

In the first chapter, I compared root bacterial communities of the plant species *Arabidopsis thaliana*, *Cardamine hirsuta* and *Arabis alpina* to the corresponding soil microbiota by pyrosequencing of 16S rRNA gene amplicon libraries. Further, *A. alpina* was used as a model system to dissect the influence of residence time of plants in the soil, plant developmental stage and growth under natural conditions on bacterial community structure using Illumina sequencing. Additionally, I isolated bacteria from roots of the three plants species and assessed their influence on plant growth. Using this experimental setup, I demonstrated that the tested Brassicaceae plant species assembled highly similar bacterial root communities and only few quantitative differences allowed to distinguish the three host plant species. By this a shared bacterial microbiota was defined that consisted of a few bacterial families. The major factors determining community composition were the compartment, soil type, time and environmental conditions. Surprisingly, plant species and plant developmental stage influenced bacterial communities only to a minor extent. Finally, several bacterial members of the root microbiota were successfully isolated and a high number of isolates positively affected root growth of *A. thaliana*, *C. hirsuta* and *A. alpina* in mono-associations as well as in synthetic communities.

In the second chapter, I isolated rhizobia from roots of *A. thaliana* and tested 22 isolates on their ability to affect plant growth under different nutrient conditions using a gnotobiotic growth assay. One Rhizobium was more closely investigated and the proliferation on plant roots, as well as phenotypic and transcriptional changes induced by this Rhizobium, were monitored in a time-resolved manner, the latter by RNA-Sequencing. Based on this approach, I established that members of the rhizobial population act as plant growth-promoting rhizobacteria that affect primary root growth under various nutrient conditions and *A. thaliana* accessions. Rhizobia were able to proliferate on the root system and induced transcriptional changes that were associated with hormone homeostasis, nutrient availability and enhanced stress tolerance.

Zusammenfassung

Pflanzen leben im Boden in Wechselwirkung mit einer Vielzahl von Organismen, wie Nematoden, Pilzen und Bakterien. Boden-Bakterien können sowohl die Rhizosphere als auch die Wurzel von Pflanzen besiedeln und beeinflussen das Pflanzenwachstum durch Modulation von Phytohormonen, Mobilisierung von Nährstoffen oder Inhibierung von Pathogenen. Durch die Entwicklung neuer Sequenziertechnologien hat das Verständnis über die Struktur von bakteriellen Gemeinschaften in Assoziation mit Pflanzen zugenommen. Jedoch bleibt weiterhin im Unklaren, welche Faktoren die Struktur von Bakteriengemeinschaften beeinflussen, wie stabil diese Gemeinschaften sind und was die Funktion von Bakterien in Wechselwirkung mit anderen Bakterien ist.

Im ersten Teil meiner Arbeit habe ich bakterielle Gemeinschaften im Boden und assoziiert mit der Wurzel von *Arabidopsis thaliana*, *Cardamine hirsuta* und *Arabis alpina* mittels Pyrosequenzierung des bakteriellen 16S rRNA Genes bestimmt. *A. alpina* diente im weiteren Verlauf der Experimente als Modelsystem zur Evaluierung, inwiefern verschiedene Faktoren, wie Bodentyp, Verweildauer der Pflanze im Boden, Pflanzenentwicklungsstadium oder Wachstum unter natürlichen Umweltbedingungen, die Struktur von Bakteriengemeinschaften beeinflussen. Zuletzt habe ich Bakterien von Wurzeln isoliert und diese auf ihren Einfluss auf das Pflanzenwachstum geprüft. Hier konnte ich zeigen, dass die verschiedenen Pflanzenspezies ähnliche bakterielle Gemeinschaften assemblieren und nur durch wenige quantitative Unterschiede zu differenzieren sind. Die Hauptfaktoren, welche die Zusammensetzung von bakteriellen Gemeinschaften beeinflussen sind der Bodentyp, die Verweildauer der Pflanze im Boden und das Wachstum unter natürlichen Umweltbedingungen. Überraschenderweise spielten die Pflanzenart und das Entwicklungsstadium der Pflanze nur eine untergeordnete Rolle für die Assemblierung bakterieller Gemeinschaften. Bakterien konnten erfolgreich vom Wurzeln isoliert werden und ein Großteil der geprüften Isolate beeinflusste die Wurzellänge von *A. thaliana*, *C. hirsuta* und *A. alpina*.

Im zweiten Teil wurden Rhizobien, eine Gruppe von Wurzel-assoziierten Bakterien, genauer untersucht. Dafür wurden Rhizobien von Pflanzenwurzeln isoliert und 22 Isolate auf ihren Einfluss auf das Wachstum von *A. thaliana* geprüft. Ein Rhizobien-Exemplar wurde sowohl auf dessen Anreicherung auf der Wurzel als auch auf die phänotypische und transkriptionelle Beeinflussung des Wurzelwachstums analysiert. Durch diese Vorgehensweise konnte gezeigt werden, dass Rhizobien das Wurzelwachstum unter verschiedensten Nährstoffbedingungen fördern und auch auf der Wurzel über die Zeit angereicht werden. Inokulation von Rhizobium stimulierte transkriptionelle Veränderungen in der Wurzel von *A. thaliana*, welche mit Hormon-Homöostase, Nährstoffaufnahme und Stresstoleranz assoziiert waren.

Chapter I

Structural and functional characterization of the bacterial microbiota of *Arabidopsis thaliana* and relative species

1. Introduction

Multicellular organisms interact with a plethora of microorganisms, such as bacteria, fungi or protozoa, and especially bacteria reach high densities within different host organisms. For example, in humans bacterial cell numbers are approximately 10 times higher than the number of cells represented by the host itself (Savage, 1977; Bäckhed et al., 2005). Also the number of genes encoded in the bacterial microbiota of humans exceeds by ~150 times the number of genes expressed by the host (Qin et al., 2010). Owing to these observations, the bacterial microbiota associated with eukaryotic host organisms is often referred to as the "second genome". This second genome confers a range of functional capacities that are otherwise inaccessible for the host, for example by degrading indigestible polysaccharides (Xu et al., 2003). Together, this highlights the importance of the bacterial microbiota for the host; however, a better understanding of the structure of bacterial communities associated with diverse hosts has become only recently available. Additionally, the mechanisms by which microorganisms influence the host in a community context are still largely unknown.

The scope of this literature review is to introduce the reader to current knowledge about the bacterial microbiota of plants as host organisms. Therefore, I will discuss bacterial communities inhabiting the soil, rhizosphere and roots of plants. I will describe the structure of these communities, the factors influencing their composition as well as the biological relevance of bacterial communities on the host plant. Additionally, this section introduces current techniques that allow the study of community structure, including an in-depth description of 16S rRNA gene-based techniques.

1.1. Structure and functions of the bacterial microbiota

1.1.1. Soil bacterial communities

Soil provides important functions for plant growth, as soil anchors roots and offers essential nutrients and water (Smith and Smet, 2012). Additionally, soil characteristics such as pH, nutrient composition and water content, are factors affecting plant performance. Soil-inhabiting microorganisms, such as bacteria, maintain soil characteristics and thereby indirectly influence plant growth. Soil represents one of the most diverse ecosystems on earth, as one gram of soil contains up to 10^9 bacteria containing 10,000 to 50,000 bacterial species (Bulgarelli et al., 2013; Christensen et al., 1999). Hence, the soil bacterial microbiota represents the start inoculum of bacteria that subsequently colonize plants comprising a large pool of bacterial-encoded functions that influence plant performance.

Abundant bacterial phyla inhabiting the soil are Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes and Proteobacteria (Fierer and Jackson, 2006). The composition of this soil bacterial biome is strongly influenced by soil pH (Fierer and Jackson, 2006; Lauber et al., 2009). Shifts in pH alter species richness and diversity, whereas geographical distance or latitude influence the bacterial soil biome only to a minor extent, as seen by investigating community composition based on the bacterial 16S rRNA gene using terminal-restriction fragment length polymorphism (T-RFLP) analyses as well as pyrosequencing (an in-depth description of techniques that allow to determine bacterial community composition can be found in Chapter I.1.4). Additionally, soil structure affects the composition of bacterial communities, as soil dominated by smaller sized particles hosts more diverse communities based on a T-RFLP analysis (Sessitsch et al., 2001). Furthermore, bacteria-bacteria interactions are believed to structure soil communities, for example, through antibiotic production (Czárán et al., 2002). A recent study tried to experimentally evaluate this hypothesis by linking antagonistic activities of bacteria in soil biomes associated with the four plant species *Andropogon gerardii*, *Schizachyrium scoparium*, *Lespedeza capitata* and *Lupinus perennis* with shifts in community composition based on pyrosequencing (Bakker et al., 2014). However, due to the complexity of factors involved in structuring bacterial communities, a direct influence of bacteria-bacteria interactions is still difficult to dissect.

Microbial communities in the soil are relevant for ecosystem stability, productivity and stress tolerance (Torsvik and Øvreås, 2002; Lau and Lennon, 2012; Wagg et al., 2014). Along this line, reducing soil biodiversity in a grassland microcosm, by fractionating communities based on microbial size, influenced ecosystem stability and resulted in a reduced plant species diversity, reduced carbon sequestration or increased phosphorus leaching (Wagg et al., 2014). Additionally, soil communities were demonstrated to affect flowering time of *Boechera stricta*, *Arabidopsis thaliana* and *Brassica rapa* based on community profiling of the bacterial 16S rRNA gene using next-generation sequencing (NGS) technologies (Wagner et al., 2014; Panke-Buisse et al., 2014). Even though the exact mechanisms by which bacteria affected flowering time were not determined in these studies, not only bacterial communities but also abiotic stress, such as drought, induces a faster flowering in *B. rapa* (Franks et al., 2007). Therefore, the influence of soil bacterial communities on flowering could be related to enhancing phenotypic plasticity of plants under abiotic stresses.

1.1.2. Rhizosphere bacterial communities

The rhizosphere is a compartment defined by soil firmly attached to the roots that is influenced by the plant through root exudation (Hiltner, 1904; Walker et al., 2003). Plants secrete ~5-20% of photosynthetically fixed carbon into the rhizosphere and thereby enrich bacteria into the rhizosphere. This phenomenon is known as the rhizosphere effect (Lochhead and Rouatt, 1955). These secreted rhizodeposits comprise carbon-containing primary and secondary metabolites that include root cells,

mucilage or root exudates. Especially root exudates are highly diverse and consist of sugars, amino acids, phenols or organic acids (Bais et al., 2006).

Root exudates are important for structuring rhizosphere communities. Adding low molecular weight carbon compounds, such as glucose, glycine and citric acid, into different soil types alters bacterial community structure as determined by pyrosequencing, where for example citric acid increases the relative abundance of Betaproteobacteria (Eilers et al., 2010). Therefore, root exudation results in a distinct taxonomic composition of rhizosphere bacterial communities compared to unplanted soil for a range of plant species, such as crops and trees (Haichar et al., 2008; Turner et al., 2013; Peiffer et al., 2013; Grayston and Prescott, 2005; Uroz et al., 2010). The most abundant phyla inhabiting the rhizosphere are Proteobacteria, while soil-enriched Acidobacteria are of a lower relative abundance (Peiffer et al., 2013). The composition of rhizosphere communities depends on the tested plant species as wheat, oat and pea host distinct rhizosphere communities using metatranscriptomics (Turner et al., 2013). This highlights the importance of plant species for structuring bacterial communities. Besides a host species-specific component, also intra-species genetic variability of the host influences rhizosphere communities, which was demonstrated for potato or maize cultivars (Van Overbeek and Van Elsas, 2008; İnceoğlu et al., 2011; Peiffer et al., 2013). These studies were based on denaturing gradient gel electrophoresis (DGGE) as well as NGS technologies. Notably, a rhizosphere effect was observed for a diverse range of plant species but not for all. *Arabidopsis thaliana* and other members of the Brassicaceae family, such as *Arabidopsis lyrata* and *Cardamine hirsuta*, display only a weak or even no rhizosphere effect based on NGS analyses (Lundberg et al., 2012; Bulgarelli et al., 2012; Schlaeppi et al., 2014). Whether this represents a distinctive feature of this particular plant lineage or is related to the smaller root system or shorter lifespan is not understood.

To establish themselves in the rhizosphere, soil bacteria need to successfully colonize the rhizosphere and compete with other microorganisms for plant-derived rhizodeposits, a trait designated as rhizosphere competence (Lugtenberg and Dekkers, 1999). Beneficial for a successful rhizosphere colonization are chemotactic movement towards root exudates (de Weert et al., 2002), competition for nutrients by siderophores (Raaijmakers et al., 1995) or production of antibiotic compounds to outcompete antagonistic bacteria (Mazzola et al., 1992). Additionally, rhizosphere-competent bacteria need to adapt to altered nutrient conditions present in the rhizosphere. This includes the ability to degrade and utilize root exudates (Garland, 1996) or to detoxify harmful substances, such as phenols (Kivistik et al., 2006).

Diverse microorganisms can successfully colonize the rhizosphere, ranging from plant pathogens to beneficial microorganisms (Raaijmakers et al., 2009). Plant pathogens negatively influence plant growth and include microorganisms such as fungi, oomycetes, nematodes or bacteria. A well described soil-borne bacterial pathogen is *Ralstonia solanacearum* that infects roots of up to 200 plant species, including crops, underlining the agricultural importance of this phytopathogen (Salanoubat et al., 2002). Bacteria that positively influence plant growth are termed plant growth-promoting rhizobacteria (PGPR)

that can increase nutrient availability, interfere with hormone homeostasis or act as biocontrol agents against phytopathogens (Kloepper et al., 1980; Lugtenberg and Kamilova, 2009). An in-depth description of the modes of action of PGPR can be found in Chapter II.1.2.

The functional relevance of rhizosphere-colonizing bacteria in a community context was demonstrated for disease-suppressive soils, as exemplified for their suppressive action against soil-borne pathogenic fungi, such as *Rhizoctonia solani* or *Fusarium oxysporum* (Mendes et al., 2011; Fujiwara et al., 2013). The rhizosphere microbiota of disease-suppressive soils was compared to non-suppressive soils using a 16S rRNA oligonucleotide microarray (Mendes et al., 2011). The suppressive and non-suppressive soils did not display qualitative but several quantitative taxonomic differences that correlated with disease suppressiveness and were associated with Gamma- and Betaproteobacteria as well as with Actinobacteria. Isolation of bacteria from disease suppressive soils enriched for bacteria with antifungal activities, predominantly Pseudomonadaceae (Gammaproteobacteria), a bacterial group well-known for their antagonistic activities against soil-borne pathogens (Cook et al., 1995b). Together, this highlights the importance of rhizosphere bacterial communities to protect plants against soil-borne pathogens.

1.2. Root bacterial communities

Not only the rhizosphere but also the root tissue is colonized by a range of microorganisms, such as fungi, protists and bacteria (Fig. 1). Compared to unplanted soil, roots host a lower diversity and density of bacteria, with estimates of 10^4-10^8 bacterial cells per gram of tissue (Bulgarelli et al., 2013). Bacteria can either be associated with the rhizoplane, i.e. the root surface, or enter the root interior. Bacteria that thrive inside plants are termed endophytes (Hardoim et al., 2008). Even though several definitions exist for endophytes, they are most commonly defined as bacteria that can be recovered from surface-sterilized roots and are visually detected inside the plant using microscopic techniques (Reinhold-Hurek and Hurek, 2011). Invasion of roots by endophytes occurs predominately via root cracks or emergence points of lateral roots (James et al., 2002; Compant et al., 2005). In few cases endophytes appear to spread systemically through the xylem to distinct parts of the plant, such as fruits (Compant et al., 2010).

The close association of bacterial communities with plants represents an apparent paradox in the field of plant-microbe interactions, as plants have evolved sophisticated mechanisms to recognize and defend themselves against colonization by microorganisms. Plants employ two distinct defense mechanisms that are well described for their effectiveness against phytopathogens and are best studied for the model plant *A. thaliana* (Jones and Dangl, 2006). A first layer of inducible defense responses is initiated by plasma membrane-localized pattern recognition receptors (PRRs) that recognize microbe-associated molecular patterns (MAMPs), referred to as MAMP-triggered immunity (MTI; Zipfel, 2014). MAMPs mainly present conserved microbe-derived molecules that are found in a range of microorganisms, such as chitin produced by fungi, β-glucan associated with the cell walls of oomycetes or bacterial flagellin (Nürnberger et al., 2004). The best studied case of MTI induced by bacterial pathogens is recognition

of flg22, an epitope of the bacterial motor protein flagellin (Felix et al., 1999). The plant receptor-like kinase (RLK) FLAGELLIN-SENSING 2 (FLS2) recognizes the 22 amino acid long minimal epitope flg22 and then activates intracellular defense responses that inhibit bacterial growth (Zipfel et al., 2004). Another example is the bacterial elongation factor EF-Tu with the minimal peptide elf18 that is recognized by the EF-Tu receptor (EFR; Zipfel et al., 2006). MAMP-treatment typically induces a transient defense response, which leads to the activation of a reactive oxygen burst or the extracellular deposition of callose (Gómez-Gómez et al., 1999; Tsuda and Katagiri, 2010).

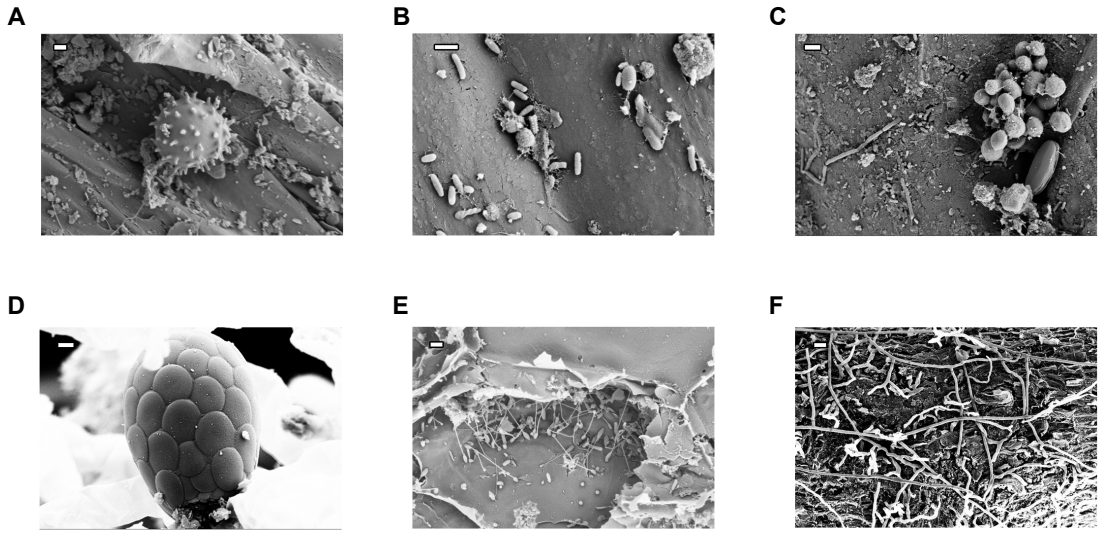


Fig. 1: Microorganisms present on the root surface of plants grown in natural soils. (A – C) *Arabis alpina* roots were grown in Cologne agricultural soil (CAS) and prepared for scanning electron microscopy (SEM) after 6 weeks of growth. Bar: 1 µM **(D – E)** *A. alpina* grown in a natural garden in Villar d'Arene (France) for up to 3 years. Bar in: **(D)** 2 µM; **(E)** 1 µM; **(F)** 20 µM.

To counteract plant immune responses pathogenic bacteria have evolved so called effector molecules that interfere with important hubs in plant defense to suppress MTI and allow bacteria to colonize plants (Weßling et al., 2014). Upon intracellular recognition of effector molecules, plants induce a second defense line that acts inside plant cells and is initiated by nucleotide-binding (NB) and leucine-rich repeat (LRR) domain containing proteins (NB-LRRs). Immunity associated with this mechanism is termed effector-triggered immunity (ETI). ETI triggers a more prolonged and robust immunity response, often inducing a localized host cell death (Jones and Dangl, 2006; Tsuda and Katagiri, 2010).

Despite this two-layered immune system, plants residing in a natural environment typically look healthy and symptomless, although the root bacterial microbiota contains a wide range of potential MAMPs. However, the mode of action by which the root bacterial microbiota evades or counteracts plant immune responses is not well understood. In addition, immune responses in roots are understudied compared to leaves, making a prediction of the interaction between plant immunity and the root bacterial microbiota difficult (Millet et al., 2010).

1.2.1. Structure of the bacterial root microbiota

The bacterial root microbiota has been studied in a selected number of plant species from the Brassicaceae lineage as well as in cannabis, poplar or rice (Lundberg et al., 2012; Bulgarelli et al., 2012; Schlaeppi et al., 2014; Winston et al., 2014; Shakya et al., 2013a; Edwards et al., 2015). By comparing the bacterial root microbiota of four Brassicaceae plant species, a taxonomically narrow and conserved bacterial microbiota was revealed based on NGS (Schlaeppi et al., 2014). These host species included *A. thaliana* and its sister species *Arabidopsis lyrata* and *Arabidopsis halleri* as well as the evolutionary more distant relative *Cardamine hirsuta*. Additionally, three *A. thaliana* accessions were included in this experimental setup allowing estimates on the influence of intra-species host genotype on community structure. Plants were grown under controlled environmental conditions in the same soil type and the root bacterial microbiota of all four plant species was determined. Additionally, *A. thaliana* and *C. hirsuta* were collected at two natural sites in Germany and their bacterial microbiota was compared to each other and to the obtained profiles of plants grown under controlled conditions.

During this investigation a major role of soil type and a minor role of intra-species variation of the host on community structure was identified. The *A. thaliana* relatives displayed more quantitative differences compared to the *A. thaliana* accessions, indicating that evolutionary divergence time is important for the structure of the bacterial microbiota. However, even among the four tested plant species only a few taxonomic groups of the root microbiota differed quantitatively. Globally, a narrow and largely conserved structure of the bacterial root microbiota was revealed among host plants that radiated from each other ~35 million years ago (Beilstein et al., 2010). Across the tested soil types, including controlled and natural environments, a shared bacterial community was identified consisting of members of the bacterial orders Burkholderiales (phylum Proteobacteria), Actinomycetales (phylum Actinobacteria) and Flavobacteriales (phylum Bacteroidetes). This shared microbiota prompts the question, whether these few taxonomic groups contribute common functions for plant growth and health (Shade and Handelsman, 2012).

Apart from members of the Brassicaceae lineage, the bacterial root microbiota of a few other plant species was investigated using NGS, including poplar, cannabis and rice (Shakya et al., 2013a; Winston et al., 2014; Edwards et al., 2015). Similar as for the Brassicaceae, the root compartments of poplar and cannabis were enriched for Proteobacteria and Actinobacteria but not for Bacteroidetes (Shakya et al., 2013a; Winston et al., 2014). In rice roots an enrichment of Proteobacteria and Bacteroidetes was detected, whereas Actinobacteria were not more abundant compared to unplanted soil (Edwards et al., 2015). This suggests that Proteobacteria represent a common phylum of the root microbiota across a range of plant species, whereas Actinobacteria and Bacteroidetes appear to be a specific trait for defined plant lineages. However, as plants in these studies were grown in different soil types using different sampling techniques and primer pairs for PCR-amplification of the 16S rRNA gene (sequencing technologies will be reviewed in Chapter I.1.4), the potential existence of a shared microbiota across distantly related plant species requires a closer analysis.

1.2.2. The bacterial root microbiota of *A. thaliana*

A. thaliana belongs to the Brassicaceae family and is a well-studied model system (Koornneef and Meinke, 2010). Advantages of *A. thaliana* are the short life cycle of few weeks, the small diploid genome and little space required for growing plants (Meinke et al., 1998). Additionally, available genome sequences, comprehensive mutant collections and simple transformation protocols make *A. thaliana* an ideal tool to test a range of biological questions (Alonso et al., 2003). *A. thaliana* does not interact with mycorrhizal fungi or engage in a mutualistic interaction with symbiotic rhizobia, allowing for a simplified view on plant-microbe interactions (Peškan-Berghöfer et al., 2004; Gough et al., 1997).

Two recent studies substantially increased our understanding about bacterial communities populating the rhizosphere and roots of *A. thaliana* based on NGS (Bulgarelli et al, 2012; Lundberg et al., 2012). The structure of the bacterial microbiota was assessed by growing eight accessions of *A. thaliana* in four soil types that were collected in the United States (US) or Germany during two seasons. In the study of Bulgarelli et al., wooden splinters (representing a lignocellulose matrix for bacterial colonization) were also implanted in the soil and the bacterial communities examined as for the root samples. This comparison allowed to distinguish between cues derived from living root cells and from the inert lignocellulose matrix derived from the plant cell wall.

Highly similar results were obtained in these two studies, even though different PCR-primer pairs, soil types and *A. thaliana* accessions were employed. Soil type and season induced major changes in the root microbiota structure, whereas host genotype influenced this structure only to a minor extent. A single bacterial species displayed quantitative differences in the relative abundance between the two accessions Shakdara (Sha) and Landsberg (Ler; Bulgarelli et al., 2012). Overall, the root compartment displayed a distinct taxonomic structure compared to soil and rhizosphere compartments. Dominant phyla associated with the root compartment included Actinobacteria, Bacteroidetes and Proteobacteria (with an overrepresentation of Betaproteobacteria). Notably, those phyla colonized roots and the wooden splinters with a different relative abundance. While living roots enriched for Actinobacteria, wooden splinters accumulated more Betaproteobacteria. The comparison between living root cells and the lignocellulose matrix indicates that root colonization by Actinobacteria requires signaling cues that are derived from living plant cells and are not needed by Betaproteobacteria. In summary, these two studies demonstrated that *A. thaliana* hosts a bacterial community with a similar taxonomic composition, despite growth of plants in different soil types and environmental conditions. Both studies confirmed that soil type explained most of the variation at the rank of bacterial phyla, whereas host genotype affects community profiles to a minor extent.

Root exudation is seen as one mechanism by which the plant influences the rhizosphere and root microbiota (Bais et al., 2006). This is supported by experiments with a transgenic *A. thaliana* line that expresses a cytochrome P450 family protein (CYP79A1) from *Sorghum bicolor*, resulting in an altered glucosinolate profile (Bressan et al., 2009). The accumulation of Alphaproteobacteria, predominantly Rhizobiaceae, differed in the glucosinolate mutant compared to wild type (WT) plants based on

community profiling using DGGE. Support for the involvement of root exudates in structuring bacterial communities comes from another study in which seven *A. thaliana* ATP-binding cassette (ABC) transporter mutants were analyzed using automated ribosomal intergenic spacer analysis (ARISA; Badri et al., 2009). The community profile of the *abcg30* transporter mutant displayed a shift in bacterial and fungal communities, as well as an altered root exudation profile, suggesting a link between the secretion of exudates and community structure.

Limited knowledge exists on the interplay between plant defense responses and bacterial community structure. To address this gap, *A. thaliana* mutants impaired in ethylene (ET), jasmonic acid (JA) or salicylic acid (SA) homeostasis, representing major defense phytohormones (Bari and Jones, 2009), were used for a comparative study together with wild type plants (Doornbos et al., 2011). Within this work bacteria were isolated from rhizosphere compartments and bacterial communities were profiled using DGGE. A reduced number of culturable rhizosphere bacteria were isolated from the tested mutants, suggesting a role of phytohormones in defining community size. However, no significant changes in the taxonomic structure of rhizosphere communities were detected, indicating that altered defense outputs linked to phytohormone homeostasis do not interfere with community structure. To further investigate the role of plant immunity in bacterial community establishment, additional mutants with defects in defense pathways were analyzed using T-RFLP (Hein et al., 2008). These included the *constitutive expresser of PR genes 1* (*cpr1*) and *nonexpresser of PR genes 1* (*npr1*). *cpr1* plants constitutively produce SA leading to a constitutive expression of SA-inducible defense genes, whereas *npr1-1* plants are unable to transduce a SA-dependent signaling that activates SA-inducible gene expression. *npr1-1* plants displayed a reduced bacterial diversity compared to WT plants and clustered apart from WT and *cpr1* (Hein et al., 2008). These results suggest that SA-dependent defense responses alter the taxonomic structure of the bacterial root microbiota. However, a limitation of these studies is that they mostly rely on low-resolution fingerprinting techniques (DGGE and T-RFLP) and one cannot pinpoint taxonomic groups that are affected by the different mutant backgrounds. Additionally, plant immunity is less well understood in roots compared to shoots, therefore it is uncertain whether the here presented mutants are of general importance for structuring the root or rhizosphere microbiota (Millet et al., 2010). Thus, further work is needed to better understand the relationship between the plant immune system and the bacterial microbiota.

While we gain a better understanding of the structure of root-associated bacterial communities, less is known about the functional role of bacteria in a community context. Almost all well-described PGPR were studied for their effect on plant performance in simplified mono-associations but not in a defined bacterial community (Raupach and Kloepper, 1998; Lugtenberg and Kamilova, 2009). One example addressing this gap represents a metagenomic analysis of the root microbiota of rice (Sessitsch et al., 2012). A large number of bacterial genes involved in nitrogen metabolism and siderophore production, molecules involved in iron scavenging, were enriched in roots of rice. The enrichment and in part also active expression of these genes indicates a potential role of the root microbiota in increasing nutrient

availability. However, more work is needed to link bacteria in a community context with potential plant growth-promoting activities.

1.3. The arctic-alpine perennial *Arabis alpina*

Arctic-alpine plants are interesting model systems to study bacterial community structure, as plant growth in the arctic-alpine environment demands an adaption to a range of abiotic stresses, including water limitation, extreme temperature shifts and low nutrient availability (Billings and Mooney, 1968; Chapin, 1983; Chapin and Shaver, 1989; Margesin and Miteva, 2011). Associations with beneficial microbes might serve as one strategy for plants to cope with these harsh environmental conditions (Barka et al., 2006; Yang et al., 2009). The cloning of bacterial 16S rRNA gene fragments and isolation of endophytes from the three arctic-alpine plant species *Oxyria digyna*, *Diapensia lapponica* and *Juncus trifidus* identified diverse bacterial communities (Nissinen et al., 2012). Abundant taxonomic groups associated with roots of arctic-alpine plants in this study were Actinobacteria, Bacteroidetes and Proteobacteria. Additionally, a high number of isolates from these three plant species were effective solubilizers of mineral phosphorus, which represents a common form of phosphorus in arctic-alpine environments. This finding suggests that one function of bacterial communities associated with arctic-alpine plants is to solubilize otherwise inaccessible nutrients. However, to our knowledge there is no comparative study between plants inhabiting distinct habitats that would allow us to examine how microbial communities adapt to extreme environmental conditions.

Arabis alpina is a perennial plant species that inhabits a broad range of arctic-alpine environments and is found in central Europe, East Africa, Central Asia and Iceland. *A. alpina* typically inhabits sites that are moist, rich in gravel and stone and often located in glacier forelands (Koch et al., 2006). Thus, *A. alpina* grows in a distinctive habitat compared to the annual *A. thaliana* and represents a perennial plant, which flowers repeatedly and potentially thrives in the soil for several years. To induce flowering a vernalization treatment, i.e. a prolonged cold treatment, is required for most *A. alpina* accessions to switch to reproductive growth (Rohde and Bhalerao, 2007; Wang et al., 2009). After flowering, perennial plants return to vegetative growth for subsequent vernalization, while annual plants complete their lifecycle after flowering. One major difference between annual and perennial plants is that in annuals all meristems switch to flowering stage, whereas in perennials this is only the case for a subset of meristems (Albani and Coupland, 2010). The close evolutionary relationship (divergence time of approximately 45 million years) between the annual *A. thaliana* and perennial *A. alpina* have made these two species model systems to study the mechanisms of flowering control (Beilstein et al., 2010). The identification of the *A. alpina pep1* (*perpetual flowering 1*) mutant allowed to further dissect the flowering control mechanisms differentiating annual and perennial plants (Wang et al., 2009). Mutant *A. alpina pep1* plants flower repeatedly without the requirement of a vernalization treatment. *PEP1* represents an orthologue of the *A. thaliana FLOWERING LOCUS C* (*FLC*) that is repressed in

A. thaliana upon vernalization treatment and thereby induces flowering. Importantly, this gene is differentially regulated between annual and perennial plants. Within *A. thaliana FLC* expression is repressed after vernalization via histone modifications and this repression is maintained after return to normal growth temperatures. Also in *A. alpina PEP1* is repressed trough histone modifications, however, these modifications are removed upon return to normal growth temperatures. By these means the differential expression of *PEP1* allows *A. alpina* to repeatedly flower and represents an important determinant of the perennial lifestyle (Wang et al., 2009).

The adaptation of *A. alpina* to an extreme, arctic-alpine environment, its perennial nature, the availability of genetic lines, as well as its close evolutionary relationship to *A. thaliana*, make *A. alpina* an ideal model system to study factors important for the assembly of root-associated bacterial communities.

1.4. Techniques to study bacterial communities

1.4.1. Amplicon-based techniques

To understand the stability and biological relevance of bacterial communities, an in-depth examination of community structure is required. Information about community structure can be obtained by culture-dependent or -independent techniques, where either bacteria as organisms or their DNA within an environment are investigated. One advantage of culture-independent over -dependent techniques is that unculturable organisms can be investigated allowing to determine the diversity within bacterial communities (Davis et al., 2005; Kent and Triplett, 2002; Hugenholtz et al., 1998). This chapter will focus on culture-independent techniques, by introducing the most commonly used marker gene as well as conventional and next-generation sequencing techniques.

Most culture-independent approaches are based on the extraction of total DNA from an environment and subsequently the PCR-based amplification of a marker gene (Hirsch et al., 2010). Relevant properties of a marker gene are the universal presence within the group of organisms to study and the availability of non-variable regions for primer design as well as variable regions for phylogenetic assignment. For bacteria the 16S rRNA gene is widely used as a marker, although other genes, such as the protein-coding gene *rpoB* that encodes for the beta-subunit of the DNA polymerase, can be applied (Woese and Fox, 1977; Giovannoni et al., 1990; Vos et al., 2012).

The 16S rRNA gene encodes for the bacterial 30S small subunit of the ribosome that is required for protein synthesis in bacterial cells and therefore is indispensable for cell functioning. The sequence of the 16S rRNA gene consists of complementary as well as variable sequence stretches, forming a secondary structure of hairpins and loops (Noller and Woese, 1981). These complementary regions (the hairpin structure) are highly conserved, as they are essential for the formation of the secondary structure, whereas the variable regions (the loop structure) are diverse between bacterial species. Therefore, the complementary regions can be used for designing primers and the variable regions for phylogenetic

analysis and taxonomy assignment. Advantages of using the 16S rRNA gene for bacterial community profiling compared to other marker genes, such as *rpoB*, are the presence of this gene across the whole bacterial community and its functional indispensability. Additionally, the 16S rRNA is believed to be weakly affected by horizontal gene transfer (Jain et al., 1999). A disadvantage of the 16S rRNA gene is that bacterial species often carry more than one copy of this gene within their genome and that copy numbers vary between bacterial species and therefore enforce a bias to community diversity (Case et al., 2007). Additionally, for several bacterial species the high conservation of the 16S rRNA gene does not allow to differentiate bacteria on or below species level (Fox et al., 1992). Alternative marker genes are single-copy, protein-coding genes such as *rpoB*. The advantage of using protein-coding genes is that these genes are typically not as conserved, allowing for a more precise taxonomic resolution, often at intra-species level. However, the lower conservation brings the disadvantage that protein-coding genes are typically not conserved enough to be used as universal primers for a broad coverage of microbial diversity (Küpfer et al., 2006; Vos et al., 2012). Upon PCR-amplification of a marker gene, PCR-amplicon libraries are subjected to either molecular community profiling or next-generation sequencing (NGS) to determine the structure of bacterial communities.

Several, nowadays called conventional, molecular community profiling methods were mainly used before the rise of NGS. The most common techniques are denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA). These techniques separate amplicons based on sequence composition that leads to a different melting behavior, variable restriction sites or variable lengths of the intergenic spacer regions when using DGGE, T-RFLP and ARISA, respectively (Fischer and Lerman, 1983; Liu et al., 1997; Fisher and Triplett, 1999). The disadvantage of these techniques is the lack of a direct link to the actual gene sequence. This can be partially overcome by Sanger-sequencing of selected fragments of DGEE, T-RFLP or ARISA that were before separated by gel-electrophoresis. Nevertheless, this approach does not allow for a high-throughput sequencing of multiple environmental samples (Hirsch et al., 2010).

The rise of NGS allows now to generate millions of sequences, derived from multiple samples in parallel that are pooled in a sequencing library, i.e. multiplexed, overcoming the limitations of conventional sequencing techniques (Parameswaran et al., 2007). This is achieved by using barcoded primers during a PCR-based amplification step, permitting in parallel sequencing of many multiplexed samples. The most commonly used techniques are pyrosequencing (454) and Illumina sequencing (Knief, 2014). With the increase in read length for paired-end Illumina sequencing this technique comes with several advantages compared to 454. One major advantage is the higher sequencing depth of 25,000,000 reads for the Illumina MiSeq compared to the 1,000,000 reads per run for the 454 GS FLX+, resulting in reduced sequencing costs (Knief, 2014). However, both techniques induce a higher error rate compared to Sanger sequencing, potentially leading to an overestimation of diversity (Shendure and Ji, 2008). In both techniques biases are introduced by degradation of DNA, primer bias, PCR- and

sequencing errors (Coissac et al., 2012; Engelbrektson et al., 2010; Patin et al., 2013). Of note, 454 and Illumina are biased by different types of errors. While 454 does not sufficiently dissect homopolymeric stretches of the template DNA, Illumina is biased by a higher number of substitutions (Shokralla et al., 2012; Knief, 2014).

After sequencing, the obtained reads are de-multiplexed, i.e. assigned to individual samples that were pooled in the sequencing library, and filtered for high quality sequences. Subsequently, all resulting high-quality sequences are typically clustered at 97% sequence similarity, thereby defining operational taxonomic units (OTUs; Caporaso et al., 2010). OTUs of the 16S rRNA gene sequence reflect a taxonomic assignment at species rank (Schloss and Handelsman, 2005). For taxonomy assignment a reference sequence is selected within the sequences clustered at 97% sequence similarity. Often the most abundant sequence in an OTU is chosen and used for taxonomy assignment. The enumeration of sequences in an OTU sequence cluster is then used to quantify the relative abundance of an OTU in the whole sequence pool (Caporaso et al., 2010).

1.4.2. Techniques to dissect functional capacities of bacterial communities

Amplicon-based techniques are instrumental to determine community structure, but these techniques give little information about the functional abilities encoded within bacterial communities. Additional methods that allow to overcome this limitation are so-called “omic”-techniques, including metagenomics, metatranscriptomics and metaproteomics.

Metagenomics uses random sequencing of DNA molecules via shotgun sequencing and analyzes the total pool of genomic DNA in environmental samples. This method allows to determine the structure of microbial communities based on taxonomically informative sequences, such as the 16S rRNA gene, and more importantly permits the determination of functional genes present in a sample. Shotgun-sequencing does not require PCR-based amplification steps and is not limited to a certain domain of life and therefore allows for an unbiased profiling of community composition giving insights into so far uncultivable species (Shakya et al., 2013b). However, compared to 16S rRNA gene-based techniques, metagenomics entails a lower coverage of sequences and this results in an underrepresentation of low abundant taxa (Ni et al., 2013). Additionally, the mere presence of a gene does not necessarily reflect active expression of this gene and another major challenge within this technique is the sequence assembly (Guttman et al., 2014).

The sequencing of RNA translated into cDNA, i.e. metatranscriptomics, monitors actively expressed genes in environmental samples and therefore provides insights into active processes in an environment. Even closer at the actual function of bacterial communities, metaproteomics can be applied to investigate proteins in natural communities. The application of all methods confronts one major difficulty when examining root communities. Root samples will contain most of DNA, RNA and proteins from the host plant. Therefore, a much higher number of sequences has to be generated to gather information about the bacterial community. A similar bottleneck applies to transcript sequencing, as metatranscriptomic

data contain a high number of ribosomal RNA reads (He et al., 2010; Turner et al., 2013). While nucleotide databases contain comprehensive collections of sequence information, the database references for metaproteomics are less well developed, and therefore this technique is often coupled with metagenomics, i.e. metaproteogenomics (Liu et al., 2012).

1.5. Thesis aim

With the advances in the past few years investigating bacterial communities of plants, we begin to understand the factors that influence community structure. However, we still lack insights about the stability and functional abilities of these communities. To address these gaps, I aim to further explore factors influencing community structure to evaluate the stability of the bacterial microbiota and to understand how bacteria associated with *A. thaliana* and related Brassicaceae species affect plant growth.

For the first aim and to address the stability of communities, I will compare the bacterial microbiota of the annual plant species *A. thaliana* and *C. hirsuta* and their perennial sister species *A. alpina.* This comparison allows to dissect the community structure on distinct host species that belong to the Brassicaceae family, spanning a divergence time of ~45 million years. Second, *A. alpina* will be used as a further model system to evaluate the stability of bacterial communities over time by growing plants for a prolonged time period under stable environmental conditions. Third, I will test whether the bacterial community composition is altered when *A. alpina* resides in different developmental stages and finally, whether similar communities assemble under natural growth conditions. This experimental system will allow to test the following hypotheses:

1) Bacterial communities of *A. thaliana*, *C. hirsuta* and *A. alpina* differ between each other. I hypothesize that *A. alpina* displays the most distinct bacterial communities, due to its distinct lifestyle compared to *A. thaliana* and *C. hirsuta.*
2) Bacterial communities are affected by time and plant developmental stage.
3) Bacterial communities differ when plants are grown under stable, environmental conditions in the greenhouse compared to variable, natural conditions.

For the second aim that tries to unravel the effect of the bacterial microbiota on plant growth, I will use a culture-dependent approach. Therefore, I will isolate bacteria from roots of *A. thaliana* and the related Brassicaceae species and subsequently test a subset of those bacterial isolates for their effects on plant growth. More specifically, bacteria will be inoculated alone or in synthetic communities to the host plant, which will be exposed to nutrient-sufficient and -deficient conditions. These two growth conditions were chosen to examine the potential plant growth-promoting abilities of the bacterial isolates under abiotic stress conditions. I will focus on root-associated bacteria that are shared between *A. thaliana*, *C. hirsuta* and *A. alpina*. These experiments will be conducted by inoculating bacterial isolates on all three plant species. I hypothesize that bacteria that are recruited by all host species, provide important functions to all host plants and that a subset of the tested bacteria represent PGPR.

2. Results

2.1. The structure of the bacterial root microbiota of *A. thaliana* and its relatives

Recent work demonstrated that plants host a distinct root-inhabiting bacterial microbiota compared to the surrounding soil biome (Lundberg et al., 2012; Bulgarelli et al., 2012; Shakya et al., 2013a; Schlaeppi et al., 2014; Edwards et al., 2015). Therefore, we begin to understand the factors that influence community structure, but little is known about the variation of these communities across different environments. This study addresses the impact of host species, residence time of plants in the soil, plant developmental stage as well as growth of plants in natural conditions on community structure. Therefore, I designed three independent sets of experiments that are referred to as (i) Brassicaceae comparison, (ii) Time course experiment and (iii) Natural site experiment (Tab. 1).

Tab. 1: Experimental design and numbers of replicates per DNA sample. Experimental setup comparing bacterial community composition on different host plants (Brassicaceae comparison), residence time of plants in the soil and plant development (Time course) and natural growth conditions (Natural site). The first two columns include the sample type, host plant and plant genotype. The numbers indicate the number of DNA samples collected from the indicated samples (for the number of plants included per DNA sample see Methods section Chapter I.4.2.3 and I.4.2.4). Brassicaceae comparison: Three batches of Cologne agricultural soil (CAS) were utilized. Time course experiment: Samples were collected at three time points (t1: 6 weeks, t2: 3 months and t3: 7 months) and the combined number of samples collected from two different soil batches (CAS8 and CAS9) is indicated. Natural site experiment: Plants were grown in CAS8 in the greenhouse (CAS_GH), French soil in the greenhouse (FS_GH) and French soil at a natural site in the French Alps (FS_NS).

		Brassicaceae comparison			**Time course**			**Natural site**		
Sample	Host plant	CAS5	CAS8	CAS9	t1	t2	t3	CAS_GH	FS_GH	FS_NS
Soil	-	3	3	3	6	6	6	4	4	3
Root	*A. thaliana* (Col-0)	3	3	3	-	-	-	-	-	-
	C. hirsuta (Ox)	3	3	3	-	-	-	-	-	-
	A. alpina (Paj)	3	3	3	6	8	8	4	5	-
	A. alpina (*pep1*)	-	-	-	6	8	8	-	-	-
	A. alpina (Gal5)	-	-	-	-	-	-	-	5	-
	A. alpina (Gal60)	-	-	-	-	-	-	-	5	5
Rhizosphere	*A. alpina* (Paj)	-	-	-	6	8	8	4	5	-
	A. alpina (*pep1*)	-	-	-	6	8	8	-	-	-
	A. alpina (Gal5)	-	-	-	-	-	-	-	5	-
	A. alpina (Gal60)	-	-	-	-	-	-	-	5	5

To address the impact of host species on community structure, the following three plant species belonging to the Brassicaceae family were chosen: *A. thaliana*, *C. hirsuta* and *A. alpina* (Fig. 2A). *A. thaliana* diverged from *C. hirsuta* ~35 and from *A. alpina* ~45 million years ago (Fig. 2B; Beilstein et al., 2010). *A. thaliana* and *C. hirsuta* are annual and cosmopolitan plants that often reside in the same habitat. Both species can easily be differentiated by their distinctive anatomy, as seen by their characteristic leaf and root geometries (Fig. 2A; Hay et al., 2014). *A. alpina* on the other hand is a

perennial plant with a more restricted growth range compared to the other two plant species, as it inhabits arctic-alpine environments, such as the French Alps (Koch et al., 2006).

These three plant species were grown under controlled environmental conditions in the greenhouse for 6 weeks to examine, whether their phylogenetic divergence correlates with the divergence of their microbiota. In total, three independent biological replicate experiments were conducted using three independent batches of a natural, microbe-rich soil, referred to as Cologne agricultural soil (CAS). The experimental setup included the three soil batches CAS5, CAS8 and CAS9 (Tab. 1 and Tab. 2). The two batches CAS5 and CAS9 were collected in fall 2010 and 2013, respectively and the batch CAS8 was prepared in spring 2013. In each independent biological replicate, four plants were grown in one pot and defined segments of roots collected from three to four pots were pooled for the subsequent harvesting procedure. Triplicate samples of these root samples were harvested from each independent biological repetition, washed twice and sonicated to deplete surface-associated bacteria and enrich for root-associated bacteria and potential endophytes (see method section Chapter I.4.2.5 for details). Additionally, bulk soil from unplanted pots was collected to gain information about the start inoculum.

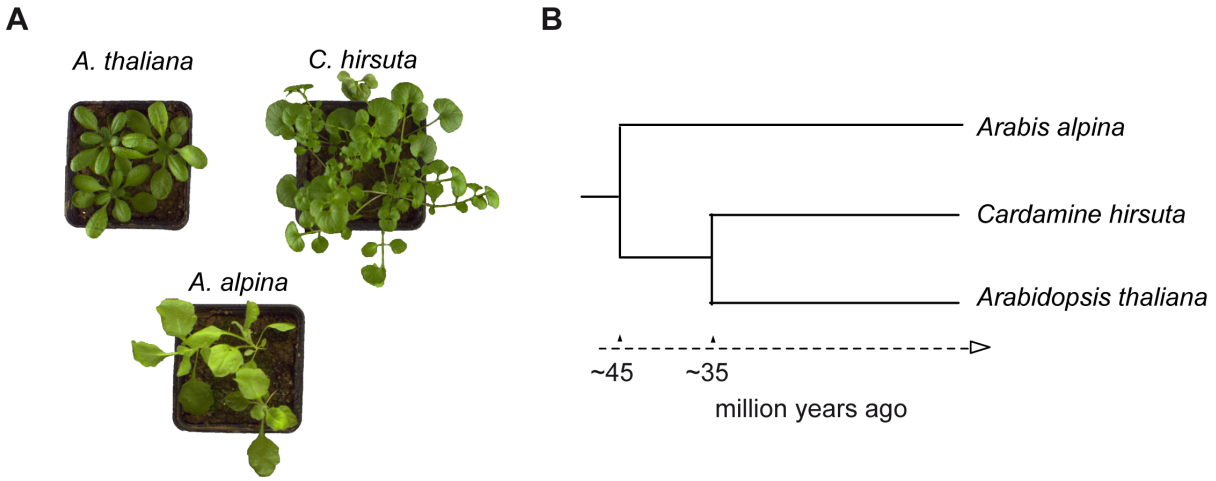


Fig. 2: Plant species utilized in this study. (A) *A. thaliana*, *C. hirsuta* and *A. alpina* grown in a natural soil for 6 weeks. **(B)** Evolutionary relatedness of tested plant species. Divergence time estimates indicated at the bottom, based on the NADH dehydrogenase subunit F and phytochrome A sequences (modified from Beilstein et al., 2010)

To validate that the sonication treatment removed soil particles and bacteria from the root surface, sonicated and non-sonicated root samples were prepared for scanning electron microscopy (SEM). This was a necessary control, as the root architecture of the three species differs. *C. hirsuta* displays a more branched and dense root system architecture compared to the other two species and insufficient removal of soil particles could result in an overrepresentation of soil- or rhizosphere-associated bacteria within the root microbiota. To examine this, two root systems with and without sonication treatment were prepared of each host species (*A. thaliana*, *C. hirsuta* and *A. alpina*) for SEM. The effectiveness of sonication was evaluated from 10 different pictures from each root specimen (Fig. 3).

The epidermis of non-sonicated roots was in ~50% of cases intact, while a partial destruction was observed in all other cases, independent of the tested plant species. The partial damage of the epidermis was possibly caused by the harvest of roots from the soil and the transfer of roots during the washing steps. The sonication treatment disrupted the root epidermis of *A. thaliana* and *A. alpina* more efficiently (~50-90%, respectively) compared to the partially removed epidermis of *C. hirsuta* (Fig. 3B). This observation needs to be considered during the subsequent analysis of bacterial communities, as insufficient removal of soil- and rhizosphere-associated bacteria could affect the root microbiota composition of *C. hirsuta*.

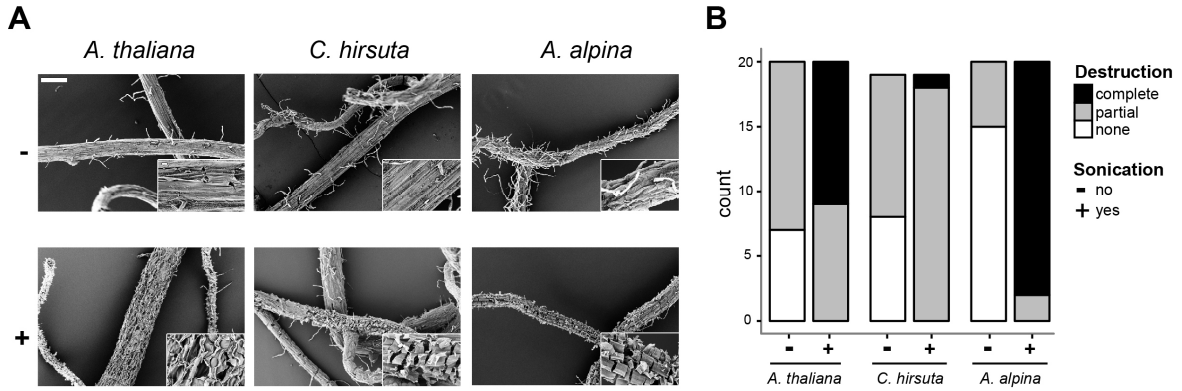


Fig. 3: Sonication treatment varies in effectiveness depending on the tested plant species. (A) Representative pictures of roots from *A. thaliana*, *C. hirsuta* and *A. alpina* with (+) and without sonication (-) treatment using scanning electron microscopy (SEM). Bar within the main picture = 100 µM. Right, lower panel of main picture = 10x magnification. **(B)** Quantification of roots with (+) and without (-) sonication treatment. Two individual roots and 10 pictures were analyzed from each root, species and treatment. Destruction levels were quantified by scoring roots for no (white), partial (grey) or complete (black) destruction. Count indicates the number of scored root systems fulfilling one of the three destruction criteria.

After harvesting soil and root samples, DNA was isolated and used as a template for a PCR-based 16S rRNA gene profiling of the bacterial community structure. A PCR was conducted by targeting the V5-V7 region of the bacterial 16S rRNA gene using the primers 799F and 1193R (Chelius and Triplett, 2001; Bodenhausen et al., 2013). PCR-amplicon libraries were sequenced using 454 pyrosequencing and the taxonomic identity and relative abundance of bacteria in the root microbiota was determined (see Methods Chapter I.4.2.8 for details).

For the 36 samples of the three tested Brassicaceae species a total of 445,354 high quality reads were generated (median of 12,370 and range of 3,544-33,643 sequences per sample). High quality reads were obtained by removing sequences shorter than 315 base pairs, a Phred score below 25 as well as chimeras and plant assigned sequences. Afterwards, OTUs were defined by clustering sequences > 97% identity using the software "*quantitative insights into microbial ecology*" (QIIME; Caporaso et al., 2010). By this means a cluster of sequences, also known as OTUs, was calculated and dependent on the region sequenced and sequence length an OTU typically represents a bacterial species (Schloss and Handelsman, 2005). The number of reads per OTU were expressed as the relative abundance of an OTU

among all sequences in a sample and OTUs were taxonomically assigned using the Ribosomal Database Project II (RDPII; Caporaso et al., 2010; Wang et al., 2007). To focus on abundant and reproducible OTUs, only OTUs with a minimum of 20 sequences in at least one sample (i.e. thresholded) were kept and all lower count OTUs removed (Schlaeppi et al., 2014). The resulting thresholded dataset will from now on be referred to as abundant community members (ACM) and the un-thresholded dataset as threshold-independent community (TIC). Within the ACM the read counts per OTU were normalized by sample size and $\log_2$-transformed values were used for the subsequent statistical analysis unless stated otherwise (Bulgarelli et al., 2012; Schlaeppi et al., 2014). The ACM consisted of 277,773 sequences (median of 7,715 and range of 2,201-26,621 sequences per sample). The TIC contained 70,005 and the ACM 443 unique OTUs that were all assigned to the kingdom Bacteria and no OTU of the kingdom Archaea was detected.

To investigate the within-sample diversity, i.e. alpha-diversity, within soil and root samples, the number of identified OTUs and the Shannon index were calculated on the TIC rarefied to 3,500 sequences (Fig. 4). The number of observed OTUs represents the number of OTUs within a sample and is also called OTU richness, which does not take into account the abundance of OTUs. However, this abundance is considered with the Shannon index. In soil samples more than twice as many OTUs were detected compared to the root compartment and samples from the three soil batches were not distinguishable. Unplanted soil displayed a significantly higher Shannon index compared to the root samples, whereas roots samples revealed no significant differences in the number of observed OTUs and Shannon index among the tested plant species. These results demonstrate that the bacterial root microbiota is less diverse compared to bacterial communities in unplanted soil and that the bacterial communities between plant species do not differ in richness and Shannon diversity.

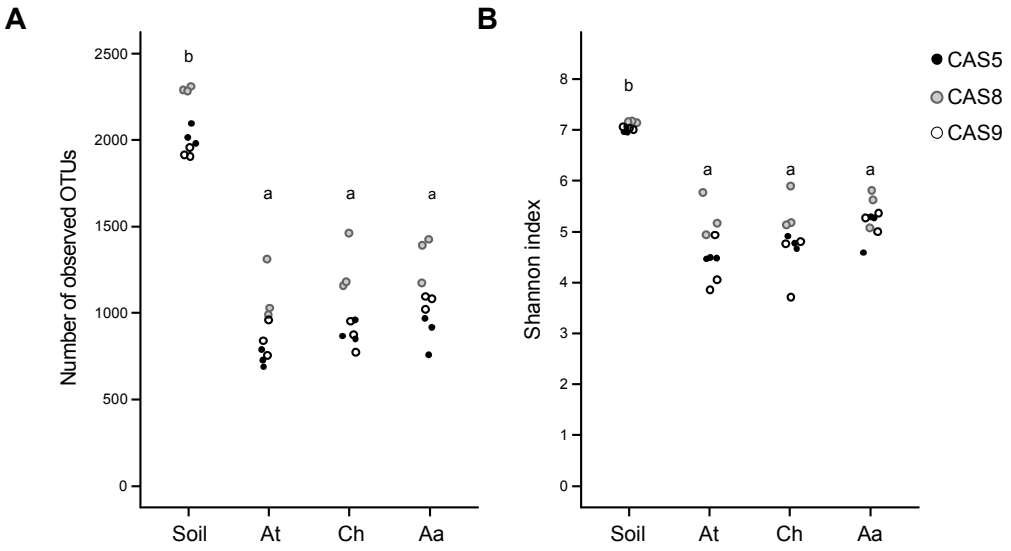


Fig. 4: The bacterial root microbiota has a lower alpha-diversity compared to unplanted soil. Alpha-diversity of bacterial communities within unplanted soil and root compartments of *A. thaliana* (At) *C. hirsuta* (Ch) and *A. alpina* (Aa) based on the threshold-independent community (TIC) rarefied to 3,500 sequences. Alpha-diversity analysis based on the number of observed OTUs **(A)** and Shannon index **(B)** for each of the three replicate experiments. Significance values based on $p < 0.05$ (Tukey's HSD).

As a first approximation of the variation in the dataset an unconstrained Principal Component Analysis (PCA) was conducted. Within a PCA samples are clustered based on the two components explaining most of the variation in the dataset and therefore this represents a good measure to determine factors that strongly influence community structure. I compared the 16S rRNA gene datasets of all three Brassicaceae species in an unconstrained PCA, where the first principal component explained 41.1% of the observed variation and largely separated the samples based on the tested compartments (Fig. 5). Root samples of the three tested plant species clustered together and distant from the soil samples. The second axis of the PCA explained 12.3% of the observed variation and separated the three soil batches. The soil batches did not cluster based on the season of harvest, as the spring batch of March 2012 (CAS8) was not more distant than the two fall batches CAS5 and CAS9 (from September 2010 and 2013, respectively). The soil batch clustered according to the point of harvest, as CAS5 and CAS9 were collected in 2010 and 2013, whereas CAS8 and CAS9 were collected in March and September of 2013. Additionally, technical replicates within the independent biological experiments clustered closely together, verifying that sample preparation introduced no strong biases. It can be concluded that the tested compartments, followed by the used soil batches, represent the major determinants of community structure, whereas variation between technical replicates and plant species play only a minor role.

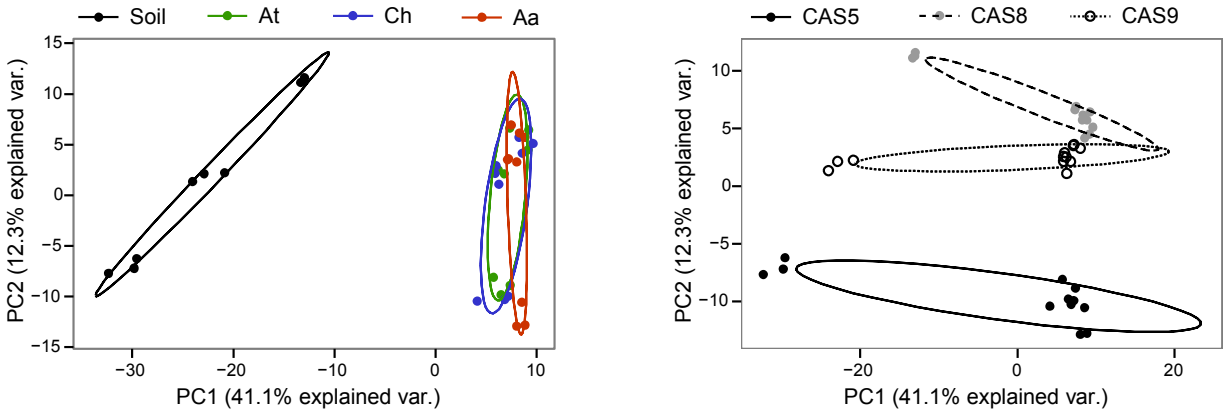


Fig. 5: Compartment and soil batch strongly influence bacterial community structure. Principal component analysis (PCA) on abundant community members (ACM) present in unplanted soil and root compartments of the three Brassicaceae plant species. The left panel highlights the separation between soil and root compartments (separated for root samples derived from *A. thaliana* (At), *C. hirsuta* (Ch) and *A. alpina* (Aa)). The right panel separates samples grown in different seasonal soil batches (CAS5, CAS8 and CAS9). The ellipse covers 69% of samples belonging to the indicated class.

In a second step of the 16S rRNA gene community analysis, the taxonomic composition of bacterial communities within the soil and root compartments was analyzed at the phylum and family rank. The most abundant phyla enriched in root compartments were Proteobacteria, Actinobacteria and Bacteroidetes (Fig. 6A). Unplanted soil displayed a distinct community structure compared to root samples as seen by a lower number of Bacteroidetes and higher relative abundance of Firmicutes and Acidobacteria. No significant differences between the three plant species were detected at the phylum rank.

At family rank a more differentiated pattern was detected for the root compared to the soil compartment (Fig. 6B). Flavobacteriaceae was the only family belonging to the Bacteroidetes and for the phylum Actinobacteria, the families Streptomycetaceae and Thermomonosporaceae showed the highest relative abundances within the root compartments. Proteobacteria were represented by numerous families enriched within the root compared to the soil compartments, such as Oxalobacteriaceae, Pseudomonadaceae and Comamonadaceae. The three plant species were differentiated by a few quantitative differences, while qualitative differences were not detected. Comamonadaceae, Burkholderiales incertae sedis and Phyllobacteriaceae were enriched in roots of *A. alpina*, Nannocystaceae depleted in roots of *C. hirsuta* and Thermomonosporaceae enriched in roots of *A. thaliana*. Importantly, *C. hirsuta* roots did not harbor a greater proportion of soil-associated bacteria compared to the roots of the two other plant species. This indicates that even though the sonication treatment was not as effective for *C. hirsuta*, no obvious biases on community structure were generated (Fig. 3).

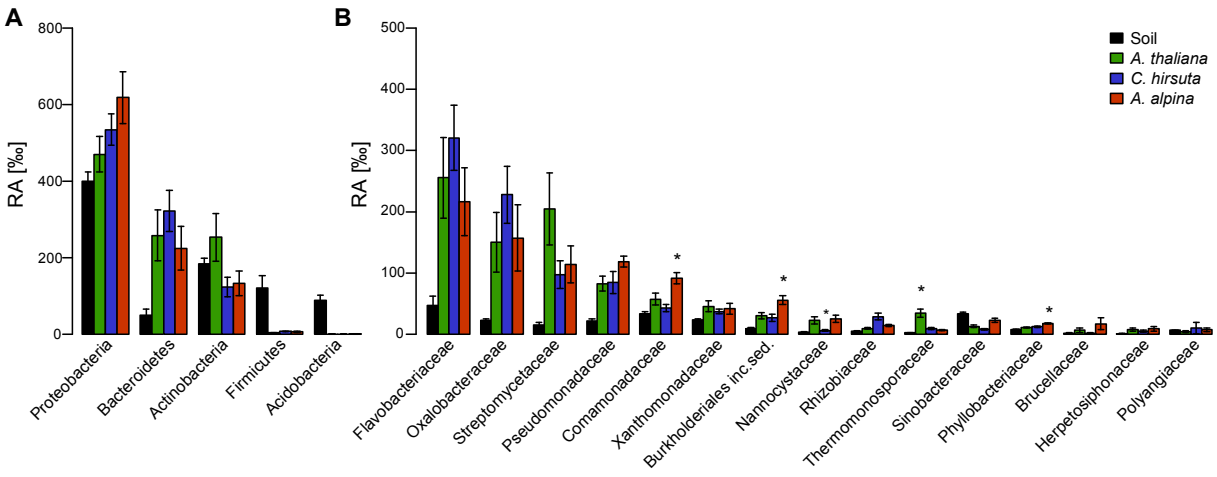
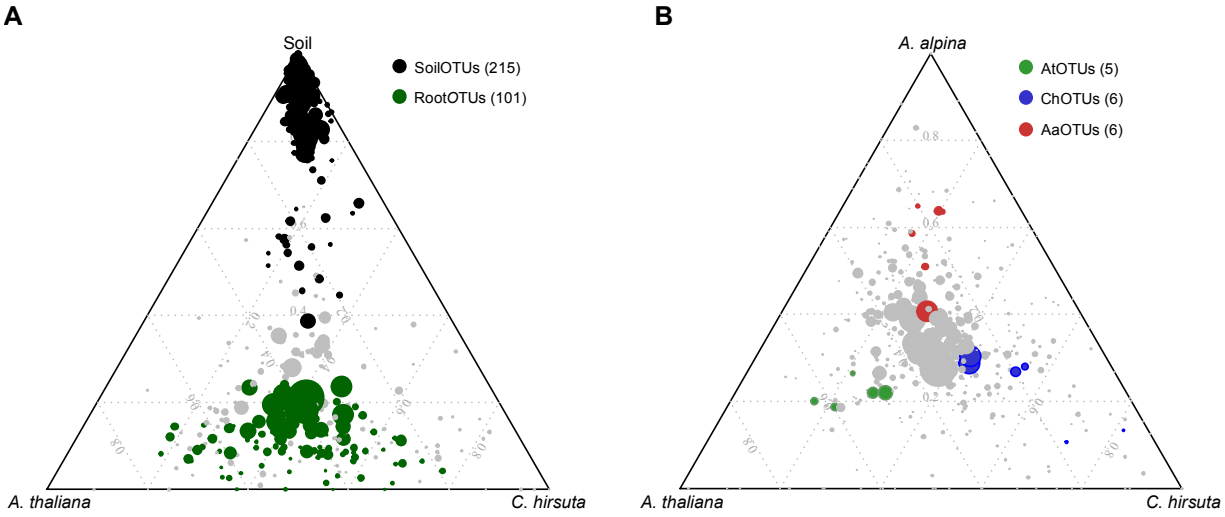


Fig. 6: Bacterial root communities display a distinct taxonomic composition compared to unplanted soil and few quantitative differences distinguish the three plant species. (A) Phylum and **(B)** family distribution of the abundant community members (ACM). Distribution depicted as relative abundance (RA) in per mile. Color-coding: Soil (black), *A. thaliana* (green), *C. hirsuta* (blue) and *A. alpina* (red). The bar graph is depicting the mean and error bars represent the standard error of the mean. Asterisk: Significant difference between *A. thaliana*, *C. hirsuta* or *A. alpina* root communities (Tukey's HSD, $p < 0.1$, FDR-corrected). Depicted are the 5 most abundant phyla and 15 most abundant families.

Next, OTUs were determined that were responsible for the clear community separation based on the tested compartment (Fig. 5). OTUs that were significantly enriched within the soil or root compartments were calculated and termed SoilOTUs and RootOTUs, respectively. Therefore, a Bayes moderated t-test was performed on the relative abundance values of the $\log_2$-transformed ACM dataset (see Chapter I.4.2.10.4 for details; based on Bulgarelli et al., 2012 and Smyth, 2005). Of the 443 observed OTUs within the ACM, 215 SoilOTUs and 101 RootOTUs were identified, while the remaining OTUs did not differ between the two compartments (Fig. 7A). These SoilOTUs and RootOTUs displayed a distinctive taxonomic structure, with a higher number of OTUs assigned to Proteobacteria and Bacteroidetes in the RootOTUs, while Actinobacteria, Firmicutes and Acidobacteria were enriched in the SoilOTUs. Overall,

this indicates that the root compartment enriches a subset of bacteria from the bacterial start inoculum present in unplanted soil.



%	SoilOTUs	RootOTUs
Proteobacteria	36.3	76.2
Actinobacteria	27.0	13.9
Bacteroidetes	0.9	7.9
Firmicutes	15.3	0
Acidobacteria	10.2	0

%	AtOTUs	ChOTUs	AaOTUs
Proteobacteria	20	100	100
Actinobacteria	80	0	0

Fig. 7: A root-enriched bacterial community can be defined and distinct plant species share most OTUs. OTUs enriched in unplanted soil and root compartments of *A. thaliana* (At), *C. hirsuta* (Ch) and *A. alpina* (Aa). Each circle depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position of each circle is determined by the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. **(A)** Root- and soil-enriched OTUs (RootOTUs and SoilOTUs), including OTUs enriched on all three plant species and OTUs specific for individual species. For simplicity only *A. thaliana* and *C. hirsuta* are depicted, but the RootOTUs also include RootOTUs enriched on *A. alpina*. **(B)** OTUs enriched on At, Ch or Aa. All enriched OTUs are based on a Bayes moderated t-test, $p < 0.05$ (FDR-corrected). Number in brackets: Total number of OTUs enriched in the respective compartment or plant species. Table below ternary plot: Taxonomic distribution (phylum rank) of compartment- or species-enriched OTUs in percent (%). For (A) only the 5 most abundant phyla are listed.

Subsequently, the three tested plant species were analyzed for similarities and differences in bacterial community composition and RootOTUs enriched on *A. thaliana*, *C. hirsuta* and *A. alpina* were determined (termed AtOTUs, ChOTUs and AaOTUs, respectively; Fig. 7B). Most OTUs could not be differentiated between the three Brassicaceae species and were located in the center of the ternary plot. Approximately 1% of the total number of observed OTUs were specifically enriched in one of the three species. These species-specific OTUs exhibited only quantitative differences, as indicated by the clustering towards the center of the ternary plot. Of note, the species-enriched OTUs followed a certain taxonomic pattern as *A. thaliana* roots were only enriched for members of the phylum Actinobacteria (family Thermomonasporaceae), whereas *C. hirsuta* and *A. alpina* enriched for diverse Proteobacteria families (Fig. 7, bottom of the plot). Especially the enrichment of Thermomonosporaceae by *A. thaliana*

correlated well with the observation that this family was enriched in roots of *A. thaliana* (Fig. 6B). In summary, these results imply that the tested plant species only weakly affect bacterial community structure and a few community members discriminate the three Brassicaceae host species.

Another OTU-based analysis aimed to identify OTUs that were enriched in all plants species and shared among the three soil batches. This putative 'shared microbiota' could potentially colonize a wide range of plants and might be relevant for overall plant performance (Shade and Handelsman, 2012). To determine the existence of a shared bacterial community, I tested for OTUs that were enriched on roots of all three plant species and in all replicated experiments using non-parametric and parametric statistics. The latter is important as not all taxonomic groups within this study followed a normal distribution (data not shown). In total 26 OTUs (designated sharedOTUs) fulfilled the above mentioned criteria and were significantly enriched in roots of all three plant species and depleted in the corresponding soil compartments as validated by Tukey's HSD, Bayesian and Mann-Whitney statistics (Fig. 8A). These sharedOTUs were represented by three bacterial phyla, with most OTUs belonging to Proteobacteria and fewer OTUs to Actinobacteria and Bacteroidetes. Proteobacteria included the families Oxalobacteriaceae, Pseudomonadaceae, Burkholderiales incertae sedis, Comamonadaceae, Xanthomonadaceae, Cystobacteriaceae and Phyllobacteriaceae. Actinobacteria and Bacteroidetes were represented by Streptomycetaceae and Thermomonosporaceae as well as Flavobacteriaceae, respectively (Fig. 8B). The 26 sharedOTUs were almost absent or only present at a low relative abundance in unplanted soil and represented in abundance the major part of the root compartment, reaching an average relative abundance of ~500 per mile (Fig. 8C). The sharedOTUs represented the most abundant OTUs in the dataset and considerable variation between different experiments was noted, as Bacteroidetes were largely absent in the root compartment of *A. alpina* grown in CAS5 compared to the other soil batches. This suggests that bacteria, especially members of the phylum Bacteroidetes, are influenced by environmental perturbations.

In conclusion for this experimental setup, the tested compartments and soil batches were the major factors influencing community structure of the bacterial root microbiota of the three tested Brassicaceae plant species. These three plant species largely share a similarly structured bacterial root microbiota and are only distinguishable at OTU or family level by a few quantitative differences. This shared bacterial community consists of a relatively simple taxonomic structure. Proteobacteria include the families Oxalobacteriaceae, Pseudomonadaceae, Burkholderiales incertae sedis, Comamonadaceae, Xanthomonadaceae, Cystobacteriaceae and Phyllobacteriaceae, while Actinobacteria and Bacteroidetes are represented by Streptomycetaceae and Thermomonosporaceae as well as Flavobacteriaceae, respectively.

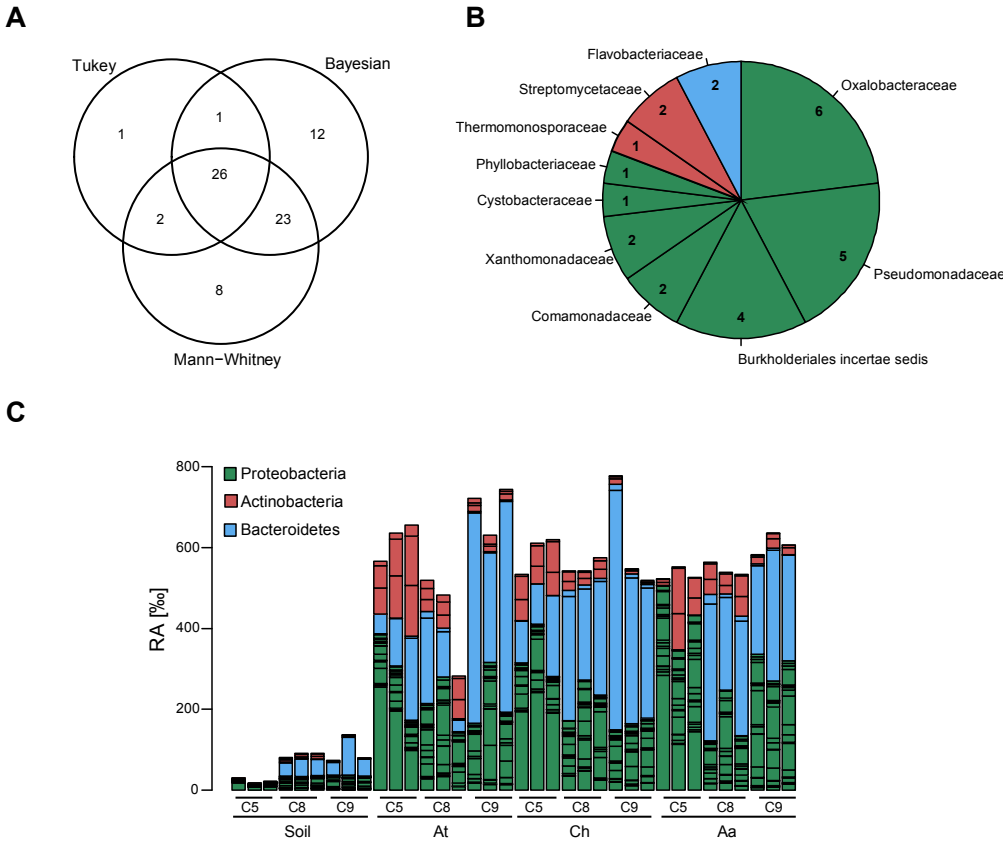


Fig. 8: A shared bacterial root microbiota assembles on three related Brassicaceae plant species. (A) Venn diagram depicting the total number of RootOTUs shared between *A. thaliana*, *C. hirsuta* and *A. alpina* based on parametric Tukey's HSD and non-parametric Bayesian and Mann-Whitney statistics. A shared bacterial community of 26 OTUs was defined (sharedOTUs). **(B)** Pie chart reporting family distribution of the 26 sharedOTUs. **(C)** Stacked relative abundance (RA) in per mile of sharedOTUs. Each segment corresponds to one of the sharedOTUs. Color-coding based on phylum distribution: Proteobacteria (green), Actinobacteria (red) and Bacteroidetes (blue).

2.2. Comparing 454 and Illumina sequencing platforms

With the development of a new Illumina sequencing protocol in our laboratory, all subsequent samples were prepared with this new protocol. The Illumina protocol allows to accommodate a higher sample number and ensures an increased sequencing depth compared to 454 pyrosequencing, providing an improved resolution for low abundance OTUs. Especially the latter is desirable, as most studies using 454 pyrosequencing failed to reach a plateau in cumulative sequencing OTU diversity, potentially underestimating the bacterial diversity present in the soil and root microbiota (Bulgarelli et al., 2012; Schlaeppi et al., 2014).

To compare the Illumina with the previous pyrosequencing pipeline, DNA from soil and root samples of the tested Brassicaceae plant species grown in CAS9 was processed with the Illumina protocol (Tab. 1) and compared to results obtained before with 454 pyrosequencing. Amplicon libraries for the Illumina pipeline were prepared in a similar manner compared to samples sequenced by pyrosequencing and afterwards sequenced with paired-end Illumina sequencing. Briefly, the V5-V7 region of the 16S rRNA gene was targeted with the primers 799F and 1193R using a modified PCR-amplification step that allows to efficiently amplify sequences with the longer primers required for the Illumina protocol (Chapter

I.4.2.8 and I.4.2.9). After sequencing, paired-end reads were assembled and all non-overlapping reads were discarded to maintain sequences with a similar fragment size. Afterwards, samples processed with pyrosequencing and Illumina were merged and an OTU table was generated using QIIME. After normalizing by sample size, thresholding and $\log_2$-transformation 44,192 and 527,649 reads were maintained among the 11 samples processed with pyrosequencing or Illumina, with a minimum number of sequences of 1,437 and 22,793 reads per sample, respectively. A total of 116 and 1,137 OTUs were observed with 454 and Illumina sequencing technologies, respectively.

Samples from the soil or root compartments revealed a high correlation towards each other based on a Spearman´s rank-correlation analysis, whereas soil samples displayed a lower correlation to root samples and vice versa. The latter was expected because of differences in bacterial community structure between the root and soil compartments (Fig. 9A, Fig. 6). Of note, comparing either soil or root samples processed with the same sequencing platform revealed a higher correlation compared to samples processed with a different sequencing platform. ~75% of the 116 OTUs detected with pyrosequencing were also recovered with Illumina (Fig. 9B). The inability to detect a subset of OTUs could be explained by biases introduced by the PCR-amplification step or the fact that pyrosequencing and Illumina are biased by different sequencing errors. Overall, the high overlap between pyrosequencing and Illumina indicates a good comparability between the two techniques.

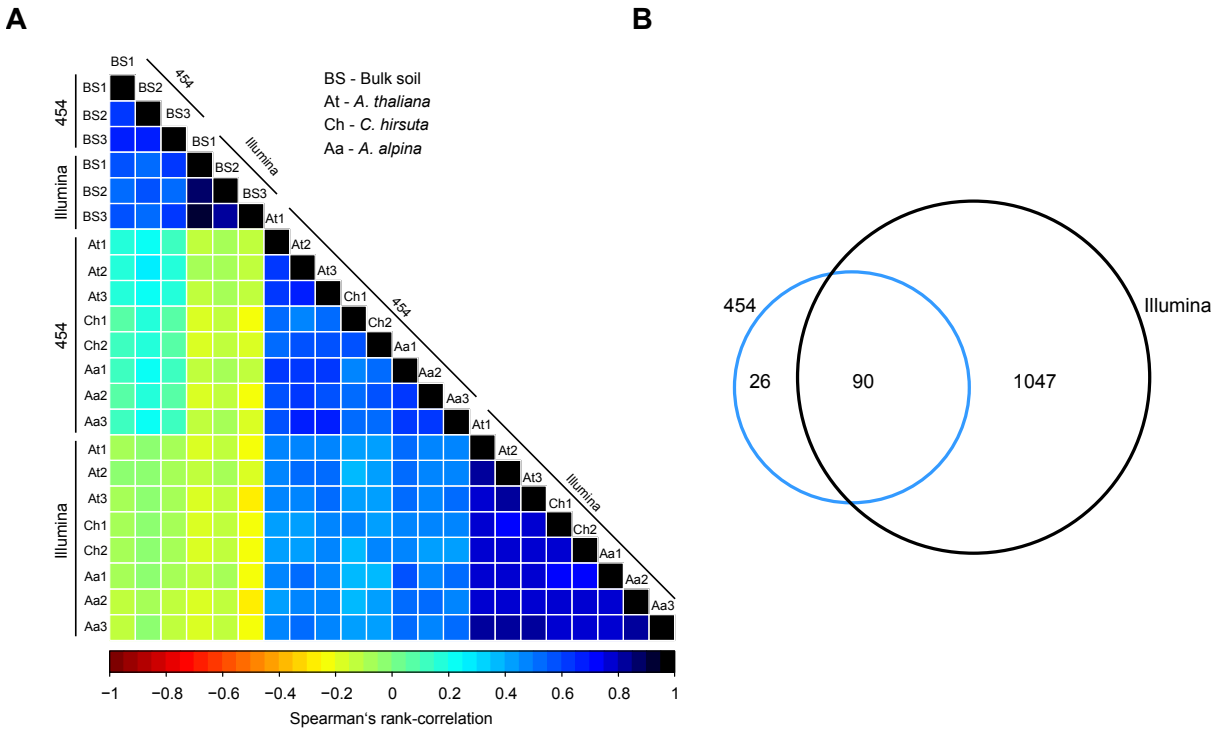


Fig. 9: 454 pyrosequencing and Illumina protocols generate comparable results. (A) Spearman's rank-correlation of sequencing data generated from bulk soil (BS) and root compartments by pyrosequencing (454) or Illumina sequencing. Root samples were isolated from the three plant species *A. thaliana* (At), *C. hirsuta* (Ch) and *A. alpina* (Aa). From each sample 2-3 independent DNA samples were sequenced (1-3). **(B)** Total number of OTUs detected by the 454 and Illumina protocol and their respective overlap. Comparison is based on the thresholded and normalized dataset.

2.3. The bacterial microbiota of perennial *A. alpina*

2.3.1. Influence of time and development on the bacterial microbiota of *A. alpina*

A time course experiment was conducted to investigate the relevance of prolonged residence time of plants in the soil, as well as plant developmental stage, and its impact on bacterial community structure of *A. alpina*. Additionally, I examined whether *A. alpina* affected rhizosphere communities. The so far investigated Brassicaceae plant species *A. thaliana* and *C. hirsuta* display only a weak or even no rhizosphere effect (Bulgarelli et al., 2012; Schlaeppi et al., 2014). The absence of a marked rhizosphere effect contrasts with observations for other plant species such as oat, wheat and pea (Turner et al., 2013). Therefore, I investigated whether the absence of a rhizosphere effect is a general feature of Brassicaceae species or whether annual plant species do not strongly affect rhizosphere communities due to their shorter survival time within the soil. Therefore, *A. alpina* was grown for a prolonged time period in the greenhouse and samples were collected at three time points over a time period of 7 months, allowing to address the presence of a rhizosphere effect as well as the stability of soil, rhizosphere and root communities over time.

Apart from the importance of residence time of plants in the soil on community structure, I also tested whether plants residing in different developmental stages display changes in bacterial community profiles. It was shown before that non-flowering *A. thaliana* secretes dissimilar root exudates compared to flowering plants and exudates are thought to be important for structuring bacterial communities (De-la -Peña et al., 2010; Badri et al., 2009). Nevertheless, recent studies on *A. thaliana* suggest that community structure is largely similar at vegetative, flowering and senescence stage (Chaparro et al., 2014; Lundberg et al., 2012). However, one limitation of these studies is the fact that plants residing in different developmental stages were not collected at the same time point. Therefore, these studies do not allow to disentangle the influence of time and plant developmental stage. Additionally, the short lifetime of *A. thaliana* might not be long enough to induce changes in community structure. The *A. alpina pep1* mutant offers a useful tool to dissect the role of plant developmental stage and residence time of plants in the soil on community structure. To dissect these components, *A. alpina* WT (accession Pajares, Paj) and *A. alpina pep1* mutant plants were included in the time course experiment (Tab. 1).

A. alpina WT and *pep1* plants were grown in the greenhouse for 7 months and samples were harvested after 6 weeks, 3 months and 7 months (Fig. 10). After 6 weeks, WT and *pep1* plants resided in the vegetative stage. After three months *pep1* plants started to flower, while WT plants remained in the vegetative stage until the end of the experiment (for details on sample numbers refer to Methods section Chapter I.4.2.3). My experimental setup included material from two plant lines, grown at three different time points in two seasonal batches of Cologne agricultural soil (CAS8 and CAS9; see also Tab. 1). In addition to the soil and root compartments, I also sampled the rhizosphere. The rhizosphere compartment is defined by soil particles that are firmly attached to roots and are collected during the first washing step (see Methods Chapter I.4.2.5 for details). A total of 106 samples were harvested and

prepared for amplicon sequencing using the Illumina protocol. Illumina sequenced samples were processed as described in Chapter I.2.2. The TIC included 8,320,272 high quality reads with a minimum and maximum number of sequences of 27,690 and 186,961, respectively. To ensure reproducibility, low abundance OTUs were removed by applying a threshold of 20 sequences within at least one sample, resulting in 6,742,856 high quality reads (ranging from 18,129 and 148,846 sequences per sample). Samples were normalized by sample size and $\log_2$-transformed. The TIC and ACM comprised 409,297 OTUs and 3,311 OTUs. Most OTUs in the ACM were assigned to the kingdom Bacteria, while 52 OTUs were classified as Archaea.

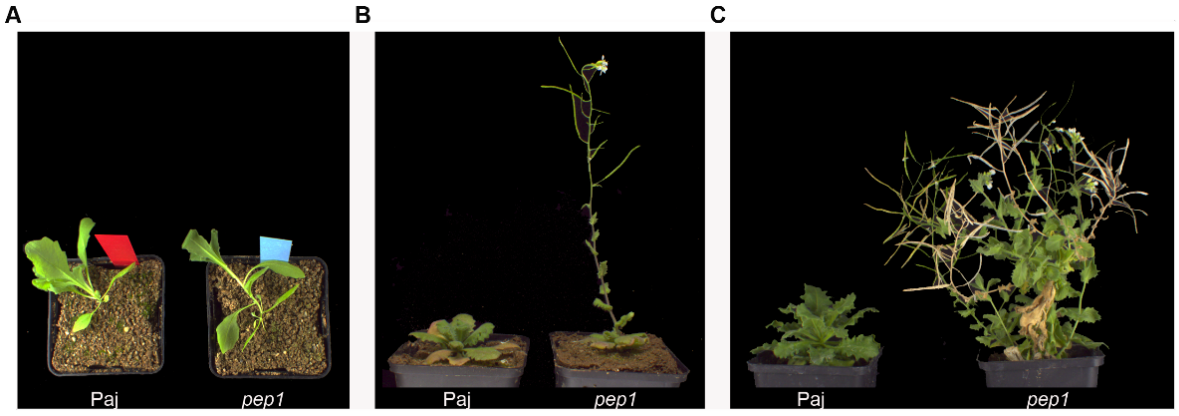


Fig. 10: Growth pattern of *A. alpina* over a time course experiment of 7 months. *A. alpina* WT (Paj) and *pep1* plants grown for 6 weeks **(A)**, 3 months **(B)** and 7 months **(C)** in Cologne agricultural soil (CAS) under controlled environmental conditions in the greenhouse.

To gain information about alpha-diversity per sample, the number of identified OTUs and the Shannon index within the tested compartments across time were calculated for the TIC rarefied to 27,000 sequences (Fig. 11). The soil and rhizosphere compartments displayed a significantly higher OTU richness, as well as Shannon index, compared to the root compartment, demonstrating a higher diversity in soil and rhizosphere samples. The difference between soil and root compartments is in accordance with previous experiments, albeit a higher number of OTUs were detected in the time course experiment compared to OTUs detected in bacterial communities associated with the three tested Brassicaceae species (maximum number of detectable OTUs of ~10,000 and ~2,000, respectively, Fig. 4A). This observation can be explained by the higher sequencing depth of samples processed with the Illumina protocol. No significant differences were detected for the soil and rhizosphere compartments over time, whereas the Shannon index was significantly higher in root communities collected at 3 and 7 months compared to 6 weeks. This suggests an increase in diversity of bacterial root communities over time.

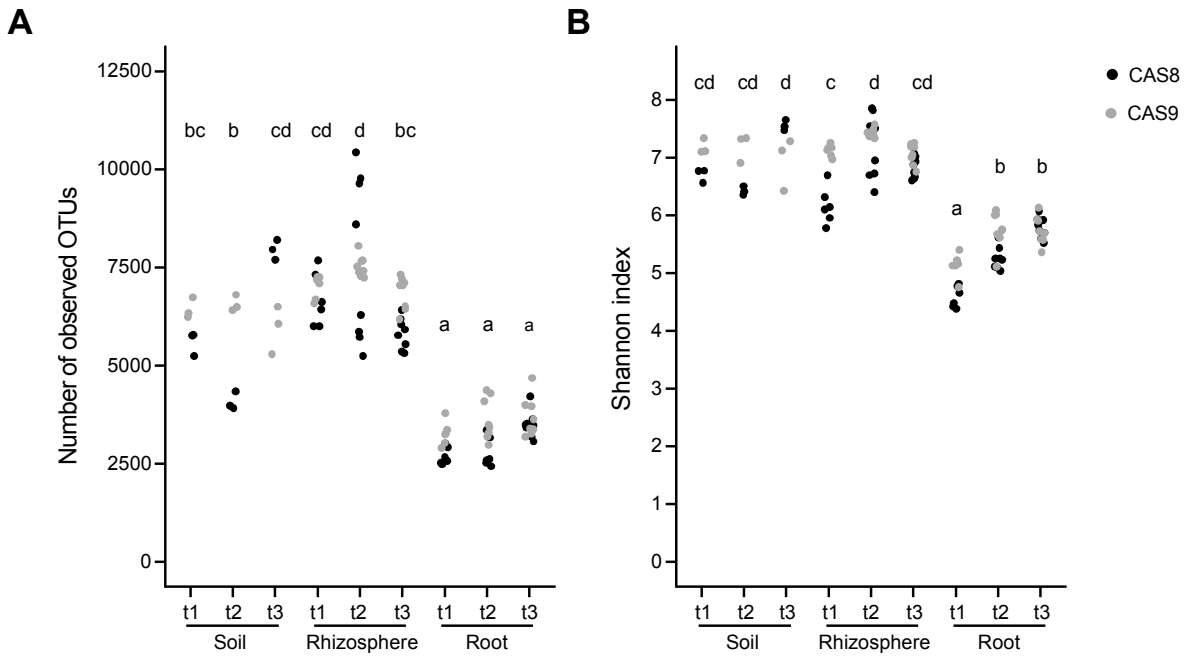


Fig. 11: Root bacterial communities harbor a reduced complexity compared to the soil and rhizosphere and residence time of plants in the soil only marginally affects alpha-diversity. Alpha-diversity analysis of *A. alpina* soil, rhizosphere and root communities sampled at t1, t2 and t3 (6 weeks, 3 months and 7 months, respectively) calculated on the threshold-independent community (TIC) rarefied to 27,000 sequences. **(A)** Number of observed OTUs **(B)** Shannon-index. Black and Grey: Plans were grown in two independent biological experiments, using two different soil batches (CAS8 and CAS9). Significance values based on $p < 0.05$ (Tukey's HSD).

To determine factors that explain the variation observed between samples, a PCA was carried out (Fig. 12). Similar to the comparative analysis of the root microbiota of three Brassicaceae species (Fig. 5), differences between compartments explained most of the observed variation (21.3%). Again, soil and root samples formed separate clusters and the rhizosphere compartment was located between these two compartments. The second principal component explained 12.6% of the observed variation and separated the soil batches CAS8 and CAS9. This is consistent with the previous observation that tested compartment and soil batch represent major determinants of bacterial community structure.

Next, the bacterial microbiota composition over time was examined at phylum and family rank (Fig. 13). On phylum rank Proteobacteria, Bacteroidetes and Actinobacteria were abundant in the root and Firmicutes and Acidobacteria in the soil compartment at 6 weeks after sowing. This resembled the composition of the root microbiota of the three tested Brassicaceae species, where plants were collected at the same age (Fig. 6). The rhizosphere compartment displayed an intermediate structure compared to the soil and root compartments. In the rhizosphere Bacteroidetes reached a similar abundance compared to the root and Firmicutes compared to the soil compartments. Over time, the taxonomic distribution of the soil communities remained stable and a more distinct pattern was observed for the root and rhizosphere compartments. In the root compartment only Actinobacteria remained stable, whereas the relative abundance of Bacteroidetes decreased in the root as well as rhizosphere compartments over time.

In the root compartment this was compensated with an increase in the relative abundance of Proteobacteria (for statistical analysis see Tab. S 1).

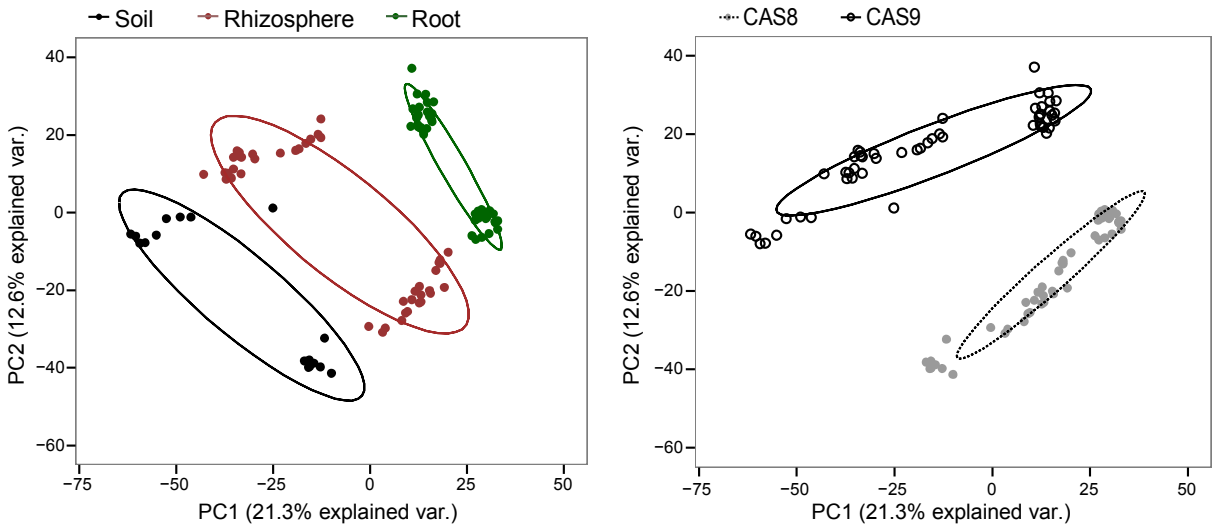


Fig. 12: Compartment and soil batch strongly influence bacterial community structure of *A. alpina*. Principal component analysis (PCA) displaying the variation within the soil, rhizosphere and root compartments of *A. alpina* plants grown in two soil batches (CAS8 and CAS9) based on the abundant community members (ACM). The ellipse covers 69% of samples belonging to the indicated class. Different time points are not indicated.

At lower taxonomic rank similar families were abundant during the time course experiment compared to the bacterial root microbiota of the three Brassicaceae plant species (Fig. 13B, Fig. 6B). The phylum Bacteroidetes was represented by Flavobacteriaceae, Actinobacteria by Streptomycetaceae and Proteobacteria by diverse families such as Comamonadaceae, Pseudomonadaceae or Burkholderiales incertae sedis. Interestingly, the family Thermomonasporaceae was not detected among the 15 most abundant families associated with *A. alpina*, consistent with the fact that this family was mostly enriched on roots of *A. thaliana* (Fig. 6B). Also on family rank quantitative differences were detected across the tested time points (Tab. S 1). For example, the relative abundance of Flavobacteriaceae decreased in the rhizosphere and root compartments over time, consistent with the reduction of Bacteroidetes observed at phylum rank. The increase of Proteobacteria at phylum rank was due to diverse low abundance families, such as Hyphomicrobiaceae, Myxococcaceae or Cystobacteriaceae. Notably, Comamonadaceae, one of the most abundant families after 6 weeks, decreased in relative abundance over time. Despite the stability of Actinobacteria at phylum rank, this taxonomic group was variable at family rank as the relative abundance of Streptomycetaceae decreased in the root compartment at 7 months compared to 6 weeks and 3 months. In summary, dynamic changes in the relative abundances of the detected phyla and families in the root and rhizosphere compartments were detected over time, whereas soil communities remained stable. Thus, the tested compartment, soil type, as well as residence time of plants in the soil, are important factors influencing the structure of the rhizosphere and root-associated bacterial microbiota.

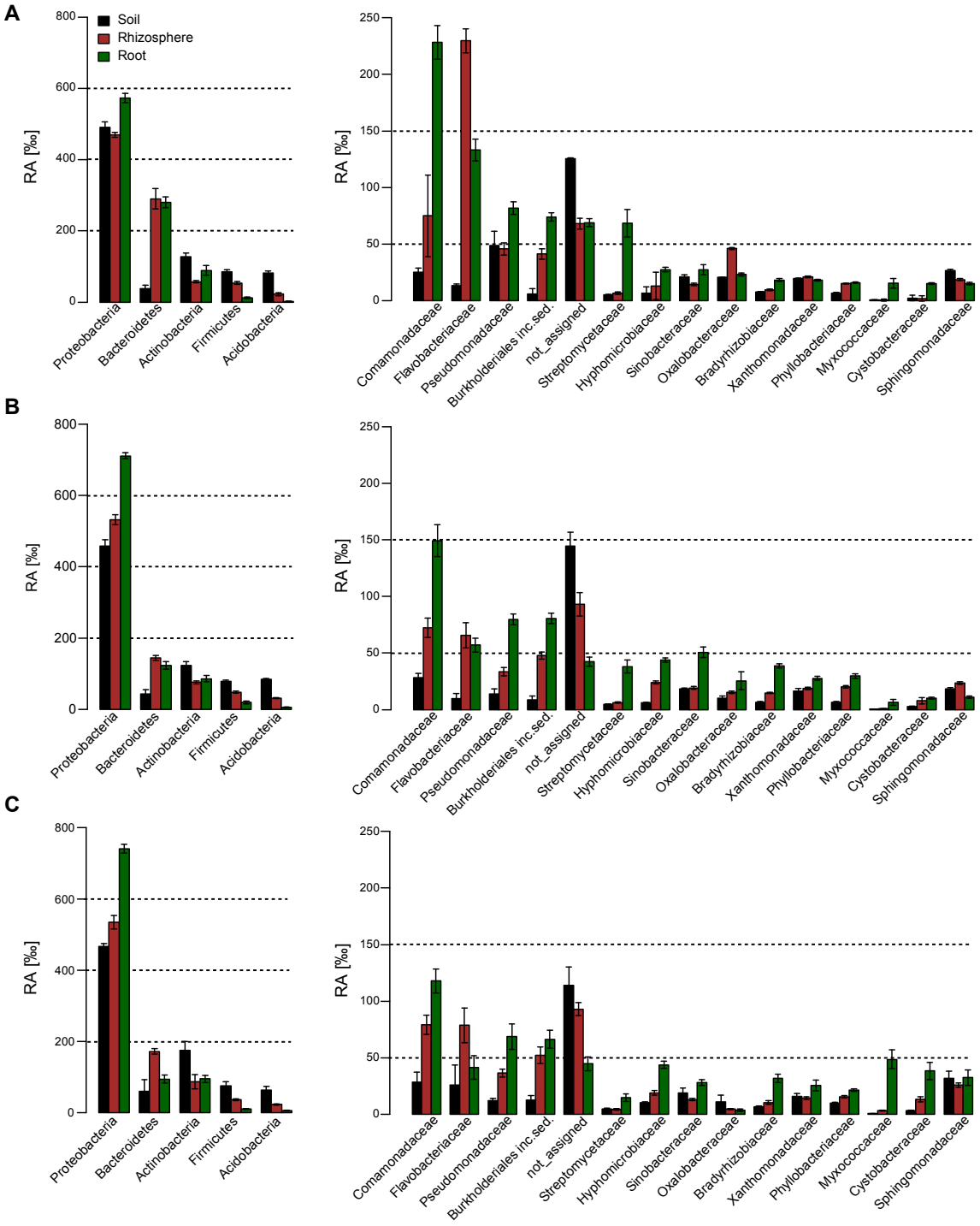


Fig. 13: Prolonged residence time of *A. alpina* in the soil influences bacterial community structure. Phylum and family (right and left) distribution of abundant bacterial taxa within the soil, rhizosphere and root compartments of *A. alpina* over time. Community structure after: **(A)** 6 weeks, **(B)** 3 months and **(C)** 7 months. Shown is the relative abundance (RA) in per mile. The bar graph is depicting the mean and the error bars represent the standard error. Only the 5 most abundant phyla and 15 most abundant families are illustrated. Summary statistics can be found in Tab. S 1.

In the next step SoilOTUs and RootOTUs were determined, as well as OTUs enriched in the rhizosphere, termed RhizoOTUs. Of the 3,311 OTUs in the ACM, 874 SoilOTUs, 425 RhizoOTUs and 519 RootOTUs were determined across all tested time points (Fig. 14A). A subset of OTUs was shared

between the soil and rhizosphere, as well as between the rhizosphere and root compartments, whereas a clear separation between the soil and root compartments existed. The clear separation of OTUs based on compartment correlated with the clustering observed within the PCA (Fig. 12). Altogether, this indicates that the rhizosphere and root microbiota of *A. alpina* contains a distinct subset of bacterial families and OTUs compared to unplanted soil.

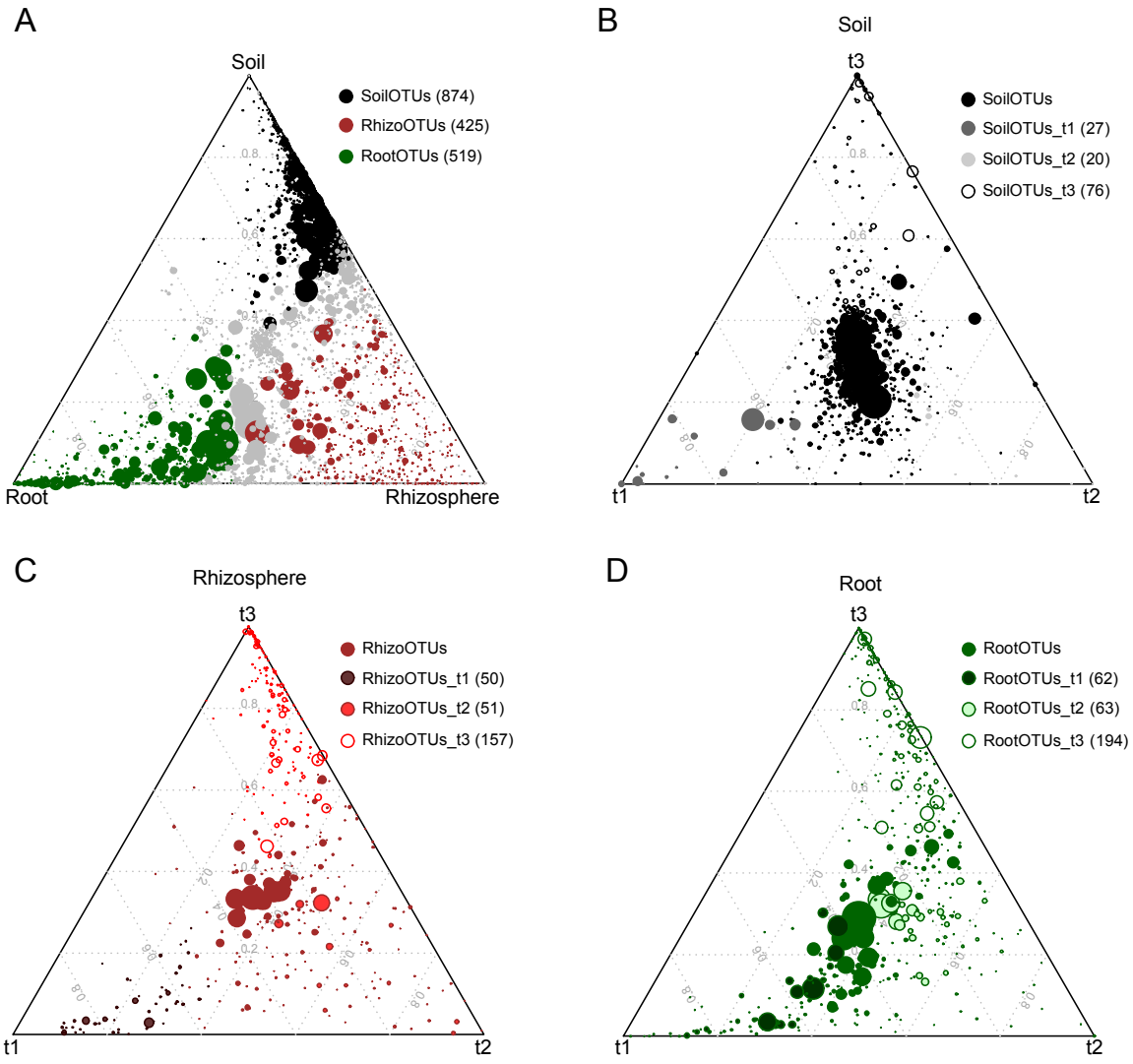


Fig. 14: Distinct soil, rhizosphere and root communities assemble on *A. alpina*, with distinct patterns over time. OTUs enriched in the soil, rhizosphere and root compartments of *A. alpina* after 6 weeks, 3 and 7 months (t1, t2 and t3, respectively). Each circle in the ternary plot depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position of each circle is determined by the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. **(A)** Soil-, rhizosphere- and root-enriched OTUs (RootOTUs, RhizoOTUs, SoilOTUs), including OTUs observed across all tested time points and OTUs specific for individual time points. Time point-specific SoilOTUs **(B)**, RhizoOTUs **(C)** and RootOTUs **(D)**. For (A) the mean of all samples of the soil, rhizosphere and root compartments independent of the time point is plotted. For B-D the mean of soil (B), rhizosphere (C) or root (D) samples at each time point is plotted. Number in brackets: Total number of OTUs enriched in the respective compartment or time point. Enriched OTUs, based on a Bayes moderated t-test; $p < 0.05$ (FDR-corrected). Ternary plot separating the time points pooled in (A) can be found in Fig. S1. Taxonomic distribution of time point-specific OTUs can be found in Tab. S 2A.

To assess the stability of bacterial communities, the SoilOTUs, RhizoOTUs and RootOTUs were individually analyzed for their dynamics over time. From the 874 SoilOTUs only 27, 26 and 76 OTUs were enriched in one of the three time points and most of these time point-specific SoilOTUs were detected at a low relative abundance (Fig. 14B). This indicates that the soil community is largely stable over the time course experiment, consistent with the stable taxonomic composition of the soil compartment over time (Fig. 13). A more differentiated pattern was observed for the rhizosphere and root compartments. Overall, a higher number of RhizoOTUs and RootOTUs were significantly enriched at individual time points. Approximately 60% of the RhizoOTUs and RootOTUs displayed a distinctive pattern over time, with the highest number of OTUs being time point-specific at 7 months (Fig. 14C and D). Hence, a small subset of the bacterial rhizosphere and root community was stable over time, while the larger part of time point-specific OTUs followed a pattern of bacterial succession. Focusing on the taxonomic distribution of these OTUs, a distinctive pattern was observed. Proteobacteria increased in relative abundance over the soil, rhizosphere and root compartments, Actinobacteria decreased and Bacteroidetes increased in relative abundance in the rhizosphere, whereas Acidobacteria were abundant in the soil (Tab. S 2A). This taxonomic shift was also observed for the time point-specific OTUs as well as across the root compartments of the three Brassicaceae plant species (Fig. 6). Together, this suggests that the tested plant species preferentially attract specific taxonomic groups into the rhizosphere and root compartments. Additionally, it can be assumed that these taxa are highly competitive rhizosphere- and root-colonizing bacteria.

The design of the time course experiment also allowed me to analyze the influence of plant developmental stage on the composition of the bacterial microbiota. Therefore, the composition of the root- and rhizosphere-associated bacterial microbiota of *A. alpina* WT and *pep1* plants were compared over time. At 6 weeks after sowing both plant genotypes were in the vegetative growth stage and no OTUs differentially accumulated on either of the two plant lines (Fig. 10, Fig. 15A). This suggests that plant genotype, i.e. mutant background, does not alter community structure. Next, the bacterial root microbiota of the *A. alpina* WT and *pep1* plants was compared at 3 and 7 months after sowing, to test for differences induced by shifts in the developmental stage. No changes in community structure were detected at 3 months and only two low abundance OTUs differentiated these two plant genotypes at 7 months (Fig. 15B and C). A similar observation was made for rhizosphere bacterial communities (data not shown). These results were unexpected, as *pep1* plants displayed strong phenotypic differences compared to WT plants starting from 3 months after sowing, as seen by a reduction in overall shoot biomass (Fig. 10). Nevertheless, differences between WT and *pep1* plants did not affect the stability of the bacterial root microbiota and this suggests that plant developmental stage does not play a dominant role in structuring the bacterial microbiota.

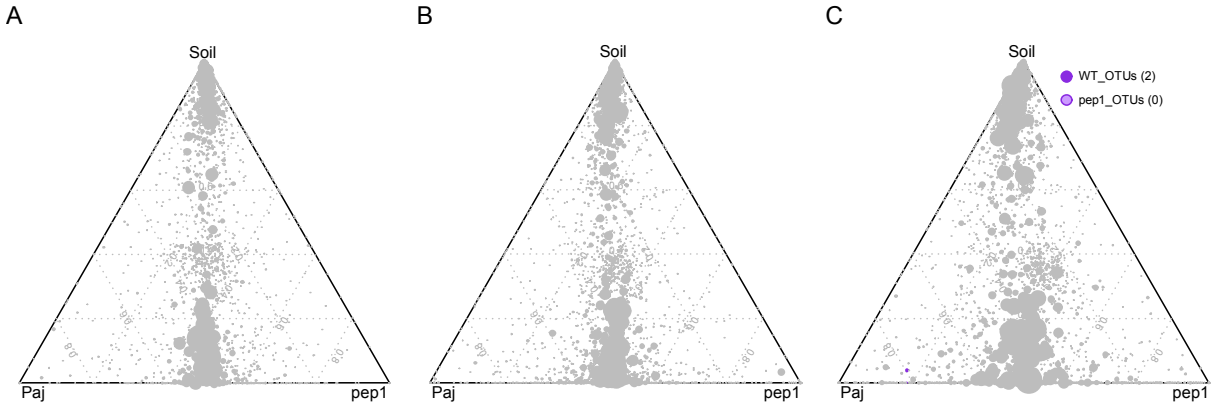


Fig. 15: Plant developmental stage does not influence bacterial root communities. OTUs enriched in soil and root compartments of the *A. alpina* wild type (Paj) and *pep1* mutant plants after 6 weeks **(A)**, 3 months **(B)** and 7 months **(C)**. Each circle depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position of each circle is determined by the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. Number in brackets: Total number of OTUs enriched in the respective plant line. OTUs enriched in the two plant lines are based on a Bayes moderated t-test; $p < 0.05$ (FDR-corrected)

2.3.2. Influence of environmental conditions on the bacterial microbiota of *A. alpina*

Summarizing previous results, a strong influence of compartment, soil batch and residence time of plants in the soil and only minor effects of plant species and plant developmental stage on community structure were detected. However, so far the experimental setup did not account for the fact that *A. alpina* is adapted to an arctic-alpine environment and that growth under controlled greenhouse settings in a nutrient-rich soil barely resembles natural growth conditions. Therefore, a third experiment (1 independent biological replicate) was conducted to evaluate the role of soil type and environmental conditions on the composition of the soil, rhizosphere and root microbiota of *A. alpina*.

In the natural site experiment root and seed material from naturally grown *A. alpina* plants (designated Galibier60; Gal60) was collected in the French Alps at the Col du Galibier (45.061 N/ 6.402 E) for community profiling. Additionally, large amounts of soil were collected in the French Alps to be used in greenhouse experiments with *A. alpina* accessions under controlled environmental conditions (Tab. 1). Soil analyses from the French and previously used Cologne soil revealed that these two soil types differed in pH and nutrient content (Tab. 2, Tab. 3). Such differences could alter the composition of soil communities, as soil pH was found to be a strong determinant for community structure (Fierer and Jackson, 2006). Therefore, this experimental system allows to test for the importance of soil type and environmental conditions for community structure.

In the natural site experiment the previously used Spanish accession Pajares (Paj) and the two French accessions Gal5 and Gal60 were included (Fig. 16). Of note, Gal5 and Gal60 do not require a vernalization treatment to flower and Gal5 started flowering, whereas Gal60 resided in the vegetative stage during the harvest. This permits to confirm the importance of plant developmental stage on community structure in a natural setting. Additionally, the *A. alpina* accession Paj was grown in CAS8

to allow cross-referencing to the time course experiment. Of note, *A. thaliana* seeds were not able to germinate in the French soil, suggesting a strong adaptation of *A. alpina* to its native soil (Fig. 16, picture of *A. thaliana* was kindly provided by Dr. S. Spaepen; details for growth conditions can be found in Methods section Chapter I.4.2.4).

In the natural site experiment a total of 59 samples were prepared for Illumina sequencing (Tab. 1) and the obtained reads were further processed as discussed above during the comparison of the pyrosequencing and Illumina protocols. The TIC yielded 3,775,993 high quality reads covering 208,726 OTUs (min. and max. number of sequences: 16,838 and 128,851). After thresholding the ACM consisted of 3,084,606 reads (min. and max. number of sequences: 14,901 and 103,981) and was represented by 2,317 OTUs, with all OTUs belonging to the kingdom Bacteria and no members of the kingdom Archaea were detected.

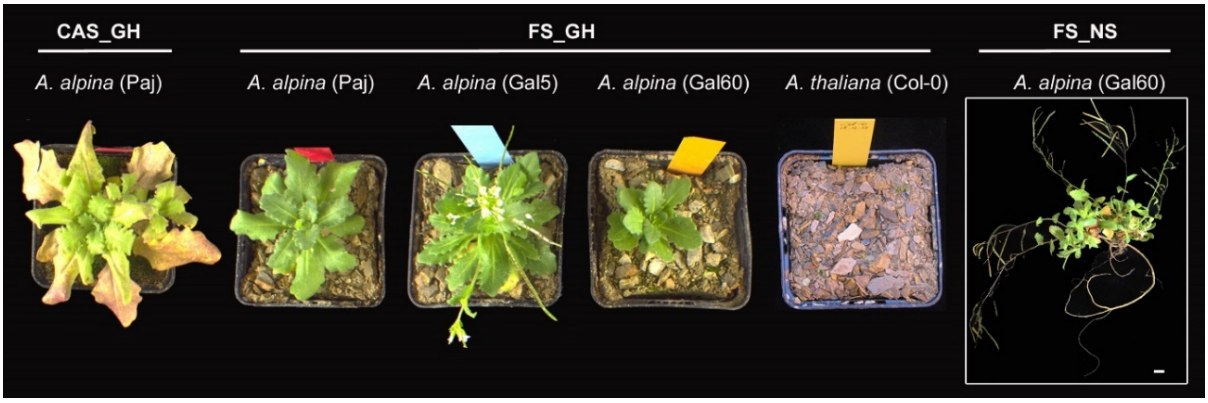


Fig. 16: Growth morphology of *A. alpina* grown in two soil types in the greenhouse or natural conditions. Plants of the Spanish accession *A. alpina* Pajares (Paj) were grown in the greenhouse in Cologne agricultural (CAS) or natural French soil (FS). Additionally, in the FS in the greenhouse (FS_GH) the French accessions Gal5 and Gal60, as well as *A. thaliana* Col-0, were included. The *A. alpina* accession Gal60 was collected at a natural site (NS) in the French Alps (Col du Galibier). From this site also the natural French soil was collected. Plants were grown in the greenhouse for 3 months. Age of the Gal60 accession in the natural environment (FS_NS) cannot be determined. The picture representing *A. thaliana* was kindly provided by Dr. S. Spaepen**.**

First, alpha-diversity was calculated across the three tested growth conditions and compartments based on the TIC rarefied to 16,000 sequences (Fig. 17). Soil- and rhizosphere-derived samples yielded a similar number of observed OTUs and Shannon index, whilst root samples displayed a reduced alpha-diversity. Approximately 4,000 OTUs were detected in the soil and rhizosphere compartments, whereas ~ 2,000 OTUs were identified in the root compartments. Samples grown under stable greenhouse conditions in the Cologne or French soil (CAS_GH and FS_GH, respectively) displayed no significant differences. In contrast, a higher number of OTUs were detected in the soil and rhizosphere compartments of plants sampled at the natural site in the French Alps (FS_NS). However, the Shannon index was undistinguishable for all tested soil types and environmental conditions, indicating that the difference for the observed number of OTUs did not affect the abundant OTUs. Globally, similar trends

in alpha-diversity were found for the natural site and time course experiment (Fig. 11), indicating a good comparability between experiments.

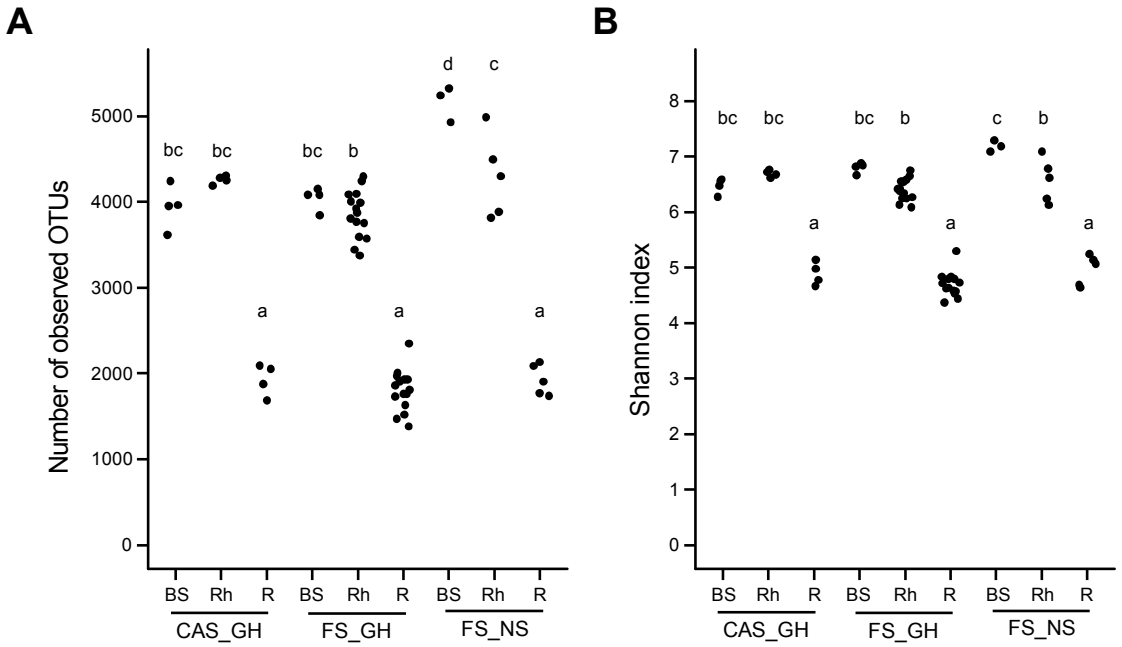


Fig. 17: Root compartments display a reduced diversity compared to soil and rhizosphere compartments independent of the tested soil type and growth conditions. Alpha-diversity analysis of *A. alpina* soil, rhizosphere and root communities sampled from the Cologne agricultural and French soil grown in the greenhouse (CAS_GH and FS_GH) as well as French soil grown at a natural site (FS_NS). Calculated on the threshold-independent community rarefied to 16,000 sequences. **(A)** Number of observed OTUs **(B)** Shannon-index. Significance values based on $p < 0.05$ (Tukey's HSD). BS: bulk soil, Rh: rhizosphere; R: root.

Next, a PCA was utilized to partition the influence of the tested compartments, soil types and environmental growth conditions on community structure (Fig. 18). Similar to the time course experiment, most of the observed variation (21.1%) was explained by differences among the tested compartments, where a clear gradient between the soil, rhizosphere and root compartments was observed. The second principal component explained 15.9% of the variation and separated samples derived from the Cologne and French soils, while samples derived from the French soil grown either in the greenhouse or collected at the natural site were undistinguishable (FS_GH versus FS_NS). Notably, the PCA revealed that the root communities converged to more similar communities from divergent start inocula. Therefore, it can be concluded that the tested compartments and soil types are of major importance for determining bacterial community structure, whereas environmental conditions are of minor importance.

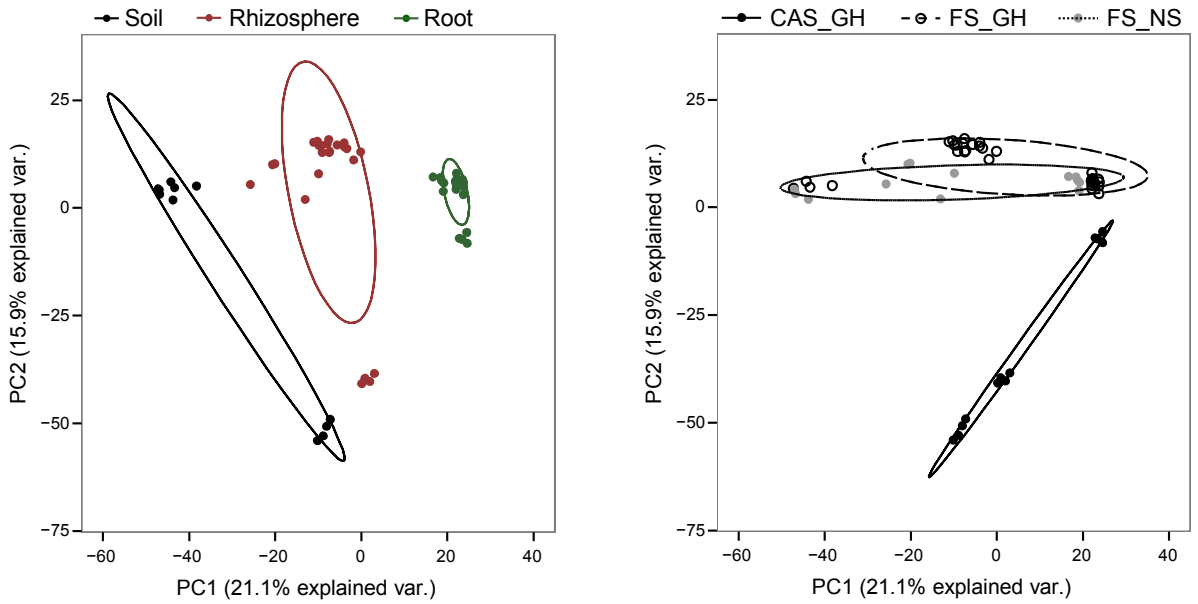


Fig. 18: Compartment and soil type strongly influence bacterial community structure. Principal component analysis (PCA) on bacterial communities inhabiting the soil, rhizosphere and root compartments of *A. alpina* plants grown in two soil types (Cologne agricultural soil (CAS) and French soil (FS)) in the greenhouse (GH) or at a natural site (NS) in France. From each sample the root, rhizosphere and soil compartments were analyzed. The ellipse covers 69% of samples belonging to the indicated class.

Next, I examined how the tested growth conditions affected the taxonomic pattern of bacterial communities at phylum and family rank (Fig. 19, Tab. S 3). Again, the most abundant phyla detected in the root compartments were Proteobacteria, Actinobacteria and Bacteroidetes, whereas Acidobacteria were enriched in unplanted soil. Additionally, the tested soil types were distinguishable by differences at phylum rank (Tab. S 3). A soil type-dependent pattern was detected for Firmicutes that were of a higher relative abundance in the Cologne soil (CAS_GH) and were almost absent from the French soil collected in the greenhouse or at the natural site (FS_GH and FS_NS). Notably, within this experiment Firmicutes were also detected in the root compartment of plants grown in the Cologne soil (CAS_GH). The detection of Firmicutes within the root compartment was unexpected but could be explained by the fact that the soil *A. alpina* was planted in was wet during the harvest procedure and might have resulted in an overestimation of Firmicutes within the root compartment. Nevertheless, the taxonomic structure of the natural site experiment strongly reflected the pattern of the time course experiment at three months (Fig. 13). For example, a lower relative abundance of Bacteroidetes in the rhizosphere and root compartments was observed both in the time course and natural site experiments. This suggests that Bacteroidetes become outcompeted after 6 weeks and that bacteria of this phylum represented less successful colonizers over time.

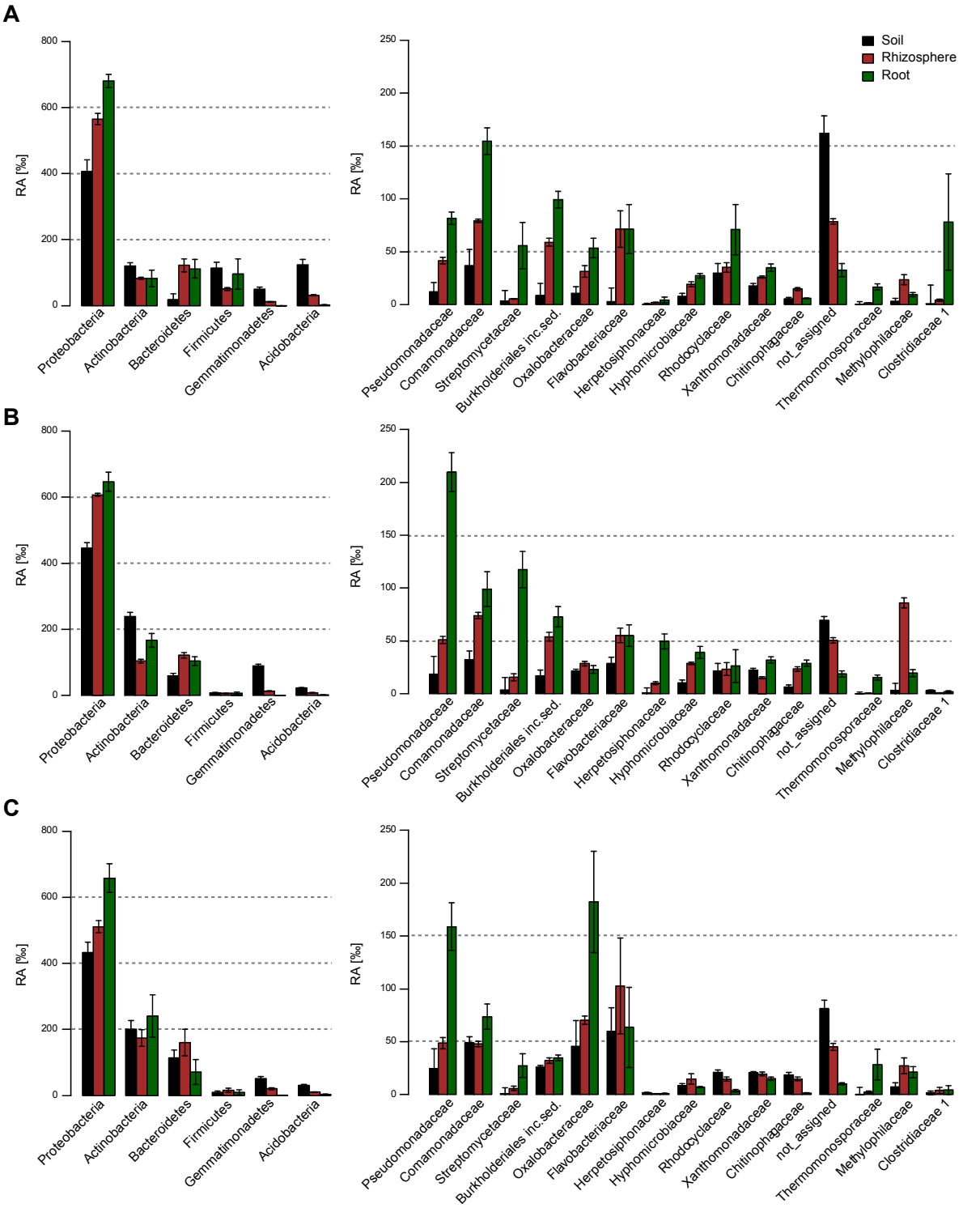


Fig. 19: Soil type and environmental conditions induce shifts in bacterial communities in the soil, rhizosphere and root compartments of *A. alpina*. Phylum (left) and family (right) distribution of abundant bacterial groups within the soil, rhizosphere and root compartments across three tested growth conditions. Growth of plants in: **(A)** Cologne agricultural soil in the greenhouse (CAS_GH), **(B)** French soil in the greenhouse (FS_GH) and **(C)** French soil at a natural site (FS_NS). Shown is the relative abundance (RA) in per mile. The bar graph is depicting the mean and error bars represent the standard error. Depicted are the 6 most abundant phyla and 15 most abundant families. Summary statistics can be found in Tab. S 3.

On family rank similar families were detected during the natural site experiment compared to previous time course experiment. The phylum Proteobacteria was represented by diverse families such as Pseudomonadaceae, Comamonadaceae or Burkholderiales incertae sedis. Additionally, Actinobacteria were represented by Streptomycetaceae and Thermomonosporaceae and Bacteroidetes by Flavobacteriaceae. The taxonomic identity of bacterial families enriched in the soil, rhizosphere and root compartments of *A. alpina* grown in Cologne soil in the greenhouse were similar to bacterial families detected during the time course experiment at three months, indicating a good comparability between experiments (Fig. 13). Few families were among the 15 most abundant families that were not detected during the time course experiment, such as Thermomonosporaceae. Differences between the time course and natural site experiment could be explained by the fact that growth conditions for plants were slightly different. For example, the soil during the time course experiment was supplemented with 50% sterilized sand, as otherwise *A. alpina* did not develop healthy but senescent leaves during a growth period of 7 months (Fig. 16, first picture).

Notably, quantitative differences were observed among the 15 most abundant families dependent on the tested growth condition (Fig. 19, Tab. S 3). Comparing bacterial communities from samples collected in the Cologne and French soils grown in the greenhouse, Pseudomonadaceae displayed a higher relative abundance in the root compartment of plants grown in the French soil. Additionally, samples derived from the French soil grown in the greenhouse (FS_GH) displayed distinctive features compared to the other two growth conditions. For example, the relative abundance of Herpetosiphonaceae was higher in the root compartment and of Methylophilaceae in the rhizosphere compartment. Additionally, Oxalobacteriaceae were enriched and Streptomycetaceae depleted in root samples grown in the French soil under natural conditions (FS_NS). Overall, this demonstrates that soil type and environmental conditions affect the structure of soil, rhizosphere and root bacterial communities on family and phylum rank.

For an in-depth analysis of OTUs that were abundant in the soil, rhizosphere and root compartments, their distribution across the tested growth conditions was calculated. In total 799, 369 and 319 SoilOTUs, RhizoOTUs and RootOTUs were identified, respectively (Fig. 20A). Approximately 70% of the SoilOTUs were significantly enriched in one of the three tested conditions (Fig. 20B). SoilOTUs from the French soil grown in the greenhouse or collected at the natural site in the French Alps (FS_GH and FS_NS) were more similar to each other and more distant from samples from the Cologne soil (CAS_GH). A similar trend was observed for the RootOTUs and RhizoOTUs, where ~85% of OTUs displayed a distinct enrichment under one of the tested conditions (Fig. 20C and D). Of note, whilst for the RhizoOTUs a subset of OTUs was shared between the French soil collected in the greenhouse and the natural site (FS_GH and FS_NS), the RootOTUs displayed a more distinctive pattern across all three growth conditions.

These results indicate that bacterial communities in the start inoculum are highly influenced by the soil type and environmental conditions and this results in a distinctive composition of root and

rhizosphere bacterial communities. However, it is remarkable that regardless of these differences on OTU level, similar taxonomic patterns for the compartment-enriched OTUs were detected (Tab. S 2B). Same as for the time course experiment an enrichment of Proteobacteria over the soil, rhizosphere and root compartments, a decrease of Actinobacteria and increase of Bacteroidetes within the rhizosphere and enrichment of Acidobacteria in the soil compartment was measured. Therefore, this taxonomic footprint seems to be stable across the tested growth conditions.

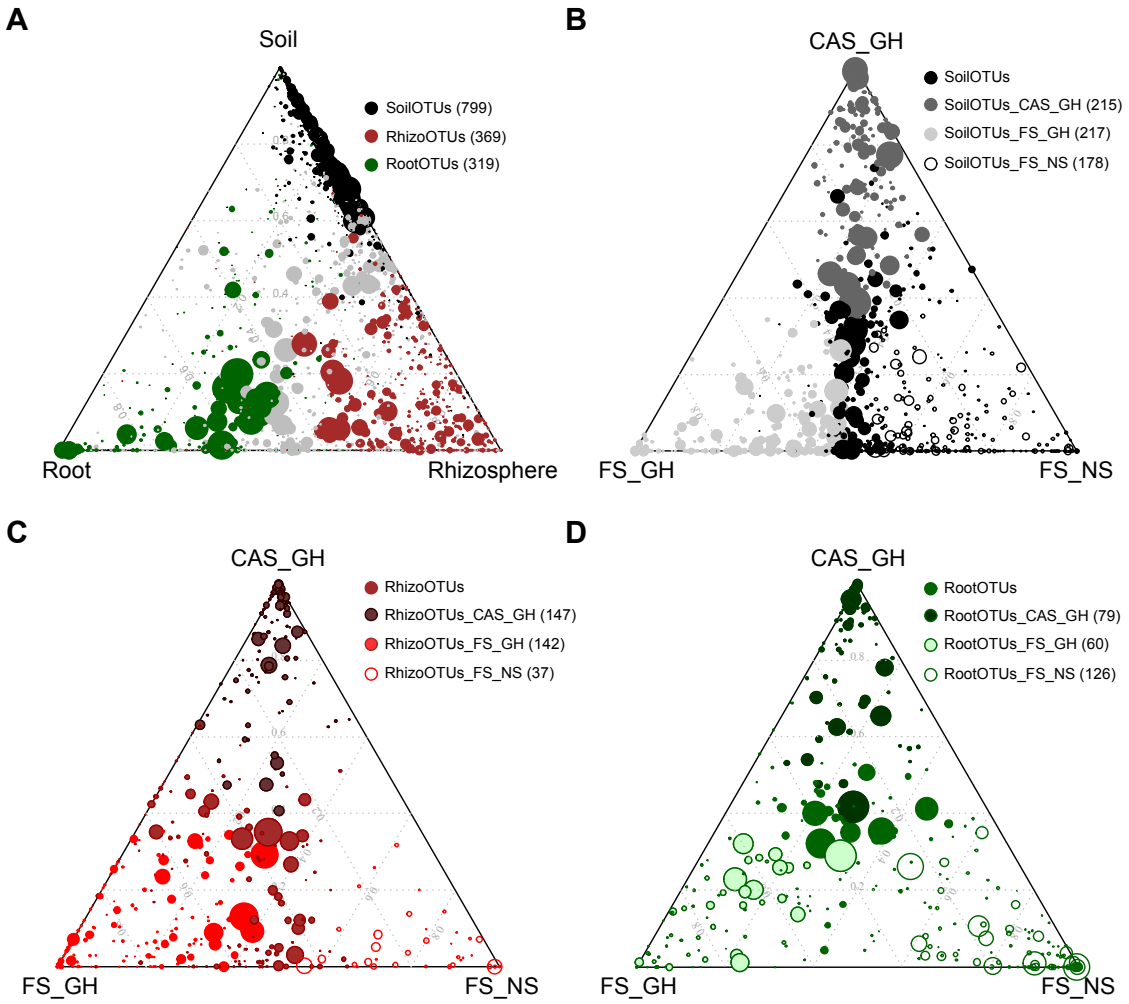


Fig. 20: Distinct bacterial communities assemble in the soil, rhizosphere and root compartments of *A. alpina*, with distinctive patterns across the tested soil types and environmental conditions. *A. alpina* was grown in two soil types (Cologne agricultural soil (CAS) and French soil (FS)) with FS samples collected in the greenhouse (GH) or natural site (NS). For each condition the root, rhizosphere and soil compartments were collected. Each circle depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position of each circle is determined by the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. **(A)** OTUs significantly enriched in the root, rhizosphere and soil compartments (RootOTUs, RhizoOTUs and SoilOTUs) summarized across all tested conditions. Growth condition-dependent SoilOTUs **(B)**, RhizoOTUs **(C)** and RootOTUs **(D)**. Number in brackets: Enriched OTUs based on a Bayes moderated t-test; $p < 0.05$ (FDR-corrected). Ternary plot that separates the used growth conditions that were pooled within (A) can be found in Fig. S 2. Taxonomic distribution of enriched OTUs is recorded in Tab. S 2B.

Finally, I determined how RootOTUs and RhizoOTUs were affected by plant host genotype as well as plant developmental stage. Therefore, I compared the bacterial communities of the three tested *A. alpina* accessions. A few OTUs were enriched on roots of the French accessions Gal5 and Gal60, while twice as many OTUs were enriched on roots of the Spanish accession Paj (Fig. 21A). A similar but weaker effect was detected for rhizosphere bacterial communities associated with the three *A. alpina* accessions (Fig. 21B). As the flowering accession Gal5 did not enrich for more OTUs than the non-flowering accessions Gal60 and Paj, it can be concluded that plant development exerts a minor influence on bacterial community composition, even below the impact of plant accession. The latter might indicate an adaption towards distinctive environmental conditions, but will require the examination of a broader range of soil types and accessions to verify this hypothesis.

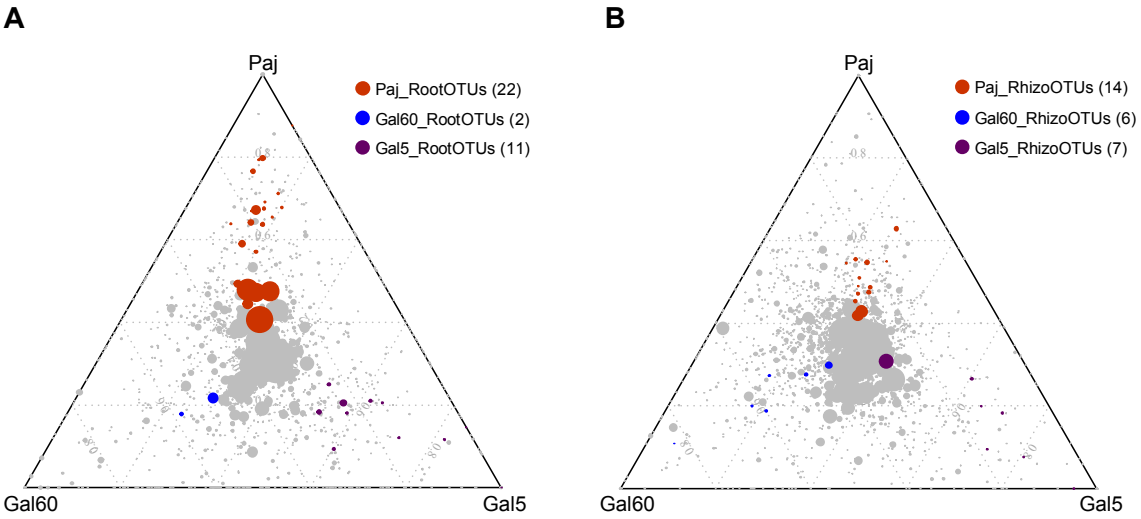


Fig. 21: Plant accession and plant developmental stage weakly affect the bacterial root microbiota in the natural site experiment. Ternary plots of OTUs within the root **(A)** and rhizosphere **(B)** compartments of the Spanish *A. alpina* accession Paj (non-flowering) and the two French accessions Gal5 (flowering) and Gal60 (non-flowering). Each circle depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position of each circle is determined by the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. Brackets: Number of OTUs enriched within individual accessions. OTUs enriched are based on a Bayes moderated t-test; $p < 0.05$ (FDR-corrected).

Summarizing the so far presented results of the culture-independent sequencing approach, the tested compartment, soil type, soil batch, time and environmental conditions are major factors determining the composition of bacterial communities associated with *A. alpina*. Notably, *A. alpina* features a rhizosphere effect in contrast to previous observations for *A. thaliana* and *C. hirsuta* (Schlaeppi et al., 2014). Plant-dependent factors on the other hand, such as plant species and plant developmental stage, affect community composition only to a minor extent. The three Brassicaceae species *A. thaliana*, *C. hirsuta* and *A. alpina* assemble a defined bacterial root microbiota, including members of the Proteobacteria, Actinobacteria and Bacteroidetes that were represented by few bacterial families. The

distinct structure of the bacterial root microbiota, compared to soil- and rhizosphere-associated communities, indicates that plants enrich for a specific subset of the bacterial community and that environmental factors, potentially related to bacteria-bacteria interactions, are important, additional assembly cues.

2.4. Isolation and functional characterization of bacteria of the root microbiota of Brassicaceae plant species

2.4.1. Isolation of bacteria from roots of three Brassicaceae plant species

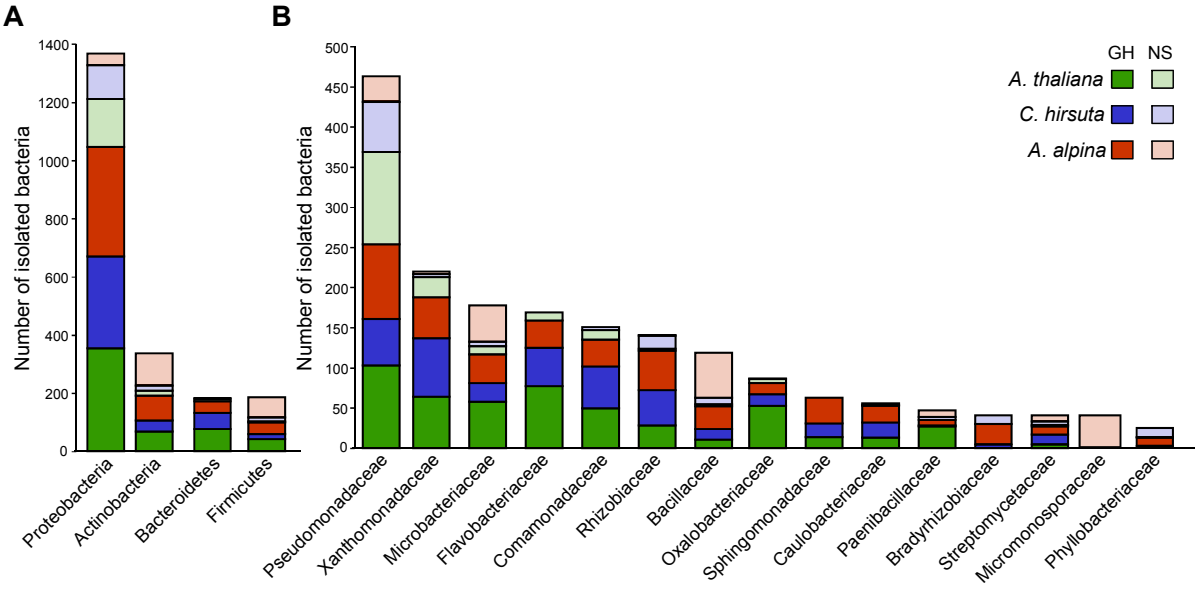
During the culture-independent approach I was able to dissect the influence of soil type, environmental conditions, residence time of plants in the soil and plant species on the composition of the bacterial root microbiota (Fig. 18, Fig. 20, Fig. 14 and Fig. 6). Regardless of the tested condition, a similar taxonomic pattern was observed for bacterial root communities, as seen by a high relative abundance of Proteobacteria and lower abundance of Actinobacteria and Bacteroidetes (Fig. 6, Fig. 13 and Fig. 19). Importantly, root-associated bacterial communities also displayed a shared pattern on lower taxonomic rank. The most abundant families were represented by Comamonadaceae, Pseudomonadaceae and Oxalobacteriaceae (phylum Proteobacteria), as well as Streptomycetaceae (phylum Actinobacteria) and Flavobacteriaceae (phylum Bacteroidetes). The presence of these families in close interaction with plants could suggest an important, yet unknown, function for plant growth (Shade and Handelsman, 2012). To examine this, I isolated bacteria from roots of the three Brassicaceae plant species and subsequently tested a subset of these bacterial isolates corresponding to the most abundant families for their effects on plant growth.

Bacteria were isolated from roots of *A. thaliana*, *C. hirsuta* and *A. alpina* that were grown in the greenhouse or under natural environments. The taxonomy of 2,095 isolated bacteria was determined by Sanger sequencing of a fragment of the 16S rRNA gene (see Methods Chapter I.4.2.11). Most bacteria were isolated from plants grown under controlled greenhouse conditions in two soil types and using two growth media, yielding 548, 429 and 553 bacterial exemplars from *A. thaliana* and *C. hirsuta* and *A. alpina*, respectively. A smaller number of bacteria was isolated from various natural sites in Germany and France, resulting in 196 (*A. thaliana*), 149 (*C. hirsuta*) and 220 (*A. alpina*) bacteria.

The isolated bacteria belonged to diverse taxonomic groups. Approximately 75% of all isolates were assigned to Proteobacteria, whereas a lower proportion of Actinobacteria, Bacteroidetes and Firmicutes was isolated (Fig. 22A). Overall, similar proportions of bacteria per phylum were isolated from the three Brassicaceae species grown in the greenhouse. *A. thaliana* and *C. hirsuta* grown under natural conditions yielded mostly bacteria assigned to Proteobacteria, whereas Actinobacteria were predominantly isolated from *A. alpina*. Regarding Actinobacteria, a total of 17, 18 and 110 bacteria were isolated from *A. thaliana*, *C. hirsuta* and *A. alpina* from the natural sites, respectively. Almost no

Bacteroidetes were recovered from plants grown at the natural sites, yielding in total only 10, 1 and 0 Bacteroidetes from *A. thaliana*, *C. hirsuta* and *A. alpina* from the natural sites, respectively.

A high diversity of bacterial taxa was isolated from roots of the three Brassicaceae plant species, as seen by the taxonomically diverse range of families being represented among the isolates (Fig. 22B). Of note, the most abundant families largely corresponded between the culture-independent sequencing and -dependent isolation approaches (Fig. 6). Isolates belonging to Proteobacteria were represented by the families Pseudomonadaceae, Xanthomonadaceae or Comamonadaceae and Bacteroidetes by Flavobacteriales. Actinobacteria belonged to the families Microbacteriaceae, Streptomycetaceae and Micromonosporaceae. Interestingly, Microbacteriaceae and Micromonosporaceae were mostly isolated from *A. alpina* grown at a natural site.



C

		overlap OTUs-Isolates		
	OTUs	**Nr. OTUs**	**%**	**Nr. of strains**
TIC	70,005	**101**	0.14	823
ACM	443	**63**	14.22	685
RootOTUs	101	**23**	22.77	307
sharedOTUs	26	**11**	42.31	165

Fig. 22: Diverse bacterial taxa can be isolated from related Brassicaceae species. Total number of isolated bacteria at **(A)** phylum and **(B)** family rank (only displaying the 15 most abundant families). Isolation of bacteria from roots grown in different batches of Cologne and Golm agricultural soil in the greenhouse (GH, intense color) or from different natural sites (NS, pale color). For simplicity the bar graphs do not differentiate isolates from different soil types or plant accessions. Color-coding: *A. thaliana* (green), *C. hirsuta* (blue) and *A. alpina* (green). **(C)** Overlap between the culture-dependent isolation effort and culture-independent sequencing effort of the bacterial root microbiota of the three Brassicaceae plant species *A. thaliana*, *C. hirsuta* and *A. alpina*. OTUs: refers to OTUs of the threshold-independent community (TIC) members, abundant community members (ACM), RootOTUs and shared OTUs of the bacterial root microbiota of the three Brassicaceae species. This comparison is only based on bacterial strains that were isolated from the three Brassicaceae species grown in Cologne agricultural soil in the greenhouse (1,112 bacterial strains). Overlap OTU-Isolates represents the number of OTUs that could be isolated, the overlap between culture-independent and -dependent approaches in % as well as the number of strains behind the number of detected OTUs.

To compare the overlap between the culture-independent sequencing approach and the culture-dependent isolation approach, the following comparison was made: A total of 1,112 bacterial strains isolated from the three Brassicaceae grown in the greenhouse under stable conditions in Cologne agricultural soil were compared to the OTUs of the TIC (70,005 OTUs), ACM (443 OTUs), RootOTUs (101 OTUs) and sharedOTUs (26 OTUs) defined by the 16S rRNA gene profiling of the three Brassicaceae plant species grown in three batches of CAS soil (Fig. 6, Fig. 8). Thereby, an overlap of 0.14%, 14.22%, 22.77% and 42.31% was detected based on the TIC, ACM, RootOTUs and sharedOTUs, respectively (Fig. 22BC). This indicates that dependent on the dataset a high number of bacterial strains detected by the culture-independent sequencing approach can be isolated, especially from the ACM.

The culture-dependent isolation approach resembled in part observations made during the culture-independent sequencing approaches. First, Proteobacteria, Actinobacteria and Bacteroidetes were isolated in similar proportions that were observed during the culture-independent sequencing approaches, especially during later time points during the time course experiment, with Proteobacteria being more than twice as abundant as Actinobacteria and Bacteroidetes (Fig. 22, Fig. 6, Fig. 13 and Fig. 19). Second, most of the isolated families were also among the families that were detected during the culture-independent sequencing approach. Only Thermomonosporaceae (phylum Actinobacteria) and Myxococcaceae (phylum Proteobacteria) could not be isolated. Third, up to 42% of the abundant sharedOTUs defined during the comparison between three plant species of the Brassicaceae family could successfully be isolated. Overall, this indicates that OTUs as well as families and phyla of the bacterial root microbiota based on the culture-independent approach can be efficiently isolated with only few exceptions. For these exceptions alternative growth conditions, such as growth at lower temperatures, may be needed or older plants need to be harvested, as Myxococcaceae were mostly enriched in root compartments of *A. alpina* during late time points (Fig. 13).

2.4.2. Functional characterization of bacteria of the shared microbiota of *A. thaliana*

A shared bacterial root microbiota associated with *A. thaliana*, *C. hirsuta* and *A. alpina* was defined based on the culture-independent community profiling of the 16S rRNA gene, consisting of a defined, narrow taxonomic composition (Fig. 8). This shared bacterial microbiota consisted of OTUs belonging to a few bacterial families of the phyla Proteobacteria, Actinobacteria and Bacteroidetes and a subset of bacterial exemplars of this community were successfully isolated (Fig. 8, Fig. 22). This shared bacterial microbiota represented an ideal starting point for functional assays, as I hypothesized that bacteria of the shared microbiota provide core services for general plant performance.

Bacterial exemplars of the shared microbiota belonging to the phyla Proteobacteria, Actinobacteria and Bacteroidetes were isolated from *A. thaliana* and subsequently tested for their plant growth-promoting activities on *A. thaliana*. To evaluate whether nutrient-limiting conditions trigger distinct responses, plant growth assays were performed under nutrient-sufficient and -deficient conditions (Gruber et al., 2013). In this study, I focused on phosphorus-limiting conditions, as plant-available

phosphorus forms are typically found in low concentrations in the soil and often limit plant growth under natural conditions (Rodríguez and Fraga, 1999). Hence, nutrient-deficient conditions were induced by reducing the phosphorus content in the media from 625 µM to 50 µM KH_2PO_4 (see Methods Chapter I.4.2.13). A detailed review about the relevance of plant-bacteria interactions under phosphorus deficiency can be found in Chapter II.1.2.1.

The reduction of the phosphorus concentration in the media resulted in a clear growth reduction of *A. thaliana*, as seen by a reduction of shoot biomass by ~40% and primary root length by ~60% as well as an increase of the total number of root hairs (Fig. 23A). Root length represented the most reproducible phenotype and was used as a major readout for subsequent experiments. Approximately 70% of the tested bacterial isolates altered primary root growth under phosphorus-sufficient and -deficient conditions, with most isolates increasing root length (Fig. 23B). Notably, the effect on root length was more pronounced under phosphorus deficiency compared to sufficiency (Two-way ANOVA; $p < 0.001$), implying that the root growth-promoting activities of the bacterial isolates or the plant's responsiveness to this service is stronger under abiotic stress conditions.

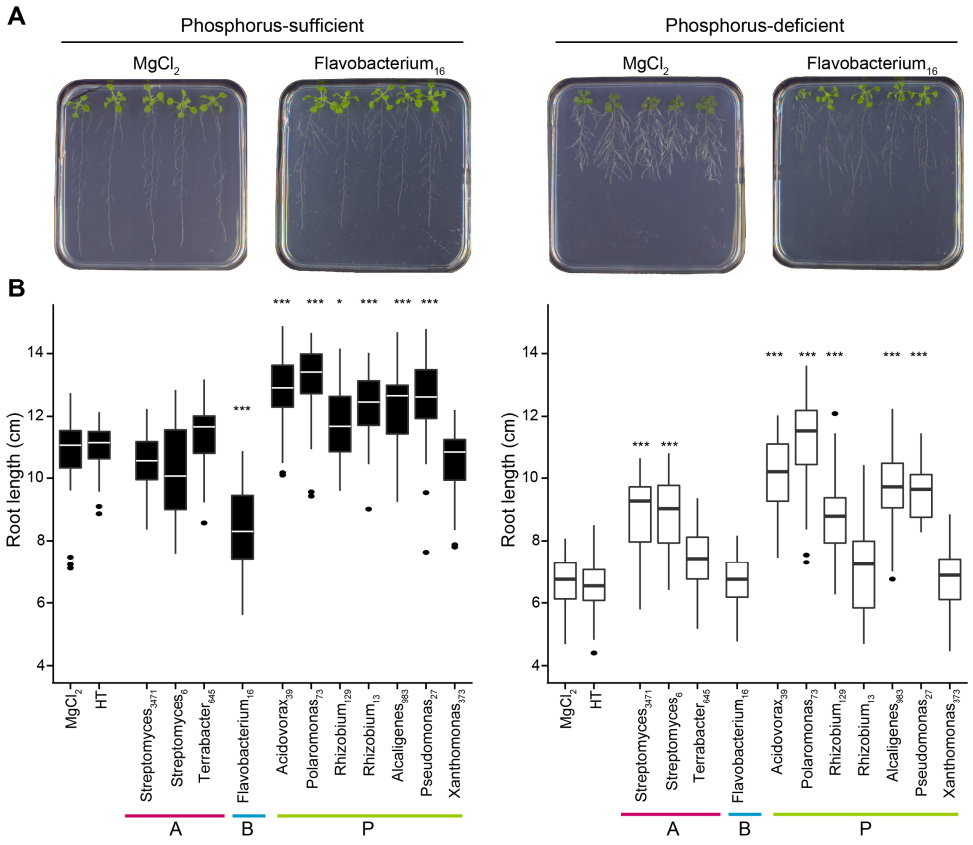


Fig. 23: The majority of bacterial isolates of the shared root microbiota affect root growth dependent on the nutrient status. **(A)** *A. thaliana* grown under phosphorus-sufficient (625 µM KH_2PO_4) or -deficient (50 µM KH_2PO_4) conditions after adding $MgCl_2$ (mock-treated) or Flavobacterium$_{16}$ at a final density of 10^5 cells per ml **(B)** Primary root length of *A. thaliana* grown under phosphorus-sufficient (black) and -deficient (white) conditions after mock treatment ($MgCl_2$ and heat-treated bacteria (HT)) or after co-inoculating bacteria belonging to Proteobacteria (P), Actinobacteria (A) or Bacteroidetes (B) with the plant. Number behind bacterium = OTU-ID. n = 30-45 plants measured in three independent biological replicates. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Dunnett's test using $MgCl_2$ as control, performed separately for the two phosphorus concentrations).

Qualitative and quantitative phenotypic differences were induced by the tested bacterial isolates under phosphorus-sufficient and -deficient conditions. For example, two of the tested Streptomyces isolates increased root length of *A. thaliana* under phosphorus-deficient conditions, but none of the tested Actinobacteria altered root length under phosphorus-sufficient conditions. This suggests that bacteria belonging to Streptomyces might be important for the plant under phosphorus-limiting conditions. Flavobacterium$_{16}$ (phylum Bacteroidetes) was the only bacterial isolate that inhibited root growth, but this effect was only detected under phosphorus-sufficient conditions. Most of the tested Proteobacteria increased root length, regardless of the nutrient status. The only exception was Rhizobium$_{13}$ that increased root length only under phosphorus-sufficient but not -deficient conditions.

In summary, the effects of the tested bacterial isolates on root growth depended on their taxonomic identity as well as on the supplied nutrient concentrations. Most of the tested bacteria appear to be tolerated or even favored by *A. thaliana*, as no negative phenotypes were detected that implied the induction of a defense response, such as the formation of anthocyanins or necrosis.

As these results indicate that a subset of the tested bacteria possess plant growth-promoting activities in binary interactions, I tested whether these activities were maintained in a community context. It is possible that the plant growth-promoting activity is positively influenced by bacteria-bacteria interactions or alternatively competition events among bacteria could negatively affect plant growth (Requena et al., 1997; Toro et al., 1997). Thus, the previously examined bacteria were tested in simple, defined synthetic communities for their effect on *A. thaliana*.

To this end five different synthetic communities were developed: two rather simple communities consisting each of one representative isolate from the phyla Proteobacteria, Actinobacteria and Bacteroidetes (referred to as triad community), as well as two more complex communities representing 4 or 7 bacterial members. More specifically, the triad communities were composed of Acidovorax$_{39}$ (Triad A) and Polaromonas$_{73}$ (Triad B) from the family Comamonadaceae (phylum Proteobacteria), Flavobacterium$_{16}$ from the Flavobacteriaceae (phylum Bacteroidetes) and Streptomyces$_{6}$ from the Streptomycetaceae (phylum Actinobacteria). The more complex Community A was designed to display an increased complexity compared to the triad community and included several bacteria belonging to the three phyla of the shared microbiota (Actinobacteria: Streptomyces$_{1369}$, Streptomyces$_{6}$, Terrabacter$_{645}$. Bacteroidetes: Flavobacterium$_{16}$. Proteobacteria: Acidovorax$_{39}$, Rhizobium$_{129}$, Xanthomonas$_{373}$). Community B represented a taxonomically more narrow community that included only bacteria belonging to the phylum Proteobacteria (Polaromonas$_{73}$, Rhizobium$_{13}$, Pseudomonas$_{27}$, Alcaligenes$_{983}$), as most bacteria assigned to this phylum increased root length in binary interactions regardless of the nutrient status (Fig. 23B). Additionally, the combination of Community A and Community B was assembled and represented the most complex community tested.

Co-inoculation of *A. thaliana* with the simpler triad communities Triad A and Triad B increased primary root length under phosphorus-deficient but not under -sufficient conditions (Fig. 24). The absence of an increase of root length under phosphorus-sufficient conditions was reminiscent of the

individual effects observed in the binary interactions (Fig. 23B). Under phosphorus sufficiency Acidovorax$_{39}$ and Polaromonas$_{73}$ increased root length, whereas Streptomyces$_{6}$ exhibited no effect and Flavobacterium$_{16}$ inhibited root growth. Hence, it appears that the net effect of the triad community (no growth promotion) results from the averaging of the positive, neutral and negative effects of their three community members. In contrast to phosphorus-sufficient conditions, under phosphorus-deficient conditions two of three triad members positively affected root growth in the binary interaction. Therefore, it appears possible that the net effect of the three individual members explains the increase of root length induced by the synthetic triad community.

Under phosphorus sufficiency the complex Community B increased primary root length, while Community A did not affect root length and the combination of both communities (Community A+B) reduced root length. Importantly, under phosphorus deficiency all complex communities increased root length, confirming the observed phenotypes of the tested bacteria under binary interactions (Fig. 23B). However, the reduced root length of *A. thaliana* after inoculating the combined Community A+B under phosphorus-sufficient conditions was unexpected when considering the results from the binary interactions. A possible explanation for the negative impact of Community A+B on root growth could be that the combination of bacteria within those communities induced bacteria-bacteria interactions that negatively affected individual community members and thereby reduced root growth. However, as Community A+B negatively influenced plant growth only under phosphate-sufficient but not -deficient conditions, this phenotype is further influenced by the nutrient status. Additional experiments are required to evaluate bacteria-bacteria interactions for a better interpretation of these results through the determination of the relative abundances of individual bacteria in synthetic communities. This could be accomplished by community profiling of the 16S rRNA gene by Illumina sequencing.

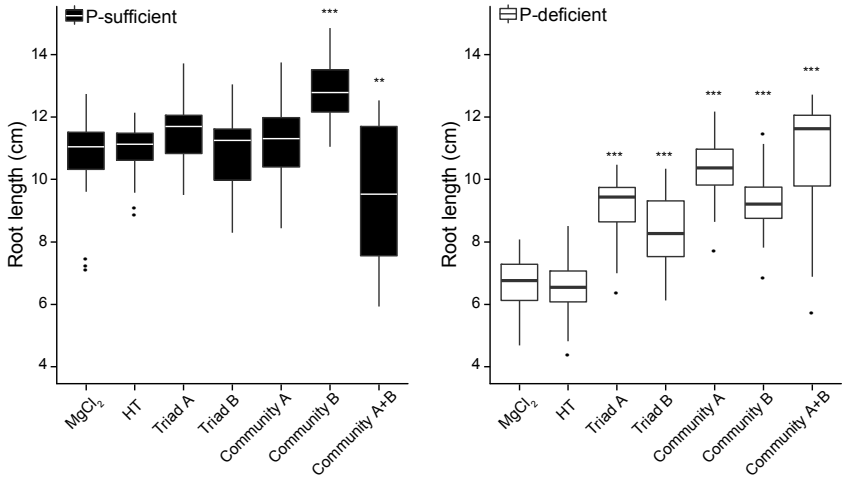


Fig. 24: Defined synthetic bacterial communities mostly maintain their ability to affect root growth under phosphorus-sufficient and -deficient conditions. Triad A = Acidovorax$_{39}$, Flavobacterium$_{16}$ and Streptomyces$_{6}$; Triad B = Polaromonas$_{73}$, Flavobacterium$_{16}$ and Streptomyces$_{6}$; Community A consists of: Streptomyces$_{1369}$, Streptomyces$_{6}$, Terrabacter$_{645}$, Flavobacterium$_{16}$, Acidovorax$_{39}$, Rhizobium$_{129}$, Xanthomonas$_{373}$. Community B consists of Rhizobium$_{13}$, Pseudomonas$_{27}$, Alcaligenes$_{983}$, Polaromonas$_{73}$: Individual bacteria were adjusted to a density of 10^5 cells per ml and afterwards bacteria were added together in equal amounts. HT = heat-treated Community A+B. n = 30-45 plants measured in three biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's test using $MgCl_2$ as control, performed separately for both nutrient conditions).

These experiments evidence that *A. thaliana* relied on bacterial growth-promoting activities to a lesser extent when grown under nutrient-sufficient conditions, whereas all inoculated bacterial communities provided a beneficial service for the host under nutrient-limiting conditions (Two-way ANOVA comparing phosphorus-sufficient and -deficient conditions; $p < 0.001$). This observation indicates that the root growth-promoting activities of the tested bacterial isolates are important for plant performance, especially under abiotic stress conditions. In summary, 9 out of the 10 tested synthetic communities at two levels of phosphorus availability maintained their root growth-promoting activities and the growth effects of the assembled communities can at least be partially predicted from the individual effects observed under binary interactions.

2.4.3. Functional role of root-associated bacteria on *A. thaliana* relatives

Numerous root-associated bacteria of the shared microbiota of related Brassicaceae species that were isolated from *A. thaliana* exerted a beneficial effect on root growth of *A. thaliana*. However, as these bacteria belong to families that were not only enriched on *A. thaliana* but also on *C. hirsuta* and *A. alpina* based on culture-independent approaches, I hypothesized that these bacteria also influence growth of other Brassicaceae (Fig. 8). Therefore, I evaluated three of the previously tested bacterial isolates in binary interactions as well as in a synthetic community on all three Brassicaceae species.

Plant morphology of the tested Brassicaceae plant species differed substantially during the growth assays as shoot biomass of *C. hirsuta* was ~ 50% higher than that of *A. thaliana* (Fig. S 3). Additionally, phosphorus deficiency reduced root length of *A. alpina* but only had a minor effect on root growth of *C. hirsuta*. To correct for such differences, root length and shoot fresh weight were normalized to the respective $MgCl_2$ treatment for each plant species, separately for each phosphorus condition. In general, all plant species displayed a normal phenotypic growth behavior in the gnotobiotic growth system, allowing to compare phenotypes induced by different bacteria on the three Brassicaceae species.

Bacterial treatment induced significant differences in primary root length of *A. thaliana*, *C. hirsuta* and *A. alpina* (Fig. 25, Fig. S 3). Acidovorax increased root length under phosphorus-deficient conditions of *A. thaliana* and *A. alpina* but not *C. hirsuta* and did not affect root length of all three plants under phosphorus-sufficient conditions. Streptomyces on the other hand decreased root length of *C. hirsuta* and *A. alpina* but not of *A. thaliana* under phosphorus sufficiency. However, under phosphorus-deficiency Streptomyces increased root length of *A. thaliana* but did not affect the other two species. Flavobacterium reduced root length of all three Brassicaceae species under phosphorus-sufficient conditions and under phosphorus deficiency only of *A. alpina*. Finally, the triad community reduced and increased root length under phosphorus-sufficient and -deficient conditions, respectively. In both cases, the strongest effect was detected for *A. alpina*. Shoot fresh weight remained mostly stable after bacterial treatment under both nutrient conditions with the exception of a significant decrease of shoot biomass of *C. hirsuta and A. alpina* but not *A. thaliana* by the Streptomyces isolate under

phosphorus-deficient conditions (Fig. 25, Fig. S 3). Additionally, shoot fresh weight of *A. alpina* decreased upon treatment with the Flavobacterium isolate under these conditions.

To conclude, bacteria of a shared bacterial root microbiota, as identified by culture-independent sequencing approaches, do not necessarily induce similar phenotypes on related plant species. This is not unexpected, as the tested bacterial exemplars were initially isolated from *A. thaliana*. It is possible that the tested bacteria were best adapted to their original host *A. thaliana* and less well adapted to other hosts. It seems plausible that bacteria provide more targeted host services and trigger stronger responses on their proper host compared to hosts to which they are not adapted. Further assays will need to include additional and more diverse synthetic communities that are composed of bacterial strains isolated from the different Brassicaceae hosts. Subsequently, reciprocal inoculations will permit to test whether the shared bacterial community originating from different hosts species also exerts similar functional capacities.

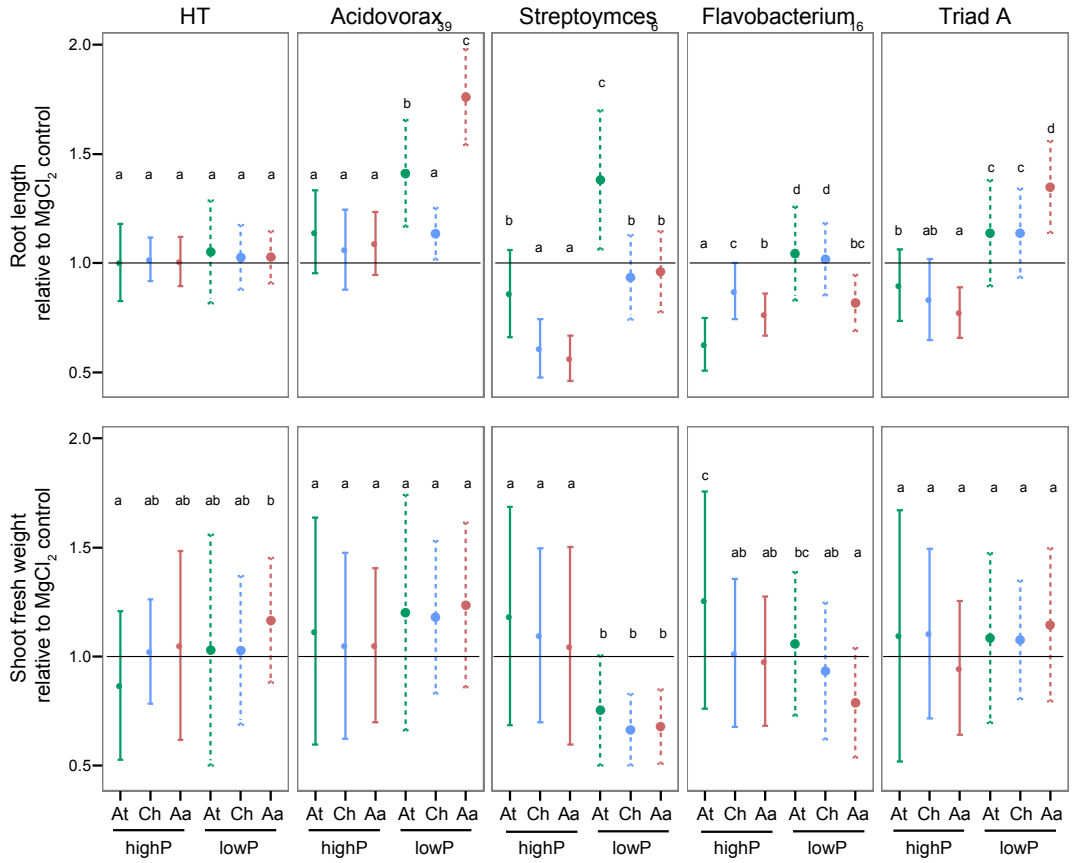


Fig. 25: Bacteria of the shared microbiota affect root and shoot growth of *A. thaliana*, *C. hirsuta* and *A. alpina*. Acidovorax$_{39}$ (phylum Proteobacteria), Streptomyces$_{6}$ (phylum Actinobacteria) and Flavobacterium$_{16}$ (phylum Bacteroidetes) were isolated from *A. thaliana* and tested on three related species (*A. thaliana* (At), *C. hirsuta* (Ch) and *A. alpina* (Aa)). Plant growth assays were performed under phosphorus-sufficient (highP) and -deficient (lowP) conditions after adding $MgCl_2$ and heat-treated (HT) bacteria as controls or Streptomyces$_{6}$, Flavobacterium$_{16}$ and Acidovorax$_{39}$ in single inocula at a final density of 10^6 cells per ml. A triad community was assembled from the three bacterial isolates by adjusting each bacterium to a density of 10^6 cells per ml and then combining bacteria in equal amounts. HT samples were generated by heat-treating the triad community. Root length was normalized to the $MgCl_2$ control of the respective host plant, separately for both phosphorus conditions (non-normalized figure can be found in Fig. S 3). Shown is the mean (dot) and the standard deviation. n = 30-45 plants measured in three independent biological replicates. Significance letters are based on Tukey's HSD using a p-value cut-off < 0.05 and performed separately for the different bacterial treatments but combined for both phosphorus concentrations.

3. Discussion

3.1. Compartment, soil type, residence time and environmental conditions are major determinants of community structure

In this study, the major factor determining bacterial community structure was the tested compartment, which is consistent with previous studies, for example on *A. thaliana*, rice and cucumber (Bulgarelli et al., 2012; Edwards et al., 2015; Ofek-Lalzar et al., 2014). Clear differences among the soil, rhizosphere and root compartments were detected in alpha-diversity, PCA clustering and taxonomic composition (Fig. 4, Fig. 5, Fig. 6). Focusing on alpha-diversity, the root samples displayed a reduced richness (number of detected OTUs) as well as a reduced diversity (Shannon index) compared to the rhizosphere and soil compartments. The major phyla present in the root compartment were with decreasing relative abundance Proteobacteria, Actinobacteria and Bacteroidetes, whereas Firmicutes and Acidobacteria were enriched in unplanted soil and excluded from the root compartment. The rhizosphere displayed an intermediate taxonomic pattern compared to the soil and root compartments, as soil-associated Firmicutes but also root-enriched Bacteroidetes were detected in the rhizosphere (Fig. 13). The differences between the compartments were evident across two distinct soil types, three soil batches, two environmental conditions, three time points (spanning 6 weeks to 7 months) and three plant species (Fig. 20, Fig. 14 and Fig. 7). Collectively, my findings suggest that the root microbiota establishment begins in the *A. alpina* rhizosphere, where a marked initial community shift occurs and continues in the root tissue, leading to the establishment of a bacterial community inside roots that is markedly distinct from the surrounding soil biota. This indicates that the three tested compartments represent distinct environmental niches, differing for example in nutrient composition, that are more successfully colonized by individual taxonomic groups. In a wider context, the observation that distinct compartments assemble different communities correlates well with other model systems, as humans display distinct gut, mouth and skin bacterial communities (Costello et al., 2009).

A. alpina not only exhibited a distinct root but also rhizosphere microbiota, as evidenced by a rhizosphere community dissimilar from the surrounding soil, exemplified by the higher relative abundance of Bacteroidetes in the rhizosphere (Fig. 12, Fig. 13, Fig. 14). The rhizosphere effect appears to be a specific trait of *A. alpina*, because the related Brassicaceae species *A. thaliana* and *C. hirsuta* displayed a weak or no differentiation of rhizosphere communities (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014). This is somewhat surprising, as the bacterial root microbiota of *A. thaliana*, *C. hirsuta* and *A. alpina* exhibited only minor quantitative differences (Fig. 6, Fig. 7). This indicates that even though the three Brassicaceae species host a highly similar root-inhabiting microbiota, there are striking differences among the tested host species in their ability to influence the rhizosphere microbiota. *A. alpina* distinguishes from *A. thaliana* and *C. hirsuta* mainly by (i) an adaptation to a more extreme environment and (ii) its perennial lifestyle. Possibly, these characteristics could explain the presence of a rhizosphere effect for *A. alpina*. Plant life in the arctic-alpine environment is challenging,

because of its rocky soil structure, low nutrient availability and harsh environmental conditions with extreme fluctuations of abiotic parameters such as temperature and UV-light irradiation (Tab. 3; Koch et al., 2006). Therefore, rhizosphere bacterial communities might support plants like *A. alpina* in balancing these fluctuations and/or increasing nutrient availability. Perennialism could also explain the observed rhizosphere effect, as perennial plants reside in the soil for an extensive period of time and have more time to actively influence the surrounding rhizosphere microbiota compared to annual plants. In support of this assumption, it was reported that the rhizosphere effect depends on succession time of plants in the soil (Tscherko et al., 2005). The rhizosphere of 16 plant species, including pioneer vegetation and resident alpine grassland, which resided in alpine soil in Austria for 4-135 years, was investigated by phospholipid fatty acid analysis and fluorimetric microplate assays to determine community composition and enzymatic activity, respectively. 4 year-old sites displayed a lower bacterial abundance and enzymatic activity in the rhizosphere compared to sites that were established since 75 and more years. This indicates that bacterial community size and activity are affected by succession time of plants in the soil. However, also annual maize was reported to influence rhizosphere communities, thus also plants with a shorter lifespan actively influence the rhizosphere microbiota (Peiffer et al., 2013). Maize differs from the tested Brassicaceae, as maize exhibits a larger root system and secretes different amounts of organic acids compared to *A. thaliana* (Gaume et al., 2001; Badri et al., 2008). The composition and levels of root exudates of *A. alpina* remain to be quantified in comparison to other Brassicaceae but could explain the differential influence on the rhizosphere microbiota. In summary, the presence of a rhizosphere effect in *A. alpina* cannot solely be explained by the perennial lifestyle or adaptation towards an extreme environment but might be associated with other, yet undiscovered, plant characteristics.

Soil type and soil batch are additional major determinants of community structure. This was evident by distinct communities harbored in the soil, rhizosphere and root samples of *A. alpina* plants grown in Cologne and French soils as well as in root communities of three Brassicaceae plant species grown in three soil batches (Fig. 18, Fig. 5). One marked difference between the Cologne and French soils was the absence of Firmicutes and low relative abundance of Acidobacteria in the French compared to the Cologne soil (Fig. 19, Tab. S 3). The importance of soil type for structuring communities is in accordance with previous findings for *A. thaliana*, other related Brassicaceae, maize or rice (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014; Peiffer et al., 2013; Edwards et al., 2015). This observation might be explained by the fact that soil defines the microbial start inoculum as well as the ‘diet’ of plants. Different soil types are characterized by differences in nutrient composition and availability (Tab. 3) and this in turn appears to be a major determinant underlying soil type-dependent differences of bacterial communities. Similarly, diet is a factor that influences gut community structure in mammals (David et al., 2014; Carmody et al., 2015). For example, the switch of human diet from an animal- to a plant-based nutrition resulted in a changed gut microbial community and was detected within one day with shifts in the taxonomic composition and metabolic activity as measured by Illumina

sequencing of the 16S rRNA gene and metatranscriptomics (David et al., 2014). Accordingly, *A. alpina* grown in French or Cologne soil encounters different nutrient conditions (Tab. 3), such as a lower plant-available phosphorus concentration in the French soil, potentially explaining the low conservation of the bacterial root and rhizosphere microbiota at OTU rank (Fig. 20).

In addition to the soil type-dependent nutrient composition, also the pH differed between the French and Cologne soils, with pH values of 7.6 and 6.7, respectively (Tab. 2). The composition of the soil microbiota was shown to be strongly influenced by soil pH (Fierer and Jackson, 2006; Lauber et al., 2009). Fierer and Jackson investigated the bacterial community structure across hundred different sites in North and South America, where species richness and diversity varied strongly between sites and these variations correlated best with shifts in soil pH. Striking differences were observed in low and high pH soils, whereas richness and diversity of bacterial communities at moderate pH ranging from 6.5 to 7.5 appeared largely similar. Thus, the pH differences between the French and Cologne soils are likely of minor importance for explaining the differences in community structure compared to the differences in nutrient content (Tab. 2, Tab. 3).

In addition to compartment and soil type, environmental conditions play a major role in the differentiation of the *A. alpina* associated bacterial community (Fig. 20). Especially the rhizosphere and root compartments of *A. alpina* grown in the French soil in the greenhouse or in the native soil in the French Alps displayed distinctive bacterial community profiles (Fig. 20). *A. alpina* plants growing in the French Alps need to cope with a nutrient-depleted soil (Tab. 3), fluctuating temperatures, freezing and a high UV-light irradiation (Chapin, 1983; Caldwell et al., 1980). Both the plant as well as microbial communities respond to these environmental conditions. For plants it was demonstrated that root exudation is influenced by temperature as well as light conditions (Rovira, 1969). Therefore, *A. alpina* grown in the French Alps might exhibit an altered exudation profile, contributing to shifts in bacterial community composition in the rhizosphere and root compartments. Bacteria also need to adapt to the arctic-alpine environment and strategies to survive under colder temperatures are the production of cold-adapted active enzymes, maintenance of the fluidity of cell membranes or the production of cryoprotectants (Margesin and Miteva, 2011). The arctic-marine bacterium *Colwellia psychrerythraea* displays distinct gene repertoires that allow this bacterium to adapt to extreme temperature regimes (Methé et al., 2005). Part of this adaptation includes a gene repertoire encoding for proteins functioning in polyunsaturated fatty acid synthesis (involved in enhancing membrane fluidity) as well as genes predicted to encode extracellular polysaccharides and enzymes (involved in cryoprotection). Therefore, only bacteria with the best adapted genetic repertoire will persist in environments with extreme temperatures, such as the French Alps, and will be subsequently recruited by the plant. In further experiments it will be interesting to isolate bacterial strains from roots of plants grown in contrasting environments and assess their ability to thrive under various abiotic stress conditions, such as freezing.

Besides soil type and environmental conditions, residence time of *A. alpina* in the soil induced shifts in the composition of root- and rhizosphere-inhabiting bacterial communities. For example,

Bacteroidetes were abundant members of the rhizosphere and root compartments of *A. alpina* at 6 weeks but at later time points the relative abundance of this phylum decreased approximately by 50% (Fig. 13, Tab. S 1). This suggests that Bacteroidetes are out-competed over time by more successful colonizers, which could be mediated by changes in microbe-microbe interactions among microbiota members, regulating community structure over time. Alternatively, *A. alpina* might counter-select Bacteroidetes at later time points, thereby inhibiting the proliferation of members of this phylum over time. Comparing the results of this work with other studies on the root bacterial microbiota, no consistent picture for the stability or dynamics of bacteria over time can be drawn. *A. thaliana* did not display distinct bacterial root communities when comparing vegetative, flowering or senescence plants that were grown under stable greenhouse conditions as well as in the field (Lundberg et al., 2012; Schlaeppi et al., 2014). A similar observation was found for root- and leaf-inhabiting bacterial communities of field-grown *Nicotiana attenuata* plants that were collected at five developmental stages (Santhanam et al., 2014). However, *N. attenuata* as well as most *A. thaliana* accessions complete their lifecycle in a short time period (typically ~ 3 months) and such a time period might not be enough to induce shifts in community composition. A different picture was observed for the rhizosphere microbiota of potato plants that were grown in the field for up to 150 days post planting (dpp), where plants residing in the seedling (30 dpp), flowering (~70 dpp) and senescence stage (~140 dpp) were sampled and processed using pyrosequencing (İnceoğlu et al., 2011). The bacterial communities associated with potato roots displayed a time-dependent community shift at phylum rank, as for example the relative abundance of Alphaproteobacteria increased in the rhizosphere compartment over time. However, also bacterial community composition of unplanted soil fluctuated over time, indicating variations in the initial start inoculum, making the interpretation of the observed community shifts in the root compartment difficult. In the human gut the stability of bacterial communities was monitored during a 5-year time period using Illumina and whole-genome sequencing (Faith et al., 2013). The composition of the gut microbiota changed over time, with gut communities reaching a more stable composition towards the end of the time course experiment, while being more variable during the first year. Interestingly, Proteobacteria and Firmicutes were unstable over time, suggesting that within humans these phyla are either less competitive or counter-selected by the host. In general, this indicates that time represents one factor that determines community composition. One limitation of this study is that it is unclear, whether the observed shifts in root and rhizosphere bacterial community composition are due to the longer residence time of plants in the soil, altered nutritional conditions in the soil over the time course experiment or whether this represents a specific feature of *A. alpina*. To dissect these possibilities, more perennial plants need to be investigated under the same growth conditions and time points, including detailed soil analyses during the time points of harvest. To test whether bacterial root and rhizosphere communities reach a stable composition after 7 months or evolve further, *A. alpina* could be tested in a multigenerational experiment.

In summary, the major determinants of bacterial community structure in this study were the tested compartment, soil type and batch, environmental conditions and residence time of plants in the soil. However, even though a large proportion of the bacterial community was affected by these factors, approximately 15% of OTUs were shared across distinctive growth conditions and these shared OTUs consisted of few taxonomic lineages (Fig. 20, Tab. S 2). The shared root microbiota of *A. thaliana*, *C. hirsuta* and *A. alpina* was dominated by members of the Proteobacteria (families Pseudomonadaceae, Comamonadaceae, Oxalobacteriaceae), followed by a lower relative abundance of Bacteroidetes (family Flavobacteriales) and Actinobacteria (family Streptomycetaceae). This indicates that plants possess a coordinated assembly mechanism for bacterial communities that is independent of soil type, environmental conditions or residence time of plants in the soil. An alternative explanation could be that this shared microbiota includes the most competitive bacteria for colonizing plant-associated habitats.

3.2. Plant species and plant development influence community structure only to a minor extent

In contrast to the strong influences of soil type, environmental conditions and residence time of plants in the soil on community structure, different Brassicaceae plant species harbored taxonomically similar bacterial root communities (Fig. 6, Fig. 7). The root microbiota of *A. thaliana*, *C. hirsuta* and *A. alpina* exhibited an overall similar taxonomic structure, with most OTUs shared between species. This is in accordance with previous studies that demonstrated a weak influence of host genotype on bacterial community composition for *A. thaliana*, rice or maize (Lundberg et al., 2012; Bulgarelli et al., 2012; Schlaeppi et al., 2014; Edwards et al., 2015; Peiffer et al., 2013).

Only a few OTUs and families significantly differed between *A. thaliana*, *C. hirsuta* and *A. alpina* (Fig. 6, Fig. 7). While *A. thaliana* enriched for Thermomonosporaceae, *C. hirsuta* was characterized by a reduced relative abundance of Nannocystaceae and *A. alpina* by a higher relative abundance of Comamonadaceae, Burkholderiales incertae sedis and Phyllobacteriaceae (Fig. 6). These differences between the three host species could be explained by many factors. First of all, the largely similar bacterial community between *A. thaliana* and *C. hirsuta* was expected, as both species co-exist in the same natural habitat. Therefore, both plant species likely have adapted similarly to the soil bacterial microbiota as well as to the nutrient composition and environmental conditions in the soil (Ratcliffe, 1961). Conversely, differences between all three species could explained by differences in root architecture, altered root exudation profiles or in the case of *A. alpina* the perennial lifestyle and adaptations to a more extreme environment. *C. hirsuta* has a more branched root system architecture compared to *A. thaliana* and *A. alpina* and this might represent a more attractive habitat for bacteria. Species differences in the bacterial root microbiota might be additionally explained because distinct plant species secrete differentially composed root exudates, as for example *Cardamine amara* does not produce methionine-derived glucosinolates (Windsor et al., 2005). Root exudates are thought to be

major signaling cues for plant-microbe interactions (Bais et al., 2006). Therefore, appears possible that differences in plant exudate profiles result in distinct microbial communities. This hypothesis should be addressed by evaluating root exudation profiles of the three Brassicaceae plant species under the same growth conditions. Finally, as *A. alpina* adapted to a distinct habitat compared to the other two Brassicaceae, the largely similar community structure was more unanticipated (Fig. 7). This finding could be explained by the fact that even tough *A. alpina* inhabits a distinct, natural habitat, bacteria are still required for the same services as for other plant species, when tested under the same growth conditions. Examples for potentially important bacterial services are enhancing nutrient availability, protecting against pathogens or increasing abiotic stress tolerance (Lugtenberg and Kamilova, 2009).

Few comparative approaches were undertaken to study the structure of the bacterial root microbiota of plant species other than the Brassicaceae, making an overall conclusion difficult. One recent study compared the bacterial root microbiota of monocotyledonous wheat and dicotyledonous cucumber (Ofek-Lalzar et al., 2014). 15.6% of the total observed variation between the bacterial communities inhabiting roots of wheat and cucumber was explained by host genotype based on a PCA, whereas *A. thaliana*, *C. hirsuta* and *A. alpina* did not separate within the PCA in this study (Fig. 5). The variation between wheat and cucumber was mainly due to *Pseudomonas* being of a high relative abundance in wheat and *Cellvibrio* in cucumber. Monocots and dicots diverged around 150-200 million years ago, while *A. thaliana* and *A. alpina* radiated approximately 45 million years ago (Wolfe et al., 1989; Chaw et al., 2004; Beilstein et al., 2010). Therefore, the comparison of wheat and cucumber suggests that a long evolutionary divergence time and the resulting differences in the genetic background and plant physiology appear to alter community structure.

The use of *A. alpina* as a model system permitted me to evaluate the influence of plant developmental stage on bacterial community structure separately of the residence time of plants in the soil. Typically, the impact of developmental stage on community structure is difficult to dissect, as most studies do not separate between residence time and developmental stage, as plants at different developmental stages are typically collected at different time points. The *A. alpina pep1* mutant represents a useful genetic tool to disentangle the influence of time and plant developmental stage on bacterial community structure. The parallel growth of *A. alpina* wild type and *pep1* plants allowed to compare bacterial communities inhabiting the root and rhizosphere of a flowering with a non-flowering plant at the same time point. Remarkably, plants residing in different developmental stages did not or only to a minor extent differ in the structure of their bacterial root and rhizosphere microbiota (Fig. 15, Fig. 21). These results permit to reject the hypothesis that bacterial community structure differs between flowering and vegetative grown plants. As root exudation of *A. thaliana* depends on plant developmental stage (Chaparro et al., 2013) one would assume stronger differences between flowering and non-flowering plants. *A. thaliana* at seedling or vegetative stage secretes a higher proportion of sugars and alcohols compared to flowering or senescent plants that in turn produce more amino acids and phenols compared to younger plants. Additionally, flowering *A. thaliana* plants secrete more defense-related proteins compared to

non-flowering plants and this could also affect bacterial community structure (De-la -Peña et al., 2010). However, the present study reveals no influence of plant developmental stage on the structure of bacterial communities. To further understand the role of root exudates for bacterial community composition, it is required to determine the exudations profiles of *A. alpina* WT and *pep1* plants, as it is so far unclear whether the *pep1* mutant displays an altered root exudation profile. However, also natural *A. alpina* accessions that resided in different developmental stages did not display distinct root or rhizosphere bacterial communities (Fig. 21), confirming the low impact of plant developmental stage on root microbiota structure. It is still possible that different developmental stages induce functional shifts within a structurally stable community. A recent study coupling a transcriptomic analysis with 16S rRNA amplicon sequencing investigated the bacterial rhizosphere microbiota of *A. thaliana* (Chaparro et al., 2014). The rhizosphere microbiota was mostly stable based on community profiling of the 16S rRNA gene, but gene expression depended on plant developmental stage. For example, expression of bacterial genes associated with streptomycin biosynthesis increased during flowering. Therefore, bacterial communities appears not to be affected by plant developmental stage on structural but potentially on functional level.

To conclude, the plant-dependent factors host species and plant developmental stage only play a minor role or do not affect community structure at all compared to the strong influence of compartment, soil type, environmental conditions and residence time of plants in the soil. The compartment-specific communities in the soil, rhizosphere and roots indicate that the plant provides an environmental niche with specific nutritional conditions for bacteria, highlighting the importance of plant-microbe interactions for community assembly. Additionally, the importance of plant-independent factors for community structure, such as soil type, residence time or environmental conditions, indicates that microbe-microbe interactions are a strong factor shaping communities and the importance of these interactions needs to be addressed in future studies.

3.3. Defining a shared Brassicaceae root microbiota

By comparing bacterial root communities of the three Brassicaceae species *A. thaliana*, *C. hirsuta* and *A. alpina*, I could demonstrate that these plant species assemble a shared bacterial root microbiota (Fig. 8). This shared microbiota comprised members of Proteobacteria, Actinobacteria and Bacteroidetes. These three phyla consisted of a few bacterial orders, including Burkholderiales (families Oxalobacteriaceae, Burkholderiales incertae sedis, Comamonadaceae), Actinomycetales (Thermomonosporaceae and Streptomycetaceae) and Flavobacteriales (Flavobacteriaceae). The taxonomic composition of this shared bacterial microbiota on Brassicaceae is consistent with a previous study of *A. thaliana* and *C. hirsuta* plants that were grown under controlled environmental conditions in the greenhouse and harvested at two natural sites in Germany (Schlaeppi et al., 2014). In this study raw sequence data from several studies on *A. thaliana* root profiles was compared to determine a shared root microbiota across studies that used distinct soil types, plant accessions and PCR-primer combinations

(Lundberg et al., 2012; Bulgarelli et al., 2012; Bodenhausen et al., 2013). This analysis compared *A. thaliana* grown at 10 different sites and using the primer combinations 799F-1193R (Schlaeppi et al., 2014; Bodenhausen et al., 2013); 799F2-1193R (Bulgarelli et al., 2012) and 1114F-1393R (Lundberg et al., 2012). This effort revealed that Actinomycetales were a common feature at higher taxonomic rank, while no common OTUs were found across all studies (Schlaeppi et al., 2014). The absence of shared OTUs across these studies could be due to several reasons, such as use of different primers, sampling procedures, start inocula as well as a combination of these factors. Not only the bacterial root microbiota of Brassicaceae plants but also that of other plant species, such as cannabis, poplar and rice, was investigated (Shakya et al., 2013a; Winston et al., 2014; Edwards et al., 2015). The root microbiota of cannabis and poplar enriched for members of the Proteobacteria and Actinobacteria, whereas in the root microbiota of rice Proteobacteria and Bacteroidetes were of a higher relative abundance compared to unplanted soil. This suggests that Proteobacteria are a conserved phylum across plant species, whereas Actinobacteria and Bacteroidetes assemble dependent on the plant lineage. For a final definition of a shared root microbiota additional plant lineages and a multitude of different growth conditions should be tested using standardized sampling and sequencing protocols.

The 26 OTUs of the shared root microbiota of *A. thaliana*, *C. hirsuta* and *A. alpina* represented about half of the total community in size, contained abundant as well as low-abundant taxonomic groups and comprised a few taxonomic lineages (Fig. 8). The observation of a shared community points towards a coordinated assembly of the bacterial root community across numerous environmental conditions. The assembly of the shared microbiota is potentially driven by two factors. First, bacteria in the soil respond to host-derived cues, such as root exudates (Bulgarelli et al., 2012; Bais et al., 2006), and second, bacteria-bacteria interactions between members of the shared microbiota allow for a competitive advantage for root colonization (Bakker et al., 2014; Kinkel et al., 2011).

The presence of a shared root microbiota associated with Brassicaceae plant species suggests an important function of these taxonomic groups for plant performance (Shade and Handelsman, 2012). Bacterial isolates with the same taxonomic assignments found among the shared Brassicaceae root microbiota included Burkholderiales, Actinomycetales and Flavobacteriales that were mostly studied in simplified binary systems, providing first insights in their functional relevance. Their functional investigation in a community context was done in the frame of this thesis (Fig. 24, see below for discussion). One described function for bacteria of the order Burkholderiales is their ability to suppress soil-borne diseases (Benítez and Gardener, 2009). For example, Pelomonas decreased growth of several plant pathogens, such as *Phytophthora sojae*, *Rhizoctonia solani* or *Alternaria solani*, indicating a potential function of Burkholderiales as biocontrol strains. Additionally, bacteria of the Burkholderiales order fix atmospheric nitrogen, transform sulfur and enhance general nutrient uptake (Hanson et al., 2012; Schmalenberger et al., 2008). Last, Burkholderiales are potential degraders of pesticides and xenobtiotics, such as atrazine or chlorinated substances (Devers et al., 2007; Mattes et al., 2008; Pérez-Pantoja et al., 2012).

Of the shared Brassicaceae root microbiota members of the Actinomycetales were the major representatives of the phylum Actinobacteria, including the families Streptomycetaceae and Thermomonosporaceae (Fig. 8). One strain, related to Thermomonosporaceae, was isolated from roots of *Casuarina equisetifolia* and was demonstrated to fix atmospheric nitrogen, potentially linking bacteria from this family to nutrient uptake mechanisms (Valdes et al., 2005). Bacteria belonging to Streptomyces have been studied in depth because of their capacity to produce a wealth of secondary metabolites, including antibiotics such as Streptomycin or Vancomycin (Schatz and Waksman, 1944; Solenberg et al., 1997). Accordingly, many Streptomyces strains enhance disease resistance against a range of fungal and bacterial pathogens such as *Botrytis cinerea*, *Alternaria brassicicola*, *Fusarium oxysporum* or *Erwinia carotovora* (Schrey and Tarkka, 2008; Seipke et al., 2012).

Finally, Flavobacteria being the third member of the shared Brassicaceae root microbiota (Fig. 8) are ubiquitous bacteria inhabiting water and glacier environments as well as being abundant in the rhizosphere, root and phyllosphere of plants (Kirchman et al., 2010; Bajerski and Wagner, 2013; Bulgarelli et al., 2012; Schlaeppi et al., 2014; Bodenhausen et al., 2013). A recent comparative genomic analysis demonstrated that plant-colonizing Flavobacteria differ extensively from their aquatic relatives (Kolton et al., 2013). The genomes of plant-associated Flavobacteria contain more genes related to the degradation of plant-related carbohydrates, such as arabinose or pectin, hinting towards an adaptation of Flavobacteria to plants as an ecological niche. Flavobacteria also represent potential biocontrol strains, as volatiles produced by *Flavobacterium johnsoniae* were effective against *Phytophthora capsici* and reduced infection on pepper (Sang and Kim, 2012). An alternative example is the type IX protein secretion system of *Flavobacterium sp*. that was required for defense against the foliar pathogen *Clavibacter michiganensis* (Kolton et al., 2014).

Together, the insights into the functions of single strains with the same taxonomic assignments of the shared Brassicaceae microbiota suggest that these bacteria might have implications in pathogen defense, nutrient availability and xenobiotic tolerance. However, while these studies were conducted using simplified binary plant-bacteria systems, it is not understood, whether these bacterial capacities are maintained in a community context and whether synergistic or additive effects can occur. To address these questions bacterial exemplars of the shared Brassicaceae root microbiota need to be tested in synthetic communities for their effect on plant performance, which presented an aim of this thesis (see below for discussion).

3.4. Members of the bacterial root microbiota are culturable

To address the potential function of the shared bacterial root microbiota, I isolated bacteria from roots of *A. thaliana*, *C. hirsuta* and *A. alpina* grown in the greenhouse and at numerous natural sites in Germany and France. In this effort 2,095 bacteria were isolated, belonging to taxonomically diverse families that were also detected using culture-independent sequencing approaches on OTU and family

rank (Fig. 6, Fig. 22). From the 101 RootOTUs and 26 sharedOTUs defined during the comparison of three related Brassicaceae species ~22% and ~42% could be isolated, respectively (Fig. 22C). The proportion of bacteria isolated from the phyla Proteobacteria, Actinobacteria and Bacteroidetes was similar to the ratio that was observed by the culture-independent approaches, especially after 3 months during the time course experiment (Fig. 13B). Additionally, most of the abundant families detected during the culture-independent sequencing approach could be isolated, expect for Thermomonosporaceae and Myxococcaceae. The ability to recover a high proportion of taxonomic groups that were detected during the culture-independent sequencing approach was unexpected, as predictions state that only a small proportion of bacteria (0.1-10%) that are detected by culture-independent approaches are culturable (Head et al., 1998; McCaig et al., 2001). However, since such estimates were typically based on soil-borne bacterial communities, they might not correlate with bacteria inhabiting plant roots. Comparing 1,264 culturable bacterial isolates with a culture-independent sequencing effort of soil bacterial communities inhabiting four sites in British Columbia an overlap of 22% could be detected (VanInsberghe et al., 2013). This is comparable to percentages of isolated RootOTUs, while the proportion of isolated sharedOTUs was even higher (~42%). Even though an overlap between culture-dependent and -independent approaches existed, differences between both methods were noticed. Qualitative differences occurred as only 22% of the RootOTUs or 42% of the sharedOTUs, as defined during the 16S rRNA gene based profiling of the three Brassicaceae species, could be isolated (Fig. 22C). Quantitative differences were often found at family rank, for example Streptomycetaceae were the third most abundant family on *A. thaliana* roots based on the comparison of the three Brassicaceae plant species, but only few bacteria of this family were isolated (43 isolates; Fig. 22, Fig. 6). Microbacteriaceae on the other hand were often isolated (179 isolates) but were not detected among the 15 most abundant families within the Brassicaceae experiment (Fig. 6). A subset of families could not be cultivated but were abundantly detected on roots of Brassicaceae using culture-independent approaches, such as Thermomonosporaceae and Myxococcaceae (Fig. 6, Fig. 22).

Qualitative as well as quantitative differences between the culture-independent and -dependent approach were expected, because plants and bacteria grown in agar plates and soil encounter distinctive growth conditions, as for example growth media is typically relatively rich in nutrients. Additionally, the culture-dependent approach introduces biases during the isolation procedure, because of media conditions or cultivation time (Davis et al., 2005). Finally, culture-independent approaches are affected by primer biases, PCR-biases or sequencing errors (Coissac et al., 2012; Engelbrektson et al., 2010; Patin et al., 2013). Differences between the culture-independent and -dependent approach were the use of different primer pairs (799F-1193R and 799-1392R, respectively), PCR-amplification protocols (see Methods section Chapter I.4.2.8 and I.4.2.11) and sequencing technologies (pyrosequencing and Sanger-sequencing, respectively). Explanations for the inability to isolate the families Thermomonosporaceae and Myxococcaceae could be an insufficient isolation depth or sub-optimal cultivation conditions. The first hypothesis is relatively unlikely, as over 2,000 bacteria were isolated and Thermomonosporaceae

reached a high relative abundance within the root compartments of *A. thaliana* (relative abundance of ~ 50 per mile; Fig. 6). Both families are generally cultivable, as they were successfully isolated during other studies (Valdes et al., 2005; Bretscher and Kaiser, 1978). A more targeted selection of growth conditions might aid in recovering lacking bacterial families and OTUs. Improved isolation conditions could include a longer incubation period at lower temperatures to avoid that slow-growing bacteria are out-competed by fast-growing ones.

3.5. Bacteria of the shared root microbiota alter root architecture of *A. thaliana*

One aim of this study was to assess the effects of bacteria belonging to the shared root microbiota on plant growth of *A. thaliana*, *C. hirsuta* and *A. alpina*. In a first step, individual bacterial strains with taxonomic assignments of the shared microbiota (belonging to Proteobacteria, Actinobacteria and Bacteroidetes) were co-inoculated with *A. thaliana.* These experiments were conducted under phosphorus-sufficient and -deficient conditions, to evaluate whether these bacteria have the ability to improve plant growth dependent on the nutrient conditions. These experiments revealed that most of the tested bacteria increase root length but not shoot biomass with a more pronounced effect on root length under phosphorus deficiency (Fig. 23, Fig. 25). The lack of improved shoot growth can be explained by the fact that plants were 24 days old when harvested and still relatively young and a positive effect on shoot biomass might take longer to be established. The gnotobiotic growth system (see Methods Chapter I.4.2.13 for details) limited the assay to an incubation time of 24 days, as plants reached the bottom of plates during the end of the experiment. For assays with a longer incubation time an additional experimental system is required. Alternative growth assays should be conducted in a more native, sterile and soil-like system, where additional growth phenotypes should be scored, such as flowering time and seed production.

While the tested bacteria did not affect shoot biomass, ~ 70% of the tested strains increased and only one strain ($Flavobacterium_{16}$) reduced primary root length (Fig. 23). Interestingly, the positive effect on root growth was more pronounced under phosphorus-deficient compared to -sufficient conditions. For example, the two tested Actinobacteria increased root length only under phosphorus-deficient conditions. This could indicate that plants preferentially recruit beneficial bacteria under phosphorus-deficient conditions to increase the amount of plant available phosphorus. However, so far no evidence exists that plants preferentially recruit beneficial bacteria at a higher relative abundance under phosphorus-deficient compared to -sufficient conditions. To address this hypothesis, the relative abundance of bacteria on the root system should be assessed using qPCR.

The increase in root length induced by many of the tested strains present changes in root system architecture that could result in a general increase of plant fitness. Increased root length, as well as enhanced lateral root number, are beneficial for a better plant performance, i.e. by increasing the access

to water and nutrients (Ho et al., 2005; Manschadi et al., 2006). Also phosphorus deficiency induces changes in root architecture that are believed to increase root surface area and thereby enhance nutrient uptake (Williamson et al., 2001; López-Bucio et al., 2002). The actual mechanisms by which the tested bacteria increase root growth is unknown. The mode of action of many PGPR involves the bacterial production of hormones as major factors affecting root architecture. For example, plant auxin homeostasis regulates root branching and root length (Casimiro et al., 2001; Dharmasiri et al., 2005) and for example *Azospirillum brasilense* is a PGPR that interferes with auxin homeostasis and decreases root length and enhances root hair formation on wheat (Spaepen et al., 2008). The hypothesis that the tested bacterial isolates manipulate plant hormone homeostasis is worthwhile to test, for example by assessing the plant growth promotion effect on plant mutants defective in hormonal pathways. Additionally, bacterial isolates could be tested for auxin production in vitro or their genomes could be analyzed for the presence of auxin biosynthesis genes.

In a second step, bacterial strains of the shared microbiota were investigated as synthetic communities to assess how bacteria affect plant growth in interaction with other bacteria. This is of particular interest, as few studies examined the action of model PGPR in a community context (Raupach and Kloepper, 1998). We have currently limited insights into how bacteria-bacteria interactions affect the outcome of plant growth-promoting effects. To address this gap, synthetic communities were assembled based on the available culture-independent analysis of the comparison of three plant species of the Brassicaceae family (Fig. 8). Three types of synthetic communities were assembled, the most simple community consisting of only three members of the phyla Proteobacteria, Actinobacteria and Bacteroidetes (Triad community), intermediate complexity communities comprising of 4 to 7 members (Community A and B), as well as the combination of all 11 bacteria (Community A+B). These synthetic communities were inoculated on *A. thaliana* and evaluated for their effects on plant growth. The inoculation with the triad and intermediate communities increased root length under phosphorus-deficient but not -sufficient conditions and the resulting effects on plant growth were similar to the phenotypes of the individual bacteria in binary interactions (Fig. 23, Fig. 24). Interestingly, when combining all 11 bacteria in one synthetic community (Community A+B), a reduction of root length was observed under phosphorus sufficiency. This result did not correlate with the changes in root length observed for bacteria in binary interactions. One explanation for this phenotype could be the establishment of unfavorable bacteria-bacteria interactions that resulted in a negative effect on root growth. Bacteria-bacteria interactions can be of a competitive nature, as some bacteria inhibit growth of other bacteria by the production of antibiotic compounds (Slattery et al., 2001). Competitive interactions could alter the community composition in such a way, i.e. by decreasing the abundance of strains with positive effects, that plant performance is negatively affected. The role of bacteria-bacteria interactions on plant performance is difficult to dissect without information about the abundance of individual members of the inoculated bacterial community on the root system. Therefore, the abundance of synthetic community members should be quantified using qPCR or Illumina sequencing. Nevertheless,

the presented results imply that the fine-tuning of microbial communities is important for their effect on plant growth and that the effects observed under binary interactions are partially maintained in more complex synthetic communities. Another limitation of this study is that a high genomic diversity can exist between strains of the same species, making the assembly of such complex communities challenging (Baltrus et al., 2011). Alternative techniques that could increase our understanding about the role of bacteria on plant performance in a community context could be "omic"-techniques, including metagenomics, metatranscriptomic or metaproteomics.

3.6. The effect of bacteria of the shared microbiota cannot be predicted for other Brassicaceae than *A. thaliana*

The bacterial strains, which were functionally examined, were initially selected, because they represented bacteria of a shared microbiota present on *A. thaliana*, *C. hirsuta* and *A. alpina*. The presence a shared bacterial community on related Brassicaceae host plants raised the question, whether bacteria of the shared bacterial root microbiota have similar roles or functions for a multitude of host species, i.e. whether they induce similar physiological changes on related Brassicaceae.

To address this question, bacterial exemplars that were isolated from *A. thaliana* were tested for their effect on *C. hirsuta* and *A. alpina* and for a comparison on their native host *A. thaliana*. These tests were conducted using an Acidovorax, a Streptomyces and a Flavobacterium isolate, both in in binary plant-bacteria interactions as well as using a synthetic community, again comparing phosphorus-sufficient and -deficient conditions (Fig. 25). All individually tested bacteria altered root length under phosphorus deficiency, as seen by longer roots upon treatment with Acidovorax of all three species, shorter roots upon inoculation with Flavobacterium only for *A. alpina*, while Streptomyces increased root length only of *A. thaliana*. Under phosphorus sufficiency, bacterial isolates induced different phenotypes, as Acidovorax did not affect root growth, Streptomyces reduced root length of *C. hirsuta* and *A. alpina* but not of *A. thaliana*, whereas Flavobacterium reduced root length of all three species. The synthetic triad community increased root length under phosphorus deficiency with quantitative differences dependent on the tested plant species. Under phosphorus sufficiency, the triad community reduced root growth of all three plant species, with the strongest effect on *A. alpina*. Therefore, the observed phenotype partially resembled the observations made for *A. thaliana*. Nevertheless, quantitative and qualitative differences were observed, depending on the tested plant species or nutrient condition.

In summary, the bacterial isolates that were selected based on the identification of a shared bacterial microbiota on related Brassicaceae using culture-independent sequencing of the bacterial 16S rRNA gene and isolated from *A. thaliana* did not induce the same phenotypic changes on other tested host species. Hence, we can conclude that individual strains possess a certain host-specificity and that isolates originally isolated from *A. thaliana* affect other Brassicaceae plant species differently. One explanation for this observation is that the definition of a shared bacterial root microbiota was based on the 16S

rRNA gene, which as a marker is insufficient to resolve sub-species variation between bacteria. The taxonomic resolution of the 16S rRNA gene is not deep enough to differentiate between stains of the same species (Fox et al., 1992). Bacteria assigned to an OTU exhibit a range of different functional abilities as discussed in Chapter II, exemplifying the plasticity of intra-OTU variability using the example of multiple strains of rhizobia OTUs. To better understand the biological relevance of the shared bacterial microbiota, additional bacterial strains belonging to the same bacterial species/OTU that originated from different host plants should be included in further experiments. This would allow to reciprocally inoculate bacterial strains and host plants with regard to isolation origin.

3.7. Conclusions and future perspectives

The development of NGS technologies facilitated the in-depth examination of the bacterial microbiota of plants. However, we still have limited insights into the factors that influence the composition of these communities, the stability of the bacterial microbiota and most importantly, their functional relevance. To increase our understanding about bacterial communities associated with plants, I have addressed two research questions. First, I investigated, the structure of the bacterial root microbiota of three distinct host plant species and their dynamics due to differences in soil type, environmental conditions and residence time in the soil. Second, I have examined the functions of bacteria isolated from the root microbiota in affecting plant growth as individual strains and, importantly, in a community setting.

For the first research question, I demonstrated that the structure of the bacterial root microbiota of related Brassicaceae plant species, spanning an evolutionary divergence of 45 million years, is largely similar. The use of *A. alpina* as a model system allowed to dissect additional factors, such as residence time in the soil, environmental conditions as well as plant developmental stage, on their influence on community structure. Compared to major influences induced by soil type, residence time and environmental conditions, I confirmed the minor role of host species and I revealed that plant developmental stage does not affect the structure of the bacterial microbiota. A surprising observation was the strong influence of *A. alpina* on rhizosphere communities that was minor or even not detected for the related species *A. thaliana* and *C. hirsuta* (Bulgarelli et al., 2012; Schlaeppi et al., 2014). The comparison between *A. alpina*, *C. hirsuta* and *A. thaliana* allowed to confirm the presence of a shared bacterial root microbiota, consisting of a relatively simple taxonomic structure (Schlaeppi et al., 2014). For the second research question, I demonstrated that individual bacterial isolates of the shared bacterial microbiota exerted beneficial effects for the plant, while only few bacteria had no effect or negatively affect plant growth. Bacterial isolates altered root growth of *A. thaliana*, *C. hirsuta* and *A. alpina*. Importantly, these phenotypes caused by individual bacterial strains were largely maintained when synthetic communities were inoculated on the three Brassicaceae host plants. However, the function of bacteria on related plant species could not be predicted by the observed phenotype on *A. thaliana* alone.

This study addressed factors influencing community structure and evaluated the function for a subset of the bacterial root microbiota with regard to their plant growth-promoting abilities. For a deeper understanding of the importance of bacterial communities for plant performance, the model system of *A. alpina* should be further developed and some limitations of the current study need to be overcome. First, the perennial nature of *A. alpina* was not fully exploited during the used experimental setup. This setup did not address the question, whether the bacterial microbiota remains stable after 7 months and the use of *A. alpina* allows to address the dynamics of the root bacterial communities over consecutive years. It would be interesting to investigate the bacterial microbiota composition across a multigenerational experiment. Second, community analysis based on a single marker gene, namely the 16S rRNA gene, permits to analyze community structure, while not revealing functional insights into the community (Fig. 26). Further investigations could couple 16S rRNA-based community profiling

with "omic"-techniques, such as metagenomics, -transcriptomics and -proteomics that allow to investigate the functional part of the community. Third, while the functional relevance of bacteria of the root microbiota for host physiology is starting to be addressed, the underlying mechanisms for colonization and plant growth-promoting activities, i.e. the involvement of molecular pathways, such as the host immune system, remain elusive. It will be essential to examine functionally distinct synthetic communities for their effect on plant performance and using a range of different stress conditions. One advance would result in utilizing multiple approaches that couple the structural analyses of a community (i.e. community profiling) with functional approaches (i.e. "omic" techniques). One example would be to couple a functional analysis from axenic growth assays with transcriptomic analyses to investigate the expressed bacterial traits and pathways in the interaction of a synthetic community with the host plant.

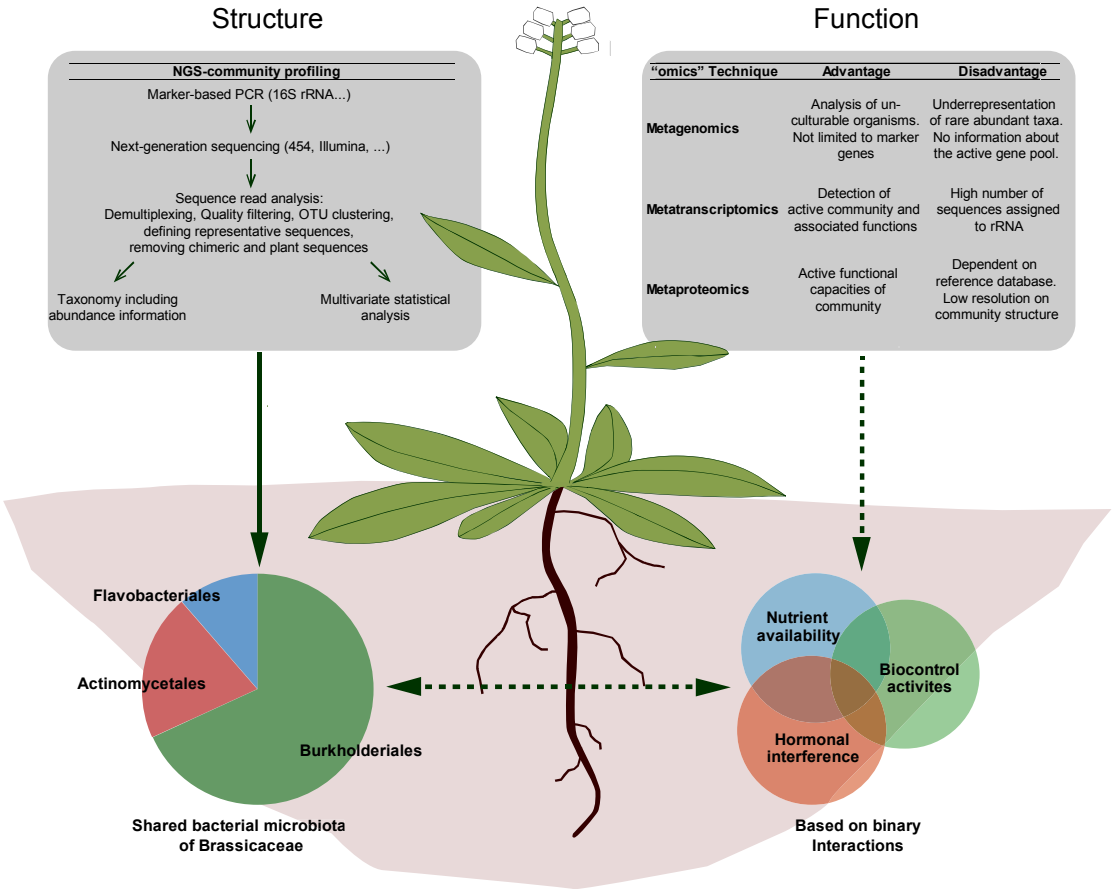


Fig. 26: Schematic representation of next-generation-sequencing (NGS) techniques and possible applications to link community structure with functional abilities of bacteria associated with plants. Marker-based NGS community profiling allows to gain insights into the structure of bacterial communities associated with different Brassicaceae and other host plants. However, the use of marker genes does not provide information about the functional potential encoded within these communities. Functional activities of bacteria were so far mostly studied based on simplified, binary interactions (Lugtenberg and Kamilova, 2009). However, the use of "omic"-techniques might provide alternatives to fill the gap between current knowledge about the structure of bacteria gained using NGS (pie chart based on the current study) and functional abilities of these bacterial communities. Closed arrow: Available information. Dashed arrow: So far absence of a direct correlation.

4. Materials and Methods

4.1. Materials

4.1.1. Storage and preparation of soil material

Soil types used in this study were collected at the following locations: 'CAS5', 'CAS8' and 'CAS9' (50.958 N/ 6.856 E, Cologne, Germany), and 'France' (45.061 N/ 6.402 E, Col du Galibier, France). Soil types were harvested at the following dates: 'CAS5' in September 2010, 'CAS8' in March 2013, 'CAS9' in September 2013 and 'France' in August 2012. At all locations soil was not exposed to agricultural use for several years. To excavate material the topsoil (10-15 cm) was removed and a layer between -15 and -30 cm collected, followed by storage of soil for one week at ambient temperature for drying (according to Bulgarelli et al., 2012). Homogenized soil was afterwards stored at 4°C until further usage. Upon usage soil was sieved with a 5 mm followed by a 2 mm mesh and afterwards distributed to 7x7x9 cm or 9x9x9 cm pots for experiments. Geochemical characterization was carried out by the "Labor für Boden- und Umweltanalytik" (Eric Schweizer AG, Thun, Switzerland) and results are listed in Tab. 2 and Tab. 3). For the *A. alpina* time course experiment 'CAS8' or 'CAS9' soil was mixed in a 1:1 ratio with autoclaved sand (at 121 °C for 20 min, stored overnight, followed by a second autoclaving under the same conditions) to ensure a sufficient aeration of plant roots for a growth period of 7 months. *A. alpina* grown in CAS that was not supplemented with sand did not survive well under these growth conditions, as seen by necrosis formation on leaves (data not shown here), and is most likely due to the adaptation of *A. alpina* to the sandy loam of the arctic-alpine soil (Tab. 2). In all other cases, soil was used without further modifications according to Bulgarelli et al., 2012.

Tab. 2: Soil parameters. Soil classification according to the Food and Agriculture Organization (FAO) of the United Nations. Listed are two soils from Cologne (CAS5 and CAS8), two soils from the French Alps (Col du Galibier and Croix de fer). Golm3 and Widdersdorf are two soil types from Germany that were used for bacteria isolation.

Soil	[1]C.org (%)	Clay (%)	Silt (%)	[2]Classification	pH
CAS5	4	21	31	Loam	6.9
CAS8	4	21	31	Loam	6.7
Col du Galibier	3	16	31	Sandy loam	7.6
Croix de fer	3.5	16	21	Sandy loam	7.8
Golm3	2.5	1	1	Sand	6.9
Widdersdorf	3.5	21	31	Loam	7.0

[1] organic carbon

[2] Soil texture classification according to FAO

Tab. 3: Nutrient content of used soil types. Listed are two soils from Cologne (CAS5 and CAS8), two soils from the French Alps (Col du Galibier and Croix de fer). Golm3 and Widdersdorf are two soil types harvested in Germany and that were used for isolation of bacterial strains.

Soil	Extract	[3]Phosphorus	[3]Potassium	[3]Magnesium	[3]Calcium	[3]Nitrate
CAS5	[1]H_20	6.3	22.6	8.9	52.1	22.2
	[2]AAE	85.3	124.8	118.5	1604.1	
CAS8	[1]H_20	1	11	5.3	31.8	12
	[2]AAE	20.7	101.9	144.5	1666.1	
Col du Galibier	[1]H_20	0.2	4.8	2	112.7	3.5
	[2]AAE	1.9	19.6	262.1	73800.8	
Croix de fer	[1]H_20	0.9	8.3	4.1	9.4	2.6
	[2]AAE	6.2	19.5	16.2	569.8	
Golm3	[1]H_20	15.1	28.2	5.4	41.6	13.7
	[2]AAE	98.6	84	39.8	768.2	
Widdersdorf	[1]H_20	5.6	32	9.5	166.5	4.7
	[2]AAE	71.6	123.8	224.6	12021.5	

[1] determined with 1:10 (w/v) water extract as a proxy for plant-available nutrients

[2] determined with 1:10 (w/v) ammonium-acetate-EDTA (AAE) extract as a proxy for reserve nutrients

[3] mg/kg

4.1.2. Plant material

Three plant species belonging to the Brassicaceae family were investigated during this study, namely *Arabidopsis thaliana* (ecotype Columbia, Col-0), *Cardamine hirsuta* (ecotype Oxford, Ox) and *Arabis alpina* (ecotype Pajares (Paj), F1-Gal5, Gal60 and mutant line *pep1* (Wang et al., 2009)). Seed material for *A. thaliana* and *C. hirsuta* was kindly provided by Dr. Klaus Schläppi (Agroscope Institute for Sustainability Sciences ISS). Seed material from *A. alpina* was supplied by Dr. Maria Albani and Dr. Jörg Wunder (Department of Plant Developmental Biology, Max Planck Institute for Plant Breeding Research). *A. alpina* Gal60 was collected at the natural site in France at the Col du Galibier together with the respective soil (Chapter I.4.1.1).

4.1.3. Oligonucleotides

Oligonucleotides used for PCR amplification are listed in Tab. 4 and were purchased from Sigma (Hamburg, Germany) or Invitrogen (Karlsruhe, Germany).

Tab. 4: Oligonucleotides used in this study.

Name	Sequence (5' - 3')
799F	AAC MGG ATT AGA TAC CCK G
1193R	ACG TCA TCC CCA CCT TCC
1392R	ACG GGC GGT GTG TRC
B5_F	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACG ACT GCG ACT GGC GAA CMG GAT TAG ATA CCC KG
B5_R1	ACG ACT GCG ACT GGC GAA CMG GAT TAG ATA CCC
B5_R2	CAG CCA TTT AGT GTC ACG TCA TCC CCA CCT TCC
B5_Index	GGA AGG TGG GGA TGA CGT GAC ACT AAA TGG CTG

4.1.4. Enzymes

DNA-free DFS Taq polymerase was purchased from Bioron (Ludwigshafen, Germany). Antarctic phosphatase and Exonuclease I were acquired from New England Biolabs (Frankfurt, Germany).

4.1.5. Chemicals and antibiotics

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Serva (Heidelberg, Germany), BD (Heidelberg, Germany) unless stated otherwise.

4.1.6. Buffers and solutions

Buffers and solutions used in this study are described within each method. If not stated otherwise, buffers were prepared in ddH_2O and aqueous solutions were sterilized by autoclaving at 121 °C for 20 min.

4.2. Methods

4.2.1. Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis in gels consisting of 1-1.5 % (w/v) agarose (Bio-Budget Technologies, Krefeld, Germany) supplied with ethidium bromide solution (1:40000) in Tris-acetate-EDTA (TAE) buffer (400 mM Tris; 10 mM EDTA; 200 mM acetic acid; pH 8.5).

4.2.2. Seed sterilization

Plants were surface sterilized by sequential washing for 20 Min with 70% ethanol and a brief washing step with 100% ethanol. After removal of ethanol, seeds were dried under the sterile hood.

4.2.3. Plant growth conditions in the greenhouse

For the comparison of the three plant species of the Brassicaceae family, surface sterilized seeds were sown at a density of four plants per pot (7x7 cm) and stratified for three to four days. Plants were grown in the greenhouse for 6 weeks under short day conditions (8/16 hours day/night with a temperature of 22°C/18°C and a relative humidity of 70%). For bulk soil samples, unplanted pots were kept under the same conditions as the plant material until harvest. After 6 weeks all plants resided in the vegetative stage. For each DNA sample 3-4 pots, including a total number of 12-16 plants, were pooled during harvest. For every species three independent DNA samples per condition were included in the analysis. In total three independent biological repetitions were conducted at independent time points using the soil batches CAS5, CAS8 and CAS9 (see also Tab. 1).

For the *A. alpina* time course experiments surface sterilized seeds were sown at a density of one plant per pot (9x9 cm) and stratified for three to four days. Plants were grown in the greenhouse for up to 7 months under long day conditions (16/8 hours day/night with a temperature of 22°C/18°C and a relative humidity of 70%). The root, rhizosphere and soil compartments were harvested after 6 weeks, 3 months and 7 months after sowing. After 6 weeks of sowing, 6-8 pots were pooled during harvest, whereas after 3 and 7 months only one pot per replicate was used for further DNA isolation. For every condition three to four DNA samples per independent biological repetition were included in the analysis. In total two independent biological repetitions were conducted at different time points using the soil batches CAS8 and CAS9 (see also Tab. 1).

4.2.4. Plant growth of *A. alpina* under natural conditions

To assess bacterial community structure under natural growth conditions two approaches were undertaken. First, plants were directly collected in the French Alps (45.061 N/ 6.402 E, Col du Galibier, France; i.e. natural site experiments) and second, soil from the same location was excavated and used for plant growth under greenhouse conditions (i.e. natural soil experiments). For the natural site experiments healthy looking plants, residing in flowering stage, were excavated by collecting at least 10 cm of the main root. Then, plants were transferred to pots including natural soil and transported to Cologne, Germany. For plant recovery, material was stored for at least one day at 4°C until harvest. Additionally, seed material was collected at the same location and included in the natural soil experiment (designated *A. alpina* Gal60).

For the natural soil experiments, sterilized seeds of the *A. alpina* accessions Paj, Gal5 and Gal60 were directly sown onto French soil and stratified for three to four days. Plants were grown in the greenhouse for 3 months under long day conditions (16/8 hours day/night with a temperature of 22°C/18°C and a relative humidity of 70%). To cross-reference this experiment to the time course experiment, the *A. alpina* accession Paj was grown for 3 months in CAS8 soil and included for further comparison. For every condition three to five independent DNA samples per condition were included in the analysis (see also Tab. 1).

4.2.5. Sampling of the root, soil and rhizosphere compartment

After harvesting, roots were mechanically separated from the adhering soil particles and a defined root segment of 3 cm starting 0.5 cm distant from the hypocotyl was sampled for the comparison of the three plant species of the Brassicaceae plant family. For the time course and natural site experiment a segment of 4.5 cm was collected. Soil particles still attached to the roots were removed by gentle tapping. Roots were collected in 15 ml falcons containing 5 ml phosphorus buffered saline (PBS)-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0, 0.02% Silwet L-77) and washed for 15 minutes at 180 rpm on a shaking platform. Roots were transferred to a new falcon, the remaining soil particles were centrifuged for 20 minutes at 15,000 x g and the pellet collected in liquid nitrogen and stored at -80°C. This fraction represents the rhizosphere compartment. After washing for a second time, roots were transferred to a new falcon tube and sonicated 10 times at 160 W with 30 second brakes (Bioruptor Next Gen UCD-300, Diagenode, Liège, Belgium). Roots were transferred to fresh PBS-S, shortly dried on Whatman® glass microfiber filters (Sigma-Aldrich, Deisenhofen, Germany), transferred to 2 ml tubes and frozen in liquid nitrogen for further storage at -80°C. Unplanted soil samples were collected from an unplanted pot after removing 0.5 cm of the top soil, immediately frozen in liquid nitrogen and stored at -80°C.

4.2.6. Preparation of root samples for scanning electron microscopy (SEM)

To evaluate the efficiency of the sonication treatment on different plant species, roots of *A. thaliana*, *C. hirsuta* and *A. alpina* were collected prior to and after sonication and stored in PBS-S until further usage (see also Chapter I.4.2.5). Root material was further processed for SEM by the Central Microscopy group at the Max Planck Institute for Plant Breeding Research, Cologne. Briefly, samples were fixed in 4% glutaraldehyde (Roth, Karlsruhe, Germany) in 1x PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) at 4°C over night. Afterwards, roots were washed twice with 1x PBS for 30 minutes and washed with increasing ethanol concentrations (30% ethanol for 30 minutes, 50% ethanol for 30 minutes, 70% ethanol for 30 minutes, 90% ethanol for 30 minutes, 96% ethanol for 60 minutes, 96% ethanol for 60 minutes). To remove remaining water, root material was incubated in dried

ethanol at 4°C over night. Afterwards, samples were dried using a critical point drier (CPD30; BAL-TEC, Wetzlar, Germany). During this procedure ethanol was exchanged 10 times against liquid CO_2. Then, samples were spattered using the Polaron Sputter Coater 7600 using a platinum target. Microscopy pictures were taken with a Supra 40VP (Zeiss, Munich, Germany).

4.2.7. DNA isolation from the root, soil and rhizosphere compartments

For DNA isolation the FastDNA ® SPIN for soil kit (MP Biomedicals, Solon, USA) was utilized. Before DNA isolation, samples were homogenized using the Precellys®24 tissue lyzer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6,500 rpm for 30 seconds. Root samples were homogenized three times and in between shortly frozen in liquid nitrogen. Rhizosphere and soil samples were homogenized once. Afterwards, DNA was extracted using the FastDNA ® SPIN for soil kit according to the manufacturer's protocol.

4.2.8. Generation of 16S rRNA gene amplicon libraries for pyrosequencing (454)

DNA concentration was assessed using the Quant-iT™ PicoGreen dsDNA assay kit (Life Technologies, Darmstadt, Germany). 40 µl of a 1:200 dilution of PicoGreen was added to 4 µl of DNA in a 96 well plate. To calculate the DNA concentration a dilution series of standard lambda DNA, ranging from 0.5 to 20 ng/µL, was included on the same plate. Fluorescence was measured using the IQ5 real-time PCR Thermocycler (Biorad, Munich, Germany; 30 sec at 25°C, 3x30 seconds at 25°C for measuring fluorescence, 30 seconds at 15°C). Afterwards, DNA was adjusted to a final concentration of 3.5 ng/µl. A PCR amplicon library was generated using the primers 799F and 1193R, spanning ~400 bp of the hypervariable region V5-V7 of the bacterial 16S rRNA gene (Tab. 4, Chelius and Triplett 2001; Bodenhausen et al., 2013). For multiplexed pyrosequencing (454) the 799F primer was modified at the 5'end with a sample-specific 6mer barcode. A *Sfi*I site for ligation of 454 adaptors was added behind the barcode (5'-GATGGCCATTACGGCCNNNNNN-799F-3'). The 1193R primer was adjusted by adding a *Sfi*I site at the 5'end for 454 ligation (5'-CCTATCCCCTGTGTGCCTTGGCAGTCGA-1193R-3').

PCRs were performed using 3 µl of DNA adjusted to 3.5 ng/µl in a total volume of 25 µl. PCR components included 1.25 U DFS-Taq DNA Polymerase (Bioron, Ludwigshafen, Germany), 1x incomplete reaction buffer, 0.3% BSA, 2 mM of $MgCl_2$, 200 µM of dNTPs and 400 nM of each primer. The PCR reaction was pipetted in a laminar flow and amplified using a touch-down protocol (Tab. 5). To minimize PCR bias, 12 independent PCR reactions were performed for each DNA sample using three independent master mixes. Quality of PCR amplicons was controlled by loading 5 µl of the individual reaction on a 1% agarose gel and affirming that no band was detected in the negative control. Afterwards, the replicated PCR-reactions were combined and purified using the Agencourt AMPure XP

PCR Purification kit (Beckman Coulter, Krefeld, Germany). Purified products were eluted in a final volume of 30 µl and the complete reaction loaded on a 1.5% agarose gel and ran for ~2 hours at 80 V. Bands with the correct size of ~400 bp were cut and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Afterwards, the DNA concentration was determined using the PicoGreen assay as described above. 200 ng DNA of each of the barcoded amplicons were pooled in one library. To minimize the total volume of the reaction and the amount of primer-dimers the Agencourt AMPure XP PCR Purification kit was applied twice and the sample eluted in a final volume of 80 µl. Obtained amplicons were further prepared for pyrosequencing by the Max Planck Genome Center Cologne as described in Bulgarelli et al., 2012.

Tab. 5: Touch-down PCR protocol to generate 16S rRNA gene amplicon libraries.

Step #	Temperature [°C]	Time [seconds]	# of cycles
1	94	120	
2	94	30	
3	58	60	5x
4	72	15	
5	94	30	
6	57	60	5x
7	72	30	
8	94	30	
9	56	60	5x
10	72	45	
11	94	30	
12	55	60	20x
13	72	60	
14	72	600	
15	15	pause	

4.2.9. Generation of 16S rRNA gene amplicon libraries for Illumina sequencing

Amplicon libraries for Illumina sequencing were prepared in a similar manner compared to pyrosequencing libraries to ensure comparability between the two techniques. In a first PCR the V5-V7 region of the bacterial 16S rRNA gene was amplified using the primers 799F and 1193R without adaptors and barcodes. PCR reactions were performed using 3 µl of DNA adjusted to 3.5 ng/µl in a total volume of 25 µl. PCR components include 1.25 U DFS-Taq DNA Polymerase (Bioron, Ludwigshafen, Germany), 1x incomplete reaction buffer, 0.3% BSA, 2 mM of $MgCl_2$, 200 µM of dNTPs and 400 nM of each primer. To minimize PCR bias three independent PCR reactions using one master mix were prepared. The PCR reaction was pipetted in a laminar flow and PCR amplified (94°C/2 minutes, 94°C/30 seconds, 55°C/30 seconds, 72°C/30 seconds, 72°C/10 minutes for 25 cycles). Afterwards, single-stranded DNA and proteins were digested by adding 1 µl of Antarctic phosphatase, 1 µl Exonuclease *I* and 2.44 µl Antarctic phosphatase buffer to 20 µl of the pooled PCR product. Samples were incubated

at 37°C for 30 minutes and enzymatic activity was deactivated at 85°C for 15 minutes. Samples were centrifuged for 10 minutes at 4,000 rpm and the supernatant was transferred to a new plate. 3 µl of this reaction was used for a second PCR with primers that included barcodes and Illumina adaptors. Therefore, the 799F primer was modified at the 5‘end (5‘-AATGATACGGCGACCACCGAGATCTACACGACTGCGACTGGCGA-799F-3’). The 1193R primer was adjusted for multiplexed amplicon sequencing by adding a 12mer barcode at the 5’ end followed by the Illumina adaptor. (5’-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNNNNNNCAGCCATTTAGTGTC-1193R-3’). PCR reactions were prepared in the same way as described above using the same protocol except the number of PCR-cycles were reduced to 10. Quality of PCR amplicons was controlled by loading 5 µl of the individual reaction on a 1% agarose gel and affirming that no band within the negative control was detected. Afterwards, the replicated reactions were combined and loaded on a 1.5% agarose gel and ran for approximately 2 hours at 80 V. Bands with the correct size of ~500 bp were cut and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). DNA concentration was determined using the PicoGreen assay as described before. 30 ng DNA of each of the barcoded amplicons were afterwards pooled in one library. A maximum of 96 samples were combined in one library. Paired-end Illumina sequencing was performed by the Max Planck Genome Center using the MiSeq sequencer.

4.2.10. Bioinformatic analysis of amplicon libraries

4.2.10.1. Sequence alignment and de-multiplexing

To process obtained reads for OTU picking, Illumina generated paired-end reads were first aligned to each other using the QIIME software package and the join_paired_ends.py script (Caporaso et al., 2010). Only sequences with a correct alignment were included in the subsequent data analysis and all non-aligned reads discarded. To de-multiplex Illumina sequences, barcoded sequences were first extracted from the aligned sequences using extract_barcodes.py and the gained output together with the aligned sequences was used in split_libraries_fastq.py.

Sequences that were generated with 454 were directly de-multiplexed after sequencing using split_libraries.py. Only sequences were retained showing no ambiguous bases, no error in the barcode sequence, a minimum quality score of 27 and a minimum sequence length of 315 base pairs (bp). Afterwards, the de-multiplexed sequence output from Illumina and 454 was concatenated for subsequent data analysis.

4.2.10.2. OTU assignment and generation of OTU table

De-multiplexed sequences were clustered at 97% sequence identity into OTUs using UCLUST using the script pick_otus.py. After retrieving OTUs a representative sequence per OTU was picked by

determining the most abundant sequence within a cluster of OTUs (pick_rep_set.py). This sequence was used for taxonomic assignment using the Ribosomal Database Project II (RDPII) classifier database and the command assign_taxonomy.py (Wang et al., 2007). For the taxonomic assignment a minimum confidence interval of 0.5 was applied. The information about the obtained OTUs and taxonomy assignment was combined in an OTU table, which was used for further downstream analysis. Reads assigned to plant chloroplasts and mitochondria were removed using filter_otus_from_otu_table.py. Chimeric sequences were filtered and excluded using Chimera Slayer and the script filter_otus_from_otu_table.py.

OTU tables for the threshold-independent dataset (TIC) and the thresholded dataset at 20 sequences within at least one sample (abundant community members; ACM) were calculated using R. To obtain information about alpha- and beta-diversity the representative sequences were aligned to a template sequence using PyNAST (align_seqs.py) and the Greengenes core dataset for sequence alignment. Afterwards, the obtained sequences were filtered to remove highly variable regions (filter_alignment.py) and the filtered alignment was used to create a phylogenetic tree using FastTree (make_phylogeny.py). Afterwards, alpha-diversity metrics (total number of observed OTUs and Shannon index) were calculated using the information of the phylogenetic tree and the OTU table of the TIC rarefied to the minimum number of sequences within the respective dataset.

To correct for different sequencing depths of independent sequencing samples the OTU table was normalized to the total sample size in R. Additionally, alpha-diversity metrics were calculated in R using the function diversity within the *vegan* package to calculate the Shannon index and the function specnumber of the *vegan* package to calculate the total number of detectable OTUs.
All further statistical analyses were conducted in R and if not stated otherwise the ACM, normalized by sample size and $\log_2$-transformed, was used for all further statistical analysis.

4.2.10.3. Principal component analysis (PCA)

To calculate major determinants of community structure within the $\log_2$-transformed ACM, a PCA analysis was conducted using the package *prcomp* in R. Results of the PCA were plotted using the package *ggbiplot*.

4.2.10.4. Defining differentially enriched OTUs

To determine OTUs enriched within the root, rhizosphere and soil compartments a similar method as described by Bulgarelli et al., 2012 was used. Linear statistics on the relative abundance (RA) values were performed using the *limma* package in R. For determining compartment-enriched OTUs (i.e. SoilOTUs, RhizoOTUs, RootOTUs) a linear model was fitted using compartment as explanatory variable. Afterwards, differentially abundant OTUs were calculated using a Bayes moderated t-test

(Smyth, 2005), correcting the resulting p-values using the Benjamini-Hochberg method. To detect compartment-enriched OTUs, the intersection of the corresponding pair-wise comparisons was calculated. A p-value cutoff < 0.05 was used.

To test for OTUs enriched in the root versus the soil compartments of different plant species of the Brassicaceae family (i.e. SoilOTUs, RootOTUs), a linear model was fitted across the comparison between the root and soil compartment. To determine the sharedOTUs more stringent statistics were applied, using Tukey's HSD, Mann-Whitney and Bayesian statistics, according to the described method in Schlaeppi et al., 2014. The intersection of OTUs that passed all three statistics defines the shared bacterial community (sharedOTUs).

To test for OTUs enriched across the three time points during the *A. alpina* time course experiment, a linear model was fitted across the two possible combinations across the three different compartments (root, rhizosphere, soil). To evaluate the impact of time on the compartment-enriched OTUs, a linear model was fitted across the 8 combinations between the different time points (t1, t2, t3) and the three tested compartments. To define OTUs enriched within plants at different developmental stages the dataset was first separated into the different time points and then a linear model was fitted across four interaction terms, separating the two plant genotypes (*A. alpina* WT and *pep1*) from the three tested compartments.

In a similar manner OTUs differentially expressed in the natural site experiments were determined, just exchanging the variable time point with the variable growth condition (FrenchSoil_Greenhouse, FrenchSoil_NaturalSite and CologneSoil_Greenhouse; FS_GH, FS_NS, CAS_GH). In this calculation the variable genotype included the accessions *A. alpina* Paj, Gal5 and Gal60.

4.2.10.5. Defining a shared community within the Brassicaceae experiment using Mann-Whitney statistics

Mann-Whitney statistics were performed on the ACM using the function wilcox_test in the library *coin*. The RootOTUs were calculated for each independent biological replicate across the different plant species based on pair-wise comparisons. For this the comparison terms "*A. thaliana*-Soil", "*C. hirsuta*-Soil" and "*A. alpina*-Soil" were used, defining AtOTUs, ChOTUs and AaOTUs, respectively. A p-value cutoff < 0.1 was used (FDR-corrected).

4.2.10.6. Defining a shared community within the Brassicaceae experiment using Bayesian statistics

Bayesian statistics were calculated using the function BayesianIUT in R, considering only OTUs that show a 30x higher support for the alternative hypothesis. For this root samples of *A. thaliana*, *C. hirsuta* and *A. alpina* were compared to the group of soil samples (combining replicate experiments).

4.2.10.7. Ternary plots

Ternary plots were calculated on the means of the log_2-transformed relative abundances per compartment within the respective accession, host species, time point or growth condition (indicated individually below each plot). For this a modified version of the function ternaryplot in the package *vcd* was used (see also Bulgarelli et al., 2012).

4.2.11. Isolation and identification of root-associated bacteria

To isolate root-associated bacteria, plants were grown and roots harvested as described above (Chapter I.4.2.5). Bacteria were isolated from different accessions of *A. thaliana*, *C. hirsuta* and *A. alpina* grown in the greenhouse or at natural sites in Germany and France. Plants in the greenhouse were grown in Cologne agricultural or Golm soil (Tab. 2). Instead of immediate freezing in liquid nitrogen, roots were transferred to a 1.5 ml tube containing 10 mM $MgCl_2$. Roots were mechanically disrupted using the Precellys®24 tissue lyzer at 5,000 rpm for 3x at 30 seconds. Afterwards, the root tissue was centrifuged for 5 minutes at 1,000 x g and the supernatant transferred to a new tube. Serial dilutions were prepared from the supernatant and plated onto flour and TWYE media (flour: 6 g/L flour, 0.3 g/L yeast extract, 0.3 g/L sucrose, 0.3 g/L $CaCO_3$, 1.8%. TWYE: 0.25 g/L yeast extract, 0.5 g/L K_2HPO_4, 1.8% agar, respectively), including 50 µg/ml Benzimidazole to inhibit fungal growth. Plates were incubated for three to four days at 28°C. Afterwards, single colonies were transferred with a sterile pipette tip to 400 µl liquid media in a 96-well format and incubated for up to 7 days at 28°C at 200 rpm. Then, 100 µl of the culture was transferred to a 96-well PCR plate and bacterial cells disrupted for 10 minutes at 100°C. Cell fragments were centrifuged for 10 minutes at 3,000 rpm and the supernatant transferred into a new plate. In a PCR reaction, used to obtain sequence information for taxonomic assignment, the primers 799F-noadaptor (5´-AACMGGATTAGATACCCKG-3') and 1392R (5´-ACGGGCGGTGTGTRC-3') were used. PCRs were performed using 3 µl of isolated bacterial DNA in a total volume of 25 µl. PCR components include 1.25 U DFS-Taq DNA Polymerase (Bioron, Ludwigshafen, Germany), 1x complete reaction buffer, 0.3% BSA, 200 µM of dNTPs and 400 nM of each primer. The PCR reaction was pipetted in a laminar flow and amplified (94°C/5 minutes, 94°C/30 seconds, 50°C/ 30 seconds, 72°C/30 seconds, 72°C/5 minutes for 35 PCR cycles). PCR quality was assessed by loading 5 µl of the amplicon on a 1% agarose gel. DNA concentration was measured using a Nanodrop photometer (PeqLab, Erlangen, Germany), adjusted to 20 ng/µl and subjected to Sanger sequencing (performed by the Max Planck Genome Center, Cologne, Germany). Obtained sequences were analyzed with BioEdit and blasted against an internal 454 sequence database (as described in Bulgarelli et al., 2012) to correlate the isolated bacteria to OTUs identified within previous culture-independent studies.

To compare the overlap between the culture-dependent isolation efforts (based on bacterial strains isolated from the greenhouse) with the results from the culture-independent sequencing effort (based on the comparison of the three plant species of the Brassicaceae family) another approach was taken. To

calculate the overlap the 70,005 OTUs of the TIC, 443 OTUs of the ACM, 101 RootOTUs and 26 sharedOTUs (Fig. 7, Fig. 8) were chosen as a references dataset against which the 1,112 bacterial strains isolated from the greenhouse of plants grown in CAS soil (Fig. 22) were clustered using pick_otus.py using QIIME. For this a sequence similarity threshold of 97% was used and clustering was performed using UCLUST.

4.2.12. Purification of root-associated bacteria

To ensure that bacteria used for further testing emerged from single colonies, isolates were streaked out at least three times by using the streak plate method. To ensure additional purity, exemplars of the rhizobial population (see Chapter II) and Flavobacteria were purified by introducing a streptomycin resistance. This was achieved by platting serial dilutions of the respective bacteria on TY media, including 50 µg/ml streptomycin. Grown colonies were afterwards transferred to media containing 200 µg/ml streptomycin and their morphology compared to colonies grown on media without antibiotics. Purified bacteria were stored in a 15% glycerol stock at -80°C.

4.2.13. Plant growth-promotion assays

Plants were sterilized as described above and stratified at 4°C for three to four days. Afterwards, seeds were transferred to plates containing ½ MS media, including sucrose (2.4 g/L Murashige & Skoog medium, 10 g/L sucrose, 8 g/L plant agar, adjusted to pH 5.5) and grown in a light cabinet (Panasonic, day 10 h, 22 °C; night 14 h, 19 °C) for six days. After the initial pre-growth phase, plants were transferred to nutrient-sufficient and -deficient containing agar plates (Tab. 6), including $MgCl_2$ and heat-treated bacteria (HT) as controls as well as bacterial dilutions.

Bacterial dilutions were prepared as follows: Bacteria were grown for 2-4 days before transfer of the seedlings in TY media at 28°C shaking at 200 rpm and then prepared on the same day as the nutrient assay. Therefore, bacteria were centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. Afterwards, 3 ml $MgCl_2$ (10 mM) was added to the pellet, bacteria were re-suspended and centrifuged to remove the supernatant. This was repeated once in order to ensure that no nutrients from the growth media or antibiotics used for selection were transferred. Afterwards, the OD_{600nm} was measured and the bacterial suspension adjusted to an optical density of 0.05. From this dilution 50 µl were taken, added to 50 ml of the previously prepared nutrient-sufficient and -deficient media and immediately poured into square petri-dishes. The medium was cooled to approximately 40°C before adding bacterial dilutions to avoid killing bacteria due to the heat treatment. Heat-treated bacteria were prepared by taking bacteria from the 0.05 dilution and heat-treatment for 30 minutes at 95°C. If not stated otherwise, heat-treated controls were generated by combining all bacteria of the respective assays. After preparing the plates, seedlings were transferred onto agar plates by adding four to five seedlings per plate and preparing three replica plates per condition. Plates were closed using Micropore surgical

paper tape (3M, Neuss, Germany) and grown in the Panasonic light cabinet for 18 days. To ensure homogeneous growth, plates were randomized and relocated at least once during the selection phase. After 18 days pictures were taken and further analyzed using ImageJ (Schneider et al., 2012). For further assays, i.e. DNA isolation, roots were separated from the shoots, quickly rinsed in sterile water, dried on filter paper and stored at -80°C until further usage. Shoot fresh weight was directly measured afterwards.

Tab. 6: Media used for testing plant growth under nutrient- sufficient and -deficient conditions. Nutrient composition for phosphorus-sufficient (625 µM KH_2PO_4) and -deficient (50 µM KH_2PO_4) conditions, as well as nitrogen-sufficient (11,400 µM) and -deficient (110 µM) conditions. Concentration of supplemented macro- and microelements indicated in µM. Protocol as published by Gruber et al., 2013

	Phosphate		Nitrogen	
Treatment (µM)	**625**	**50**	**11400**	**110**
$MgSO_4.7H_2O$	750	750	750	750
KH_2PO_4	625	50	625	625
NH_4NO_3	10300	10300	1000	10
KNO_3	9400	9400	9400	90
$CaCl_2.2\ H_2O$	1500	1500	1500	1500
$CoCl_2.6\ H_2O$	0.055	0.055	0.055	0.055
$CuCl_2.2\ H_2O$	0.053	0.053	0.053	0.053
H_3BO_3	50	50	50	50
KI	2.5	2.5	2.5	2.5
$MnCl_2.4\ H_2O$	50	50	50	50
$Na_2MoO_4.2\ H_2O$	0.52	0.52	0.52	0.52
$ZnCl_2$	15	15	15	15
Na-Fe-EDTA	75	75	75	75
MES (pH 5.5)	1000	1000	1000	1000
KCl	-	575	-	-
Agar	1%	1%	1%	1%

4.2.14. Statistical analysis for plant growth-promotion assays

All statistical tests were conducted using R. If the respective dataset was compared to a control, ANOVA was carried out followed by a Dunnett's post hoc test. If multiple comparisons were analyzed, a Tukey's HSD test was conducted after ANOVA. Dunnett's test and Tukey's HSD analyses were performed using the *multcomp* package. Boxplots were generated using R and the package *ggplot2*. Within the boxplot the median, first and third quartile are plotted. The ends of the whiskers represent the lowest point, still within 1.5 inter-quartile-range (IQR) of the lower quartile, and the highest points still within 1.5 IQR of the upper quartile.

Chapter II

Root-associated rhizobial members:

Plant growth-promoting properties on *A. thaliana*

1. Introduction

Plant growth and development have been extensively studied, giving insights into distinctive aspects of plant life ranging from seed germination over flowering to senescence. Plants that reside in the soil interact with a plethora of microorganisms such as nematodes, fungi, oomycetes or bacteria. However, only recently scientists have investigated the bacterial microbiota associated with plants and tried to address the relevance of plant-microbe interactions for plant growth in a community context.

Plant-microbe interactions were mostly studied on pathogenic or mutualistic bacteria that display distinctive, visible and reproducible phenotypes. However, mutualism, i.e. the relationship between two organisms that both benefit from each other (Boucher et al., 1982), often does not result in obvious phenotypes on the plant and therefore is easily overlooked. The development of next-generation sequencing technologies offered new possibilities to address mutualistic interactions associated with host organisms. Mutualistic bacteria are fundamental for plant growth and health as they increase nutrient availability, interfere with plant hormones and control phytopathogens (Lugtenberg and Kamilova, 2009). Such beneficial bacteria are also termed plant growth-promoting rhizobacteria (PGPR). The best studied mutualistic interaction is between rhizobia and legume plants that is termed symbiosis. Much is known about the colonization process as well as the morphological and transcriptional changes induced by rhizobia (Oldroyd et al., 2011). However, even though rhizobia are associated with non-legumes plants, such as *A. thaliana* (Bulgarelli et al., 2012), much less is known about their biological relevance.

Within this literature review I will introduce the reader to general principles of root colonization by PGPR, followed by a description of mechanisms that bacteria employ to affect plant growth. Then, the mutualistic interaction between plants and rhizobia will be discussed in depth.

1.1. Colonization of plant roots by bacteria

The most important trait of PGPR is their ability to successfully inhabit the rhizosphere and subsequently colonize plant roots, while effectively competing with surrounding microorganisms, a trait designated as rhizosphere competence (Lugtenberg and Dekkers, 1999). However, only a subset of soil-borne bacteria successfully colonizes roots. One group of soil-borne bacteria colonizes the rhizosphere or root surface, i.e. rhizodermis, but does not invade the plant interior. The second group includes endophytes, defined as bacteria that colonize the interior of plant tissues and in some cases even spread systemically into distal parts of the plant (Hardoim et al., 2008). Bacterial traits required for a successful root

colonization are recognition of plant-secreted compounds, bacterial motility, competition with other microorganisms and suppression of plant defense reactions.

Plants secrete an array of carbon-containing primary and secondary compounds into the rhizosphere, ranging from sugars to organic acids and enzymes, collectively termed root exudates (Rovira, 1969). Exudates can either repel or attract neighboring bacteria and recognition of attractants by bacteria can induce motility towards the root surface by inducing flagella-driven chemotaxis (de Weert et al., 2002). In *Escherichia coli* chemotaxis is regulated by an external stimulus that activates the bacterial histidine-kinase CheA, which then phosphorylates the regulatory proteins CheY and CheB (Hess et al., 1988). This phosphorylation allows these two proteins to interact with the flagella switch protein, eliciting a change in the direction of rotation of bacterial flagella. *Pseudomonas fluorescens cheA* mutants display a reduced motility and colonization of tomato roots (de Weert et al., 2002), consistent with the importance of flagella-driven chemotaxis for root colonization. Similarly, mutating genes encoding for flagella renders bacteria immotile and alters root colonization. Non-motile mutants of *P. fluorescens* display a reduced colonization rate of potato roots and lower numbers of colony forming units (CFU) are recovered the more distant root samples are collected from the initial inoculation site (Weger et al., 1987).

Motility of bacteria is also controlled by cell density-dependent quorum sensing. This coordinated activity among individual bacteria controls a range of additional processes, such as antibiotic production and transcriptional regulation (Kievit and Iglewski, 2000). Briefly, neighboring bacteria perceive diffusible signaling molecules, such as N-acyl homoserine lactones (AHLs), which are produced by a range of plant-associated bacteria, and that induce a signal transduction cascade as well as transcriptional changes (Cha et al., 1998; Whitehead et al., 2001). To test the importance of AHL-dependent quorum sensing for plant growth, an AHL-degrading lactonase was heterologously expressed in *Serratia plymuthica*, an abundant bacterium within the rhizosphere of strawberry or lettuce (Müller et al., 2009). Degradation of AHLs by the AHL-lactonase in transgenic *S. plymuthica* strains reduced the biocontrol activity against *Verticillium dahliae*, increased IAA production and swimming motility as well as impaired enzyme production. Additionally, the role of *phzI* for rhizosphere competence of *Pseudomonas chlororaphis* was revealed (Chin-A-Woeng et al., 2001). *phzI* encodes for a quorum sensing transcriptional regulator and mutating this gene impaired antibiotic production and thereby reduced antifungal activity of *P. chlororaphis* against the fungal pathogen *Fusarium oxysporum*. However, mutating *phzI* did not affect motility and colonization of tomato roots. Therefore, quorum sensing appears to be involved in bacterial competition with other microorganisms in the rhizosphere, a trait that is also an important biocontrol activity (Mazzola et al., 1992; Raaijmakers et al., 1995). As biocontrol activities are important for indirectly promoting plant growth, this trait will be discussed in depth in Chapter II.1.2.3.

Apart from motility also bacterial surface molecules, such as lipopolysaccharides (LPS), are believed to be important for root colonization. Supporting this assumption, LPS mutants of *P. fluorescens*

WCS417r display a reduced colonization of tomato (Duijff et al., 1997) as well as a reduced systemic resistance against wilt of radish induced by *F. oxysporum* (Leeman, 1995).

After colonizing the root surface, endophytes have to surpass additional barriers to invade the plant interior. Relevant bacterial traits for the colonization of the root interior are the presence of cell wall-degrading enzymes, such as cellulases and polygalacturonases, and mechanisms to interact with and suppress plant defense reactions (James et al., 2002; Compant et al., 2005). Endophytes can enter the plant passively through root cracks, sites of lateral root emergence and root tips, whereas active colonization relies on bacterial proteins, such as cell wall-degrading enzymes (James et al., 2002; Compant et al., 2005).

Residing in close proximity to plants, root-associated bacteria likely encounter plant defense reactions that bacteria need to either suppress or avoid. The induction of plant defense by phytopathogens is well characterized but less understood for PGPR (for a more detail description of plant defense reactions induced by phytopathogens refer to Chapter I.1.2). Inoculation with PGPR induces morphological changes that are generally associated with defense reactions. For example, inoculation with *Herbaspirillum seropedicae* thickens cortical cell walls of rice (James et al., 2002). Likewise, *Burkholderia sp*. strain PsJN induces an accumulation of phenolic compounds into the cell wall and strengthening of the exodermis and cortical cells of grapevine (Compant et al., 2005). This indicates that PGPR are recognized by the plant and induce defense reactions. However, how endophytes evade defense responses is not well understood.

1.2. The role of the bacterial root microbiota on plant health

After successful colonization of plant roots, PGPR can positively affect plant growth and health. Plant growth-promoting effects that are mediated by PGPR can be divided into direct and indirect effects. Direct effects influence plant growth by enhancing nutrient availability and interfering with plant hormones. Indirect effects affect plant growth by inhibiting the growth of phytopathogens and stimulating defense reactions and will be referred to as biocontrol activities. PGPR are believed to assert beneficial effects on plant growth through these direct and indirect mechanisms, thereby enhancing the phenotypic plasticity of the plant, i.e. the ability of the plant to react in multiple ways to environmental changes or abiotic and biotic stress conditions (Sultan, 2000; Goh et al., 2013).

1.2.1. Nutrient availability

Nutrient availability is a major factor influencing plant growth that is affected by diverse soil characteristics such as pH, mineral composition or microbial activities (Silver et al., 1994; Lucas et al., 1993; Lipson et al., 1999). PGPR positively affect plant growth under nutrient-limiting conditions by mobilizing inaccessible nutrients, increasing nutrient uptake or triggering plant developmental processes

that enhance nutrient uptake (Marschner et al., 2011). PGPR influence the availability of major elements within the soil such as of nitrogen, phosphorus, iron or sulfate.

Nitrogen can be found in diverse organic and inorganic forms that are distributed heterogeneously within the soil. Even though nitrogen is the most abundant element within the atmosphere, low nitrogen concentrations in the soil often limit plant growth (Koerselman and Meuleman, 1996). Plants enhance nitrogen uptake by two major strategies; increasing the root surface area and interacting with microorganisms (López-Bucio et al., 2003; Linkohr et al., 2002; Dakora and Phillips, 2002). Biological nitrogen fixation (BNF), which is extensively studied on mutualistic rhizobia, will be reviewed in detail in Chapter II.1.3.1. Briefly, the bacterial nitrogenase complex converts atmospheric nitrogen into ammonium during BNF and thereby supplies a readily available nitrogen source to the plant in exchange for carbon as an energy source. Under nitrogen deficiency inoculation with *Klebsiella pneumoniae* reduces growth retardation of wheat, confirming the connection between PGPR and nitrogen availability (Iniguez et al., 2004). This plant growth-promoting activity was linked to the ability of *K. pneumoniae* to fix atmospheric nitrogen, as mutating the bacterial gene *nifH*, encoding for a subunit of the nitrogenase complex, resulted in the loss of plant growth-promoting activities on wheat. Besides BNF, bacteria also influence other parts of the nitrogen cycle. For example, soil-borne bacteria mineralize organic nitrogen to ammonium that can be nitrified by other bacteria to nitrate (Verstraete and Focht, 1977; Zumft, 1997). However, for most PGPR genetic evidence for a link between enhanced nitrogen availability by PGPR and plant growth is lacking.

Phosphorus is another abundant element in the soil. Even though the soil contains large pools of phosphorus, often only ~ 0.1 % is available for the plant, while the remainder resides in for the plant inaccessible organic and inorganic forms (Achal et al., 2007). 20-80% of phosphorus exists in the organic form, mostly as phytic acid (Lim et al., 2007). The remaining phosphorus resides in diverse inorganic forms that bind minerals, such as calcium in alkaline soils or iron and aluminium in acidic soils, that are sparingly soluble (Haynes, 1982). Plants adapt to phosphorus deficiency by altering root architecture, utilizing glycolipids as phosphorus-free membrane lipids, acidifying the environment or secreting enzymes (Niu et al., 2013; Ma et al., 2001; Härtel et al., 2000; Hinsinger and Gilkes, 1996; Dinkelaker et al., 1989; Tadano et al., 1993). Additionally, phosphorus deficiency induces a transcriptional reprogramming in plants like rice or *A. thaliana*, thereby affecting regulators of plant phosphorus homeostasis, including transcription factors and phosphate transporters (Secco et al., 2013; Rouached et al., 2010). Under phosphorus sufficiency the negative regulator SPX DOMAIN GENE 1 (SPX1) binds the transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1), thereby inhibiting the up-regulation of genes involved in phosphorus homeostasis in *A. thaliana* (Puga et al., 2014). This binding is abolished under phosphorus deficiency, thereby inducing the expression of starvation genes such as *SPX1* or *PHOSPHATE TRANSPORTER 1* (*PHT1*).

Not only plants but also bacteria utilize insoluble organic phosphorus forms by several mechanisms, for example by the secretion of extracellular phosphatases such as phytases and acid phosphatases

(Rodríguez and Fraga, 1999; Duff et al., 1994; Richardson and Hadobas, 1997). Plants utilize organic phosphorus, for example phytic acid, only to a limited extent, as biomass of *Danthonia richardsonii* is drastically reduced when grown in agar plates or sand-vermiculite media supplying phytate as sole phosphorus form (Richardson et al., 2001). However, co-inoculating *D. richardsonii* with phytate-mineralizing soil bacteria, such as *Pseudomonas sp.* CCAR59, increases plant biomass to levels of plants grown under phosphorus-sufficient conditions. Inorganic phosphorus on the other hand can be solubilized by bacteria through the secretion of protons or organic acids that acidify the surrounding environment (Illmer and Schinner, 1992; Halder et al., 1990). To correlate the ability of bacteria to mobilize inorganic phosphorus with a plant growth-promoting activity, phosphorus-solubilizing bacteria (PSB) were tested for their effect on lettuce and maize (Chabot et al., 1996a). Inoculation of both plants with PSB, such as *R. leguminosarum* bv. *phaseoli* strain P31, increased total biomass and phosphorus content of lettuce and maize dependent on the soil used for growing plants. However, the genetic connection between mobilization of phosphorus and growth-promoting activities by PGPR is still lacking (Freitas et al., 1997).

Iron is another element that is predominately found as Fe^{3+} oxides within the soil. Fe^{3+} oxides are mostly insoluble under neutral pH, thereby limiting plant growth (Guerinot and Yi, 1994). Plants have developed two main strategies to acquire iron: Strategy I utilized by monocots and Strategy II used by dicots (Marschner and Römheld, 1994). Strategy I depends on the acidification of the environment that induces the solubilization of Fe^{3+}. Subsequently, Fe^{3+} is reduced to Fe^{2+} through the FERRIC REDUCTION OXIDASE 2 (FRO2) and Fe^{2+} is assimilated by the IRON-REGULATED TRANSPORTER 1 (IRT1) in *A. thaliana* (Robinson et al., 1999; Vert et al., 2002). Gene expression of *IRT1* and *FRO2* is regulated by the FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1 (FIT1; Colangelo and Guerinot, 2004). Strategy II plants secrete low molecular weight, iron-binding compounds into the rhizosphere, so called phytosiderophores, that chelate Fe^{3+} and are taken up by iron transporters, such as YELLOW STRIPE-LIKE (YSL) transporters found in many crop plants (Römheld and Marschner, 1986; Curie et al., 2009). Soil bacteria, such as *Pseudomonas sp.* or *Bacillus sp.*, produce siderophores and acidify the environment and thereby increase overall iron availability (Rashid et al., 2012; Zhang et al., 2009). *Bacillus subtilis* GB03, a ubiquitous soil-borne PGPR, increases plant biomass and iron content of *A. thaliana* (Zhang et al., 2007, 2009). *B. subtilis* acidifies the rhizosphere by secretion of protons and subsequently results in iron uptake by inducing the expression of the plant genes *IRT1* and *FRO2*. This increases the iron concentration dependent on the transcription factor FIT1. Therefore, acidification can be mechanistically linked to the plant growth-promoting activity of PGPR, such as *B. subtilis*, whereas involvement of bacterial siderophores in plant iron uptake was so far not proven.

The element sulfur is integrated into plant biomolecules such as cysteine, peptides and co-enzymes. Sulfur deficiency results in a world-wide decrease of crop production, therefore being of agricultural importance (Zhao et al., 1999). Plants primarily utilize inorganic sulfur, whereas most sulfur within the

soil is found in organic forms that are mainly inaccessible for the plant (Kertesz, 2000). Therefore, bacteria that are capable of metabolizing organic sulfur forms, such as sulfonates and sulfate-esters, are essential to enhance overall sulfur availability in soils.

1.2.2. Interference with plant hormones

Besides enhancing nutrient availability, PGPR affect plant growth by interfering with plant development- and defense-related phytohormones, such as auxins, ethylene, cytokinins, abscisic acid or gibberellins. PGPR alter plant hormone homeostasis by interfering with hormone biosynthesis and signaling as well as through the direct production of hormones (Glick et al., 2007; Lugtenberg and Kamilova, 2009).

Auxins influence diverse developmental processes in plants, such as root growth, apical dominance and flowering, by regulating cell division and cell enlargement (Davies, 1995). Plant development can also be influenced by auxin-producing PGPR such as *Azospirillum brasilense*. Inoculation of the auxin-producing bacterium *A. brasilense* on wheat decreases primary root length and increases root hair number (Dobbelaere et al., 1999). Auxin production by *A. brasilense* was directly linked to this root growth-promoting phenotype by mutant analyses. *ipdC* encodes for a key enzyme in indole-acetic acid (IAA) biosynthesis and mutating this gene in *A. brasilense* reduces auxin production by 90% and abolishes the effects of *A. brasilense* on root architecture. PGPR affect auxin homeostasis not only by directly synthesizing auxin but also indirectly, for example by producing nitrite oxide (NO) or interfering with auxin transport (Creus et al., 2005; Mathesius et al., 1998).

Ethylene also interferes with plant development by inhibiting root elongation and inducing senescence or fruit ripening (Bleecker and Kende, 2000). Additionally, ethylene regulates abiotic stress responses and systemic resistance against phytopathogens (Alonso et al., 1999; Pieterse et al., 1998). Bacteria alter plant ethylene homeostasis only to a limited extend by the direct production of ethylene, more relevant is the interference with ethylene biosynthesis. In plants, ethylene is synthesized from S-adenosyl-L-methionine via its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthase and ACC oxidase (Bleecker and Kende, 2000). The PGPR *Pseudomonas putida* expresses the bacterial gene *acdS,* encoding for an ACC deaminase that degrades ACC, thereby reducing ethylene concentrations in *Lycopersicon esculentum* under abiotic stress and thus enhancing plant biomass (Grichko and Glick, 2001). A genetic link between ethylene degradation by bacteria and plant growth promotion was constructed with *Pseudomonas putida* GR12-2 *acdS* mutants that were unable to utilize ACC (Glick et al., 1994). Compared to wild type *P. putida* strains the bacterial *acdS* mutants were unable to affect primary root length of canola seedlings, thereby linking the bacterial *acdS* gene with plant ethylene homeostasis and plant growth.

Cytokinins are phytohormones that regulate cell division and cell differentiation and thereby influence shoot and primary root growth (Riefler et al., 2006). *Bacillus megaterium* UMCV1, a PGPR inhabiting the rhizosphere of bean and *A. thaliana*, increases shoot biomass and reduces primary root

length by affecting cell elongation and proliferation (Ortíz-Castro et al., 2008). This phenotype depends on cytokinin signaling, as revealed by a loss of a phenotype when inoculating *B. megaterium* on *A. thaliana* cytokinin receptor mutants. Other PGPR, such as *Paenibacillus polymyxa*, actively produce cytokinins, however, the synthesis of cytokinins was not yet correlated with a plant growth-promoting activity (Timmusk et al., 1999).

Other hormones produced by PGPR are abscisic acid (ABA) and gibberellins (Forchetti et al., 2007; Tien et al., 1979). In plants, ABA regulates abiotic stress responses, such as enhancing drought tolerance. Drought increases ABA concentrations, causing stomata to close and this reduces water loss (Shinozaki and Yamaguchi-Shinozaki, 1997). *A. brasilense* decreases ABA content in *A. thaliana* by two-folds, demonstrating that PGPR are able to influence ABA homeostasis (Cohen et al., 2008). However, a direct link between ABA-production by PGPR and enhanced stress tolerance of plants was so far not described. Gibberellins control diverse plant developmental processes, such as leaf and root development, by affecting cell division and cell expansion (Hooley, 1994; Ogas et al., 1997). A range of PGPR produce gibberellins and are believed to enhance the tolerance of plants towards drought stress, however, direct genetic evidence for this hypothesis is lacking (Bottini et al., 2004; Joo et al., 2004).

1.2.3. Biocontrol activities

Biocontrol activities of PGPR protect plants against phytopathogens by limiting growth and infection of pathogens and thereby indirectly promote plant growth. PGPR exert biocontrol activities by competing for nutrients and environmental niches with pathogenic microorganisms, for example via siderophore production (Kloepper et al., 1980). Additional bacterial traits related to biocontrol activities are antibiotic production or interference with plant defense reactions (Griffin et al., 2004; Haas and Keel, 2003; Handelsman and Stabb, 1996). Most PGPR produce a wealth of secondary metabolites, including compounds with broad-spectrum antimicrobial activities that enable PGPR to restrict the growth of phytopathogens. Examples include 2,4-diacetylphloroglucinol and phenazines that are produced by diverse Pseudomonas species (Raaijmakers and Weller, 1998; Thomashow and Weller, 1988). For example, 2,4-diacetylphloroglucinol production by *Pseudomonas* spp. correlated with reduced disease symptoms of wheat challenged with the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Raaijmakers and Weller, 1998).

Additionally, biocontrol PGPR can elicit an induced systemic resistance (ISR) response in plants and thereby indirectly inhibit growth of phytopathogens (van Loon et al., 1998). Inoculation of plants with ISR-inducing PGPR enhances resistance against subsequent infections by pathogens across all tissues, the best characterized example is ISR induced by the bacterium *P. fluorescens* WCS417r (Pieterse et al., 1996). WCS417r activates the *A. thaliana* transcription factor MYB DOMAIN PROTEIN 72 (MYB72) and this subsequently induces a systemic spreading of immune responses to distal parts of the plant, as well as a resistance against a range of pathogens, such as *Pseudomonas syringae* or *Botrytis cinerea* (Ent et al., 2008). The plant transcriptional co-activators MYC2 and NPR1 are also involved in this

process as well as the phytohormones jasmonic acid (JA) and ethylene (Ent et al., 2008; Pozo et al., 2008; Pieterse et al., 1998). ISR does not constantly induce plant defense but primes the plant for a subsequent challenge by pathogens, allowing for a faster response towards pathogen attacks.

1.3. Rhizobia as plant growth-promoting rhizobacteria

Rhizobiaceae are well characterized PGPR that engage in a mutualistic interaction with diverse plant species and include bacterial species within the α- and β-Proteobacteria. α-Proteobacteria are represented by the following genera: Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium and Azorhizobium (Peter et al., 1996). Genera within the β-Proteobacteria include Burkholderia and Cupriavidus (Masson-Boivin et al., 2009). For simplicity bacteria belonging to the family Rhizobiaceae will from now on collectively referred to as rhizobia. The best studied case of mutualism is the interaction between rhizobia and legumes, a diverse plant family consisting of around 18,000 species. (Graham and Vance, 2003). This interaction will from now on referred to as symbiosis. A wealth of information about the colonization, infection process and beneficial effect on plant growth was gathered studying rhizobia in symbiotic interaction with legume plants (Oldroyd et al., 2011); however, only limited information is available about the impact of rhizobia on non-legume host plants.

1.3.1. Rhizobia and their interactions with legume plants

The symbiotic interaction between rhizobia and legumes is well studied. This chapter familiarizes the reader with the molecular mechanisms induced by the plant that allow rhizobia to colonize roots and that are induced by rhizobia in legumes resulting in phenotypic as well as transcriptional changes.

Legumes secrete chemical signaling cues, such as flavonoids, to attract rhizobia under nitrogen deficiency (Firmin et al., 1986; Coronado et al., 1995). Rhizobia respond to these molecules by inducing the expression of nodulation genes (*nod* genes) that are essential for the initial root colonization as well as for the later nodule formation (Peters et al., 1986). The first layer of recognition between plants and rhizobia is the interaction of the rhizobial activator protein NodD with flavonoids. Activated NodD binds to conserved promoter elements (*nod* boxes) of other rhizobial *nod* genes, thus controlling their expression. NodD induces the *nodABC* gene cluster and subsequently the NodABC proteins synthesize lipochitooligosaccharides (LCOs), the so called Nod factors (Dénarié et al., 1996). LCOs consist of a backbone of chitin-oligosaccharide derivatives that carry diverse modifications depending on the rhizobial exemplar. Different *nod* genes structurally modify LCOs and these modifications are believed to represent important cues for rhizobia-plant recognition (Price et al., 1992; Mergaert et al., 1997). Additional factors involved in root colonization and nodulation are exopolysaccharides (EPS), LPS and capsular polysaccharides (CPS) that are involved in cell-to-cell communication and interaction with legumes, by masking rhizobia from plant defense reactions (Skorupska et al., 2006). For example, the

Sinorhizobium meliloti sinR and sinI loci are required for EPS synthesis and nodulation in alfalfa, indicating an involvement of surface polysaccharides in nodule formation (Marketon 2002).

Plants perceive LCOs produced by *Mesorhizobium loti* through Nod factor receptors, such as the LysM-type receptor kinases NFR1 and NFR5 of *Lotus japonicus* (Radutoiu et al., 2003). Recognition of Nod factors by legume plants induces multiple physiological processes, including the curling of root hair tips and root hair colonization by rhizobia (Bauer, 1981). Rhizobia enter root hairs through infection threads that grow in the root hair and are formed by cell wall invaginations. Within the infection threads rhizobia reach and subsequently inhabit nodule primordia, specialized plant organs that are formed by reinitiating the cell cycle of differentiated cells within the root cortex. During nodulation rhizobia are released from the infection thread by endocytosis and then surrounded by the peribacteroid membrane, forming so called bacteroids. Within bacteroids, BNF occurs through the rhizobial nitrogenase enzyme complex, converting atmospheric nitrogen into ammonium that can be utilized by plants in exchange for carbon as energy source. This complex consists of two proteins, the dinitrogenase and the dinitrogenase reductase. Rhizobial genes involved in BNF are the *nif* genes, including *nifH* encoding for a Fe-protein and *nifD* and *nifK* for subunits of the dinitrogenase (Chen et al., 2003). Notably, the nitrogenase-complex functions only in an oxygen-free environment. Therefore, the bacteroid represents the ideal compartment for BNF, as the plant cell limits oxygen diffusion and internal oxygen is bound by plant-derived leghaemoglobin (Robson and Postgate, 1980; Hunt and Layzell, 1993).

Not unexpected, legumes induce diverse transcriptional changes during nodule development. Examples include genes encoding for cell wall-modifying proteins, extensins or a peroxidase expressed by the legumes *Pisum sativum*, *Vigna unguiculata* and *Medicago truncatula*, respectively (Scheres et al., 1990; Arsenijevic-Maksimovic et al., 1997; Cook et al., 1995a). Other proteins associated with nodulation are enzymes involved in phenylpropanoid biosynthesis, such as the chalcone synthase encoded by *M. truncatula*, which produces flavonoids and is essential to induce nodule formation upon recognition of the symbiont *Sinorhizobium meliloti* (Wasson et al., 2006). The legume *M. truncatula* induces the expression of defense-related genes during the early nodulation phase of *S. meliloti* (Lohar et al., 2006). Therefore, it is essential for rhizobia to evade plant defense reactions. EPS surface polysaccharides are proposed to enable rhizobia to evade the detection by the plant immune system. Supporting this assumption, *Rhizobium meliloti* and *S. meliloti* mutants that lack EPS result in an enhanced callose deposition and phenol accumulation in nodules and induce a higher number of defense-related genes in *Medicago sativa* and *M. truncatula*, respectively (Niehaus et al., 1993; Jones et al., 2008). Additionally, the structure of the flg22 peptide, which is recognized by the plant receptor-like kinase FLS2, is diverged in *R. meliloti* compared to phytopathogens (Felix et al., 1999). This divergence is believed to mask *R. meliloti* from recognition by the plant immune system. Supporting this assumption, treatment of *Lotus japonicus* with purified flagella from *Mesorhizobium loti* does not stimulate defense responses, suggesting that the divergence of flg22 masks rhizobia from recognition by the plant (Lopez-Gomez et al., 2011). Even though plants do not respond to treatment with rhizobial flagellin, *S. meliloti*

still induces the expression of defense-related genes in *M. truncatula* during early phases of nodulation, whereas these genes were repressed at later time points (Lohar et al., 2006). This early up-regulation indicates that the plant still recognizes colonization by rhizobia and the later down-regulation of defense-related genes suggests that rhizobia escape or counter-act defense responses. Accordingly, a recent study demonstrated that application of Nod factors from *Bradyrhizobium japonicum* suppresses defense responses in soybean by reducing reactive oxygen species (ROS) production (Liang et al., 2013). Additionally, many rhizobia possess functional type-3-secretion systems (T3SS) that secrete proteins into the plant cell and are believed to modulate defense responses (Deakin and Broughton, 2009). However, as rhizobial T3SS mutants show contrasting phenotypes on plant hosts, the role of T3SS during symbiosis is not yet uncovered.

1.3.2. Rhizobia and their interactions with non-legume plants

Compared to the detailed studies on symbiosis, much less is known about the interaction between rhizobia and non-legume plants. However, the bacterial root microbiota of diverse Brassicaceae plants displays a high abundance of rhizobia (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014) and rhizobia were effectively isolated from non-legumes such as *A. thaliana* or wild rice (Berge et al., 2009; Peng et al., 2008). Notably, symbiotic rhizobia also colonize and promote growth of non-legumes, including rice (Yanni et al., 1997; Chi et al., 2005). For example, *S. meliloti* strain 1021, a symbiont of alfalfa, effectively colonizes rice and displays an endophytic behavior by spreading systemically within the plant based on a microscopic analysis of bacteria labelled with green fluorescent protein (GFP; Chi et al., 2005). Additionally, *S. meliloti* enhances photosynthetic activity as well as the total root and shoot area of rice. This indicates that symbiotic rhizobia colonize and exert plant growth-promoting activities on non-legume plants.

Until now, no evidence exists that rhizobia require the presence of *nod* genes for the colonization of non-legume plants, indicating that other mechanisms are required for the colonization process compared to legumes. For example, *Azorhizobium caulinodans* ORS571 *nodD* and *nodC* mutants did not display an altered colonization of *A. thaliana* roots compared to wild type strains (Gough et al., 1997). Therefore, neither perception of flavonoids by NodD nor synthesis of LCOs by NodC appear to be required for colonization of *A. thaliana* by symbiotic rhizobia, which were originally isolated from *Sesbania rostrata*. Even though Nod factors appear not to be essential for the colonization of non-legumes, non-legumes respond to application of Nod factors. Treating *A. thaliana* and tomato with Nod factors purified from the soybean symbiont *B. japonicum* reduces plant defense reactions, as seen by a decreased production of reactive oxygen species, kinase activation and callose deposition (Liang et al., 2013). The *A. thaliana* chitin receptor LYK1/CERK1 represents a structurally related protein to the Nod factor receptor NFR1 of soybean. Mutating *A. thaliana* LYK1/CERK1 and treating these mutants with Nod factors failed to repress immune responses, indicating the presence of a conserved recognition process for chitin-like compounds.

An increasing body of evidence suggests that rhizobia not only colonize non-legumes but also improve plant growth by enhancing nutrient uptake, interfering with hormone homeostasis and controlling proliferation of phytopathogens. For example, inoculation of several symbiotic rhizobia, such as *Rhizobium leguminosarum* bv. *trifoli* and *Bradyrhizobium* sp.*,* on rice increased yield and nutrient uptake by up to 20% (Biswas et al., 2000). However, this plant growth-promoting activity was not correlated with BNF as measured by nitrogen-15-based studies, indicating that the functional capacities of symbiotic and non-symbiotic rhizobia in regard to nitrogen availability differ. Additionally, the symbiotic rhizobium *R. leguminosarum* colonizes maize and lettuce and significantly increases plant biomass correlating with the ability of this strain to mobilize insoluble phosphorus (Chabot et al., 1996b, 1996a). Notably, many symbiotic rhizobial strains, including *R. leguminosarum* bv. *trifoli* and *R. meliloti*, mobilize phosphorus in vitro, suggesting that rhizobia are generally important for increasing phosphorus availability (Halder and Chakrabartty, 1993). Additionally to nutrient uptake, the plant growth-promoting capacities of rhizobia also correlate with hormone homeostasis. Symbiotic *S. meliloti* strain 1021 colonizes rice and significantly increases shoot biomass, root volume, shoot nitrogen content and grain yield and this correlates with an increase in root and leaf IAA and gibberellin content (Chi et al., 2005). Apart from affecting plant nutrition and hormone levels, symbiotic rhizobia increase growth of non-legumes indirectly by biocontrol activities. *Rhizobium etli* G12 colonizes roots of potato and reduces gall formation induced by the nematode pathogen *Meloidogyne incognita* (Hallmann et al., 2001). However, the mechanisms behind this biocontrol activity were not uncovered. Another example is biocontrol activity induced by *R. leguminosarum* bv. *phaseoli* RPE6 against *Rhizoctonia solani* that correlated with the production of phenol by this rhizobial strain (Mishra et al., 2006).

1.4. Thesis aim

Rhizobia are ubiquitous bacteria that are associated with diverse plant species, belonging to legume (van Rhijn and Vanderleyden, 1995; Martínez-Romero et al., 1991) and non-legume plants (Bulgarelli et al., 2012). However, most investigations of rhizobia were concentrated on their symbiotic interaction with legumes. The nodule formation on legumes induced by rhizobia, their functional capacity to fix atmospheric nitrogen as well as the underlying molecular mechanisms are well understood (Oldroyd et al., 2011). Intriguingly, even though rhizobia are abundant on non-legume plants, only limited knowledge exists about their function and the molecular mechanisms employed. Therefore, my first aim is the assessment of the biological relevance and functional variability of the rhizobial population on the non-legume plant *A. thaliana*. My second aim addresses the question, whether rhizobia that colonize *A. thaliana* also trigger molecular responses in the plant.

First, I will isolate and taxonomically identify several exemplars of the naturally occurring rhizobial population inhabiting roots of *A. thaliana* in cooperation with other members of our laboratory. To assess the functional properties of rhizobia, isolated rhizobial exemplars will be tested for low complexity phenotypes such as calcium phosphate mobilization and auxin production. Second, rhizobia will be inoculated on *A. thaliana* and tested for their plant growth-promoting activities under nitrogen- and phosphorus-sufficient as well as -deficient conditions. I hypothesize that a subset of the tested rhizobial population will exert a plant growth-promoting activity on *A. thaliana*.

To understand, whether rhizobia induce a molecular response in roots of *A. thaliana* an RNA-sequencing (RNA-Seq) approach will be employed. Therefore, I will investigate how inoculation of one Rhizobium affects plant growth of *A. thaliana* and whether this Rhizobium proliferates on the root system in a time-resolved manner. To monitor changes within the root transcriptome, I will select different time points after inoculation of *A. thaliana* with Rhizobium to perform an RNA-Seq experiment. I hypothesize that rhizobia will be recognized by the plant and induce shifts in gene expression. Ideally, these shifts can be used to uncover how *A. thaliana* responds towards rhizobia on a molecular level. In case the newly isolated rhizobia represent novel PGPR, changes within the root transcriptome will be useful to link the observed phenotypic changes to the molecular mechanisms triggered by rhizobia.

2. Results

2.1. Isolation and taxonomic assignment of *A. thaliana* associated rhizobia

This study addresses the question whether rhizobia, a class of bacteria well described in symbiotic interaction with legumes, offer beneficial roles for non-legume plants. Additionally, I was interested in intra- and inter-species variations on phylogenetic and phenotypic level, as such variations could provide important tools to dissect the mechanisms behind plant growth-promoting activities.

To analyze rhizobia by a population-based approach, bacteria were isolated from roots of two non-legume plant species grown in four soil types (for details see Methods Chapter II.4.2.3). The isolation background included two host plants (*A. thaliana* and *C. hirsuta*) and four soil types (natural soil collected in Cologne and Golm with plants grown in the greenhouse and in Widdersdorf and France with plants collected at a natural site; Tab. 7).

Tab. 7: Isolation background of rhizobia. Soil type: naturally collected soil used for plant growth. Brackets: genotype of the plant species used for bacteria isolation. Asterisks: rhizobia were isolated from plants grown and collected from a natural site, therefore no information about the plant genotype can be added.

Bacteria ID	Plant species	Soil type
R129_A	*A. thaliana* (Sha)	Cologne
R129_B	*A. thaliana* (Sha)	Cologne
R129_C	*A. thaliana* (Sha)	Cologne
R129_D	*A. thaliana* (Sha)	Cologne
R129_E	*A. thaliana* (Sha)	Cologne
R129_F	*A. thaliana* (Sha)	Cologne
R129_H	*A. thaliana* (Sha)	Golm
R129_I	*A. thaliana* (Sha)	Golm
R129_J	*A. thaliana* (Sha)	Golm
R129_K	*A. thaliana* (Sha)	Golm
R129_L	*A. thaliana* (Ler)	Cologne
R129_M	*C. hirsuta* *	Widdersdorf
R129_N	*C. hirsuta* *	France
R219_B	*A. thaliana* (Sha)	Cologne
R219_F1	*A. thaliana* (Sha)	Cologne
R219_B2	*A. thaliana* (Sha)	Cologne
R13_A	*A. thaliana* (Sha)	Cologne
R13_B	*A. thaliana* (Sha)	Cologne
R13_C	*A. thaliana* (Sha)	Cologne
R13_D	*A. thaliana* (Sha)	Cologne
R13_E	*A. thaliana* (Sha)	Golm
R13_F	*A. thaliana* (Sha)	Golm

Taxonomic identity of all bacterial isolates was determined by Sanger sequencing of a partial 16S rRNA gene fragment (~500 bp) and blasting of this fragment against an internal database based on Bulgarelli et al., 2012 (for details see Methods Chapter II.4.2.4). To avoid contamination, isolated bacteria assigned to rhizobia were purified by introducing a natural occurring streptomycin resistance

into the genome by subsequent growth on media with increasing streptomycin concentrations. By this approach 22 rhizobial exemplars were isolated and purified. Based on Sanger sequencing of the 16S rRNA gene and defining a bacterial species by applying a threshold > 97% sequence identity, the isolated rhizobia were assigned to three species (designated R129, R13 and R219). Next, the genomes of all 22 rhizobia were sequenced using Illumina or PacBio sequencing platforms (for details see Methods Chapter II.4.2.6). To confirm the phylogenetic relationship of the rhizobial population, all exemplars were analyzed based on the full 16S rRNA gene sequence (~1,500 bp) retrieved from the rhizobial genomes compared to the shorter fragment previously subjected to Sanger sequencing. To place the newly isolated exemplars into an evolutionary framework, publicly available genomes from symbiotic rhizobia were included in this comparison (Fig. 27).

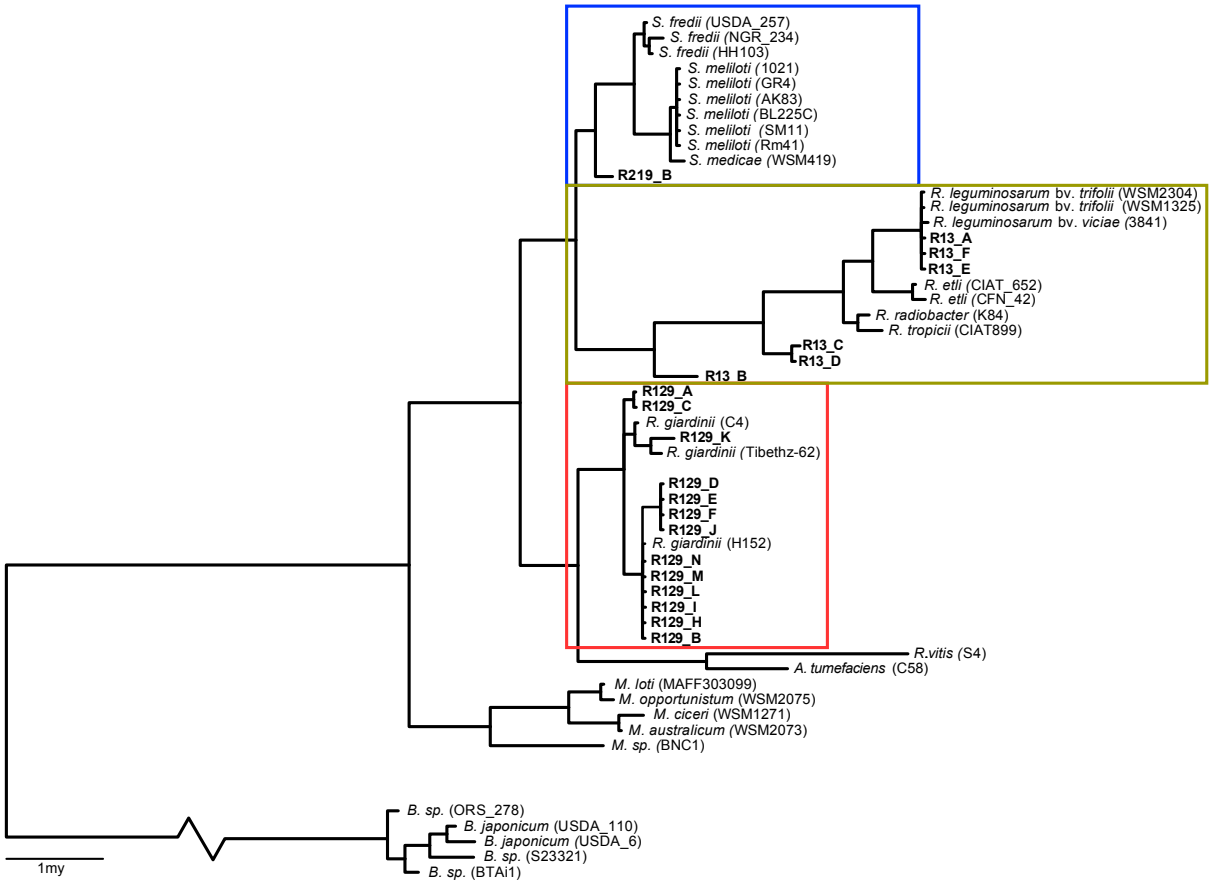


Fig. 27: *A. thaliana* associated rhizobial members represent three bacterial species that are closely related to symbiotic rhizobia. Phylogenetic relationship of rhizobial exemplars based on a Bayesian tree and as inferred from 16S rRNA gene information retrieved from rhizobial genomes. Bradyrhizobium was used as outgroup. Scale given in million years (my). S: Sinorhizobium. R: Rhizobium. A: Agrobacterium. M: Mesorhizobium. B: Bradyrhizobium. Brackets: Exemplar ID. Bold: rhizobia used within this study. Italics: publicly available genomes. Genome analysis and figure preparation by Ruben Garrido Oter.

Clustering of the genomic 16S rRNA gene confirmed the separation of the newly isolated rhizobia into three distinctive species. Furthermore, an evolutionary relationship between *A. thaliana* associated rhizobia to published symbiotic rhizobia existed, as those bacterial groups clustered together. The closest related symbiotic rhizobia were *R. giardinii*, *R. leguminosarum* and *S. meliloti* that co-clustered with

R129, R13 and R219, respectively. Apart from an inter-species variation, also members belonging to the same bacterial species displayed variations within the 16S rRNA gene, as for the species R129 three distinct sub-branches were observed. However, this observation did not correlate with the isolation background, as rhizobia isolated from different plant species or soil types co-clustered. For example, the exemplars R129_B and R129_H that were isolated from Cologne and Golm soil co-clustered based on the 16S rRNA gene sequence.

In summary, we could successfully isolate several exemplars from three rhizobial species from *A. thaliana* and a related Brassicaceae plant species, representing a diverse rhizobial population based on the 16S rRNA gene and whole genome information (Ruben Garrido Oter, personal information).

2.2. In vitro characterization of phenotypic traits within the rhizobial population

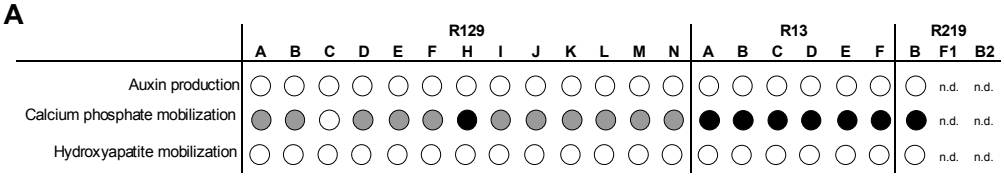
To assess, whether the here isolated rhizobia comprised novel PGPR, the rhizobial population was characterized for the presence of described plant growth-promoting activities in vitro. These traits included the production of auxin and mobilization of calcium phosphate (CaP) and hydroxyapatite (HA; for details see Methods Chapter II.4.2.7 and II.4.2.8).

First, the rhizobial population was screened for auxin production, as influencing auxin homeostasis is a well described trait by which PGPR affect plant growth, which is also employed by symbiotic rhizobia (Datta and Basu, 2000). None of the tested rhizobia produced auxin in vitro, with all exemplars producing ~1 µg/ml auxin (Fig. 28A, B). This concentration was below the described auxin concentration produced by other symbiotic rhizobial exemplars such as *R. meliloti* (Datta and Basu, 2000). However, the absence of auxin production in vitro does not exclude that rhizobia affect auxin homeostasis by other mechanisms, for example by interfering with auxin transport.

Another trait employed by PGPR is increasing nutrient availability by mobilizing insoluble phosphorus forms (Rodríguez and Fraga, 1999). Thus, rhizobia were tested for their ability to mobilize CaP and HA, two inorganic phosphorus forms (Fig. 28A and C). 19 out of 20 tested rhizobia mobilized CaP to various extents and all lacked the ability to mobilize HA (data not shown). A strong mobilization of CaP correlated with a halo formation and complete clearing of the media. Mobilization of CaP ranged from no to strong mobilization (R129_C and R13_E, respectively). Additionally, a high diversity was observed among the rhizobial population for this trait. R129 exemplars displayed a broad range of CaP mobilization abilities, ranging from no over intermediate to strong mobilization. This resembled the variations detected on 16S rRNA gene level. However, no obvious correlation between phylogenetic and phenotypic variation could be detected for this particular trait. For example, R129_H and R129_I clustered closely together based on the 16S rRNA gene (Fig. 27) but displayed different potentials to mobilize CaP (Fig. 28). Additionally, rhizobia were tested for their ability to utilize the two organic phosphorus forms, phytate and phosphonate and two sulfate deposits, toluene- and pentene-sulfonate

(Dr. A. Schmalenberger, personal communication). A similar tendency as for CaP was observed, as the rhizobial population exhibited a range of abilities to mobilize these nutrients, overall indicating a high phenotypic variation among the rhizobial population.

A



B

Strain	IAA (µg/ml)	SD	reference
R129_A	0.73	0.03	this study
R129_B	0.54	0.23	this study
R129_C	0.62	0.01	this study
R129_D	1.02	0.32	this study
R129_E	1.28	1.04	this study
R129_F	0.35	0.18	this study
R129_H	0.52	0.43	this study
R129_I	0.48	0.43	this study
R129_J	0.53	0.52	this study
R129_K	1.45	0.62	this study
R129_L	1.45	0.28	this study
R129_M	0.82	0.40	this study
R129_N	0.65	0.52	this study
R13_A	0.78	0.38	this study
R13_B	0.44	0.26	this study
R13_C	0.34	0.42	this study
R13_D	0.72	0.37	this study
R13_E	0.52	0.26	this study
R13_F	0.82	0.55	this study
R219_B	0.97	0.03	this study
Azospirillum brasilense	35.00	n.d.	Dr. Spaepen (personal communication)
Rhizobium phaseoli	17.60	n.d.	Datta et al., 2000
Rhizobium leguminosarum	2.00	n.d.	Datta et al., 2000
Rhizobium meliloti	20.00	n.d.	Datta et al., 2000

C

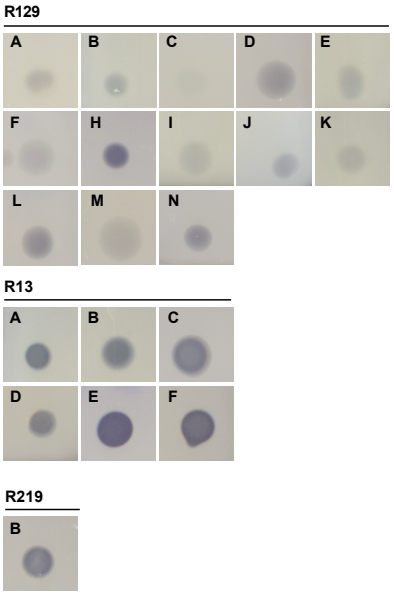


Fig. 28: The tested rhizobial members mobilize calcium phosphate but do not produce auxin in vitro. (A) Overview of phenotypic traits exhibited by the rhizobial population (tested species include R129, R13 and R219). White circle: No phenotype. Grey circle: Intermediate phenotype. Black circle: Strong phenotype. **(B)** In vitro indole-acetic acid (IAA) production measured by the Salkowski assay, including published information from plant growth-promoting rhizobacteria. **(C)** Mobilization of calcium phosphate (CaP). A strong mobilization correlates with a clearing of the media and halo formation. For better visualization bacterial colonies were rinsed with water before taking the picture. n = 2. SD = Standard deviation. n.d. = no data available.

2.3. Plant-dependent characterization of rhizobia under nutrient-sufficient and -deficient conditions

The ability of members of the rhizobial population of *A. thaliana* to mobilize insoluble phosphorus and sulfate forms raised the question, whether the rhizobial population associated with *A. thaliana* positively affects plant growth dependent on the nutrient conditions. Therefore, rhizobial exemplars were tested for a growth-promoting activity under nutrient conditions sufficient for plant growth (i.e. nutrient-sufficient), when limiting phosphorus or nitrogen concentrations or when supplying only insoluble phosphorus sources (i.e. nutrient-deficient). Since symbiotic rhizobia colonize legumes dependent on

the nitrogen concentration (Coronado et al., 1995), plant growth promotion was first assessed under nitrogen-sufficient and -deficient conditions. Second, I examined whether the ability of rhizobia to mobilize phosphorus correlated with increasing plant growth under phosphorus deficiency (for details see Methods Chapter I.4.2.13 and II.4.2.9).

Nutrient deficiency reduces plant growth and allows to test whether rhizobia alleviate this growth retardation (Gruber et al., 2013). Limiting either the phosphorus or nitrogen concentration in the media used for growing plants reduced growth of *A. thaliana* in the absence of R129_E (Fig. 29). Phosphorus-deficiency reduced shoot biomass and primary root length and increased lateral root length. Nitrogen deficiency on the other hand caused a distinct phenotype compared to phosphorus deficiency. Under nitrogen deficiency biomass was severely reduced and a less prominent effect on primary root length was observed compared to phosphorus deficiency. Co-inoculating *A. thaliana* together with the rhizobial exemplar R129_E increased root length under all tested nutrient conditions, almost recovering root length under nutrient deficiency to levels observed under nutrient-sufficient conditions. Additionally, R129_E increased the density of fine root hairs under nitrogen- and phosphorus-deficient but not under nutrient-sufficient conditions. The actions of R129_E on plant growth were restricted to altering root architecture as no effect on shoot biomass was detected under any of the tested nutrient-conditions.

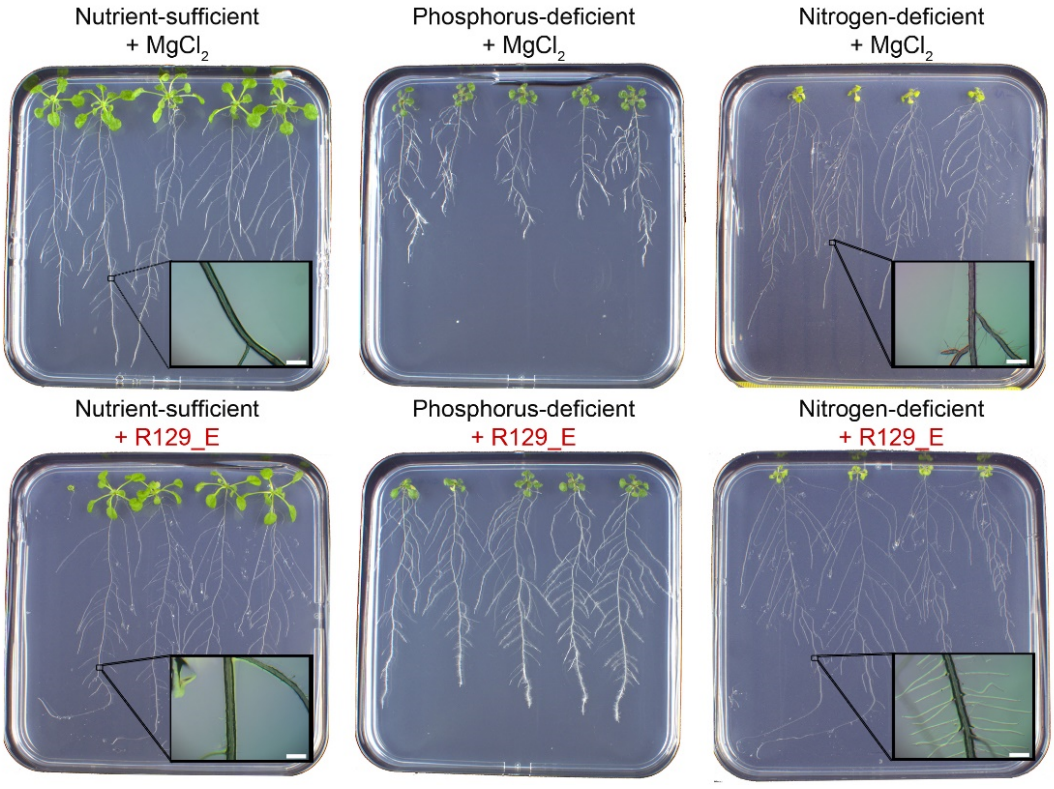


Fig. 29: Rhizobial exemplar R129_E alters root architecture of *A. thaliana* dependent on the nutrient concentration. Growth phenotype of *A. thaliana* 18 days post inoculation (dpi) with $MgCl_2$ or R129_E at a final density of 10^5 cells/ml. Right lower panel of the plate: magnification of a section of the main root. Bar = 500 µm (for magnification).

To evaluate, whether the ability of R129_E to enhance root growth was conserved across the rhizobial population, a subset of the population was tested on their effect on *A. thaliana* first under nitrogen-sufficient and -deficient conditions. To allow for a fast-screening procedure, primary root length and shoot biomass were used as main phenotypic readouts.

6 of the 8 tested rhizobial exemplars increased primary root length under both nitrogen concentrations (Fig. 30A). Comparing the effect under nitrogen-sufficient and -deficient conditions, all exemplars displayed a similar pattern, but roots were significantly longer under nitrogen deficiency (Two-way ANOVA comparing nitrogen-sufficient and -deficient conditions: $p = 0.04$). Notably, the tested exemplars displayed qualitative differences, even among bacteria of the same species. Inoculation with R129_E increased root length, whereas R129_A induced no effects on root growth compared to non-treated control plants. Apart from R129_A and R129_C all tested rhizobial exemplars increased root length, with quantitative differences observed between exemplars as seen for R13_A and R13_C under nitrogen deficiency. The observed qualitative and quantitative differences were not due to the isolation background, as the exemplars R129_A and R129_E were isolated from the same background but displayed qualitative differences.

As isolation background of the rhizobial population did not explain the observed quantitative and qualitative differences, I assessed whether an absence or lower abundance of a subset of rhizobial exemplars on the root system explained these results. This question was addressed by measuring the relative abundance of rhizobia on *A. thaliana* roots by quantitative PCR (qPCR). Therefore, the 16S rRNA gene was amplified with primers specifically targeting the rhizobial 16S rRNA gene. Afterwards, their relative abundance was normalized by amplifying the plant chloroplast 16S rRNA gene with plant-specific primers (for details see Methods section Chapter II.4.2.10). All rhizobial exemplars that increased root length also proliferated on the root system by two to three log-units (Fig. 30C). Interestingly, R129_A and R129_C, the two rhizobial exemplars that did not increase root length, were either not detected on the root system or only at a low relative abundance. This indicates that the presence of rhizobia on the root system correlates with their ability to increase root length. However, it is unclear whether the absence of R129_A and R129_C on the root system is due to repelling activities of the plant or the inability of those two rhizobia to colonize roots or the media. Additionally, no obvious correlation between the relative abundance of rhizobia on the roots and the observed quantitative differences in root length existed. For example, R219_B increased root length by ~ 30% under nitrogen-sufficient and by ~ 80% under nitrogen-deficient conditions. However, R219_B did not differentially proliferate on roots grown under nitrogen sufficiency and deficiency. This indicates that the observed quantitative differences induced on root growth cannot be explained by a differential proliferation of rhizobia on the root system. Finally, none of the tested rhizobia increased shoot or root biomass under nitrogen-sufficient or -deficient conditions (Fig. 30B and D). This suggests that the plant growth-promoting activities of the rhizobial population is restricted to altering root architecture.

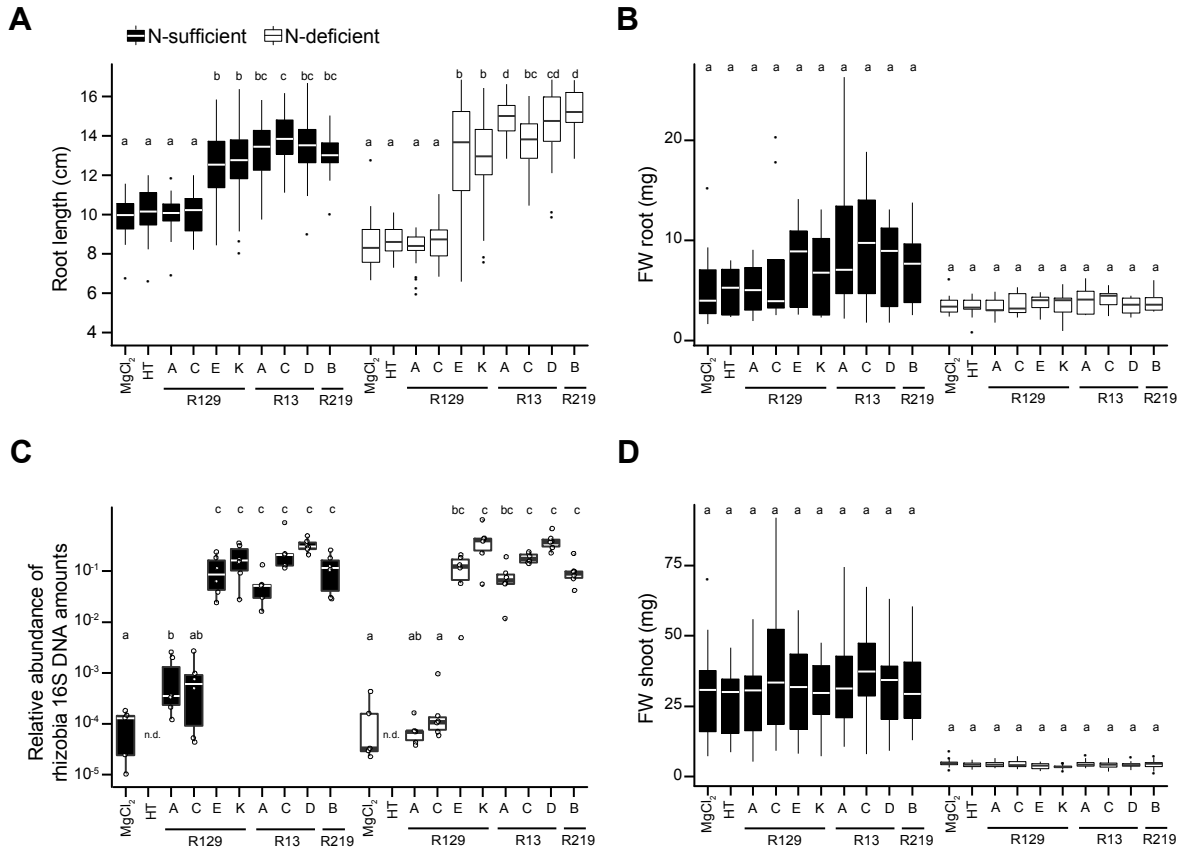


Fig. 30: Rhizobial exemplars increase primary root length under nitrogen-sufficient and -deficient conditions. Plant growth-promoting activities of rhizobia on *A. thaliana* under nitrogen (N)-sufficient and -deficient conditions. Plants were mock-treated ($MgCl_2$ and HT (heat-treated)) or treated with rhizobial exemplars (including the species R129, R13 or R219) at a final density of 10^5 cells/ml. **(A)** Primary root length (cm), **(B)** Root fresh weight (mg), **(C)** Relative abundance of rhizobia determined by quantifying the 16S rRNA gene of rhizobia that was normalized by the plant chloroplast 16S rRNA gene and log_{10}-transformed. Open circles represent individual measurements. **(D)** Shoot fresh weight (mg). (A/B/D): n = 25-30 plants measured in three independent biological replicates. (C): n = 6 measurements performed in two independent biological replicates. Significance letters calculated separately for each nitrogen concentration (Tukey's HSD, $p < 0.05$). n.d. = no data available.

Next, I evaluated whether the ability of members of the rhizobial population to mobilize phosphorus correlated with a plant growth-promoting activity under phosphorus-deficient conditions. Therefore, all 22 rhizobial exemplars were evaluated for their effect on *A. thaliana* under phosphorus deficiency. Apart from testing for a plant growth-promoting activity, this will also allow to evaluate the phenotypic variability within the rhizobial population.

Similar as for nitrogen-sufficient and -deficient conditions, 21 out of 22 rhizobia increased primary root length under phosphate deficiency (Fig. 31). Again, rhizobia displayed qualitative as well as quantitative differences (Tab. S 4). Qualitative differences were detected for R129_C that did not increase root length compared to all other rhizobial exemplars, similar to the observed phenotype for this exemplar under nitrogen deficiency. In contrast to the absence of a phenotype under the tested nitrogen conditions, R129_A induced a slight but significant effect on root growth. Additionally,

quantitative differences among the rhizobial population were detected (pairwise statistical analysis can be found in Tab. S 4) and a few rhizobial exemplars induced a different root growth phenotype under phosphorus-sufficient compared to -deficient conditions. For example, R13_C increased root length under phosphorus sufficiency but induced a less pronounced effect under phosphorus deficiency. Of note, R13_C did not induce quantitative differences dependent on the nutrient concentration under the previously tested nitrogen conditions. Also, R129_K increased primary root length under nitrogen sufficiency but only weakly affected root length under phosphorus deficiency. Overall roots were significantly longer after bacterial treatment under phosphorus-deficient compared to -sufficient conditions (Two-way ANOVA comparing phosphorus-sufficient and -deficient conditions: $p < 0.001$). This indicates that the major part of the rhizobial population affects root growth with quantitative and qualitative differences depending on the nutrient status.

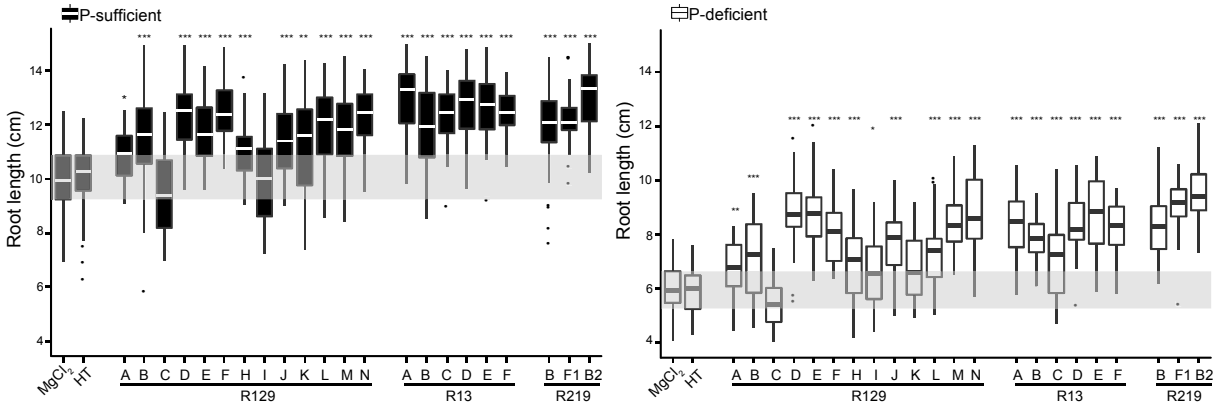


Fig. 31: Rhizobial exemplars affect root growth under phosphorus-sufficient and -deficient conditions. Plant growth promotion of rhizobia on *A. thaliana* was tested under phosphorus (P)-sufficient and -deficient conditions. Plants were either mock-treated ($MgCl_2$ or HT (heat-treated)) or treated with rhizobia (including the species R129, R13 or R219) at a final density of 10^5 cells/ml (R = Rhizobia) and primary root length (cm) was scored afterwards. n = 30-45 plants measured in three independent biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's test using $MgCl_2$ as control, performed separately on both phosphorus conditions). Statistical analysis based on pairwise comparisons can be found in Tab. S 4.

A limitation of the used gnotobiotic growth system so far is the fact that phosphorus deficiency was induced by reducing the concentration of soluble phosphorus within the media (from 625 µM to 50 µM KH_2PO_4). Therefore, the root growth-promoting activity of rhizobia cannot be directly linked to their potential to mobilize insoluble phosphorus forms (Fig. 28). To overcome this limitation, two rhizobial exemplars were tested for their effect on plant growth when supplying only insoluble CaP or HA as sole phosphorus source for the plant. R129_E and R13_F exhibited contrasting CaP mobilizing capacities in vitro (Fig. 28C). Therefore, those two rhizobia represented ideal exemplars to assess, whether their different ability to mobilize CaP correlated with different growth-promoting activities.

Both rhizobial exemplars significantly increased root length independent of the supplied phosphorus source and no qualitative differences were observed between the two exemplars (Fig. 32A). Additionally, none of the tested exemplars increased shoot biomass under the tested conditions (Fig. 32B). However,

it has to be noted that supplying CaP only marginally reduced shoot biomass, whereas CaP application strongly reduced primary root length under mock-treatment. This suggests that *A. thaliana* is able to utilize CaP by itself to a certain extent, not allowing to induce a full phosphorus deficiency response. Therefore, a wider range of organic and inorganic phosphorus forms needs to be examined to gain a better overview about the ability of rhizobia to enhance plant growth in relation to phosphorus mobilization. Nevertheless, these results suggest that at least under the tested conditions rhizobia do not actively provide the plant with insoluble phosphorus forms by mobilization of CaP or HA.

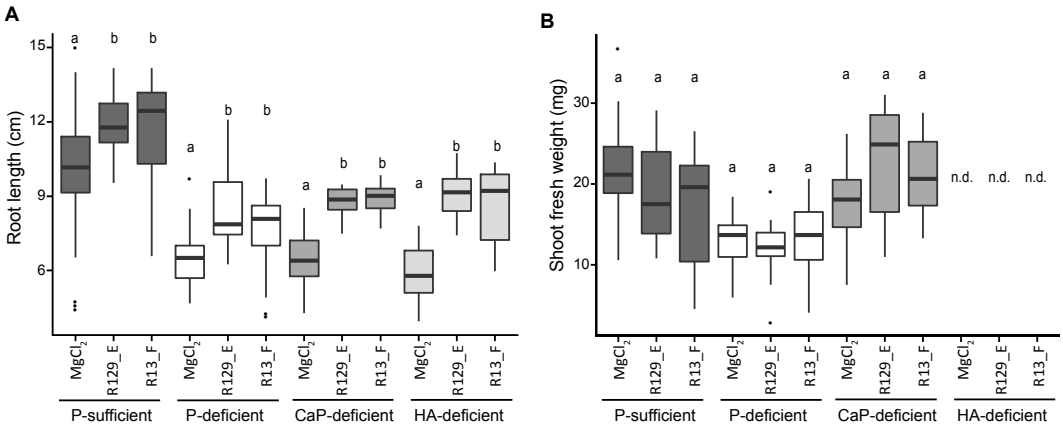


Fig. 32: R129_E and R13_F increase root length but not shoot biomass *of A. thaliana* grown on insoluble phosphorus forms. (A) Primary root length (cm) and **(B)** shoot fresh weight (mg) of *A. thaliana* grown under phosphorus-sufficient (black) and -deficient (white) conditions or after supplementing only the insoluble phosphorus forms calcium phosphate (CaP, dark grey) and hydroxyapatite (HA, light grey). *A. thaliana* was mock-treated ($MgCl_2$) or treated with R129_E and R13_F at a final density of 10^5 cells/ml (R = Rhizobia). n = 15-30 plants measured in two independent biological replicates. Significance values based on $p < 0.05$ (Tukey's HSD, performed separately for different nutrient treatments). n.d. = no data available due to low shoot biomass (< 5 mg); however, rhizobia did not visually increase shoot biomass. Representative pictures can be found in Fig. S 4.

Next, I examined whether two rhizobial exemplars affect root growth of other *A. thaliana* accessions or whether the observed phenotype represents a specialized effect triggered only on the *A. thaliana* accession Col-0. Therefore, the additional *A. thaliana* accessions Bay-0, Fei-0 and Dra-2, adapted to diverse geographic locations, were investigated for their response towards rhizobial treatment (Nordborg et al., 2005; Sulpice et al., 2009). The exemplars R129_E and R13_C were chosen for this comparison, as those two exemplars induced the same phenotypic changes under phosphorus sufficiency and displayed quantitative differences under phosphorus deficiency (Fig. 31). Both rhizobial exemplars were tested for their plant growth-promoting activities on Col-0, Bay-0, Fei-0 and Dra-2 under phosphorus-sufficient and -deficient conditions. Ideally, this will allow to understand whether the root growth-promoting activities are conserved across *A. thaliana* accessions.

R129_E and R13_C significantly increased root length but not shoot biomass of all tested *A. thaliana* accessions under both tested phosphorus concentrations (Fig. 33A and B). R129_E and R13_C induced quantitative differences on root growth under phosphorus-deficient but not -sufficient conditions on all

four tested accessions, even though only the effect on Col-0 and Bay-0 passed statistics. This suggests that the effect of rhizobia on root growth is conserved across different *A. thaliana* accessions, whereas quantitative differences were only partially stable. This suggests that the root growth-promoting activities of the rhizobial population might represent an overall important activity that potentially can be observed on a range of other plant species.

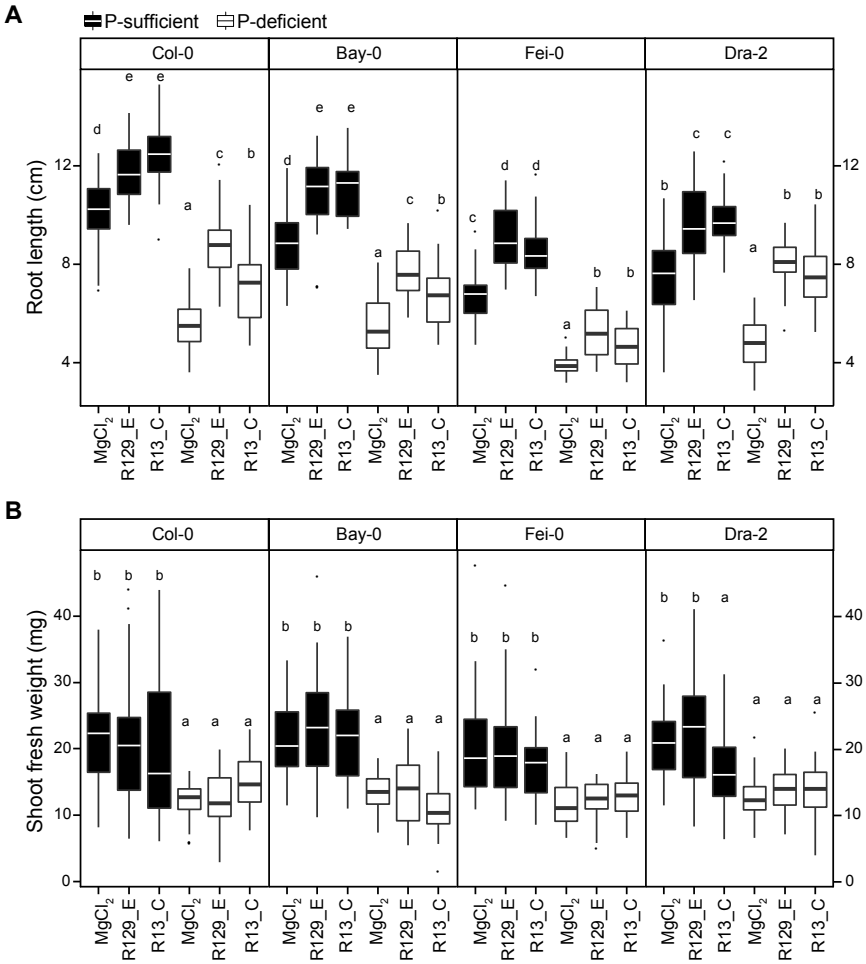


Fig. 33: R129_E and R13_C induce similar phenotypic changes on four different *A. thaliana* accessions. (A) Primary root length (cm) and **(B)** shoot fresh weight (mg) of *A. thaliana* grown under phosphorus (P)-sufficient (black) and -deficient (white) conditions after mock treatment ($MgCl_2$) and after adding R129_E or R13_C at a final density of 10^5 cells/ml (R = Rhizobia). Plant growth-promoting abilities were tested on the *A. thaliana* accessions Col-0, Bay-0. Fei-0 and Dra-2. n = 30-45 plants measured in three independent biological replicates. Significance values based on $p < 0.05$ (Tukey's HSD, performed separately for the four tested *A. thaliana* accessions).

2.4. Root transcriptional changes induced by R129_E

From previous analyses it becomes apparent that the rhizobial population associated with *A. thaliana* potentially provides a range of functional capacities linked to plant growth under various nutrient conditions. However, the molecular mechanisms by which non-symbiotic rhizobia affect plant growth remained elusive.

To better understand the phenotypic changes induced by rhizobia, the phenotypic and transcriptional changes induced in roots of *A. thaliana* were determined in a time-resolved manner. Therefore, *A. thaliana* was co-inoculated with R129_E and the effect on root growth and the relative abundance of rhizobia on the root system were monitored during 6 time points between 0 to 18 days post inoculation with R129_E (dpi).

R129_E increased primary root length first at 12 dpi under phosphorus deficiency, whereas root length increased under phosphorus sufficiency only at 16 dpi (Fig. 34A, Tab. S 5). Root length increased steadily during the remaining time course experiment under both nutrient conditions. Notably, root length of mock-treated samples reached a plateau under phosphorus deficiency starting from 12 dpi. However, inoculating *A. thaliana* with R129_E resulted in a constant increase in root length under phosphorus deficiency until 18 dpi. This suggests that rhizobia might be important for plant growth by enhancing the phenotypic plasticity of plants under abiotic stress conditions.

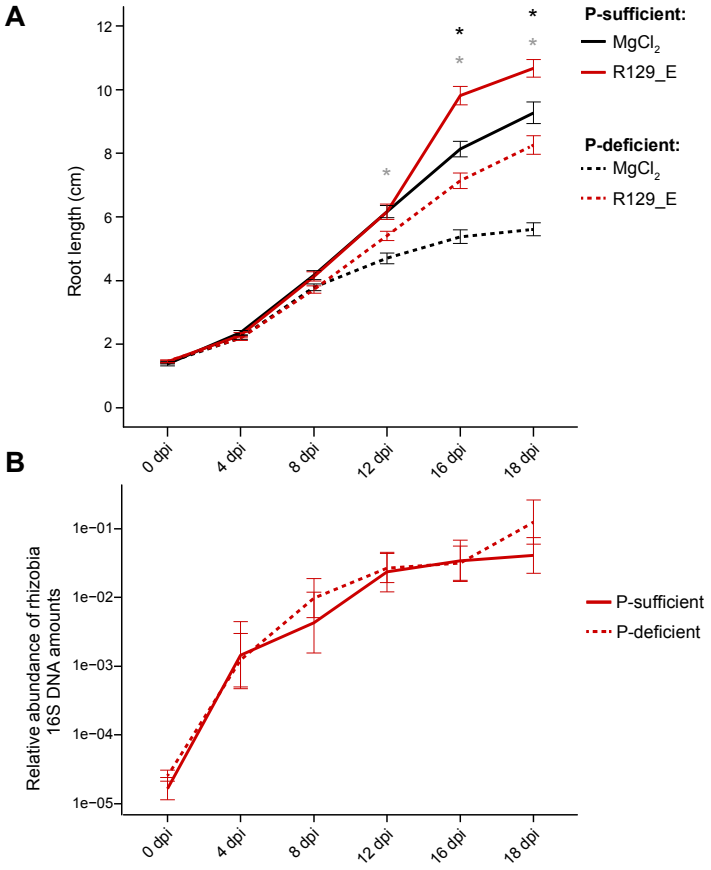


Fig. 34: R129_E induces an earlier increase of primary root length under phosphorus deficiency with no correlation with the relative abundance of bacteria on the root system. (A) Primary root length (cm) of *A. thaliana* grown under phosphorus (P)-sufficient and -deficient conditions after mock treatment ($MgCl_2$) or adding R129_E at a final density of 10^5 cells/ml. Root length was measured 0, 4, 8, 12, 16 and 18 days post inoculation (dpi). n = 30-45 plants measured per time point, representing three independent biological replicates. Black asterisk: Significant difference after rhizobial treatment under P-sufficient conditions. Grey asterisk: Significant difference after rhizobial treatment under P-deficient conditions. Statistics based on Tukey's HSD and $p < 0.05$. **(B)** Relative abundance of R129_E on *A. thaliana* roots as determined by qPCR. Abundance of rhizobia was determined by quantifying the rhizobial 16S rRNA gene, normalizing by the plant chloroplast 16S rRNA gene and log_{10}-transformation. n = 6, representing two independent biological replicates. Line graph is based on the mean value of all data points and error bar depicts the standard error. Summary statistics for (A) can be found in Tab. S 5.

To decipher, whether the earlier phenotype induced by R129_E under phosphorus deficiency correlated with an earlier accumulation of bacteria on the root system, the relative abundance of R129_E on the root system was quantified using qPCR as described before (Fig. 34B). R129_E proliferated strongly during early time points on the root system (0 to 12 dpi), while reaching a plataeu during later time points (12 to 18 dpi). No differences in the relative abundance of R129_E on the root system between phosphorus-sufficient and -deficient conditions were detected, again arguing for the fact that the relative abundance of rhizobia on the root system does not correlate with phenotypic differences under phosphorus-sufficient and -deficient conditions.

Next, transcriptional changes induced in roots of *A. thaliana* upon inoculation with R129_E were monitored in a time-resolved manner under phosphorus-sufficient and -deficient conditions using RNA-Sequencing (RNA-Seq). Five roots from plants grown in one agar plate were pooled for one RNA sample and in total three replicates per treatment were sampled. In total 48 root samples were collected from $MgCl_2$ and R129_E treated plants grown under phosphorus-sufficient and -deficient conditions at 4, 8, 12 and 16 dpi. Samples were subjected to RNA-Seq and the obtained reads were further analyzed. In average 17-34 million reads were generated per sample, with 93-97% of sequences aligning to the *A. thaliana* genome after quality filtering (Tab. S 6). The remaining sequences were thresholded to remove less-reproducible genes, normalized using the trimmed mean of M-values (TMM) method and log_2-transformed resulting in a counts per million (cpm)-transformed dataset. Differentially expressed genes (DEGs) were calculated using the limma package (p-value < 0.05 (FDR-corrected) and log_2-fold change > 1 and < -1; for details see Methods Chapter II.4.2.11 and II.4.2.12).

To assess the integrity of RNA samples during the time course experiment, the expression pattern of described house-keeping genes was analyzed, as those genes are expected to be stable across variable conditions (Czechowski et al., 2005). Gene expression of house-keeping genes, such as *ACTIN 2* (*ACT2*) or *POLYUBIQUITIN 10* (*UBQ10*), was stable over time, bacterial and nutrient treatment, indicative of sample integrity within this experiment (Fig. 38).

In a first step the cpm-transformed dataset was investigated by a hierarchical cluster analysis to obtain an overview about sample behavior across the tested conditions (Fig. 35). Samples collected at different time points clustered apart, with samples at 16 dpi clustering most distantly. Therefore, time represented the strongest determinant of transcriptional changes within this experiment. The next factor that explained differences within gene expression was determined by R129_E treatment, as starting from 4 dpi R129_E inoculated root samples clustered apart from mock-treated samples. Phosphorus deficiency induced only minor changes in gene expression, as a separation of samples based on phosphorus deficiency was only detected at 16 dpi. Altogether, this indicates that root transcriptional changes were predominately influenced by time and R129_E treatment and minor changes were induced by phosphorus treatment, with the strongest effects detected after 16 dpi.

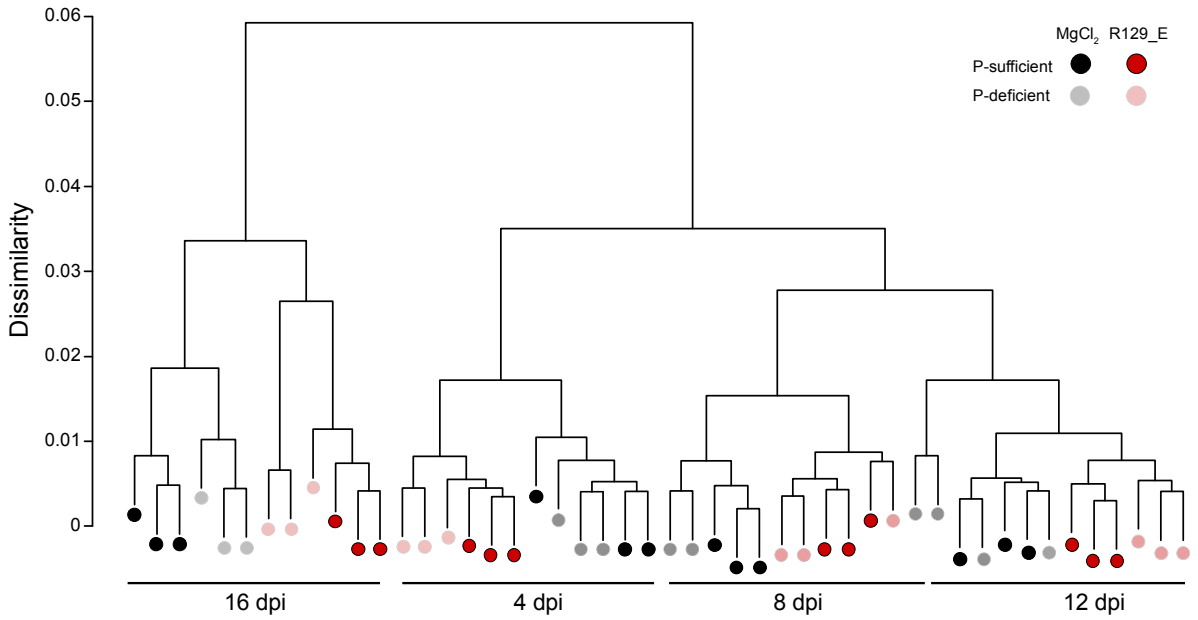


Fig. 35: R129_E induces transcriptional changes in *A. thaliana* roots under phosphorus-sufficient and -deficient conditions. Cluster-Dendrogram based on hclust and depicting transcriptional changes in roots of *A. thaliana* that were either mock-treated ($MgCl_2$) or treated with R129_E a final density of 10^5 cells/ml. Root samples for the RNA-Sequencing experiment were harvested at 4, 8, 12 and 16 days post inoculation (dpi) under phosphorus-sufficient and -deficient conditions. Analysis was performed on the counts per million (cpm) transformed dataset.

To obtain an overview about the total number of DEGs, all DEGs induced or repressed by inoculation with R129_E (i.e. bacterial treatment) or phosphorus deficiency (i.e. nutrient treatment) were calculated and compared to each other. To ensure reproducibility, only genes exhibiting a log_2-fold change above 1 (i.e. up-regulated) or below -1 (i.e. down-regulated) were considered within the analysis. In total 1,530 and 922 DEGs were significantly affected by bacterial and nutrient treatment, respectively (Fig. 36A, B). The ratio between up- and down-regulated DEGs upon bacterial treatment was similar (812 versus 718), whereas nutrient treatment induced more genes (746 versus 176). Comparing transcriptional changes across time, the highest number of DEGs was observed at 16 dpi, both for bacterial and nutrient treatment. This correlated with the observation that samples harvested at 16 dpi clustered apart from samples harvested at earlier time points (Fig. 35). From 4 to 12 dpi bacterial treatment resulted in a lesser number of DEGs compared to 16 dpi, whereas almost no DEGs were affected by nutrient treatment at these time points. Again, this observation correlates with the cluster analysis, where nutrient limitation only displayed a consistent clustering at 16 dpi.

To assess whether similar gene sets were targeted upon bacterial and nutrient treatment, all DEGs that were up- and down-regulated during the respective treatment were filtered for genes that were similarly affected by bacterial and nutrient treatment (Fig. 36C, D). Briefly, only few genes overlapped across the two treatments (179 up- and 23 down-regulated), whereas a higher proportion of DEGs was specifically induced upon bacterial or nutrient treatment. Therefore, bacteria treatment induced distinct transcriptional changes compared to nutrient treatment.

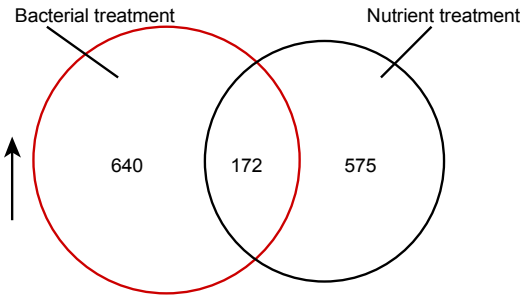
A

	↑	↓	P-sufficient ↑	P-sufficient ↓	P-deficient ↑	P-deficient ↓
Total	812	718	495	476	607	499
4 dpi	141	105	62	52	122	87
8 dpi	238	201	198	94	114	171
12 dpi	150	126	98	75	117	95
16 dpi	626	524	336	360	478	322

B

	↑	↓	$MgCl_2$ ↑	$MgCl_2$ ↓	R129_E ↑	R129_E ↓
Total	747	176	406	99	559	149
4 dpi	2	0	1	0	1	0
8 dpi	7	0	7	0	0	0
12 dpi	0	0	0	0	0	0
16 dpi	742	176	400	99	558	149

C



D

Fig. 36: The highest number of DEGs is detected after 16 days post inoculation and R129 and bacterial and nutrient treatment induce distinct transcriptional changes. (A) Differentially expressed genes (DEGs) that were significantly up- or down-regulated upon treatment with R129_E (= bacterial treatment). Depicted are the total numbers of DEGs and DEGs that depend on phosphorus (P)-sufficient or -deficient conditions. **(B)** DEGs significantly up- or down-regulated under P-deficient conditions (= nutrient treatment). Depicted are the total numbers of DEGs and DEGs that depend on $MgCl_2$ and R129_E treatment. **(C/D)** DEGs overlapping between bacterial and nutrient treatment for all up- or down-regulated genes (C and D, respectively). Comparison was conducted for the total number of DEGs (i.e. no separation across different time points was made). Up-regulated = $\log_2$-fold change > 1. Down-regulated = $\log_2$-fold change < -1. p-value < 0.05 (FDR-corrected). ↑: Up-regulated DEGs. ↓ Down-regulated DEGs.

Next, DEGs affected by bacterial treatment were analyzed for enrichments in gene ontology (GO) terms compared to the *A. thaliana* reference genome using the agriGO analysis toolkit to determine functional categories affected by bacterial treatment (Fig. 37; Du et al., 2010). These GO terms were analyzed separately based on time point, nutrient treatment and up- and down-regulation of DEGs.

Only few GO-categories were enriched among genes that were up-regulated by bacterial treatment (Fig. 37A). At 4 dpi the detected GO terms were related to responses towards stimuli such as carbohydrates, auxins or chemicals. Corresponding gene categories included auxin-responsive GH3 family proteins or BTB and TAZ (BT) domain proteins. Enrichment of those GO terms declined during 8 dpi and disappeared at 12 and 16 dpi. Of note, these terms were mainly enriched during phosphorus sufficiency. At 16 dpi most GO-term categories were enriched under phosphorus deficiency and only a few terms were detected under phosphorus sufficiency. Under phosphorus deficiency GO terms were related to phosphorus starvation responses, lipid biosynthesis or developmental processes. Genes belonging to those terms encoded for phosphorus transporters (PHT2 and PHT5), monogalactosyldiacylglycerol synthases (MGD2) or root hair-specific genes (RHS12). Additionally, the phosphorus starvation genes *PHT2* and *SPX1* were up-regulated and this is consistent with the

hypothesis that R129_E does not increase phosphorus availability for the plant (Fig. 32). The detection of root hair-specific genes on the other hand correlated with the observed increase in root hair density under nutrient deficiency (Fig. 29). Under phosphorus sufficiency only few GO terms related to rRNA modifications and oxidation-reduction processes were significantly up-regulated by bacterial treatment after 16 dpi, including genes encoding for cytochrome P450 family proteins and small nucleolar RNAs (snoRNAs).

Focusing on the down-regulated genes more GO terms were detected, belonging to a variety of functional categories (Fig. 37B). For simplicity only the most strongly enriched GO terms (Top50) based on the total number of genes normalized to the *A. thaliana* reference dataset were considered (Fig. 37B). Across time no consistent pattern was observed, as GO terms detected at 8 dpi vanished at 12 dpi and re-appeared at 16 dpi. This indicates a dynamic response of *A. thaliana* towards bacterial treatment. Enriched GO terms were assigned to organic cyclic substances and toxin metabolic processes. Additional GO terms were associated with stress and defense responses, including terms related to defense reactions, callose deposition, respiratory burst or response to chitin. Defense-related GO terms were first detected under phosphorus deficiency at 8 dpi and only at 16 dpi under phosphorus sufficiency. Gene categories encoded for a zinc-binding-dehydrogenase, several WRKY transcription factors as well as ethylene-responsive factors (ERFs). Additionally, GO terms assigned to diverse stimuli were enriched under both nutrient conditions. These terms belonged to hormonal stimuli, response to stress and response to general stimuli. Categories assigned to stress responses did not focus on one particular stress but ranged from water over light to drug stress. Many transporters were included in those GO terms such as multidrug and toxic compound extrusion (MATE) efflux family proteins. Again, the most pronounced effect on gene expression for down-regulated genes was detected during late time points (16 dpi) and only few GO terms were affected at earlier time points (4-12 dpi) such as homeostatic responses and phenylpropanoid metabolic processes.

A limitation of the GO-term analysis is that this analysis is biased towards the number of genes included within the comparison. This possibly explains why most GO terms were enriched at 16 dpi, as most genes were differentially regulated at 16 dpi. Therefore, I next determined genes that were consistently up- or down-regulated across all time points. In total only a few genes were consistently regulated upon treatment with R129_E and most genes were specifically enriched at individual time points (Fig. S 5). One example for conserved genes up-regulated across both nutrient-conditions was *CIPK13*, encoding for a CBL-interacting serine-threonine-protein kinase. Another example for a conserved down-regulated gene across nutrient conditions was *AT5G16980*, encoding for a zinc-binding dehydrogenase.

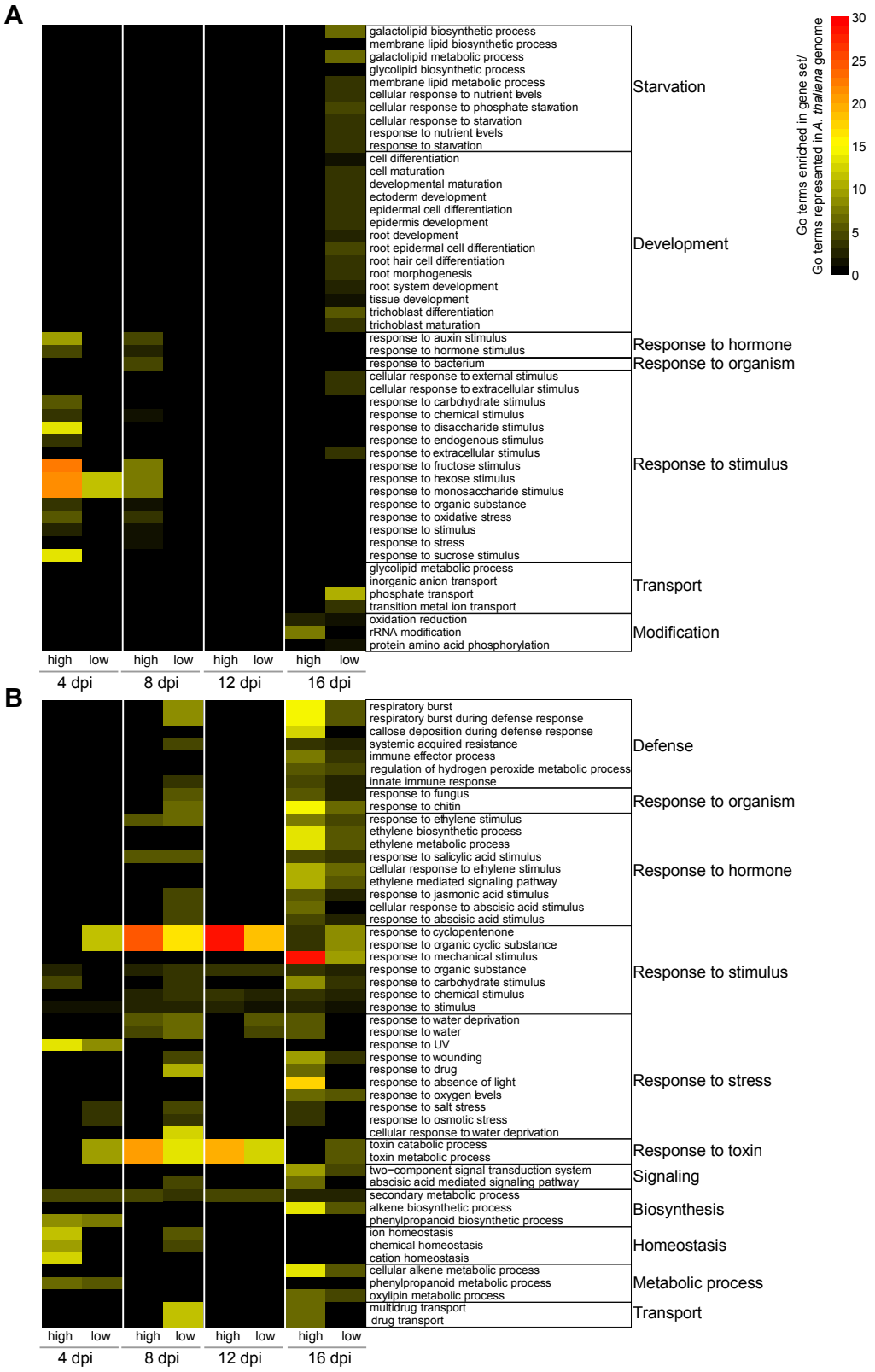


Fig. 37: R129_E induces transcriptional changes in roots of *A. thaliana* that correlate with an enrichment of diverse gene ontology terms. Gene ontology (GO) term enrichment analysis of differentially expressed genes (DEGs) that were either up-regulated (A) or down-regulated (B) upon treatment with R129_E under phosphorus-sufficient (high) or -deficient (low) conditions at 4, 8, 12 and 16 days post inoculation (dpi). GO-term analysis was performed using agriGO ($p < 0.05$; Fisher's exact test, FDR-corrected). The *A. thaliana* Col-0 tair10 genome was used as a reference. Only the top50 GO terms are represented within the heatmap. GO terms were manually sorted according to functional category.

Based on these results, it is so far difficult to correlate DEGs with the observed root growth-promoting activities of R129_E. However, this is not unexpected, as the response towards bacterial treatment was dynamic over time, making a correlation difficult. Therefore, individual DEGs were manually examined for gene categories that could be related to the recognition of rhizobia by the plant or to the plant growth-promoting activities.

To assess whether nutrient deficiency induced phosphorus starvation marker genes and whether this stress was alleviated by R129_E, the expression of phosphorus starvation markers was analyzed (Fig. 38). As expected, phosphorus deficiency induced several starvation markers without bacterial treatment, such as *SPX1* or *PHT2*, at late time points (12 and 16 dpi). The expression of those starvation markers was still induced or even increased upon R129_E treatment, as seen for *PHT2*. Therefore, R129_E did not alleviate phosphorus stress, which is in accordance with the GO-term analysis (Fig. 37). Apart from a response related to phosphorus homeostasis, DEGs also included genes categories related to iron and nitrogen homeostasis. For example, the iron-related *FRO2* and *IRT1* were up-regulated and the high affinity *nitrate transporter 2.7* (*NRT2.7*), *nitrite reductase 1* (*NIR1*) and sulfate transporters (*SULTR2.1* and *SULTR4.2*) were down-regulated upon treatment with R129_E. This points towards a general role of R129_E related to nutrient homeostasis.

R129_E not only up-regulated DEGs related to nutrient homeostasis but also DEGs that are possibly involved in a signal transduction cascade. These DEGs encoded for BTB AND TAZ DOMAIN (BT) domain proteins, several WRKY transcription factors (WRKY38, 70, 54, 48, 59, 62) as well as a receptor kinase (AT5G41300). A large number of genes were up-regulated as early as 4 dpi and remained stable until 16 dpi, independent of the nutrient status. Few genes were specifically induced under phosphorus deficiency with the most prominent group assigned to root hair-specific genes (e.g. *RHS12*). Of note, no subset of genes was not expressed under mock treatment and only up-regulated upon treatment with R129_E.

Genes that were down-regulated by R129_E independently from phosphorus deficiency, included WRKY transcription factors (*WRKY18*, *31*, *33*, 40*)* and genes encoding for NAC transcription factors. Additionally, two nitrilases (*NIT2* and *NIT4*) and the *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*) were down-regulated. Most genes were constantly down-regulated starting from 4 dpi, whereas the expression pattern of other genes varied across time. For example, *NIT4* followed the expression pattern of mock-treated samples, albeit on a lower level. *WRKY31* gene expression on the other hand displayed a variable pattern. *WRKY31* was down-regulated at 4-8 dpi under both nutrient conditions and then gene expression increased only under phosphorus deficiency and was stable under phosphorus sufficiency. This indicates that bacterial treatment induces shifts in gene expression depending on the nutrient status. However, several gene categories displayed no consistent pattern, i.e. members within these gene families were up- as well as down-regulated. Examples were the mentioned WRKYs, genes encoding for GH3-family proteins, glutathione-S-transferases and cytochrome P450 family proteins.

In summary, treatment with R129_E induced transcriptional changes in roots of *A. thaliana* that were not related to an alleviation of phosphorus stress but potentially to nutrient availability, hormone homeostasis or balancing stress responses.

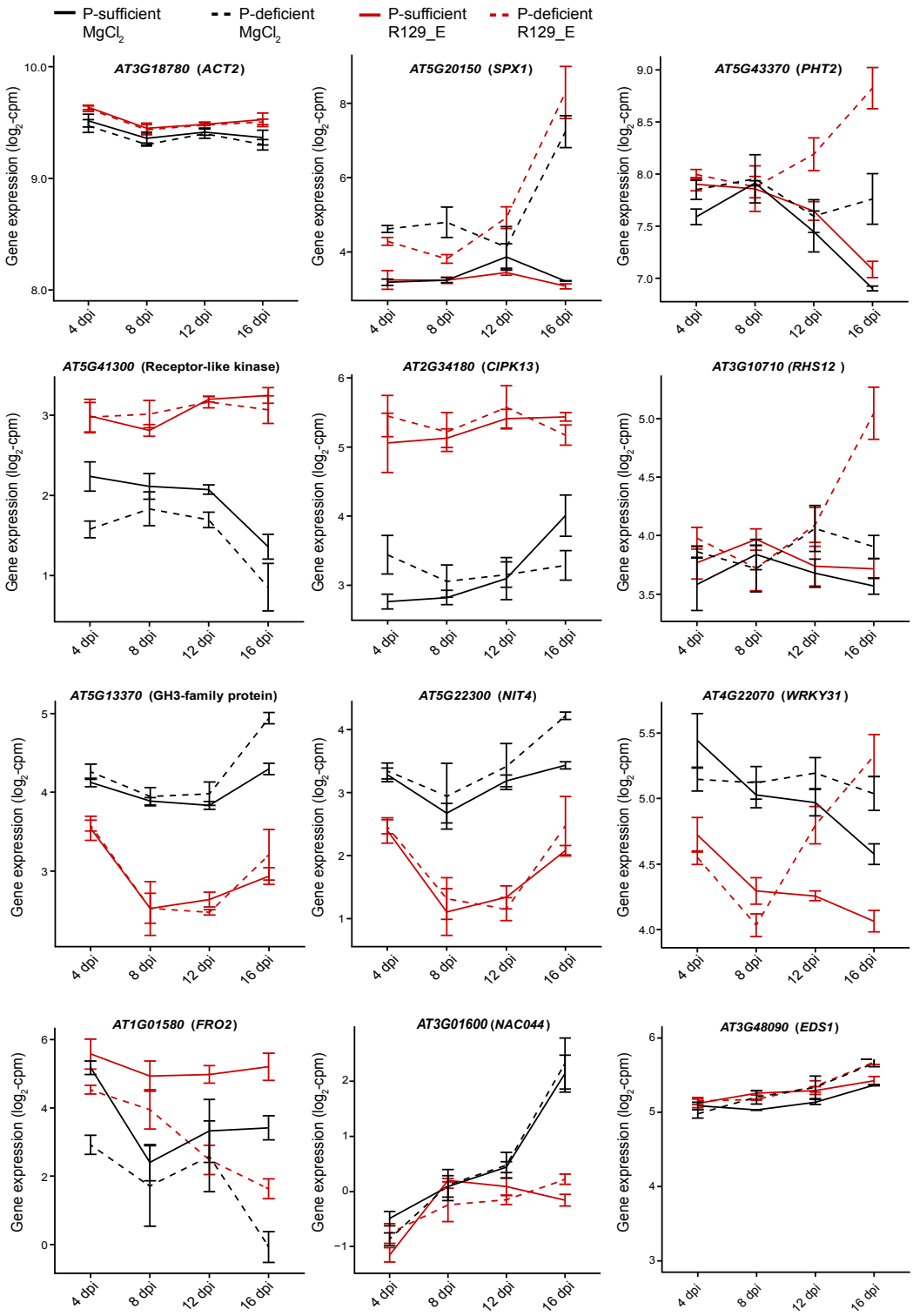


Fig. 38: Gene expression pattern for selected plant genes upon treatment with $MgCl_2$ or R129_E. Expression pattern of selected genes at 4, 8, 12 and 16 days post inoculation (dpi) with $MgCl_2$ (black) or R129_E (red) under phosphorus (P)-sufficient (solid line) or -deficient conditions (broken line). The line graph shows the mean and standard error of three biological replicates. Depicted are the log_2-counts per million (cpm).

3. Discussion

3.1. Population based-analysis of rhizobia reveals a high degree of phylogenetic and phenotypic variation

3.1.1. Phylogenetic variation within the rhizobia population

Rhizobia are well characterized in symbiotic interaction with legumes and the phenotypic and molecular changes induced by rhizobia on legumes are understood in great detail. Recent studies that investigated the root microbiota of non-legumes plants, such as of *A. thaliana* or cannabis, demonstrated that rhizobia are also abundant inhabitants of non-legumes plants (Bulgarelli et al., 2012; Schlaeppi et al., 2014; Winston et al., 2014). However, despite the presence of rhizobia on non-legume plants their biological relevance is not well understood.

To better understand the function of rhizobia on non-legume host plants, we isolated a population of 22 rhizobial exemplars from non-legume plants grown in different natural soils and further characterized those bacteria (Tab. 7). Sanger sequencing of the 16S rRNA gene allowed to determine the taxonomic identity of all exemplars and blasting of the 16S rRNA gene sequence against published sequences of symbiotic rhizobia allowed to identify their phylogenetic relationship. This comparison placed the newly isolated rhizobia into three distinct bacterial species that co-clustered with the symbiotic rhizobia *R. giardinii*, *R. leguminosarum* and *S. meliloti* (Fig. 27).

Based on the 16S rRNA gene a high phylogenetic variability existed among the 22 exemplars, as for example members of R129 formed three distinct clusters. However, this clustering did not correlate with the isolation background of rhizobia, as rhizobial exemplars from *A. thaliana* and *C. hirsuta* or Cologne and Golm soil co-clustered (Tab. 7, Fig. 27). This finding was unexpected, as I hypothesized that bacteria co-evolve with their host plants or environmental properties within the soil and that for this reason exemplars from the same isolation background behave more similar. However, the presented results indicate that this was not the case for the here tested rhizobial population. This is reminiscent of a recent genomic comparison of a nodule-inhabiting *R. leguminosarum* population of *Vicia sativa* and *Trifolium repens* (Kumar et al., 2015). A phylogenetic analysis based on 305 conserved core bacterial genes placed a total of 72 *R. leguminosarum* isolates into 5 distinct clusters. Notably, these clusters did not correlate with host origin or metabolic capacities, such as metabolizing 95 different carbon sources. This indicates that closely related isolates of *R. leguminosarum* display a high phylogenetic variability that does not correlate with isolation origin or functional activities. Similar as for the *R. leguminosarum* population, a high phylogenetic diversity existed within members of R129 and R13 based on the 16S rRNA gene (Fig. 27). However, the use of the 16S rRNA gene for defining bacterial species is often critically discussed within the research community. A species is defined on structural level as bacteria sharing 16S rRNA gene sequences > 97% sequence identity (Schloss and Handelsman, 2005). However, bacteria possessing almost identical 16S rRNA genes can represent distinct species. Three bacterial

exemplars of the genus Bacillus possess 16S rRNA genes with sequence identities > 99% and therefore would be classified as the same species (Fox et al., 1992). However, these bacteria were proven to represent distinct species based on DNA-DNA hybridization and functional analyses, such as growth of bacterial isolates at different temperatures (Nakamura, 1984). Therefore, it is essential not only to rely on the 16S rRNA gene for taxonomic assignment but also on phenotypic characteristics ideally supplemented with detailed genome analyses.

3.1.2. Phenotypic variation within the rhizobia population

Importantly, the high phylogenetic variability among the rhizobial population was also reflected on phenotypic level. A high variability was visible for low complexity phenotypes, such as mobilization of insoluble phosphorus and sulfate forms, as well as for complex traits such as root growth and proliferation of rhizobia on the root system.

Focusing on low complexity phenotypes, several rhizobia mobilized insoluble phosphorus and sulfonate forms to varying degrees and none of the tested rhizobia produced auxin in vitro (Fig. 28; Dr. A. Schmalenberger; personal communication). The inability to produce auxin is in contrast to symbiotic rhizobia, as for example *Rhizobium phaseoli* produces high amounts of auxin in vitro (Beltká et al., 1980; Sinha and Basu, 1981; Datta and Basu, 2000). During symbiosis, auxin homeostasis is relevant during nodulation and rhizobia are believed to influence this homeostasis by altering auxin transport (Wasson et al., 2006). However, as auxin production of this rhizobial population was only monitored in vitro, rhizobia should be tested for an effect on auxin homeostasis in further experiments. Therefore, plant growth-promoting activities of rhizobia should be evaluated on plant mutants impaired in auxin biosynthesis, signaling and transport.

The ability of *A. thaliana* associated rhizobial exemplars to mobilize for the plant inaccessible nutrients, such as CaP, coincides with the ability of symbiotic rhizobia, such as *R. meliloti* and *Bradyrhizobium* sp., to mobilize this insoluble phosphorus form (Halder et al., 1990; Halder and Chakrabartty, 1993). This indicates that CaP mobilization seems to be a more general feature of the rhizobial lineage and that rhizobia could potentially enhance the availability of inorganic phosphorus. Additionally, the degree of CaP mobilization varied between individual exemplars, i.e. a phenotypic variability for this trait existed. This result indicates that rhizobia might be important PGPR that enhance the phosphorus availability for the plant. Ideally, a population-based analysis of rhizobial genomes will be useful to entirely understand the genetic potential among this clade of bacteria. However, as CaP mobilizing rhizobia did not increase shoot biomass of *A. thaliana* grown on CaP as sole phosphorus form (Fig. 32), no direct evidence for the hypothesis that rhizobia enhance the availability of inorganic phosphorus for the plant exists. As *A. thaliana* appears to be able to partially mobilize CaP by itself, as no strong decrease of shoot biomass was observed when plants were grown on CaP as sole phosphorus source, this hypothesis needs to be evaluated using a broader range of plant inaccessible nutrients.

Additionally, the ability of rhizobia to mobilize and supply insoluble phosphorus forms to the plant might be only detected under more natural conditions or alternatively might be required for enhancing rhizosphere competence of rhizobia within the soil.

Focusing on the phenotypic variability among the rhizobial population related their root growth promoting activities, most rhizobia increased primary root length of *A. thaliana* under nitrogen- and phosphorus-sufficient as well as -deficient conditions (Fig. 30, Fig. 31). Quantitative differences were observed among exemplars of all three bacterial species and qualitative differences were only observed for members of R129. Similar to the described phylogenetic variation, these differences could not be explained by an adaptation to the isolation background, as rhizobial exemplars isolated from Cologne and Golm soil increased primary root length to a similar extent. Therefore, I hypothesized that the observed differences were related to a differential proliferation of rhizobia on the root system. To test this hypothesis, the relative abundance of rhizobia on roots of *A. thaliana* was quantified using qPCR. No obvious correlation between the increase of primary root length and relative abundance of rhizobia on the root system existed (Fig. 30A and C). However, also for this trait the rhizobial population displayed qualitative differences, as R129_A was not able to proliferate on the root system in contrast to most other tested exemplars. Similar to the discussion on the phylogenetic variability, these results demonstrate that the definition of a species based on sequence similarity alone is not sufficient to differentiate species at a phenotypic scale. It was demonstrated before that bacteria differentiate below species level for phenotypic traits. Host range of *P. syringae* is a well-studied example for this phenomenon. *P. syringae pv. tomato* is a pathogen of tomato that is not able to infect beans that in turn can be attacked by *P. syringae pv. phaseolicola*, a member of the same species (Joardar et al., 2005). This highlights that bacteria of the same bacterial species functionally adapt to their respective host plants. Similarly, 11 strains of *Brevundimonas alba* possessed the same 16S rRNA gene but utilized 59 tested carbon substrates to different degrees, hinting towards an adaptation to different environmental niches (Jaspers and Overmann, 2004). Such a phenotypic variability is not only observed among bacteria but also among other microorganisms such as for the pathogenic fungi *Botrytis cinerea* (Kerssies et al., 1997). More than 30 isolates of *B. cinerea* were isolated from rose flowers during the course of a whole year. These isolates were taxonomically identified based on the random amplified polymorphic DNA (RAPD) method and evaluated in their pathogenicity against gerbera and rose plants by scoring lesion formation. The RAPD based phylogenetic analysis revealed a high diversity among the isolated strains and the percent of lesions varied between 14%-166% compared to the reference strain *B. cinerea* Bc16. Therefore, strains from the same isolation background did not induce similar disease phenotypes, indicative of the high phenotypic variability that exists among microorganisms.

Altogether, this indicates that in order to link phylogeny with phenotypes and choosing bacteria of interest for further studies, the 16S rRNA gene does not provide a sufficient resolution. Therefore, several different independent exemplars of the same bacterial species should always be evaluated to first understand the variation within the bacterial species of interest and second to be able to assess the

robustness of a given phenotype. Ideally, this can be coupled with genome sequencing, as this could allow correlate variability on structural (i.e. presence or absence of genes) and functional scale (i.e. presence or absence of phenotypes).

3.2. Rhizobia actively proliferate on the root system of *A. thaliana*

The ability to colonize plant roots is a crucial trait for soil-borne PGPR that is essential to affect plant growth (Lugtenberg and Dekkers, 1999). To examine the presence of this trait within the rhizobial population, the relative abundance of 8 rhizobial exemplars colonizing roots of *A. thaliana* under nitrogen-sufficient and -deficient conditions was quantified (Fig. 30). Additionally, the relative abundance of R129_E was monitored in a time-resolved manner under phosphorus-sufficient and -deficient conditions (Fig. 34).

First, all rhizobial exemplars that increased root length were also detected on the root system using qPCR (Fig. 30). The presence of rhizobia on the root system was required to increase root length, as for example R129_A did not proliferate on roots and did not increase root length. As this exemplar could not be re-isolated from the agar (data not shown), the inability to increase root length could be explained by an inability of this exemplar to survive within the media. Alternatively, the plant might have actively suppressed growth of this exemplar. Both hypotheses could be verified by quantifying the relative abundance of rhizobia without the presence of the plant within the media. Interestingly, the observed quantitative differences for the root growth phenotype did not correlate with the relative abundance of rhizobia on the root system. Therefore, differences in the relative abundance of rhizobia on the root system did not account for the phenotypic variability observed for the root growth-promoting phenotype.

Second, the proliferation of rhizobial exemplars on the root system was not affected by the nitrogen or phosphorus concentration in the media (Fig. 30, Fig. 34B). This contrasts with previous studies describing that symbiotic rhizobia colonize legumes dependent on the nitrogen availability. White lupin increases the secretion of flavonoids under nitrate deficiency and this is hypothesized to serve as a signaling cue for rhizobia, eliciting colonization processes specifically under nitrogen-limiting conditions (Wojtaszek et al., 1993). Along that line, high nitrate or ammonia concentrations decrease the proliferation of *R. trifolii* on white clover (Dazzo and Brill, 1978). The absence of a nitrogen-dependent colonization of non-symbiotic rhizobia suggests that alternative communication mechanisms exist between non-legume plants and their corresponding rhizobial exemplars. This hypothesis is supported by the observation that colonization of rice roots by *R. leguminosarum* bv. *trifolii* did not depend on the nitrogen concentration in the soil (Yanni et al., 1997). To confirm the presence of an alternative communication system, more rhizobia could be tested on a broader range of non-legume plants and the responses of rhizobia towards root exudates should be evaluated.

Third, the presence of an active communication between the rhizobial population and *A. thaliana* is supported by the observation that R129_E does not over-accumulate on the root system. R129_E proliferated rapidly on the root system at earlier time points during a time-resolved growth assay, with

the highest increase in relative abundance at 0-4 dpi, whereas at 16-18 dpi proliferation of R129_E reached a plateau (Fig. 34B). During the time course experiment R129_E accumulated moderately by two to three log-units on the root system, correlating with the observation that green fluorescent protein (GFP)-tagged R129_E covered only a small subset of the total root area (Rott, 2013). Phytopathogens on the other hand, such as *P. syringae* DC3000, accumulate to a much higher extent on *A. thaliana* leaves and increase up to 6 log-units (Whalen et al., 1991). This suggests that *A. thaliana* controls proliferation of R129_E on the root and indicates the presence of active communication mechanism between plants and rhizobia. An active control of root colonization represents a useful mechanism for plants to prevent bacteria from over-accumulating on the host plant, as otherwise bacteria might trigger immune responses and inhibit plant growth. An alternative explanation for the plateau during the time course experiment could be that bacteria communicate among each other by quorum sensing and thereby coordinate cell density (Miller and Bassler, 2001).

Finally, not only the relative abundance but also the localization of Rhizobium was investigated across the root axis using GFP-labelled variants of R129_E (Rott, 2013). GFP-labelled R129_E was rarely detected inside the roots and more commonly localized along the root epidermis, preferentially at the beginning of the differentiation zone (Dr. T.R. Nakano, personal communication; Rott, 2013). Therefore, R129_E does not appear to be an endophyte of *A. thaliana*, even though it cannot be excluded that growth of rhizobia in an artificial agar system prevents a successful endophytic colonization. As passive root colonization often occurs through cracks at emergence points of lateral roots (James et al., 2002), rhizobia could be co-inoculated with *A. thaliana* in a more soil-like substrate, to prove whether rhizobia represent true endophytes.

The inability to detect R129_E inside the roots raises the question, whether their effect on root growth depends on a direct contact between rhizobia and roots or on the secretion of volatiles, hormones or other mobile compounds. For example, bacterial volatiles produced by *B. subtilis* increase leaf surface area as well as alter gene expression (Zhang et al., 2007, 2009), demonstrating that no direct contact between the plant and bacteria is required to induce an effect on plant growth. To address this question bacteria of the rhizobial population could be co-inoculated with *A. thaliana* in a split plate system, i.e. not allowing direct contact between rhizobia and the plant. This would allow to address whether the root growth-promoting phenotype depends on a direct contact with the bacterium.

3.3. Rhizobia modify root architecture of *A. thaliana*

As discussed before, the rhizobial population not only colonized roots of *A. thaliana* but also affected root growth. A high proportion of the tested rhizobial exemplars increased primary root length and root hair density. This effect on root length was detected using two concentrations of nitrogen and phosphorus, two insoluble phosphorus forms and four *A. thaliana* accessions (Fig. 30, Fig. 31, Fig. 32 and Fig. 33). The presence of this phenotype across a range of conditions and *A. thaliana* accessions suggests that this phenotype represents a conserved trait among the rhizobial population that potentially

is induced on a broader plant host range. In contrast to the conserved effect on root growth, rhizobia did not affect shoot biomass. The absence of an effect on shoot biomass under nitrogen deficiency indicates that *A. thaliana* associated rhizobia do not fix atmospheric nitrogen compared to symbiotic rhizobia and thereby provide ammonia to the plant, which is consistent with the absence of the bacterial *nifH* gene within genomes of the here tested rhizobial population (R.G. Oter, personal communication).

The increase of root length was mostly independent from the nutrient status, as all exemplars increased root length under phosphorus-sufficient as well as -deficient conditions. However, it has to be noted that roots were significantly longer under nutrient-deficient compared to -sufficient conditions (Fig. 30, Fig. 31). Additionally, root length increased earlier under phosphate-deficient compared to -sufficient conditions, but this phenotype did not correlate with a higher relative abundance of rhizobia on the root system (Fig. 34). Altogether, this indicates that the root growth-promoting phenotype of the rhizobial population or the responsiveness of *A. thaliana* to this service is stronger under abiotic stress conditions. This correlates with the observation that the density of root hairs increased specifically under nutrient deficiency (Fig. 29). Also symbiotic rhizobia induce distinct phenotypic changes on legumes, especially by altering root development (Bauer, 1981). On the legume alfalfa symbiotic *R. meliloti* induces nodulation, as well as root hair curling, the latter being essential for root colonization by rhizobia (Ardourel et al., 1994). Notably, symbiotic rhizobia also induce changes in root architecture on non-legumes. *R. leguminosarum* bv. *trifolii*, a symbiotic rhizobia on clover, increases root length of rice under nitrogen-sufficient as well as -deficient conditions without significant differences dependent on the nitrogen concentration (Yanni et al., 1997). This suggests that the effect on root architecture is conserved across symbiotic and non-symbiotic rhizobia. To verify this a broader taxonomic range of rhizobia, including symbiotic rhizobia, could be tested on diverse non-legume plants.

An increase in primary root length and root hair density can be functionally linked with an increase in surface area that is in turn believed to enhance nutrient availability (López-Bucio et al., 2003). This correlates with the observation that root hair density was only increased under nutrient deficiency and primary roots were longer compared to phosphorus-sufficient conditions (Fig. 29, Fig. 30 and Fig. 31). Alternatively, the biological relevance of the root growth-promoting phenotype might be related to stabilizing plant growth and enhancing phenotypic plasticity under suboptimal conditions. This coincides with the observation that R129_E alleviates root growth arrest under phosphate deficiency (Fig. 34A). It was hypothesized that enhanced phenotypic plasticity induced by PGPR might allow the plant to more rapidly adapt to changing environmental or to abiotic stress conditions (Goh et al., 2013). PGPR affect this plasticity either directly by influencing hormone and nutrient homeostasis or indirectly via biocontrol mechanisms. Altering plasticity of root growth could in turn enhance plant fitness in changing environments, as the root system is essential for acquisition of water and nutrients, anchoring of plants in the soil and providing an interface for the interaction with soil microorganisms (Smith and Smet, 2012).

The inability to monitor plant fitness and the use of an artificial system for plant growth are limitations of the current study. The growth system in petri dishes does not allow to grow plants for a prolonged time period to produce seeds. Therefore, no correlation between changes in root growth and plant fitness can be drawn. The hypothesis that an increase in root surface area correlates with an increase in plant fitness needs to be verified in a more suitable experimental system. Such a system should employ a sterile soil-like matrix and allow to grow plants for a prolonged time to score additional fitness parameters, such as seed production. Another limitation of this study is the detachment of rhizobial members from the community context found under natural conditions. As mentioned before, rhizobia are abundant members of the root microbiota of *A. thaliana* along with other bacterial families, such as Comamonadaceae, Streptomycetaceae or Flavobacteriaceae (Bulgarelli et al., 2012; Schlaeppi et al., 2014). Therefore, it is unclear whether the observed effect on root growth will be maintained in a community context or whether bacteria of the root microbiota alter colonization and rhizosphere competence of rhizobia. To investigate the colonization, as well as the effect on plant growth, fluorescently labelled rhizobia could be evaluated in competition with other bacteria belonging the root microbiota of *A. thaliana*.

3.4. Rhizobium R129_E induces transcriptional changes in roots of *A. thaliana*

The rhizobial population affected root growth of *A. thaliana* under various different growth conditions, but the molecular mechanisms behind this phenotype remained elusive. One likely mechanism that is affected by rhizobia is hormone homeostasis, as root growth is tightly regulated through phytohormones such as auxins and cytokinins (Davies, 1995; Riefler et al., 2006). However, none of the 22 rhizobia produced auxins in vitro and R129_E still increased root length of *A. thaliana* cytokinin biosynthesis and signaling mutants (Fig. 28, Dr. T.R. Nakano, personal communication). For this reason, it appears unlikely that rhizobia act via these two hormonal pathways, at least in a direct way.

To unravel the molecular mechanisms employed by R129_E, a transcriptome analysis was carried out in a time-resolved manner. Therefore, *A. thaliana* was inoculated with $MgCl_2$ or R129_E under phosphorus-sufficient and -deficient conditions. RNA from root samples was isolated at 4, 8, 12 and 16 dpi and subjected to RNA-Seq. Additionally, root length and relative abundance of R129_E was quantified throughout the experiment. This allowed to demonstrate that R129_E induced not only phenotypic but also transcriptional changes in roots as revealed by the RNA-Seq experiment (Fig. 34, Fig. 35). Gene expression of *A. thaliana* was mainly influenced by the time point of sampling and bacterial treatment, whereas phosphorus deficiency only had a minor impact.

Gene expression in roots of *A. thaliana* was mostly influenced by the time point of sampling, as the four time points clearly clustered apart, with the most distinct sub-cluster and highest number of DEGs being detectable at 16 dpi (Fig. 35, Fig. 36). Furthermore, samples at 4 dpi clustered apart from samples

collected at 8 and 12 dpi, which were more similar to each other. Overall the strong influence of time on gene expression was not unexpected, as the plant underwent multiple developmental changes, as seen by an increase in total biomass over time. Subsequently, developmental processes are influenced by diverse molecular mechanisms such as hormone homeostasis (Davies, 1995). The distinct clustering at 16 dpi was more unexpected, as I anticipated to detect DEGs correlated with the observed root growth-promoting activities and plant-microbe recognition processes much earlier, i.e. before the first phenotypic changes induced by R129_E were visible. However, it is likely that changes in gene expression related to root growth occur only in a small number of cells, such as the root meristem (Nawy et al., 2005). Thus, transcriptional changes linked to root growth might be only induced in a small number of cells, resulting in a dilution by cell types unrelated to the observed phenotype. For example, transcriptional changes induced by hypoxia occur in a cell type-specific manner (Mustroph et al., 2009). Under hypoxia root cells enrich for genes related to anaerobic responses, whereas shoot cell types express more mRNAs involved in galactolipid biosynthesis. Therefore, cell type-specific gene expression could potentially mask molecular mechanisms induced by R129_E that are linked to root growth. To unravel these mechanisms, transcriptional changes within the root meristem could be evaluated. Several techniques allow to isolate individual cell types, such as ribosomal-affinity purification, fluorescence-activated cell sorting or laser capture microdissection, representing valuable tools to better understand the here observed phenotypes (Mustroph et al., 2009; Birnbaum et al., 2003; Casson et al., 2005).

In contrast to the strong influence of time on gene expression, phosphorus deficiency induced only marginal transcriptional changes, as samples affected by nutrient treatment only consistently clustered at 16 dpi and only few DEGs were detected at earlier time points (Fig. 35, Fig. 36). Phosphorus deficiency typically induces early transcriptional changes in *A. thaliana*, starting after 4 hours upon removal of phosphorus from the media (Hammond et al., 2003; Wu et al., 2003). However, this initial starvation response is strongly reduced after 28 hours. This indicates that *A. thaliana* responds to phosphorus deficiency with an early, unspecific stress response and adapts to the applied stress at later time points and then only induces a lower number of DEGs directly related to an adaptation towards phosphorus deficiency. This observation correlates with the inability to detect a prominent response towards phosphorus-limitation at 4-12 dpi (Fig. 35). The fact that *A. thaliana* responds to phosphorus deficiency at 16 dpi in our experiment might indicate a second induction of a stress response towards phosphorus deficiency. Under prolonged phosphorus deficiency the plant might sense that the regulation of pathways for increasing phosphorus uptake did not alleviate phosphorus stress, thus inducing a second phase of transcriptional changes. Additionally, treatment with R129_E did not alleviate this phosphorus-dependent stress response, as phosphate starvation genes, such as *SPX1*, were not down-regulated upon bacterial treatment (Fig. 38). In the case of the phosphate transporter PHT2, R129_E even induced gene expression. This suggests that changes in root growth induced by R129_E are unrelated to an adaptation to phosphorus deficiency.

Even though R129_E did not alleviate phosphorus stress, R129_E clearly induced transcriptional changes starting from 4 dpi (Fig. 35). This correlates with the observation that R129_E already proliferated on the root system between 0-4 dpi (Fig. 34B). However, as other PGPR, such as *A. brasilense,* induce transcriptional changes as early as 1 dpi (Spaepen et al., 2014), it is likely that the initial recognition process between R129_E and *A. thaliana* is not detected within this time course experiment. Early recognition of bacteria or bacterial signaling molecules by the plant often occurs through receptor proteins (Gust et al., 2012). Therefore, the up-regulation of receptor-like protein kinase-related and LRR-protein kinase family proteins by R129_E might be related to the recognition of R129_E by the plant (Fig. 38). Notably, this resembles recognition processes during symbiosis, where legumes perceive Nod factors through LysM-type receptor kinases (Radutoiu et al., 2003). Therefore, these receptors might represent potential candidates for further mutant analyses. Apart from an induction of receptor-like proteins, R129_E did not induce well-known genes related to defense responses, such as *PHYTOALEXIN DEFICIENT 4* (*PAD4*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) or *PATHOGENESIS-RELATED GENE 1* (*PR1;* Glazebrook, 2001). In contrast, R129_E down-regulated defense-related genes, such as genes encoding for glycosyl transferases, peroxidases or glucosinolate biosynthesis proteins (Bennett and Wallsgrove, 1994; Almagro et al., 2009). Finally, R129_E did not induce the ISR-marker genes *MYC2* and *NPR1*. Therefore, R129_E did not trigger a plant defense response during the tested time points and the root growth-promoting activities of R129_E appear to be unrelated to biocontrol activities related to ISR. The absence of a defense response upon bacterial treatment could be explained by the (i) masking of bacterial MAMPs, (ii) active down-regulation of defense processes or (iii) no induction of defense genes in *A. thaliana* upon recognition of R129_E. However, from the here presented data it cannot be elucidated, which mechanisms are employed by bacteria or plants and an earlier analysis of defense marker genes and mutant lines impaired in defense responses will be required to address this question.

Even though defense-related genes were not among the detected up-regulated DEGs, R129_E differentially regulated genes encoding for the protein-kinase MPK3, BT proteins and several WRKY and NAC transcription factors. These genes might represent a signal-cascade induced upon bacterial recognition and could be related to transcriptional changes affecting nutrient availability, hormone homeostasis or adaptation to stress.

R129_E differentially regulated genes related to nutrient availability that are involved in nutrient homeostasis and transport. Along that line, R129_E induced genes encoding for two iron transporters (IRT1, IRT2), a peptide transporter (PTR3) and the ferric chelate reductase FRO2. Additionally, R129_E down-regulated genes encoding for the nitrate transporter NRT2.7 and nitrate reductase NIR1. The up-regulation of *IRT2* and *FRO2* hints for an involvement of R129_E in iron homeostasis and resembles the response of *A. thaliana*, when treated with volatiles produced by *B. subtilis* GB03 that linked the plant growth-promoting activities of *B. subtilis* with an alleviation of iron-deficiency (Zhang et al., 2009). A link between R129_E and iron homeostasis could be evaluated by testing the effect of

R129_E on plant growth under iron-deficiency and correlated with the ability of R129_E to acidify the surrounding media and produce siderophores. It will be also interesting to assess whether a direct contact between rhizobial exemplars and the plant is required or whether the plant growth-promoting abilities of rhizobia correlate with volatile production. Additionally, R129_E not only seemed to interfere with iron but also with nitrogen homeostasis, as bacterial treatment down-regulated *NRT2.7* at earlier time points and *NIR1* at later time points. However, as discussed for iron homeostasis, no nitrogen deficiency was induced, therefore the root growth-promoting abilities of R129_E cannot be directly linked to nitrogen homeostasis.

Apart from nutrient availability, R129_E induced transcriptional changes related to hormone homeostasis, as GO terms assigned to auxin responses were enriched at 4 dpi (Fig. 37A, Fig. 38). Genes encoding for auxin-related proteins included nitrilases, GH3 family proteins and SAUR-like auxin-responsive proteins. The two nitrilases NIT2 and NIT4, where gene expression was down-regulated upon bacterial treatment during all time points, hydrolyze indole-3-acetonitrile to IAA (Vorwerk et al., 2001; Bartel and Fink, 1994). Additionally, *A. thaliana nit1* mutants display a shorter primary root (Normanly et al., 1997), thereby potentially linking the observed root growth phenotype in this study to changes in auxin homeostasis. However, the role of nitrilases in auxin homeostasis is still discussed, as these enzymes appear to play a more dominant role in cyanide detoxification compared to auxin biosynthesis (Piotrowski, 2008). Another group of DEGs related to auxin homeostasis encode for GH3 proteins that conjugate amino acids to auxin and thereby control the active auxin pool (Staswick et al., 2005). Also genes within this group, such as *WES1*, are involved in root growth and stress tolerance, again drawing a potential link to R129_E affecting root growth and interfering with auxin homeostasis (Park et al., 2007). The observation that rhizobia do not produce auxins in vitro (Fig. 28) indicates that an influence on auxin homeostasis is not mediated by direct auxin production but rather by alternative means. An alternative way to influence auxin homeostasis could be by NO production, as NO affects the IAA signaling pathway as well as root growth and NO is also produced by PGPR such as *Azospirillum spp.* (Pagnussat et al., 2002; Lamattina et al., 2003; Creus et al., 2005). Alternatively, rhizobia could influence auxin homeostasis by interfering with auxin transport, as was proven for *R. leguminosarum* bv. *trifolii* interacting with white clover (Mathesius et al., 1998). The relevance of auxin transport for the nodulation process of white clover was based on GH3:*gusA* expression analyses upon treating plants with the auxin transport inhibitor naphthylphthalamic acid (NPA). A similar approach could be taken to unravel the role of R129_E on auxin homeostasis in *A. thaliana*.

R129_E not only affected auxin-related genes but also GO terms related to ethylene homeostasis, as seen by a differential regulation of biosynthesis genes (*ETO3*, *ACS11*) and down-regulation of ethylene-responsive factors (*ERF13;* Fig. 37B). However, as ethylene also mediates stress-related responses (Alonso et al., 1999), it is unclear whether these genes are related to changes in root growth or with stress-related responses. No apparent genes involved in cytokinin or gibberellin homeostasis were affected by bacterial treatment. Therefore, the strongest correlation between root growth and hormone

production was observed for auxin homeostasis and should be verified in future experiments testing plant mutants in auxin biosynthesis, signaling and transport and their responses towards inoculation with R129_E.

As discussed before, rhizobia might be involved in balancing stress responses in plants by enhancing phenotypic plasticity, allowing a faster adaptation to changes in the environment. This correlates with the observation that R129_E down-regulated GO terms related to stress response pathways (Fig. 37). DEGs were detected that encoded for NAC-transcription factors, cytochrome P450 family proteins or glutathione-S-transferases, which were predominantly down-regulated and are often associated with stress responses (Nakashima et al., 2012; Narusaka et al., 2004; Edwards et al., 2000). An example for an up-regulated gene was the protein kinase *CIPK13*, where members of this gene family were directly linked to an increased stress tolerance (Fig. 38). Heterologous expression of *CIPK6* from *Cicer arietinum* in tobacco increased and similarly knockdown of *CIPK6* in *A. thaliana* decreased salt tolerance (Tripathi et al., 2009). Growing *A. thaliana* in agar plates could induce a stress for the plant, as roots are exposed to an unnatural high dose of light and the agar matrix could affect water availability (Yokawa et al., 2011; Ghashghaie et al., 1991). This could also explain, why often GO terms related to UV or water stress were induced by the plant, which were in turn often down-regulated by R129_E (Fig. 37). Therefore, it will be important to examine, how rhizobia affect plants grown under various stress conditions, using growth systems that allow to evaluate additional parameters such as plant fitness.

In summary, the rhizobial population isolated from *A. thaliana* represent novel PGPR that not only effectively colonize plant roots but also induce morphological and transcriptional changes. The morphological changes were linked to a root growth-promoting activity that was conserved across different nutrient conditions as well as *A. thaliana* accessions. Abiotic stress, such as nitrogen deficiency, enhanced this root growth-promoting activity, indicating a relevant role of the rhizobial population to balance abiotic stress conditions. Additionally, R129_E induced transcriptional changes that were related to hormone homeostasis, nutrient availability and stress responses. However, the direct link between the root growth-promoting activities of R129_E and the transcriptional changes induced in *A. thaliana* still remain elusive and require further investigations.

3.5. Conclusions and future perspectives

The goal of the second part of this dissertation was to assess the function of the rhizobial population associated with non-legumes and to decipher the molecular responses induced in *A. thaliana* roots upon recognition of Rhizobium. As presented in the introductory chapter, a wealth of information exists about the colonization, function and molecular mechanisms elicited by symbiotic rhizobia on legumes (Oldroyd et al., 2011). Conversely, few studies address the biological relevance of non-symbiotic rhizobia interacting with non-legume plants. Therefore, I wanted to investigate: (i) how high is the phylogenetic and functional variability of the rhizobial population associated with *A. thaliana*, (ii) do these rhizobia represent novel PGPRs and (iii) does inoculation with Rhizobium trigger transcriptional responses in the plant.

I could demonstrate that rhizobia associated with the roots of *A. thaliana* possess plant growth-promoting activities and increase root length of *A. thaliana* under various conditions, such as sufficient nutrient supply, phosphorus and nitrogen deficiency as well as on four *A. thaliana* accessions. Of note, this root growth-promoting activity was mostly conserved across 22 members of the rhizobial population, even though individual exemplars affected root growth to varying degrees. Additionally, rhizobia effectively colonized roots and this, together with the root growth-promoting activities induced by the rhizobial population, establishes these non-symbiotic rhizobia as PGPR on *A. thaliana*. Inoculation with Rhizobium induced not only phenotypic but also transcriptional changes within the plant as was determined by RNA-Seq. Of note, the latter did not involve an induction of defense marker genes and conversely, recognition of Rhizobium induced transcriptional changes associated with interference with hormone homeostasis, nutrient availability and balancing stress responses.

This study could thereby verify that rhizobia are biological relevant not only during symbiosis with legumes but also represent PGPR on the non-legume plant *A. thaliana*. The current study only delivers first insights into the role of rhizobia as PGPR on non-legume plants and some unanswered questions remain. First, even though rhizobia were demonstrated to withhold plant growth-promoting activities, the molecular mechanisms by which rhizobia affect root growth remained elusive. To address this gap a comparative analysis of rhizobia and other bacterial isolates from the root microbiota of *A. thaliana* should be employed by utilizing bacteria that display opposite phenotypes on root growth. Ideally, such opposite phenotypes will allow to dissect bacterial genes that correlate with phenotypic and transcriptional changes within the plant. Second, we still lack evidence about how symbiotic rhizobia and non-symbiotic rhizobia diverged and how such a specialized process as symbiosis evolved. Again, this could be addressed by using a comparative genomic approach comparing symbiotic and non-symbiotic rhizobial exemplars. Finally, even though rhizobia alter root growth, the biological relevance and molecular basis of this phenotype remains unclear. Even though it is likely that changes in root growth are connected to increased nutrient availability, this needs to be validated by measuring nutrient content in different plant organs under nutrient deficiency as well as testing the root growth-promoting activity of R129_E on plant mutants of genes that were differentially regulated during this study (Fig.

39). Additionally, it should be tested whether a direct contact between bacteria and the plant is required or whether R129_E acts on the plant indirectly through volatiles, NO or LCOs. Despite these limitations, the current study provides novel insights into plant growth-promoting activities of non-symbiotic rhizobia as well as the induced molecular responses in *A. thaliana* roots (Fig. 39). Ideally, these insights will in future allow to establish rhizobia as a new model system on non-legume plants and to decipher how rhizobia evolved as PGPR on distinct host plants.

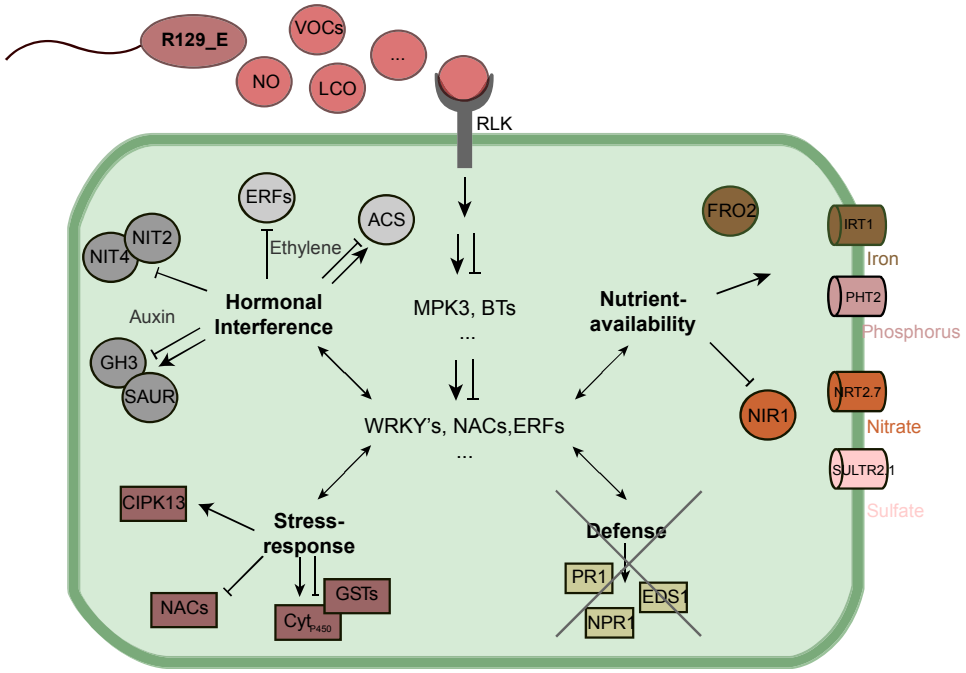


Fig. 39: Putative model for action of R129_E on gene expression in *A. thaliana* roots. Plants recognize R129_E either directly or indirectly via signaling molecules such as volatiles (VOCs), nitric oxide (NO) or lipochitooligosaccharides (LCOs). Recognition induces a transcriptional reprogramming in the plant that affects the expression of genes encoding for kinases (MPK3), BT proteins as well as NAC- and WRKY-transcription factors. This transcriptional cascade potentially induces changes correlating with the root growth-promoting activities of the rhizobial population, such as hormonal interference, nutrient availability or stress responses. Conversely, the recognition of rhizobia does not induce a defense response. Examples of genes induced (→), repressed (⟞) or not affected (x) are indicated. RLK: Receptor-like kinase. BT: BTB AND TAZ DOMAIN. ACS: 1-aminocyclopropane-1-carboxylic acid synthase. ERF: ethylene-responsive factor. Cyt_{P450}: cytochrome P450. GST: glutathione-S-transferase.

4. Materials and Methods

4.1. Materials

4.1.1. Storage and preparation of soil material

See Chapter I.4.1.1.

4.1.2. Plant material

Seed material from *Arabidopsis thaliana* (ecotypes Col-0, Fei-0, Dra-2, Bay-0) and *Cardamine hirsuta* (ecotype Oxford, Ox) was kindly provided by Dr. Klaus Schläppi (Agroscope Institute for Sustainability Sciences ISS). Plant growth assays in this chapter were exclusively performed on *A. thaliana*. The *A. thaliana* accession Col-0 was standardly employed except for Fig. 33.

4.1.3. Bacterial strains

Rhizobial exemplars employed during this study are listed in Tab. 7. Indicated are the plant genotype used for isolation and the soil type used for plant growth. Each individual exemplar was isolated from an independent root; therefore, each strain represents an independent colonization event. With the exception of isolates R129_L, R129_M and R129_N, all rhizobial exemplars were isolated and their respective genomic DNA prepared by Dr. Matthias Rott.

4.1.4. Antibiotics

Antibiotics used in this study are listed in Tab. 8. Antibiotics were prepared in 1,000 x concentrated stock solutions and stored at -20°C. Benzimidazole was added to the media to prevent fungal growth.

Tab. 8: Used antibiotics and fungicides.

Antibiotic/fungizide	Final concentration	Source
Streptomycin	200 µg/ml	Sigma (Hamburg, Germany)
Benzimidazole	50 µg/ml	Sigma (Hamburg, Germany)

4.1.5. Growth media, solutions and buffers

Media was sterilized by autoclaving at 121 °C for 20 min. Antibiotics and fungicides were added after the media cooled down to ~50°C. Heat-labile compounds were sterilized using filter sterilization units (0.22 µM filter) prior to addition to the media. Media used for cultivation of root-associated bacteria are listed in Tab. 9. Buffers and solutions used in this study are described within each method. If not stated otherwise, buffers were prepared in ddH_2O and aqueous solutions were sterilized by autoclaving at 121 °C for 20 min.

Tab. 9: Growth media used in this study.

Growth medium	Growth purpose for:	Reference
Tryptone yeast (TY)	basal medium, Rhizobia	Beringer, 1974
Tap water yeast extract (TWYE)	Actinobacteria	Coombs and Franco, 2003
Flour	Actinobacteria	Coombs and Franco, 2003
Yeast extract mannitol (YEM)	Rhizobia	Vincent, 1970

4.1.6. Oligonucleotides

Oligonucleotides used for PCR amplification are listed in Tab. 10 and were purchased from Sigma (Hamburg, Germany) or Invitrogen (Karlsruhe, Germany).

Tab. 10: Oligonucleotides used in this study.

Name	Sequence (5' - 3')	Reference
799F1	AAC MGG ATT AGA TAC CCK G	Chelius and Triplett, 2001
1193R	ACG TCA TCC CCA CCT TCC	Bodenhausen et al., 2013
1392R	ACG GGC GGT GTG TRC	Stahl et al., 1988
16SRhizo1-F	CGA GCT AAT CTC CAA AAG CCA TC	this study
16SRhizo1-R	TGA CCC TAC CGT GGT TAG CTG	this study
At16S-F	CAG GCG GTG GAA ACT ACC AAG	this study
At16S-R	TAC AGC ACT GCA CGG GTC GAT	this study

4.1.7. Enzymes

DNA free DFS Taq polymerase was purchased from Bioron (Ludwigshafen, Germany).

4.2. Methods

4.2.1. Seed sterilization

Plants were surface sterilized by sequential washing for 20 Min with 70% ethanol and a brief washing step with 100% ethanol. After removal of ethanol, seeds were dried under the sterile conditions.

4.2.2. Growth of plants for bacteria isolation

Surface sterilized seeds were sown at a density of 4 plants per pot (7x7 cm) filled with diverse naturally collected soils (Tab. 2, Tab. 3) and stratified for three to four days. *A. thaliana* was grown in different batches of Cologne ('CAS') or Golm ('Golm') agricultural soil under short day conditions (8/16 hours day/night, a temperature of 22°C/18°C and a relative humidity of 70%). After 6 to 8 weeks plants were prepared for harvesting. Additionally, *C. hirsuta* was collected in a natural habitat with the following locations: 'Dümpelfeld (50.45012819440579 N/ 6.936978399753571 E, Dümpelfeld, Germany) and

'France' (Saint Eva, France; no coordinates available). Bacteria were isolated within one week after harvest of plant material from natural sites.

4.2.3. Isolation of bacteria from naturally grown plants

After harvest roots were separated from the adhering soil particles and a defined root segment was gathered (3 cm in total, starting 0.5 cm distant from the hypocotyl). Soil particles still attached to the roots were removed by gentle tapping. Roots were collected in 15 ml falcons containing 3 ml PBS-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0, 0.02% Silwet L-77) and washed for 15 minutes at 180 rpm on a shaking platform. Roots were washed twice, transferred to a new falcon tube and sonicated for 10 times at 160 W with 30 seconds breaks (Bioruptor Next Gen UCD-300, Diagenode, Liège, Belgium). Roots were transferred to fresh PBS-S, shortly dried on Whatman® glass microfiber filters (Sigma-Aldrich, Hamburg, Germany) and transferred to 2 ml tubes. After adding 500 µl $MgCl_2$ and 2 mm metal beads, roots were disrupted at 5,000 rpm for 3 x 30 seconds (Bioruptor Next Gen UCD-300, Diagenode, Liège, Belgium). The homogenate was centrifuged for 5 minutes at 1,000 x g and the supernatant transferred to a new tube. Serial dilutions of the supernatant were prepared and 100 µl plated onto diverse growth media (see Tab. 9). Plates were incubated for 3-7 days at 28°C until a sufficient number of colonies was observed.

4.2.4. PCR amplification of the 16S rRNA gene of isolated bacteria

Single bacterial colonies were transferred to 400 µl liquid media (see Tab. 9), distributed in a 96-deepwell plate using a sterile tip and incubated for seven days at 28°C and 200 rpm. Afterwards, 100 µl of the culture was transferred to a 96-well PCR plate and bacteria cells disrupted for 10 minutes by heat treatment at 100°C. Cell fragments were centrifuged for 10 minutes at 3,000 rpm and the supernatant, including bacteria and DNA, was transferred to a new plate. In a PCR reaction, used to obtain sequence information for taxonomy assignment, the primers 799F1 and 1392R were used (Tab. 10). PCRs were performed using 3 µl of isolated bacterial DNA in a total volume of 25 µl. PCR components include 1.25 U DFS-Taq DNA Polymerase (Bioron, Ludwigshafen, Germany), 1x complete reaction buffer, 0.3% BSA, 200 µM of dNTPs and 400 nM of each primer. The PCR reaction was pipetted in a laminar flow and amplified (94°C/5 minutes, 94°C/30 seconds, 50°C/30 seconds, 72°C/30 seconds, 72°C/5 minutes for 35 PCR cycles). PCR quality was assessed by loading 5 µl of the amplicon on a 1% agarose gel. The amplicon was purified using an isopropanol precipitation protocol and the DNA concentration measured by Nanodrop photometer. Afterwards, the DNA concentration was adjusted to 20 ng/µl and subjected to Sanger sequencing. Obtained sequences were analyzed with BioEdit (Hall, 1999) and blasted against an internal pyrosequencing database (as described in Bulgarelli et al., 2012) to correlate isolated bacteria to OTUs identified during previous sequencing analyses. To ensure that bacteria used

for further testing emerged from single colonies, isolates were at least streaked out three times by using the streak plate method. To ensure additional purity, rhizobial exemplars were further purified by introducing a streptomycin resistance. This was achieved by platting serial dilutions of the respective bacteria on TY media, including 50 µg/ml streptomycin. Grown colonies were afterwards transferred to media containing 200 µg/ml streptomycin and their morphology compared to colonies grown on media without antibiotics. Purified bacteria were stored in a 15% glycerol stock at – 80°C.

4.2.5. Growth of bacterial isolates

Unless stated otherwise, rhizobia were grown in 5 ml liquid TY media (5 g/L peptone, 3 g/L yeast extract), including 200 µg/ml streptomycin for three to four days at 28°C and shaking at 200 rpm.

4.2.6. Isolation of genomic DNA from bacterial isolates

To isolate high quality genomic DNA for Illumina and PacBio sequencing, bacteria were grown for two to three days in TY media, including 200 µg/µl streptomycin. Afterwards, bacterial cultures were centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. Lysing Matrix E beads (MP Biomedicals, Solon, USA) were added to the remaining pellet together with 500 µl buffer A (200 nM $NaCl_2$, 200 mM Tris, 20 mM EDTA at pH 8.0), 210 µl SDS (20%) and 500 µl Phenol/Chloroform/Isoamylalkohol (25:24:1, pH 7.9). Samples were mechanically disrupted using the TissueLyser II (Qiagen, Hilden, Germany) for two minutes at maximum speed, chilled for two minutes on ice and then again disrupted using the same settings. Afterwards, samples were centrifuged at 6,800g at 4°C for 20 minutes and the upper phase was transferred to PLG tubes (5 Prime, Hilden, Germany). To the upper phase a Phenol/Chloroform/Isoamylalkohol (25:24:1, pH 7.9) solution was added, the tube inverted and centrifuged at maximum speed for 5 minutes. Afterwards, the liquid phase was collected and 600 µl isopropanol (cooled to -20°C) and 60 µl sodium acetate (3 M, pH 5.5) was added. Samples were stored at -20°C for at least an hour or overnight and afterwards centrifuged at maximum speed for 30 minutes at 4°C. The obtained pellet was washed in 500 µl ethanol (100%) and centrifuged at 4°C for three minutes at maximum speed. Afterwards, samples were dried in a vacuum concentrator and re-suspended in 80 µl EB buffer (10 mM Tris-HCl, pH 8.5; Qiagen, Hilden, Germany) by incubating the samples for 5 minutes at 50°C. To avoid RNA contamination, 20 µl RNAse A (100 mg/ml, Qiagen, Hilden, Germany) was added and incubated at 37°C for 30 minutes. To ensure high quality of obtained DNA, a further purification was carried out using the QIAamp DNA micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality of DNA was assessed using a Nanodrop photometer and gel electrophoresis. Illumina and PacBio sequencing of genomic DNA was carried out by the Max-Planck Genome Center Cologne. Sequence analysis was conducted by Ruben Garrido Oter.

4.2.7. Screening for auxin production

Rhizobia were screened for their auxin production using the Salkowski assay (Gordon and Weber, 1951). Therefore, bacteria were grown as stated before, centrifuged at 4,000 rpm for 20 minutes and 600 µl of the supernatant was transferred to a new tube. As standard indole-acetic-acid (IAA) was prepared in a dilution series ranging from 5 µM to 200 µM in TY media. Then, 600 µl Salkowski reagent (12 g/L $FeCl_3$, 7.9 M H_2SO_4) was added to the bacterial supernatant, as well as to the standard, and incubated in the dark for 30 minutes at 4°C. Afterwards, the optical density was measured at 550 nm and the auxin production was calculated against the measured absorbance of the standard.

4.2.8. Mobilization of insoluble phosphorus sources in vitro

Calcium phosphate (CaP) and hydroxyapatite (HA) mobilization assays were performed to test bacteria for their capacity to mobilize insoluble phosphorus forms. Bacteria were grown in TY media for 2-4 days, washed twice in $MgCl_2$ as described above and adjusted to a final OD_{600nm} of 0.05. 20 µl from this bacterial suspension were spotted onto CaP medium (0.5 g/L yeast extract, 10 g/L dextrose, 5 g/L calcium phosphate, 0.5 g/L ammonium sulfate, 0.2 g/L potassium chloride, 0.1 g/L magnesium sulfate, 0.0001 g/L manganese sulfate, 0.0001 g/L ferrous sulfate, 15 g/L agar). To test for HA mobilization the same media was used, except CaP was exchanged with 5 g/L hydroxyapatite. Plates were incubated for 7 days at 28°C and afterwards stored for two weeks at room temperature until pictures were taken. To better visualize mobilization of CaP and HA, bacterial colonies were rinsed of with water after taking a first picture.

4.2.9. Plant growth assays

Plant growth assays were performed as described in Chapter I.4.2.13. Magnification of the main root in Fig. 29 was taken with an Axio Scope.A1 microscope (Zeiss, Munich, Germany).
To correlate the ability of rhizobia to mobilize CaP with their plant growth-promoting abilities, plant growth assays were performed supplying only insoluble phosphorus sources (Fig. 32). Therefore, KH_2PO_4 was replaced by either 625 µM CaP or HA. Otherwise the plant growth assays were performed as described in Chapter I.4.2.13.

4.2.10. Time course experiment and quantitative PCR

The time course experiment was conducted as described in Chapter I.4.2.13. Instead of analyzing and isolating root material only after 18 dpi, additional sampling steps were performed at 4, 8, 12 and 16 dpi. Additionally, pictures were taken directly after transfer of seedlings (0 dpi). DNA from root samples was prepared by adding Lysing Matrix E beads to the root samples (MP Biomedicals, Solon, USA), followed by a mechanical disruption by the TissueLyser II (Qiagen, Hilden, Germany) for 30 seconds

at 20 beats per second. Afterwards, samples were chilled in liquid nitrogen for a few seconds and the bead beating step was repeated one to two times until the complete root material was disrupted. Afterwards, DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured using a Nanodrop photometer and the concentration was adjusted to 5 ng/µl. Rhizobia were amplified using primers that target the rhizobial 16S rRNA gene sequence using quantitative PCR (qPCR). Therefore, two primer pairs that specifically target the 16S rRNA gene of rhizobial exemplars employed in this study were designed using the Primer 3 and Amplify 3X programs (according to Dr. S. Hacquard). Primer sequences can be found in Tab. 10. Absence of cross-annealing was checked against the genome of the rhizobial exemplars and the *A. thaliana* genome (TAIR) using the BLASTN algorithm. 5 ng of total genomic DNA was used for the qPCR reaction and the 16S rRNA gene was amplified using the 1X iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) after adding 1.6 M of primers (95°C: 3 min, 95°C: 10 sec, 60°C: 30 sec, 72°C: 30 sec at 40 cycles). For normalization the *A. thaliana* chloroplast 16S rRNA gene was amplified in a similar way using plant-specific primers that do not cross-hybridize with the bacterial 16S rRNA gene (Tab. 10). Afterwards, the relative abundance was determined using the protocol described in (Pfaffl, 2001).

4.2.11. RNA-Seq sampling and sequencing

For a transcriptional analysis, root material of plants treated with $MgCl_2$ or R129_E was collected at 4 dpi, 8 dpi, 12 dpi and 16 dpi as described before (Chapter II.4.2.10). Therefore, triplicate samples of $MgCl_2$ or R129_E inoculated plants were grown under phosphorus-sufficient and -deficient conditions, resulting in 48 samples. To isolate RNA, root material was mechanically disrupted by Lysing Matrix E beads to root samples (MP Biomedicals, Solon, USA). Afterwards, samples were homogenized at 20 beats per second for 30 seconds using the TissueLyzer II (Qiagen, Hilden, Germany). The homogenization step was repeated for two to three times until the complete root material was disrupted. Afterwards, RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, United States). In the subsequent Illumina sequencing only samples were included that displayed a minimal degradation and a RNA integrity number (RIN) above seven. Preparation of Illumina sequencing libraries was conducted by the Max Planck Genome Center in Cologne using an input of 1.5 µg of total RNA. Sequences were generated using the Illumina HiSeq2500 platform. Roughly 20,000,000 million reads per sample with a length of 100 bp were generated and further analyzed (Tab. S 6). Obtained sequences were quality filtered by trimming the Illumina adaptor and discarding every sequence below a phred score of 25 using the FASTX toolkit. High quality sequences were mapped to the *Arabidopsis* genome (tair10 annotation file) using the software Tophat2 and Bowtie2. From this a count table for each gene and sample was generated using BEDtools suite and function coverageBED and used as further input in R for further statistical analysis.

4.2.12. Statistical analysis of RNA-Seq dataset

For the statistical analysis of sequences obtained by RNA-Seq, all genes with less than 100 reads across all samples were discarded to ensure reproducibility. Afterwards, the thresholded dataset was log_2-transformed and normalized by the function *voom* based on the trimmed mean of M-values (TMM) method (R package limma) resulting in log_2-transformed counts per million (cpm) dataset. A linear model was fit to each gene with the function *lmFit* using the following comparisons: Nutrient_treatment, Bacteria_treatment and Timepoint. Bacteria_treatment separated R129_E and $MgCl_2$ treated samples, Nutrient_treatment separated phosphorus-sufficient and -deficient samples and Timepoint separated samples collected at 4, 8, 12 and 16 dpi. Differentially expressed genes (DEGs) affected by Bacteria_treatment were calculated across the interaction term $MgCl_2$ versus R129_E across time using a moderated f-test over 8 contrasts. Four contrasts define the interaction between R129_E and $MgCl_2$ across phosphorus-sufficient and four contrast across phosphorus-deficient conditions. DEGs across Nutrient_treatment were calculated across the interaction term phosphorus-deficient versus -sufficient conditions across time using a moderated f-test over 8 contrasts. Four contrasts define the interaction of phosphorus deficiency against $MgCl_2$ and 4 contrast across R129_E treated samples. In all cases the obtained p-values were adjusted for false discoveries caused by multiple hypothesis testing via the Benjamini-Hochberg (BH) procedure. In total 7,412 out of 22,515 genes exhibited significant differences across interaction type and time point (false discovery rate (FDR) < 0.05). DEGs were defined by considering genes with a log_2-fold change above 1 or below -1 for up- or down-regulated genes, respectively. Gene expression levels are represented as gene-wise standardized log_2-transformed counts per million.

To construct a Cluster-Dendrogram log_2-transformed samples were compared to each other by calculating the Spearman distance between samples and clustering samples using hclust in R (Fig. 35). To test for Gene Ontology (GO) term enrichments among differentially expressed genes, the agriGO-GO Analysis Toolkit and Database for Agricultural Community (http://bioinfo.cau.edu.cn/agriGO/) was used.

4.2.13. General statistical analysis

All statistical tests were conducted using R. If the respective dataset was compared to a control, ANOVA was carried out followed by a Dunnett's post-hoc analysis. If multiple comparisons were analyzed, a Tukey's HSD test was conducted after ANOVA. Dunnett's test and Tukey's HSD analyses were performed using the *multcomp* package in R. All plots were generated using R and the package *ggplot2*. Within the boxplot the median, first and third quartile are plotted. The ends of the whiskers represent the lowest point, still within 1.5 inter-quartile-range (IQR) of the lower quartile, and the highest points still within 1.5 IQR of the upper quartile.

Abbreviations

%	percent
µ	micro
w/v	weight per volume
454	pyrosequencing
Aa	*Arabis alpina*
ABA	abscisic acid
ABC	ATP-binding cassette transporter
ACC	1-aminocyclopropane-1-carboxylate
ACM	abundant community member
AHL	N-acyl-homoserine lactone
ANOVA	analysis of variance
ARISA	automated ribosomal intergenic spacer analysis
At	*Arabidopsis thaliana*
ATP	adenosintriphosphat
BNF	biological nitrogen fixation
BSA	bovine serum albumin
CaP	calcium phosphate
CAS	Cologne agricultural soil
CFU	colony forming unit
Ch	*Cardamine hirsuta*
Col	*Arabidopsis thaliana* ecotype Columbia
cpm	counts per million
CPR1	constitutive expresser of PR genes 1
CPS	capsular polysaccharides
DEG	differentially expressed gene
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	desoxynucleotide
dpi	days post inoculation
EDTA	ethylendiaminetetraacetic acid
EFR	elongation factor Tu receptor
EPS	exopolysaccharides
ERF	ethylene-responsive factors
ET	ethylene

ETI	effector triggered immunity
ETS	effector triggered susceptibility
FAO	Food and Agriculture Organization
FDR	false discovery rate
FIT1	Fe-deficiency-induced transcription factor
FLC	flowering locus C
FLS2	flagellin-sensing 2
FRO2	ferric reduction oxidase 2
FS	French soil
Gal	Galibier
GFP	green fluorescent protein
GH	greenhouse
GO	gene ontology
h	hour
HA	hydroxyapatite
HT	heat-treated
IAA	Indole-3-acetic-acid
IRT1	iron-regulated transporter 1
ISR	induced systemic resistance
IQR	inter-quartile-range
JA	jasmonic acid
LCO	lipochitooligosaccharide
Ler	*Arabidopsis thaliana* ecotype Landsberg erecta
LPS	lipopolysaccharides
MAMP	microbe-associated molecular pattern
MATE	multidrug and toxic compound extrusion
min	minute
ml	milliliter
MPIPZ	Max Planck Institute for Plant Breeding Research
MS	Murashige and Skoog
MTI	MAMP-triggered immunity
NB-LRR	nucleotide binding leucine-rich repeat
n.d.	no data available
NGS	next-generation sequencing
NO	nitrite oxide
nod	nodulation
NPR1	nonexpresser of PR genes 1

NS	natural site
OD	optical density
OTU	operational taxonomic unit
Ox	*Cardamine hirsuta* ecotype Oxford
Paj	*Arabis alpina* ecotype Pajares
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PEP1	perpetual flowering 1
PGPR	plant growth-promoting rhizobacteria
pH	negative decimal logarithm of H^+ concentration
PR1	pathogenesis related gene 1
PRR	pattern recognition receptor
PSB	phosphorus-solubilizing bacteria
pv.	pathovar
QIIME	quantitative insights into microbial ecology
qPCR	quantitative PCR
RA	relative abundance
RDP	Ribosomal Database Project
RIN	RNA integrity number
RNA	ribonucleic acid
ROS	reactive oxygen species
rpoB	RNA polymerase beta-subunit encoding gene
rRNA	ribosomal ribonucleic acid
RT	room temperature
SA	salicylic acid
SAR	systemic acquired resistance
SD	standard deviation
SE	standard error
sec	second
SEM	scanning electron microscopy
Sha	*Arabidopsis thaliana* ecotype Shakdara
SIP	stable isotope probing
TAE	Tris-acetate-EDTA
Taq	*Thermophilus aquaticus*
TIC	threshold-independent community members
TMM	trimmed mean of M-values

T-RFLP	terminal-restriction fragment length polymorphism
Tris	tris-(hydroxymethyl)-aminomethan
T3SS	type-3-secretion system
TWYE	tap water yeast extract
TY	tryptone yeast
U	unit
UV	ultraviolett
V	Volt
W	Watt
WT	wild type
YEM	yeast extract mannitol

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Supplementary Figures

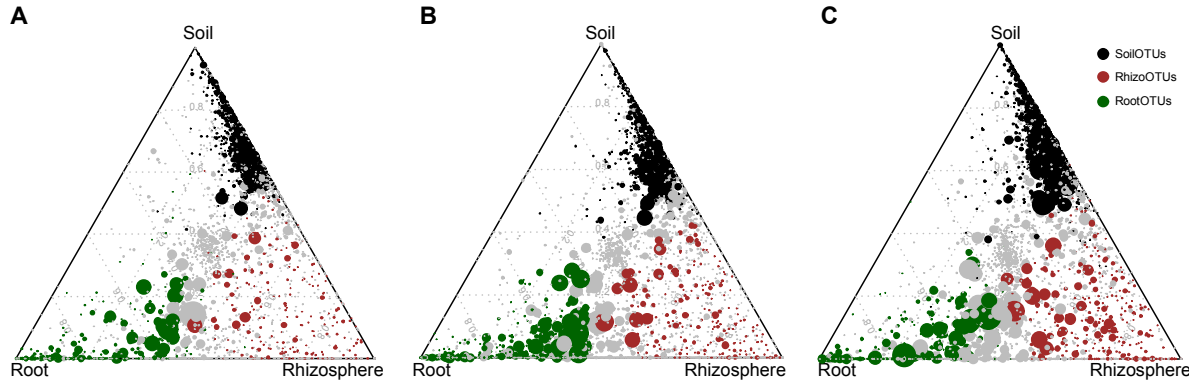

Fig. S1: A distinct root, rhizosphere and soil bacterial microbiota assembles over time. OTUs enriched in the soil, rhizosphere and root compartments of *A. alpina* after 6 weeks, 3 and 7 months (t1, t2 and t3). Each circle in the ternary plot depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position indicates the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. Depicted is the community structure at t1, t2 and t3 (**A-C**, respectively). Enriched OTUs include OTUs that are specifically enriched at individual time points and OTUs that are stable over time ("sharedOTUs"). Compartment-enriched OTUs based on $p < 0.05$ (FDR-corrected). OTUs are only color-coded when they are enriched at the depicted time point, i.e. at t1 only RootOTUs are color-coded that are enriched within roots at t1 but not t2 and t3.

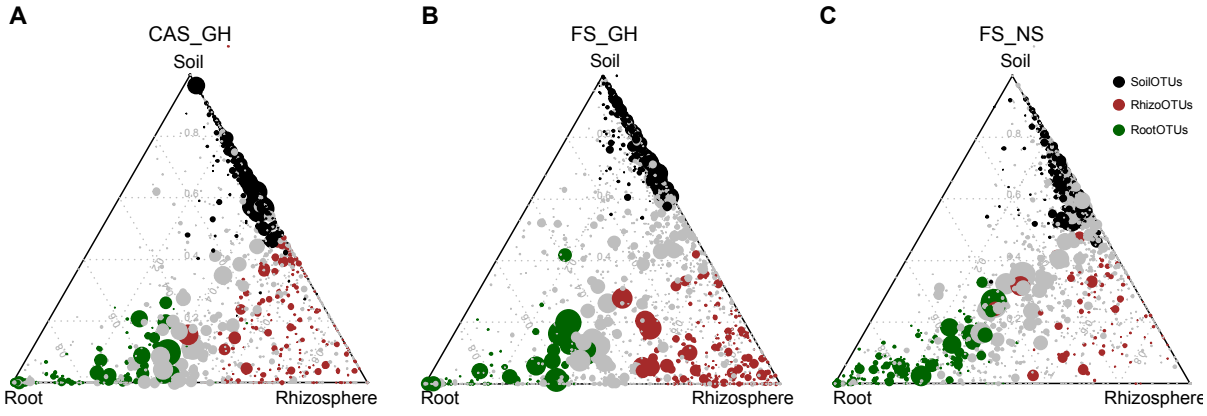

Fig. S 2: Distinct root, rhizosphere and soil bacterial communities assemble under distinctive growth conditions. *A. alpina* was grown in two soil batches (cologne agricultural soil (CAS) and French soil (FS)) with FS samples collected in the greenhouse (GH) or natural site (NS). For each condition the root, rhizosphere and soil compartments were collected. Each circle depicts one individual OTU. The size of the circle reflects the relative abundance (RA). The position indicates the contribution of the indicated compartments to the RA. The dotted grids and numbers inside the plot represent 20% increments of contribution from each compartment. RootOTUs, RhizoOTUs and SoilOTUs enriched in CAS_GH **(A)**, FS_GH and **(B)** and FS_GH. Enriched OTUs include OTUs that are specifically enriched during individual growth conditions and OTUs that are stable over growth conditions ("sharedOTUs"). Enriched OTUs based on $p < 0.05$ (FDR-corrected). Only OTUs are color-coded that at the depicted growth conditions, i.e. in CAS_GH only RootOTUs are color-coded that are enriched within roots at CAS_GH but not FS_GH and FS_NS.

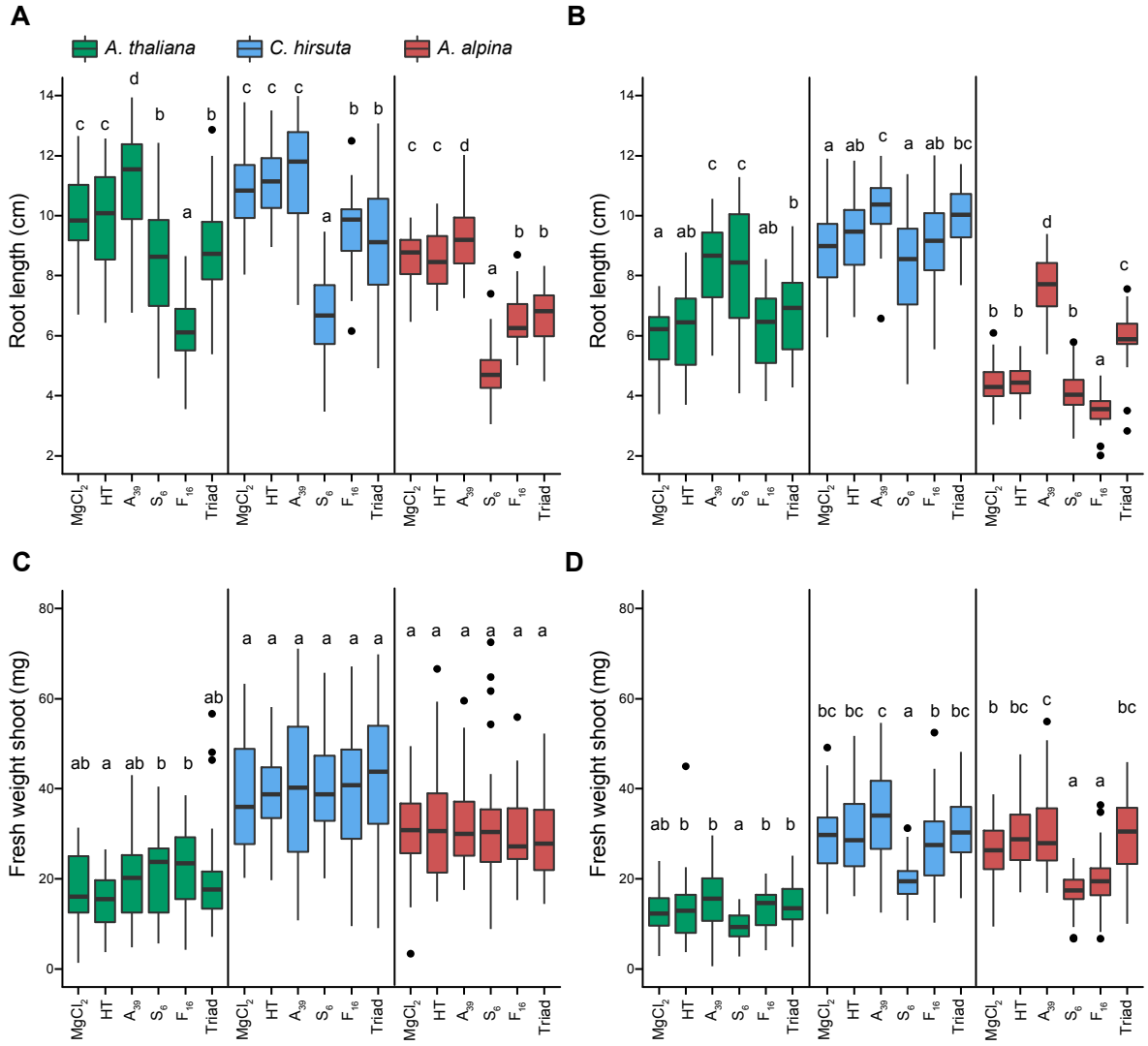

Fig. S 3: Bacteria of the shared microbiota affect growth of *A. thaliana*, *C. hirsuta* and *A. alpina*. Acidovorax$_{39}$ (A_{39}), Streptomyces$_6$ (S_6) and Flavobacterium$_{16}$ (F_{16}) were isolated from *A. thaliana* and tested for their effect on plant performance on three related plant species (*A. thaliana* (green), *C. hirsuta* (blue) and *A. alpina* (red)). Plant growth assays were performed under phosphorus-sufficient (**A and C**) or -deficient (**B and D**) conditions, after adding $MgCl_2$ or heat-treated (HT) bacteria as mock control and Streptomyces$_6$, Flavobacterium$_{16}$ and Acidovorax$_{39}$ in single inoculum at a final density of 10^6 cells per ml. A synthetic triad community was assembled by first adjusting all three bacteria that were tested in binary interactions to a density of 10^6 cells per ml and combining them afterwards in equal amounts. Measured parameters: Primary root length in cm (**A and B**) and shoot fresh weight in mg (**C and D**). n = 30-45 plants measured in three independent biological replicates. Significance letters are based on Tukey's HSD using a p-value cut-off < 0.05 and performed separately for the different plant species and nutrient conditions.

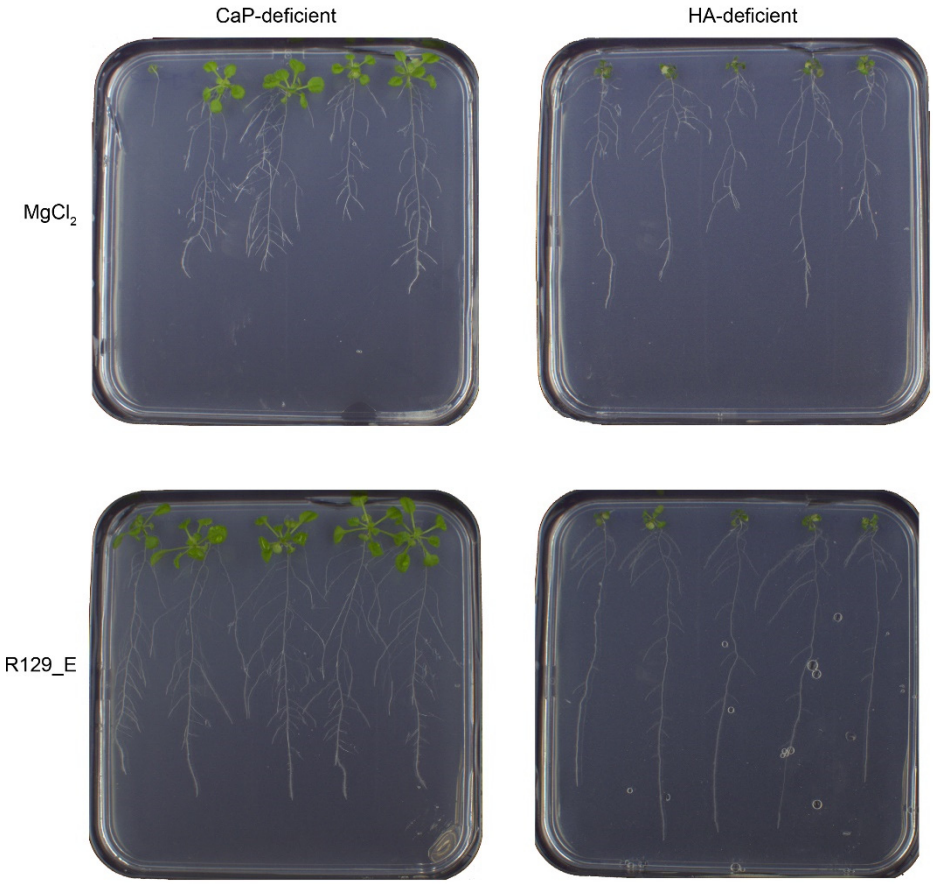

Fig. S 4: R129_E alters root architecture of *A. thaliana* independent of the used phosphorus sources. Growth phenotype of *A. thaliana* 18 days post inoculation (dpi) with $MgCl_2$ or R129_E at a final density of 10^5 cells/ml. Only insoluble phosphorus was supplied, either as calcium phosphate (CaP) or hydroxyapatite (HA).

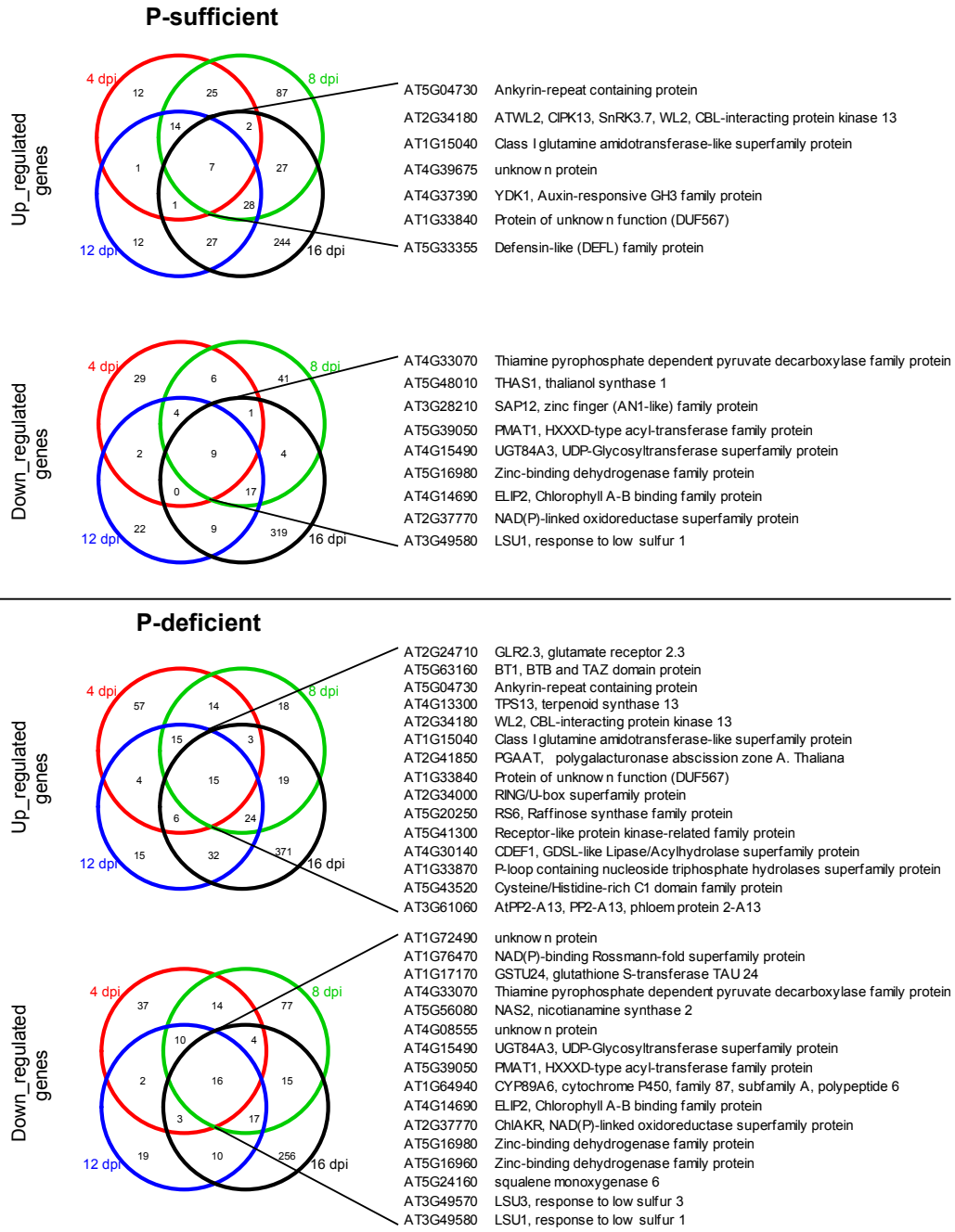

Fig. S 5: Differentially expressed genes upon treatment with R129_E. Up- and down-regulated genes 4, 8, 12 and 16 dpi with R129_E compared to mock treatment under phosphorus (P)-sufficient and -deficient conditions. Genes included in this comparison exhibit a log2-fold change above 1 (up-regulated) or below -1 (down-regulated) and a p-value < 0.05 (FDR-corrected).

Supplementary Tables

Tab. S 1: Summary statistics of phyla and families within the time course experiment across different compartments and time points. Depicted p-values are based on Tukey's HSD (FDR-corrected). Statistics were performed separately for each compartment. Only phyla and families are depicted that are also plotted in Fig. 13. t1: 6 weeks, t2: 3 months, t3: 7 months.

PHYLUM	Root compartment			Rhizo compartment			Soil compartment		
	t2-t1	t3-t1	t3-t2	t2-t1	t3-t1	t3-t2	t2-t1	t3-t1	t3-t2
Proteobacteria	< 0.001	< 0.001	0.43	< 0.001	< 0.001	1.00	0.59	0.75	1.00
Bacteroidetes	< 0.001	< 0.001	0.15	< 0.001	< 0.001	0.96	1	0.75	0.94
Actinobacteria	1.00	1	1	< 0.001	< 0.001	0.57	1	0.26	0.08
Chloroflexi	0.58	1	0.21	< 0.001	< 0.001	0.82	0.55	0.50	0.08
Firmicutes	0.52	0.90	0.11	0.64	< 0.001	0.03	1	0.66	1
Acidobacteria	< 0.001	< 0.001	1	< 0.001	0.07	0.01	1	0.63	0.53

FAMILY	Root compartment			Rhizo compartment			Soil compartment		
	t2-t1	t3-t1	t3-t2	t2-t1	t3-t1	t3-t2	t2-t1	t3-t1	t3-t2
Comamonadaceae	0.91	0.21	0.02	1	0.68	0.46	1	1	1
Flavobacteriaceae	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	1	1	1
Pseudomonadaceae	1.00	0.01	0.02	0.04	0.20	0.83	0.19	0.09	1
Burkholderiales_ incertae_sedis	0.69	0.73	0.08	0.20	0.24	1	1	0.41	0.68
not_assigned	0.004	< 0.001	1.00	< 0.001	< 0.001	0.70	1	1	0.60
Streptomycetaceae	0.05	< 0.001	< 0.001	1	0.01	0.01	1	1	1
Hyphomicrobiaceae	< 0.001	< 0.001	1.00	< 0.001	< 0.001	< 0.001	1	0.02	0.01
Sinobacteraceae	< 0.001	< 0.001	< 0.001	< 0.001	0.27	< 0.001	1	0.87	1
Oxalobacteraceae	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	0.25	1
Bradyrhizobiaceae	< 0.001	< 0.001	0.02	< 0.001	0.62	< 0.001	1	0.92	1
Xanthomonadaceae	0.88	0.89	0.26	0.69	< 0.001	0.01	1	0.71	1
Phyllobacteriaceae	0.18	0.87	0.01	< 0.001	1	< 0.001	1	0.03	0.01
Myxococcaceae	< 0.001	< 0.001	< 0.001	0.26	< 0.001	< 0.001	1	1	1
Cystobacteraceae	0.01	< 0.001	< 0.001	< 0.001	< 0.001	0.003	1	0.18	0.56

Tab. S 2: OTUs enriched in the time course and natural site experiment. Depicted are the total number of OTUs and the phylum distribution (in %) of these OTUs. OTUs enriched within the root, rhizosphere and soil compartments (RootOTUs, RhizoOTUs and SoilOTUs) independent of time and soil type were determined. Additionally, OTUs that showed a time point-specific or soil type-specific enrichment are indicated. **(A)** OTUs within the time course experiment. **(B)** OTUs within the natural site experiment. t1 = 6 weeks, t2 = 3 months, t3 = 7 months. C8_GH: plants grown in Cologne agricultural soil in the greenhouse; FS_GH: plants grown in French Soil in the greenhouse; FS_NS: plants grown in French Soil at the natural site.

A

		Number of OTUs	Proteobacteria	Actinobacteria	Bacteroidetes	Firmicutes	Acidobacteria
TOTAL	RootOTUs	519	74.2	13.1	7.7	0.4	0.6
	RhizoOTUs	425	44.2	2.6	28.5	1.6	1.2
	SoilOTUs	874	41.1	14.1	2.6	8.7	12.8
RootOTUs	t1	62	71.0	14.5	9.7	0	0
	t2	63	68.3	7.9	7.9	1.6	1.6
	t3	194	79.9	14.9	4.6	0	0
RhizoOTUs	t1	50	58.0	0	36.0	4.0	0
	t2	51	49.0	0	25.5	5.9	0
	t3	157	37.6	3.2	28.0	0.6	1.9
SoilOTUs	t1	27	74.1	0	3.7	0	11.1
	t2	20	40.0	0	15.0	5.0	5.0
	t3	76	64.5	13.2	6.6	1.3	3.9

B

		Number of OTUs	Proteobacteria	Actinobacteria	Bacteroidetes	Firmicutes	Acidobacteria
TOTAL	RootOTUs	319	65.5	23.2	5.0	2.5	0.9
	RhizoOTUs	369	53.7	4.9	13.8	4.3	0
	SoilOTUs	799	39.0	17.5	5.6	2.4	9.5
RootOTUs	C8_GH	79	74.7	6.3	8.9	5.1	0
	FS_GH	60	70.0	11.7	10.0	1.7	0
	FS_NS	126	54.8	39.7	0.8	2.4	2.4
RhizoOTUs	C8_GH	147	50.3	4.1	10.2	8.2	0
	FS_GH	142	59.2	3.5	14.8	1.4	0
	FS_NS	37	45.9	13.5	27.0	5.4	0
SoilOTUs	C8_GH	215	40.5	9.3	0.5	5.6	22.8
	FS_GH	217	48.8	18.4	5.1	1.4	2.8
	FS_NS	178	27.0	10.7	16.9	1.7	6.7

Tab. S 3: Summary statistics of phyla and families detected in the natural site experiment across different compartments and growth conditions. Depicted p-values are based on Tukey's HSD (FDR-corrected). Statistics were performed separately for each compartment. Only phyla and families are depicted, which are also plotted in Fig. 13. C8: CAS soil, FS: French soil, NS: Natural site, GH: Greenhouse.

PHYLUM	Root compartment			Rhizo compartment			Soil compartment		
	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH
Proteobacteria	1	1	1	0.06	0.03	< 0.001	0.02	0.17	0.79
Actinobacteria	0.32	0.12	1	0.30	< 0.001	0.002	< 0.001	< 0.001	0.25
Bacteroidetes	1	0.36	0.33	1	0.89	0.74	0.02	0.001	0.21
Firmicutes	0.008	0.02	1	< 0.001	0.003	0.09	< 0.001	< 0.001	1
Gemmatimonadetes	1	0.04	0.03	1	0.06	0.05	< 0.001	1	0.003
Acidobacteria	0.34	0.38	0.005	< 0.001	< 0.001	0.08	< 0.001	< 0.001	0.10

FAMILY	Root compartment			Rhizo compartment			Soil compartment		
	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH	FS_GH-C8_GH	FS_NS-C8_GH	FS_NS-FS_GH
Pseudomonadaceae	0.003	0.06	0.53	0.49	0.84	1	0.36	0.06	0.57
Comamonadaceae	0.29	0.16	1	0.74	< 0.001	< 0.001	0.61	0.12	0.05
Streptomycetaceae	0.86	0.58	0.04	0.27	1	0.12	1	0.002	0.006
Burkholderiales_ incertae_sedis	0.40	0.01	0.024	0.82	0.01	0.01	< 0.001	< 0.001	0.02
Oxalobacteraceae	0.06	0.02	< 0.001	1	0.001	< 0.001	0.03	0.001	0.03
Flavobacteriaceae	1	1	1	1	1	0.89	0.38	0.04	0.41
Herpetosiphonaceae	< 0.001	0.33	< 0.001	0.002	0.54	< 0.001	< 0.001	< 0.001	0.01
Hyphomicrobiaceae	1	0.002	< 0.001	0.10	0.32	< 0.001	0.68	1	1
Rhodocyclaceae	0.06	0.02	0.78	0.27	0.32	1	0.05	0.05	1
Xanthomonadaceae	1	0.02	0.02	< 0.001	0.15	0.10	0.12	0.43	1
Chitinophagaceae	< 0.001	0.002	< 0.001	0.13	1	0.08	0.69	0.001	0.01
not_assigned	0.16	0.01	0.23	0.002	0.001	0.66	< 0.001	0.001	0.29
Thermomonosporaceae	1	1	1	0.26	1	0.15	1	0.28	0.29
Methylophilaceae	0.27	0.20	1	< 0.001	1	< 0.001	0.90	0.09	0.33
Clostridiaceae 1	0.06	0.10	1	0.01	0.49	0.17	0.003	0.17	0.17

Tab. S 4: Summary statistics for root growth-promoting activities of rhizobia under different phosphorus conditions. Statistics for phosphorus-sufficient **(A)** and -deficient **(B)** conditions. n: total number of samples, SD: standard deviation and SL: significance letter based on Tukey's HSD performed separated for each nutrient condition (p-value < 0.05). HT: Heat-treated.

A

Sample	Nutrient	n	Mean	SD	SL
$MgCl_2$	sufficient	118	10.01	1.15	ab
HT	sufficient	125	10.15	1.13	ad
R129_A	sufficient	42	10.84	0.95	bcde
R129_B	sufficient	43	11.58	1.90	efh
R129_C	sufficient	34	9.47	1.58	a
R129_D	sufficient	40	12.26	1.21	ghjk
R129_E	sufficient	40	11.67	1.26	efhi
R129_F	sufficient	43	12.47	1.06	hjl
R129_H	sufficient	41	11.03	1.02	cf
R129_I	sufficient	40	10.01	1.51	ac
R129_J	sufficient	42	11.45	1.37	efh
R129_K	sufficient	29	11.13	1.86	cdfg
R129_L	sufficient	40	11.92	1.53	fj
R129_M	sufficient	37	11.80	1.50	efhj
R129_N	sufficient	41	12.29	1.10	ghjk
R13_A	sufficient	39	13.48	1.82	l
R13_B	sufficient	40	12.12	1.71	ghjk
R13_C	sufficient	36	12.49	1.24	hjl
R13_D	sufficient	44	12.78	1.36	jl
R13_E	sufficient	42	12.63	1.21	ijl
R13_F	sufficient	40	12.43	0.84	hjl
R219_B	sufficient	39	12.07	1.74	fj
R219_F1	sufficient	43	12.31	1.07	hjl
R219_B1	sufficient	39	13.22	1.34	kl

B

Sample	Nutrient	n	Mean	SD	SL
$MgCl_2$	deficient	117	5.93	0.90	ab
HT	deficient	124	5.94	0.84	ab
R129_A	deficient	42	6.70	1.09	cd
R129_B	deficient	36	7.20	1.46	cdef
R129_C	deficient	42	5.43	0.90	a
R129_D	deficient	42	8.87	1.31	jl
R129_E	deficient	42	8.78	1.33	jl
R129_F	deficient	35	8.06	1.17	fgij
R129_H	deficient	43	6.82	1.45	cd
R129_I	deficient	39	6.66	1.19	bd
R129_J	deficient	36	7.59	1.20	dgh
R129_K	deficient	43	6.34	1.54	bc
R129_L	deficient	35	7.41	1.27	dg
R129_M	deficient	44	8.37	0.97	hjk
R129_N	deficient	39	8.64	1.45	ikl
R13_A	deficient	35	8.38	1.23	gjk
R13_B	deficient	40	7.81	0.85	eghi
R13_C	deficient	40	7.04	1.50	cde
R13_D	deficient	45	8.34	1.03	gjk
R13_E	deficient	35	8.67	1.45	ikl
R13_F	deficient	41	8.30	0.91	gjk
R219_B	deficient	43	8.25	1.08	gjk
R219_F1	deficient	39	9.09	0.96	kl
R219_B1	deficient	44	9.51	1.06	l

Tab. S 5: Summary statistics for effect of R129_E on plant growth over a time course experiment under phosphorus-sufficient and -deficient conditions. n: total number of samples, SD: standard deviation and SL: significance letter based on Tukey's HSD (p-value < 0.05). dpi: days post inoculation.

Sample	Nutrient	Timepoint	n	Mean	SD	SL
$MgCl_2$	sufficient	0 dpi	117	1.38	0.28	a
$MgCl_2$	sufficient	4 dpi	120	2.37	0.40	b
$MgCl_2$	sufficient	8 dpi	117	4.17	0.75	e
$MgCl_2$	sufficient	12 dpi	92	6.17	0.94	h
$MgCl_2$	sufficient	16 dpi	57	8.13	0.91	j
$MgCl_2$	sufficient	18 dpi	76	9.31	1.52	k
R129_E	sufficient	0 dpi	120	1.45	0.26	a
R129_E	sufficient	4 dpi	120	2.28	0.45	b
R129_E	sufficient	8 dpi	118	4.13	0.76	de
R129_E	sufficient	12 dpi	95	6.16	1.19	h
R129_E	sufficient	16 dpi	57	9.85	1.11	l
R129_E	sufficient	18 dpi	70	11.29	1.26	m
$MgCl_2$	deficient	0 dpi	119	1.42	0.23	a
$MgCl_2$	deficient	4 dpi	119	2.21	0.37	b
$MgCl_2$	deficient	8 dpi	118	3.78	0.56	cd
$MgCl_2$	deficient	12 dpi	82	4.70	0.76	f
$MgCl_2$	deficient	16 dpi	63	5.38	0.84	g
$MgCl_2$	deficient	18 dpi	76	5.61	0.87	g
R129_E	deficient	0 dpi	116	1.46	0.23	a
R129_E	deficient	4 dpi	112	2.19	0.35	b
R129_E	deficient	8 dpi	119	3.71	0.63	c
R129_E	deficient	12 dpi	103	5.41	0.74	g
R129_E	deficient	16 dpi	59	7.13	0.93	i
R129_E	deficient	18 dpi	78	8.31	1.32	j

Tab. S 6: Overview of generated sequences for transcriptional analysis of *A. thaliana* inoculated with $MgCl_2$ or R129_E: RNA from *A. thaliana* roots harvested at 4, 8, 12 and 16 days post inoculation (dpi) with either $MgCl_2$ or R129_E under phosphorus (P)-sufficient or -deficient conditions was subjected to RNA-Sequencing. Depicted are the number of reads after different steps within the data analysis. Quality filtering included discarding sequences below a quality score of 25 and reads below 75% of bases with a quality score of 25 (FASTX-toolkit). Alignment to the *A. thaliana* reference genome was performed using the TAIR10 index.

			Total number of reads	Reads after quality filtering	Number of reads aligned to At	% of reads aligned to At
$MgCl_2$	P-sufficient	4 dpi	17,798,740	17,124,590	15,937,951	93
		8 dpi	34,223,518	32,453,537	31,363,015	97
		12 dpi	16,963,781	16,238,341	15,267,806	94
		16 dpi	24,672,820	23,178,965	21,928,183	95
	P-deficient	4 dpi	19,263,906	18,647,695	17,753,907	95
		8 dpi	18,811,358	17,696,486	16,766,905	95
		12 dpi	18,780,157	17,732,919	16,424,857	93
		16 dpi	26,479,826	25,201,465	24,112,064	96
R129_E	P-sufficient	4 dpi	23,412,309	22,362,359	20,550,738	92
		8 dpi	18,290,156	17,507,533	16,751,448	96
		12 dpi	25,015,867	23,846,778	22,354,797	94
		16 dpi	17,209,315	16,371,872	15,670,249	96
	P-deficient	4 dpi	21,579,980	20,272,383	19,366,630	96
		8 dpi	20,000,547	19,288,669	18,431,206	96
		12 dpi	20,776,709	19,759,676	18,807,697	95
		16 dpi	19,322,997	17,950,867	16,786,160	94

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Britta & Bernd

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Erklärung

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Köln, April 2015 Nina Dombrowski

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