Leibniz-Gemeinschaft

Final report

Chemical Communication in the Rhizosphere

Leibniz-Institute: Leibniz Institute of Plant Biochemistry
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Content

Executive summary

The aim of this network project was to test the emerging hypothesis in rhizosphere biology that plant genetic variability constitutes a primary determinant of root exudate synthesis and secretion, which in turn alters physico-chemical soil properties for optimal nutrient acquisition and specifically remodels root-associated microbial communities in mutualistic interactions and defense strategies. Major objectives of the research represented (i) comprehensive analysis of genetic variability of plant root exudate composition, (ii) analysis and correlation of microbial community dynamics in relation to genetically determined patterns of root exudation, and (iii) analysis of modulation of root exudate patterns by soil microorganisms and limited nutrient availability.

The genetic variability of root exudate composition is demonstrated for 19 *Arabidopsis thaliana* accessions covering the genetic diversity of this plant species. The bacterial rhizosphere community has been analyzed for the same *A. thaliana* accessions in two soil types. The results demonstrate specific effects of each accession on its microbial rhizosphere community. Using transgenic *A. thaliana* lines overexpressing a maize terpene synthase, the effect of terpene production on the rhizosphere microbial communities was studied. It has been shown for different *A. thaliana* genotypes that root exudate composition is altered during phosphate starvation. Metabolic and growth responses of the 19 *A. thaliana* accessions have been analyzed for *Raoultella terrigena*, *Kosakonia radicincitans*, *Verticillium dahliae* and *Verticillium longisporum*. Early responses of *Medicago truncatula* to *Rhizophagus irregularis* and *Aphanomyces euteiches* were studied and the effects on exudate composition demonstrated.

Objectives

The aim of this network project was to test the emerging hypothesis in rhizosphere biology that plant genetic variability constitutes a primary determinant of root exudate synthesis and secretion, which in turn alters physico-chemical soil properties for optimal nutrient acquisition and specifically remodels root-associated microbial communities in mutualistic interactions and defense strategies. Major objectives of the research represented (i) comprehensive analysis of genetic variability of plant root exudate composition, (ii) analysis and correlation of microbial community dynamics in relation to genetically determined patterns of root exudation, and (iii) analysis of modulation of root exudate patterns by soil microorganisms and limited nutrient availability.

Research Task 1: Comprehensive Analysis of Genetic Variability of Plant Root Exudate Composition

Work Package IPB: Dierk Scheel, Stephan Schmidt

In order to sample root exudates from *Arabidopsis thaliana* under sterile conditions, a two-step hydroponic cultivation system was established (Fig. 1). Seeds were germinated on bottom-cut agar-filled PCR tubes in closed plastic boxes. After three weeks, individual precultivated plants were transferred to brown 50 ml glas bottles with medium, which was exchanged weekly and analyzed by liquid chromatography coupled mass spectrometry (LC-MS). After three weeks main cultivation roots and shoots were also harvested.

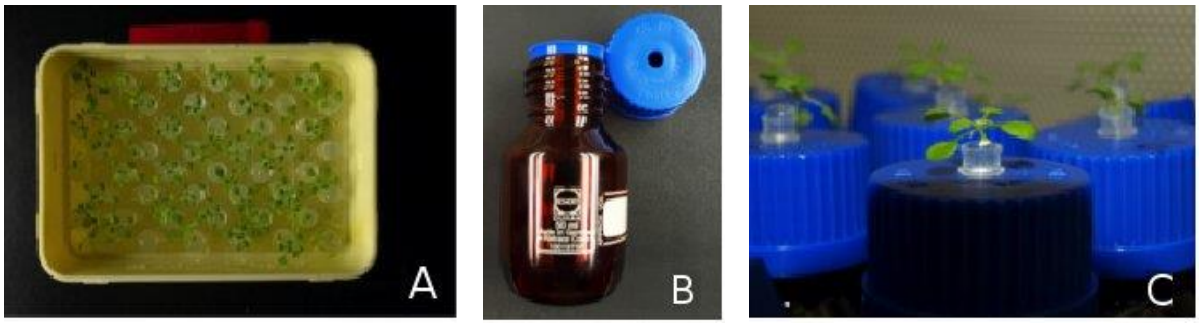

Fig. 1: Hydroponic System for *Arabidopsis thaliana*
A. Precultivation, B: bottles for main cultivation, C: main cultivation

To establish a workflow for non-targeted metabolite profiling of the semipolar fraction of root exudates and structurally identify the main component *Arabidopsis thaliana* Col0 was used. A total of 103 compounds were detected and annotated by elemental composition. More than 90 of these compounds were structurally characterized or classified and 42 were structurally identified. These compounds belong to the following substance classes: aromatic amino acids, nucleosides, dipeptides, glucosinolates and glucosinolate breakdown products, coumarins, cinnamic acid derivatives, spermidine conjugates and lignol-derived products (Fig. 2).

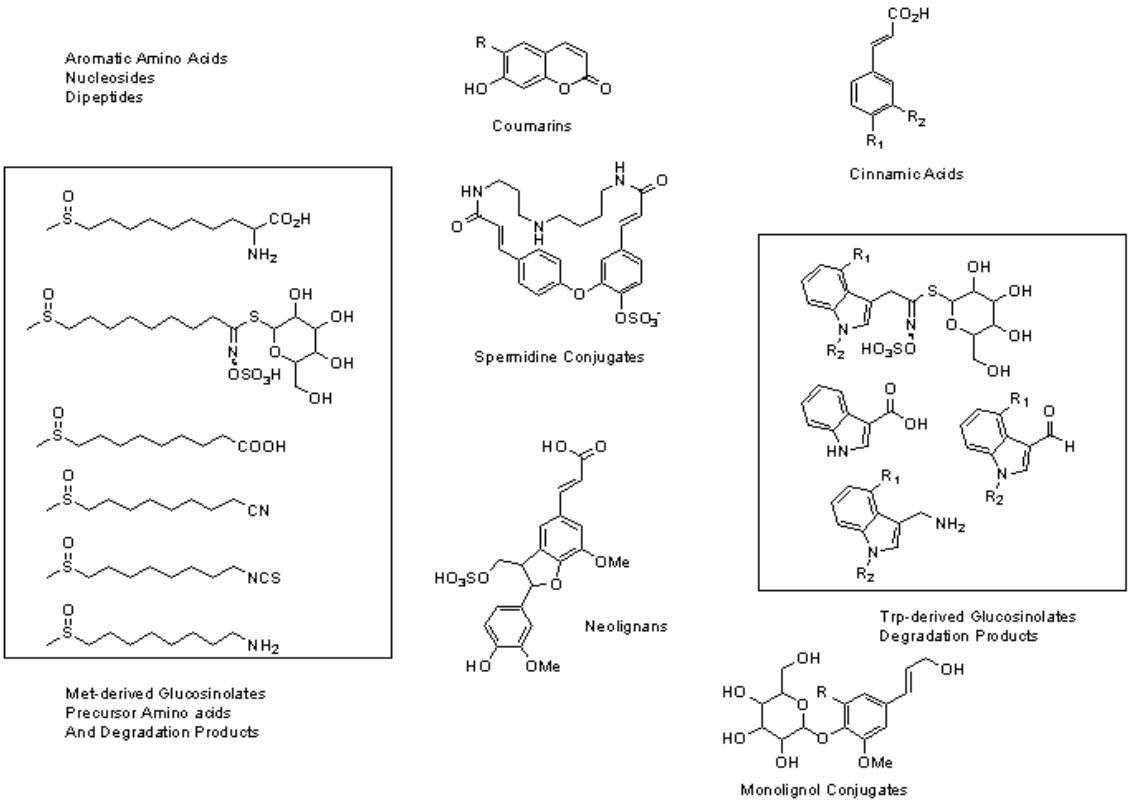

Fig. 2: Compound classes found in root exudates of *Arabidopsis thaliana* (Strehmel *et al.* 2014)

The optimized growth and analysis approach was then applied to the 19 parental accessions of the Arabidopsis MAGIC collection (Kover *et al.* 2009) in order to determine the natural variation of root exudate composition in *A. thaliana*. The different accessions displayed distinct metabolite profiles, which were further analyzed in the context of the sequence information of the 19 genomes (Clark *et al.* 2007, Nordborg *et al.* 2005).

Hierarchical clustering revealed relationships in the exudate metabolite patterns, which were only partly reflected by the root metabolite profiles and the genetic distances between accessions (Fig. 3). However, chemically related root- and exudate-specific metabolites were detected. Hydrolysis products of selected glucosinolates, coumarins and lignols were correlated with their respective glycosylated forms in roots. A user-friendly tool for a fast identification of the association between genetic and metabolite patterns was developed (Fig. 4). Strong effects, i.e. metabolite absence and nonsense mutations, were observed in the biosynthetic pathways of indolic glucosinolates and hydroxycinnamic acid conjugates: Neoglucobrassicin and a sulfated cyclic didehydro-di(coumaroyl)spermidine conjugate were not detected due to premature stop codons in the CYP81F4 monooxygenase and spermidine coumaroyl-CoA coumaroyltransferase genes, respectively.

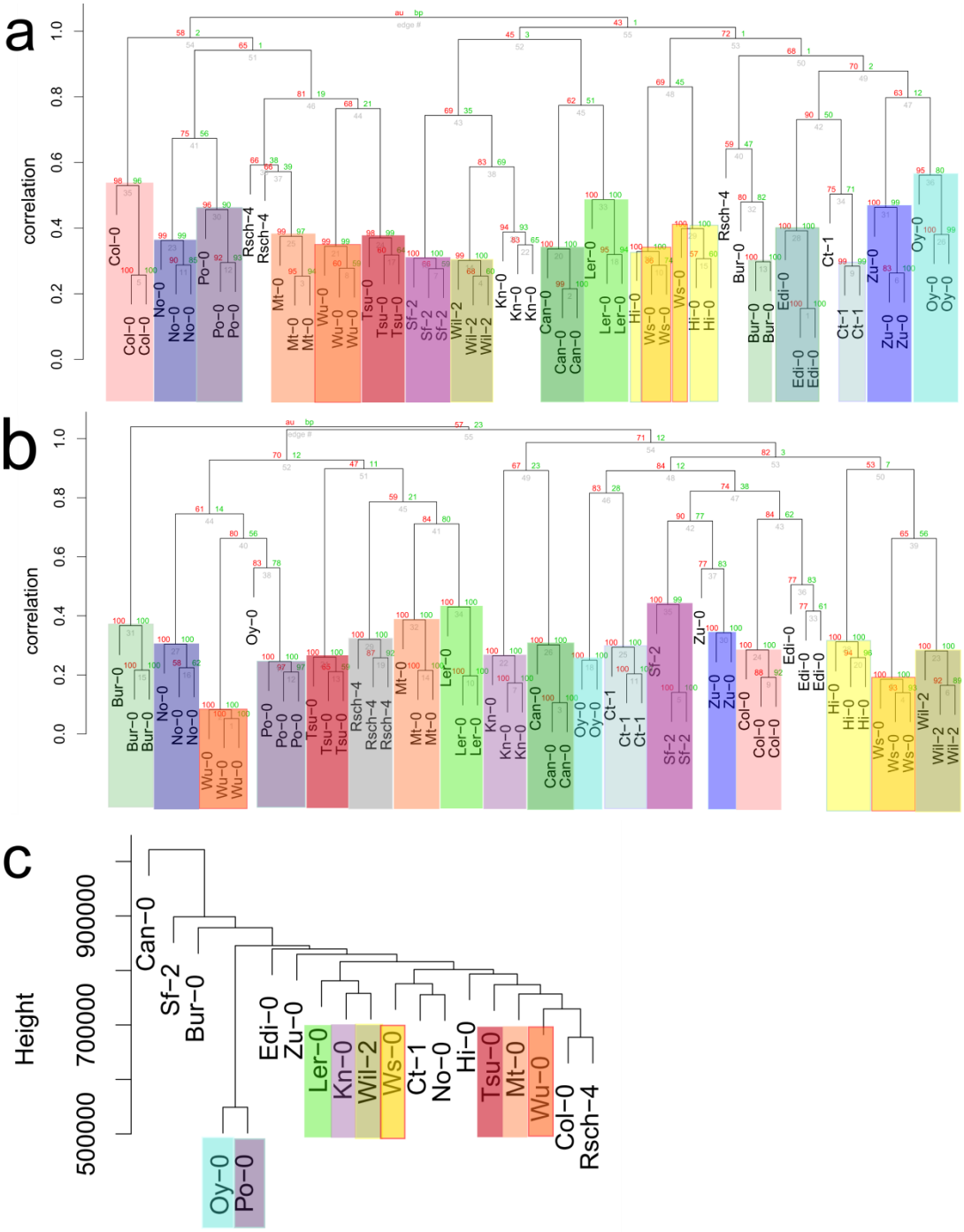

Fig. 3: Hierarchical clustering of metabolic features from (a) exudates and (b) roots and of (c) genetic distances. a+b) Features were obtained by UPLC/ESI(-)-QTOF-MS from exudate (a) or in-silico pooled root (b) samples and filtered by ANOVA ($p<0.01$) and a fold-change >2.0 (a) or >3.0 (b). Exudates were corrected for batch effects using surrogate variable analysis. Remaining features were subjected to average linkage clustering with 1-cor as a distance measure. Clusters with an AU≥0.95 were colored. c) Variant tables of the 19 genomes project were reduced to coding regions, as annotated by TAIR. The sum of all mismatches was used as a distance matrix for average linkage clustering. Similarities to the metabolic clusters were colored.

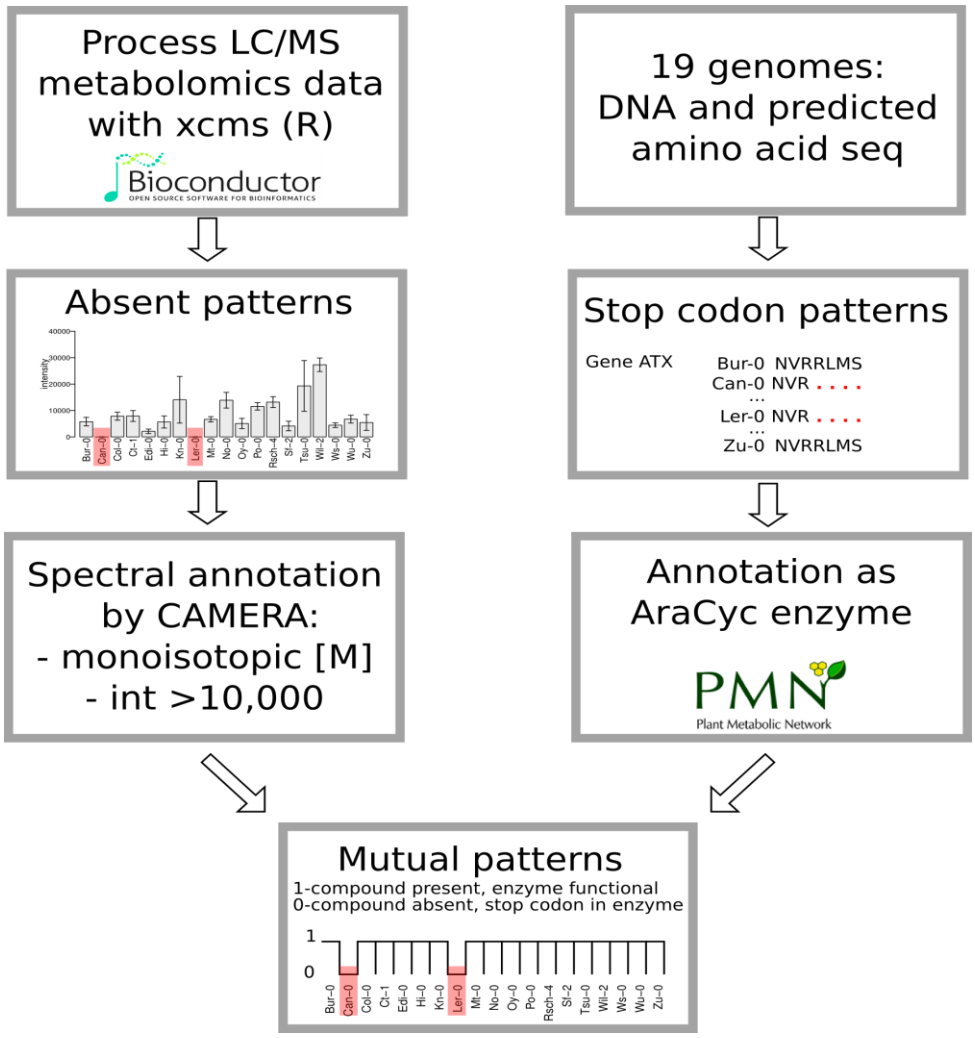

Fig. 4: Workflow for matching metabolic patterns of absence with stop codons in genes annotated as AraCyc enzymes. For the metabolic data, 1352 out of 1950 features were absent in at least one accession in the negative exudate data set. 61 of them were annotated as monoisotopic peak [M] by CAMERA. The remaining 48 features that passed the intensity threshold were matched to patterns of stop codons. Approximately 31,000 stop codons were detected. Out of the 3113 AraCyc enzymes, roughly one third (942) displayed a prematurely ended amino acid sequence possibly representing non-functional enzymes that can be causative for metabolite absence.

Research Task 2: Impact of Root Exudation on Microbial Community Dynamics

Work Package UFZ: François Buscot, Tesfaye Wubet, Carla Porges

Plants shape the microbial community of their rhizosphere via the composition and concentration of their root exudates. This project part investigated whether such rhizosphere effects vary specifically among parent lines of the same plant species. Therefore, 19 Lines of Arabidopsis from different world regions were cultivated and the composition of the bacterial and fungal communities of their rhizosphere was determined and compared using a molecular biological approach. To tackle the related mechanisms correlations with the exudation profile and the origin of the lines were analyzed.

We cultivated plants of the 19 MAGIC parent lines of *Arabidopsis thaliana* in pots with two distinct soil types (9 Replicates per line and soil type, with one replicate consisting in one pot with 10 plants each). The plants were harvested at flowering onset stage, and DNA of the

rhizosphere soil was extracted. Controls consisted in soil of the two types from pots without plants harvested at beginning and end of the treatments.

The DNA extracts were used for PCR targeting the V4 region within the 16s rRNA gene. After purification by gel electrophoresis, the composition of the bacterial community was determined in all samples by Pair End Illumina sequencing. A bioinformatics pipe line was implemented to trim the sample barcode and the primer regions as well as sequences of low quality. Furthermore the sequences were filtered for dimers. Afterwards the forward and reverse reads were joined and the sequences were checked for chimera. Subsequently the sequences were clustered into operational taxonomic units (OUT) with a similarity level of 97%. The taxonomic identity was determined using the SILVA reference database. All OTU that did not belong to the domain bacteria were removed. For the following statistical analysis, which was carried out using R, only those OTU where used that where present in at least 3 replicates.

In accordance with previous work (Bulgarelli et al. 2012), we showed that the soil type had a prominent role over the rhizosphere in shaping the soil bacterial community. However, an ANOSIM pairwise comparison showed a significant power of the 19 Arabidopsis lines to significantly influence the richness and composition of their rhizosphere microbial communities. The biome and annual precipitation of the point of origin, as well as the flowering time of the 19 accessions had a significant effect on the bacterial communities in the rhizosphere. Moreover the rhizosphere bacterial communities of the respective accession from the two soils were found to converge significantly compared to the controls without plant (Fig. 5). These results have potential importance to implement agricultural practices that are more sustainable and better use biodiversity. The analyses concerning the fungal community are still ongoing.

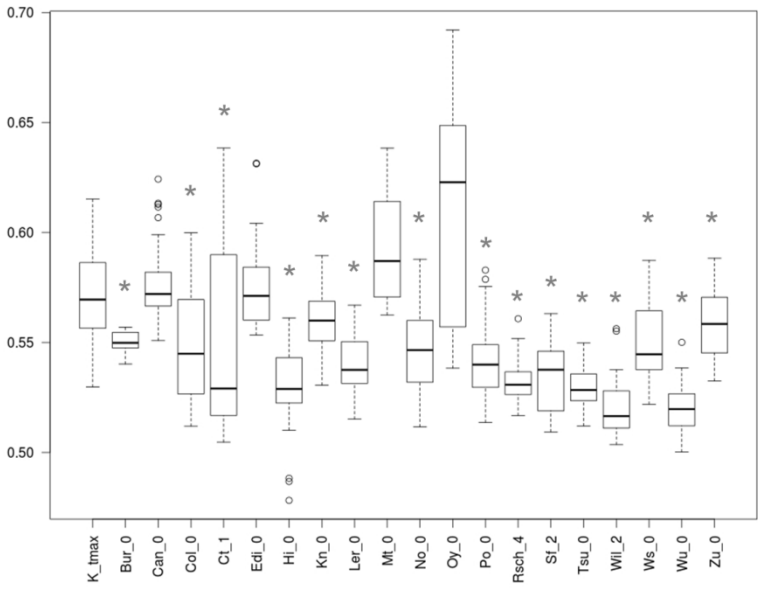

Fig. 5: Similarities of the bacterial communities of the rhizosphere of 19 Arabidopsis lines cultivated on two different soil types as compared to the communities of the same soils without plants. All values over 0,5 indicate a convergence of the rhizosphere communities. * $p < 0.05$.

Work Package MLU: Jörg Degenhardt, Gabriela Vacca

Effect of terpene production on rhizosphere microbial communities

Terpenes are produced by plants and emitted in the environment where they play important roles in plant defense, both above and below ground. The role of terpene in the rhizosphere remains less understood. We aim to understand whether the volatile terpenes condition the rhizosphere and alter the ratio of beneficial to pathogenic soil organisms. We hypothesize that terpenes emitted from roots affect the rhizosphere microbial community and condition this community to the advantage of the plant. To test this hypothesis, we determined the influence of terpene emission characterizing the bacterial communities using 454 pyrosequencing of the 16S rRNA gene in the rhizosphere of two transgenic plant lines. The first is a maize line overexpressing a caryophyllene synthase which mimics the release of a caryophyllene signal after herbivory (Degenhardt et al., 2009). The second is an Arabidopsis line expressing the terpene synthase TPS8 which is responsible for the emission of a complex terpene blend from maize roots (Fontana et al.2011).

We characterized the rhizosphere microbial community composition of wild type and terpene-releasing transgenic plants. We used an Arabidopsis transgenic plant that overexpresses a maize terpene synthase. This terpene synthase is responsible for the production of a complex volatile blend composed of 54 terpenes that is usually found in roots (Fontana et al, 2011). The transgenic Arabidopsis plants produce similar concentrations of that terpene blend in roots and release it into the rhizosphere. The maize transgenics only emit the sesquiterpene hydrocarbon (E)-ß-caryophyllene. This compound is often induced in maize roots after attack by root herbivores (Degenhardt et al., 2009). Both transgenic plants allow us to determine the effect of terpenes released by maize roots, once in the context of the Arabidopsis rhizosphere and once in the environment of the maize root. Our design permitted us to test the influence of terpene on the rhizosphere microbial community across the two models plants using DNA and RNA as template.

Microbial diversity and community composition

We generated 384,695 raw reads from 50 samples and obtained a total of 284,130 reads after removal of poor quality (9.5%) and potentially chimeric sequences (7.2%) from a subsample of 341,450 quality sequences representing 6829 sequences per sample. Sequences reads were binned at >97% sequence identity using CD-HIT-EST to define OTUs, and we identified a total of 13,449 unique bacterial OTUs. Comparison of the microbial community composition based on the whole matrix and a matrix excluding the low abundant OTUs (< 3 sequences) using a procrustes test indicated a significant correlation ($R = 0.998$ and $P = 0.001$) of the two matrices. Consequently, our analysis and results presented were focused on the abundant community members represented by 4171 OTUs.

In both Arabidopsis and Maize we observed a slight variation on the observed microbial diversity in both DNA and RNA based communities. Analysis of the taxonomic composition indicated that the majority of OTUs belonged to Proteobacteria (25.72%, 8.63%, 6.75%, and 5.32% in the Alpha-, Beta-, Gamma-, and Deltaproteobacteria subphyla, respectively), Actinobacteria (20.37%), Acidobacteria (11.72%), Bacteroidetes (6.87%), Chloroflexi (6.06%), Firmicutes (2.51%) and Gemmatimonadates (2.49%), while 1.39% of the OTUs were not classified at the phylum level. We found similar patterns of the rhizosphere microbial community composition in both arabidopsis and maize with slightly different percentage abundances in the DNA and RNA based communities of the terpene secreting and control plants.

Terpene effect on bacterial communities

We examined the microbial communities that are differentially abundant in response to the presence or absence of terpene secretion. SIMPER analysis results showed an overall dissimilarity of 52.97% in arabidopsis and 50.38% in maize rhizosphere communities. Fisher's exact test with Bonferoni correction at $P < 0.01$ was used to identify differentially abundant OTUs in terpene producing and control plants (Table 1). For spacial constraints, we will focus on the data from the Arabidopsis rhizosphere in this report. The most significant differences induced by the presence of terpenes occured in the active (rRNA-based) populations of Ara-

bidopsis and Maize. These differences demonstrate that terpenes in the rhizosphere of both plants affect specific members of the resident community.

Terpene degradation- Plants harbor a distinctive microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms. The effect of the complex terpene blend can be observed at the genus level. Some of the bacterial species relatively enriched by terpene are closely related to bacteria known for their ability to transform geosmin (Sphingopyxis, Massilia, Pseudomonas) (Eaton and Sandusky, 2010; Xue et al, 2012), or triterpenes (Solirubrobacter, Sphingobacteriaceae, Mucilaginibacter) (An et al, 2011; Son et al, 2013; Lee et al., 2013). Since structural similarities exist between plant terpenes and the heavier molecules found in petroleum (Chianelli, 1994) it is likely that some of these bacteria have a capability for biodegradation of such materials. In fact, many of these strains like *Pseudomonas cruciviae* are able to grow on ß-caryophyllene and longifolene as the sole carbon and energy sources. Pseudomonas strains have also been implicated in biotransformation of the more widely studied monoterpenes (Bicas et al. 2008; Kleinheinz et al. 1999).

Terpene and biological nitrogen transformation- A shift from nitrogen fixing bacteria to nitrifiers was observed due to terpene presence. Common habitants of the rhizosphere known due to their nitrogen fixation capacity and plant growth promoting ability as Devosia, Rhizobium, Azospirillum, Nostoc, Cellvibrio (Kuykendall et al., 2005) were less abundant in samples from terpene producing transgenic plants than in control plants (Table 1). There is increasing evidence that plant secondary metabolites, such as terpenes or phenolic compounds, affect soil C and N transformations. Certain monoterpenes have been found to inhibit net N mineralization and net nitrification in soil (White 1986; Kanerva et al., 2008). Further investigation is required to clarify the role of terpene in biological nitrogen cycle in the rhizosphere.

Balance of pathogens to beneficial bacteria within the community

Terpene affected certain bacteria considered as beneficial, because they show plant-protective effects as plant growth promoting bacteria (Sphingobium, Shpingobacteriaceae) (Innerebner et al 2011, Ahmed et al., 2014) (Table1). On the other hand, bacteria considered as pathogens like Sphingomonas and Acidovorax (Buonaurio et al, 2002; Gardan et al, 2003) were found in lower relative abundance in presence of terpene. The sensibility to hydrophobic antibiotics that is common to the representatives of the Sphingomonas genus might be responsible for the sensitivity to terpenes in this study. The results demonstrate a conditioning or the rizosphere that alters- and in most cases reduces- the activity of strains.

Antibiotic effect *in situ*- OTUs belonging to the genus Pseudomonas were found in lower relative abundance in presence of the terpene blend from Arabidopsis in comparison with control plants (Table 1). Since the transgenic Arabidopsis plants emit fifty four structurally diverse sesquiterpenes, the sensitivity of Pseudomonas to certain terpenes could be an alternative method to control pathogenic bacteria from this genus. Being Gram-negative bacteria, most Pseudomonas spp. are naturally resistant to penicillin and the majority of related beta-lactam antibiotics (Rian and Ray, 2004). Their resistance to most antibiotics is attributed to efflux pumps, which pump out some antibiotics before the antibiotics are able to act. Terpenes act by disrupting microbial cytoplasmic membrane, which thus loses its high impermeability for protons and bigger ions; if disturbance of membrane integrity occurs, then its functions are compromised not only as a barrier but also as a matrix for enzymes and as an energy transducer (Sikkemat al., 1994; Turina et al., 2006).

Concluding remarks

Metasequencing analysis demonstrated that terpenes in the rhizosphere modulate a reservoir of beneficial and pathogenic bacteria. Biotechnological implication of this research can be extended to the use of terpene-producing transgenic plants to control of pathogens and promote plant growth. A manuscript publishing these data is in preparation.

Phylum	Genus (or higher)	OTU	C	T	p-values	Description
Actinobacteria	Microlunatus	**Otu00489**	50	12	1.23E-04	isolated from rhizosphere, produce surface active compounds
Bacteroidetes	Flavobacterium	**Otu00018** **Otu00021** **Otu00034**	609 481 277	331 309 190	8.86E-20 7.60E-09 1.61E-03	associated to arabidopsis root and leaves, sensitive to germacrene
Deinococcus-Thermus	Deinococcus	*Otu00270* **Otu00270**	45 66	3 5	2.78E-07 1.32E-12	highly resistant to environmental hazards
Firmicutes	Bacillus	**Otu00020**	154	87	9.39E-04	sensitive to terpenes
Alfa-Proteobacteria	Devosia	**Otu00001**	1028	706	1.49E-15	nitrogen fixation, degrade AC
	Rhizobium	**Otu00008**	413	303	8,41E-04	nitrogen fixation
	Sphingobium	*Otu00278* **Otu00278** **Otu00254**	55 60 73	8 6 19	1.38E-06 3.01E-10 1.26E-06	plant protective, degrade AC
Gamma-Proteobacteria	Pseudomonas	**Otu00009** **Otu00093**	1009 153	503 61	3.33E-42 1.19E-08	pathogen
Actinobacteria	Solirubrobacter	**Otu00060**	34	83	4.02E-03	transform triterpene
Bacteroidetes	Flavobacterium	*Otu00013* **Otu00880**	314 2	428 38	1.03E-02 9.01E-07	associated to arabidopsis root and leaves degrade AC
	Sphingobacteriaceae	**Otu00028**	35	88	1.35E-03	transform triterpene, plant growth promoting bacteria
Beta-Proteobacteria	Aquabacterium	*Otu00330* **Otu00330**	14 16	58 56	8.98E-05 1.35E-03	rhizoremediation-fuel oil, r-strategist

Table1: Differential abundance of the most significantly ($p<0.01$) affected microorganisms due to terpene presence in Arabidopsis rhizosphere. Sequenced OTUs from RNA samples in bold and from DNA samples in italic, C: Number of sequences in control plants without terpene; T: Number of sequences in terpene producing plants. AC: aromatic compounds

Research Task 3: Genetic Variability of Root Exudates during Abiotic and Biotoc Challange

Research Task 3.1: Root Exudation in Response to Nutrient Availability

Work Package IPB: Steffen Abel, Jörg Ziegler

The objective of the project was the analysis of root exudate composition during phosphate (Pi) starvation in different *Arabidopsis* genotypes, which phenotypically differ in Pi starvation sensitivity. These phenotypic differences are manifested in the local Pi starvation response, which is determined by changes in root system architecture, whereas changes in the systemic Pi starvation response, which is determined mainly by metabolic alterations in order to retain Pi homeostasis have not been reported. These genotypes provided us with tools to elucidate the effect of changes in root system architecture, i.e. the local response, on root exudation independently of systemic responses. A sterile hydroponic system was developed, which allowed the analysis of root exudate composition during the local Pi deficiency response. This was achieved using a split system, in which the upper part of the root was still supplied with sufficient Pi, whereas the lower part of the root was exposed to Pi deficient conditions. Exudates were only collected from the lower part of the roots. Unchanged levels of metabolic markers such as arginine, malate, or citrate confirmed the absence of the systemic response after Pi deficiency, whereas decreases in primary root growth at the root tip confirmed the presence of the local response. The wild type Col-0 accession and the hypersensitive mutant *phosphate deficiency response 2 (pdr2)* showed about 25 % and more than 50% root growth inhibition, respectively, whereas root growth of the insensitive double mutant *low phosphate root 1 and 2 (lpr1/2)* was unaffected. Non-targeted LC-QTOF-MS profiling of apolar metabolites revealed that treatments and genotypes could be clearly distinguished based on their respective root exudate composition. In contrast to Col-0 and *lpr1/2*, the hypersensitive mutant pdr2 showed a decreased number of metabolites in root exudates after Pi deprivation. PDR2 is an ER resident P5 type ATPase of unknown substrate specificity. Since misexpression also leads to perturbations in the expression of various transport proteins, it is discussed to play a role in the secretory pathway, which could explain the impairment of *pdr2* in root exudation.

Several coumarins could be identified among the signals showing differential intensities after Pi deficiency. The role of coumarins as facilitators of iron uptake during iron deficiency is well established as well as the antagonistic effects of iron and Pi deficiency. Targeted coumarin profiling revealed the depletion of some coumarins after Pi deficiency, which are known to strongly accumulate after iron deficiency. However, other coumarins accumulate after iron and Pi deficiency. This points to a differential regulation of their stress-induced accumulation.

Several dimeric and trimeric oligolignols, constituents of lignin, could be identified, which accumulate after Pi deprivation. Accumulation was highest in *lpr1/2*, and lowest in hypersensitive *pdr2*. The oligolignol content in root exudates was inversely correlated to the extent of lignification in roots after Pi deficiency, *pdr2* showed very strong lignification, whereas in *lpr1/2* lignification was not detectable. This indicates a strong connection between the capability to polymerize oligolignols to lignin, thereby exuding less oligolignols, and root growth inhibition. It is possible that the laccase-like ferroxidases LPR1 and LPR2 are involved in Pi starvation induced lignification, since *lpr1/2* seem to be impaired in oligolignol polymerization, leading to decreased root lignification accompanied by increased oligolignol accumulation in root exudates.

Research Task 3.2: Root Exudation in Response to microbial Challenge

Work Package IPK: Sivasenkar Lingam, Nicolaus von Wirén

Since the recovery of metabolites in root exudates from hydroponically-grown Arabidopsis plants was so far too low for comprehensive MS-based metabolite analysis (Goals 2.1 & 2.3.1), we gave priority to the quantitative assessment of plant growth promotion by the plant growth-promoting bacterium *Raoultella terrigena* (Goal 2.3.2) and conducted an additional transcriptome analysis for the identification of Arabidopsis genes involved in the interaction or communication with *R. terrigena.*

Based on a transcriptome study conducted with RNA from roots and shoots of *Raoultella*-inoculated Arabidopsis plants, 22 highly responsive genes were identified that were either strongly up- or down-regulated in response to *R. terrigena* inoculation. T-DNA insertion lines with deletions in the corresponding genes were ordered, verified for homozygousity, and analyzed for their responsiveness to *R. terrigena*. However, all of the 22 investigated mutants did not show any impairment in growth promotion. Thus, this approach failed to identify plant genes involved in the plant-bacterial communication, but may indicate that Arabidopsis genes required for plant growth promotion by *R. terrigena* are redundant, since only single deletion mutants were assessed.

In a forward-genetics approach to identify plant genes underlying the positive growth response to *R. terrigena*, 19 parental accession lines and their recombinant inbred lines (RILs) of the Arabidopsis MAGIC collection were grown on full nutrient supply on agar plates and subjected to inoculation by the plant growth-promoting rhizobacterium *R. terrigena*. Control plants were not inoculated. Plant growth promotion was quantitatively assessed by determination of root and shoot dry weights as well as total root lengths using the WinRhizo software after root systems had been scanned. For each line 6 independent plants were assessed.

Among the 19 parental lines Wu-0 turned out as most responsive accession line to *R. terrigena*, whereas the lines Rsch-4 and Oy-0 were among the least affected lines (Fig. 6). Regarding these three traits the contrasting lines differed by a factor of 2.5 - 4. (Table 2).

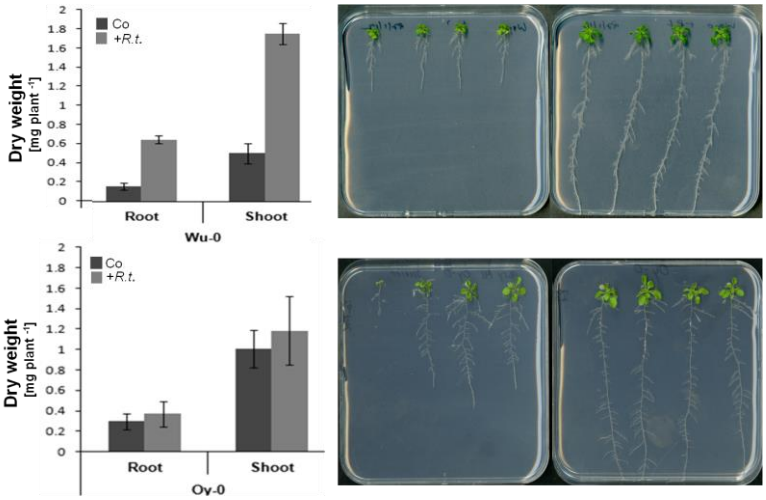

Fig. 6. Root and shoot dry weights and growth phenotype of the two parental accession lines Wu-0 and Oy-0. Plants were either not inoculated or inoculated with a *R. terrigena* suspension of 108 cfu mL-1 from the exponential growth phase and cultivated for a period of 17 days.

94 recombinant inbred lines (RILs) of the MAGIC population with a mixed genetic constitution of the three contrasting parents were then selected and again characterized for changes in shoot and root dry weight or primary root length after *R. terrigena* inoculation.

Table 2. Genotypic variation among 19 parental lines of the Arabidopsis MAGIC population regarding shoot and root dry weight or total root length in response to inoculation with *R. terrigena*. To quantify the responsiveness to *R. terrigena*, ratios were calculated by dividing the mean value from inoculated samples by the mean value from control samples. Ratios are arranged in descending order.

Line	Shoot dry wt (mg) (ratio inoculated / control)
1.Wu-0	3.47
2. Zu-0	2.77
3. Wil-2	2.49
4. Ws-0	2.46
5. Tsu-0	2.06
6. Col-0	2.0
7. Bur-0	2.0
8. No-0	2.0
9. Po-0	1.98
10. Mt-0	1.97
11. Edi-0	1.75
12. Rsch-4	1.61
13. Sf-2	1.60
14. Kn-0	1.58
15. Can-0	1.51
16. Hi-0	1.41
17. Ct-1	1.40
18. Ler-0	1.33
19. Oy-0	1.17

Line	Root dry wt (mg) (ratio inoculated /control)
1.Wu-0	4.02
2.No-0	2.73
3.Kn-0	2.15
4.Mt-0	1.95
5.Can-0	1.90
6.Zu-0	1.88
7.Col-0	1.40
8.Bur-0	1.32
9.Ct-1	1.32
10. Po-0	1.24
11. Sf-2	1.20
12.Wil-2	1.20
13.Ws-0	1.12
14.Oy-0	1.05
15.Ler-0	1.09
16.Rsch-4	0.82
17.Tsu-0	0.68
18.Edi-0	0.54
19.Hi-0	0.29

Line	Total root length (cm) (ratio inoculated / control)
1.Wu-0	3.32
2.Ct-1	2.50
3.Edi-0	2.31
4.Col-0	2.2
5.Kn-0	2.02
6.Ler-0	2.2
7.Zu-0	1.80
8. Po-0	1.74
9. Sf-2	1.74
10.Hi-0	1.73
11.No-0	1.55
12.Oy-0	1.52
13.Can-0	1.52
14.Wil-2	1.51
15.Mt-0	1.46
16.Ws-0	1.38
17.Bur-0	1.24
18.Rsch-4	1.16
19.Tsu-0	0.79

Unexpectedly, we observed an uncoupling of root and shoot growth promotion in the screened RIL population. While shoot dry weight was significantly increased upon inoculation in 92 lines, 25 lines did not show a significant increase in root dry weight, suggesting that shoot growth promotion was at least partially independent of root growth promotion by *R. terrigena*. Furthermore, 18 lines did not increase primary root length in response to *R. terrigena.*

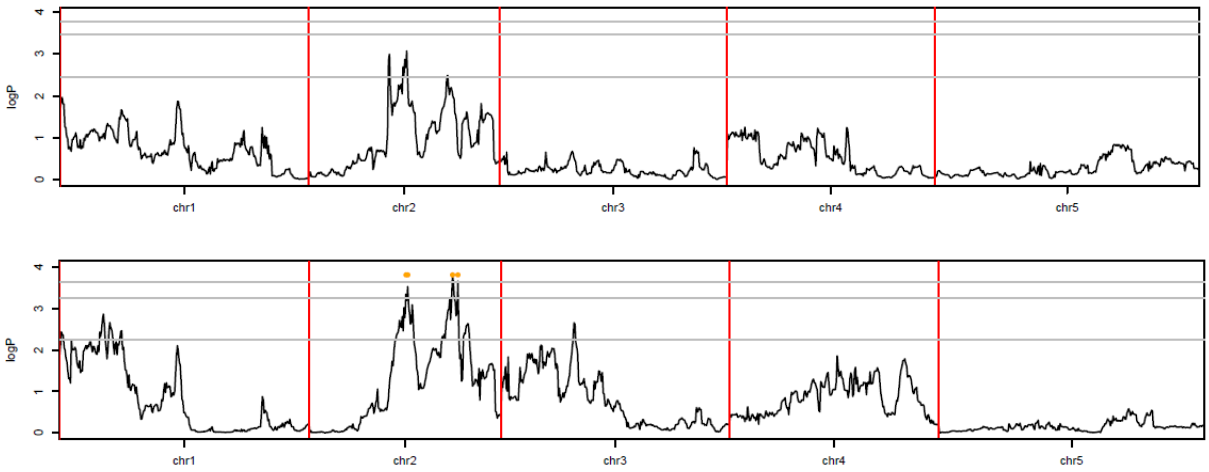

Figure 7. LogP values for significant associations between gene loci on chromosomes (chr) 1 to 5 and shoot dry weight (top) or root dry weight (bottom) of Arabidopsis plants inoculated with *R. terrigena*. LogP values close to or > 3.5 are labeled by an orange dot.

In order to track down the genomic loci and genes responsible for the differential responsiveness of these Arabidopsis RILs to *R. terrigena*, a QTL analysis was conducted for shoot and root dry weights in cooperation with AG Scheel at IPB Halle. However, QTL analysis did not indicate reliably a major locus being responsible for the responsiveness to *R. terrigena* (Fig. 7). There is just a weak indication (elevated logP score) for three gene loci on chromosome 4 that affect root dry weight in inoculated plants. However, such weak scores require verification with more lines in an independent experiment.

One possible reason for not being able to identify major QTLs for growth traits in inoculated plants may be due the limited quantitative variation in the recorded traits (variation by factor 2.5 - 4) and/or the limited number of replicas from one line, as individual plants from the same accession line still varied considerably. Furthermore, the number of RILs used in the present analysis may have been too small. Although 94 lines were considered to represent a reasonably good number for QTL analysis in the MAGIC population, genetic diversity among the selected lines may still have been too small to yield significant effects. Alternatively, the trait "responsiveness to Raoultella" may not be defined by one or several major QTLs but just based on many gene loci contributing with smaller-sized effects to growth responsiveness.

Analysis of early responses of *Medicago truncatula* to an arbuscular mycorrhizal fungus in comparison to the root pathogen *Aphanomyces euteiches*

Work Package IPB: Bettina Hause, Dorothée Klemann

Plants in nature are subjected to a variety of microorganism, among them beneficial fungi as well as pathogenic fungi and oomycetes. The focus of this project part was to analyze the early response of the model legume *Medicago truncatula* to treatments with a symbiont (spores of the arbuscular mycorrhizal fungus [AMF] *Rhizophagus irregularis*) and with a pathogen (zoospores of the causal agent of root rot, *Aphanomyces euteiches*). Thereby, we aimed to identify symbiosis-specific changes in the pattern of root exudates and root volatiles, but also of transcripts in comparison to co-cultivation with the pathogen.

To analyze AMF-induced changes in the transcript pattern, *M. truncatula* seedlings grown on agar plates were treated with both types of spores for two hours. The bottom two-cm of roots were used for transcript profiling using the "whole transcriptome array chip" (Affymetrix). Data analysis revealed that 56 genes are up- or down-regulated by treatment with AMF, whereas more than 250 genes are differentially regulated by the pathogen. However, most of both groups of genes are regulated in a similar direction compared to the non-treated control. Among the few genes regulated in opposite direction, some were identified whose gene product might be involved in signal perception, such as a putative LysM-domain containing protein and a putative protein kinase. Additionally, one gene encoding a putative sesquiterpene synthase was specifically up-regulated in roots after treatment with the pathogen. Differential expression of these genes was validated by quantitative RT-PCR. To perform functional analysis of these genes by a silencing approach in transgenic roots, artificial microRNAs (amiRNAs) were generated according to Devers et al. (2013). Additionally, several *Tnt1*-insertion lines were ordered. Among them, one affected in the sesquiterpene synthase encoding gene was identified. Infection of this mutant with *A. euteiches* resulted in the loss of three volatile sesquiterpenes and was accompanied by higher infection rates in comparison to the wild type.

To analyze metabolic changes, *M. truncatula* plants grown on hydroponic nutrient solution as established during the first two years of funding did not deliver sufficient material to get reliable results. Therefore, we switched to an aeroponic cultivation system, which was provided by the IGZ in Großbeeren. Plants were cultivated for six weeks in the aeroponic system and treated with either spores of the two microorganisms for 12 hours. LC-MS analysis of the root exudates showed more than 90 and about 70 substances, which were exudated at significantly higher levels by treatment with the AMF and the pathogen, respectively (Fig. 8). To get deeper insights into the response of mature root systems and to compare metabolite pattern

with transcriptional changes described above, the roots harvested from aeroponic cultivation were processed for transcript profiling. The analysis of all these data sets will allow a comprehensive characterization of the early response of *M. truncatula* roots to the contact with one symbiotic as well as one pathogenic soil-born microorganism.

Additionally, one aim of the project was to get first insights into the genetic variability of *Medicago truncatula* and its impact on the interaction with arbuscular mycorrhizal fungi (AMF). Therefore, a nested core collection of 32 *M. truncatula* inbred lines was analyzed in terms of root system architecture under different phosphate supply and of mycorrhization rate after inoculation with *Rhizophagus irregularis* or after cultivation in a natural soil containing different AMF species. Although the lines differed drastically in the root phenotype, they did not show clear differences in the mycorrhization rate.

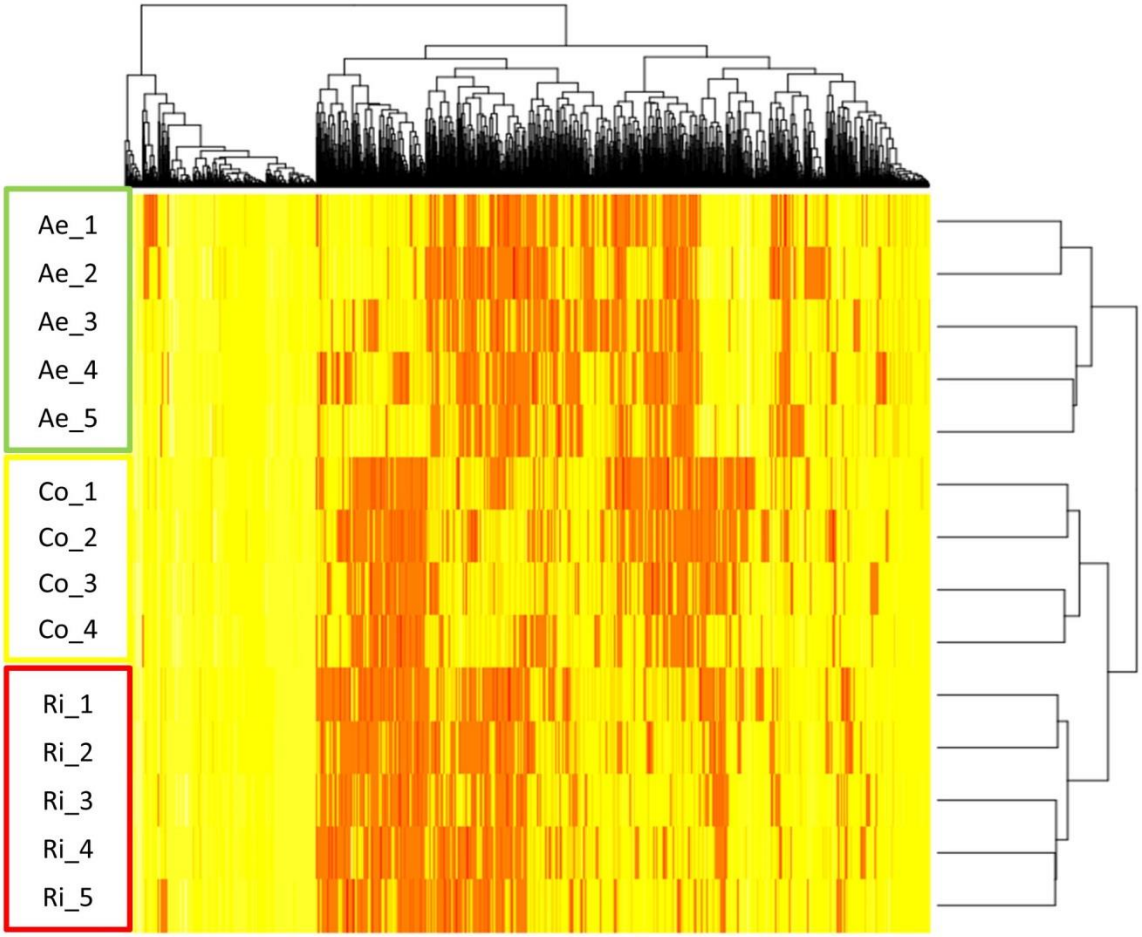

Figure 8: Heatmap of LC-MS data of root exudates of differently treated *M. truncatula* roots. Plants were cultivated in an aeroponic system for six weeks and either treated with spores of the pathogen *A. euteiches* (Ae), the AMF R. *irregularis* (Ri) or stayed non-treated (Co). Shown are compounds exhibiting at least 75 % abundance per treatment and an at least two-fold change (Oneway ANOVA, p≤0.05). Note that biological replicates of one treatment group together.

Work Package IGZ: Rita Grosch, Silke Ruppel, Monika Schreiner, Katja Witzel

Within the framework of the project, studies on the influence of plant natural genetic variability on the interaction with beneficial or pathogenic microorganisms were performed at the IGZ. Methods were successfully established for inoculation of *A. thaliana* plants with the growth-promoting endophyte *Kosakonia radicincitans* (formerly *Enterobacter radicincitans*) or

with the pathogenic Verticillium fungi *V. dahliae* and *V. longisporum*, including monitoring the colonization via quantitative polymerase chain reaction (qPCR) and plant growth at axenic conditions (Fig. 9). In order to study the interaction between plants and microorganisms, we have used a method of indirect inoculation where the bacterial or conidial suspension was pipetted onto the pot surface to allow a more natural colonization of host plants. The method for the collection of root exudates of inoculated and non-inoculated plants was therefore optimized for this experimental system and validated. Six-week old plants, grown on sand and watered with nutrient solution, were gently uprooted and roots were incubated for one hour in water and then transferred to a fresh batch of bi-distilled water for further 4 hours (Fig. 10A). The medium was filtered through a mixed cellulose ester membrane filter to remove any cellular debris and external microorganisms. The exudate from approximately 25 plants was pooled into a single sample. Metabolite profiles of roots after exudate collection were compared to those harvested without exudate collection to investigate whether the osmotic pressure of bi-distilled water causes a non-specific release of metabolites (Fig. 10B). Non-targeted LC-MS analysis revealed a marginal influence of exudate collection procedure on the root metabolite pattern with 98 (equals 3.5 %) significantly altered entities out of 2,800 detected ones. A targeted analysis enabled the profiling of intact glucosinolates (Fig. 10C) and their respective breakdown products in exudates to assess also low abundant secondary metabolites.

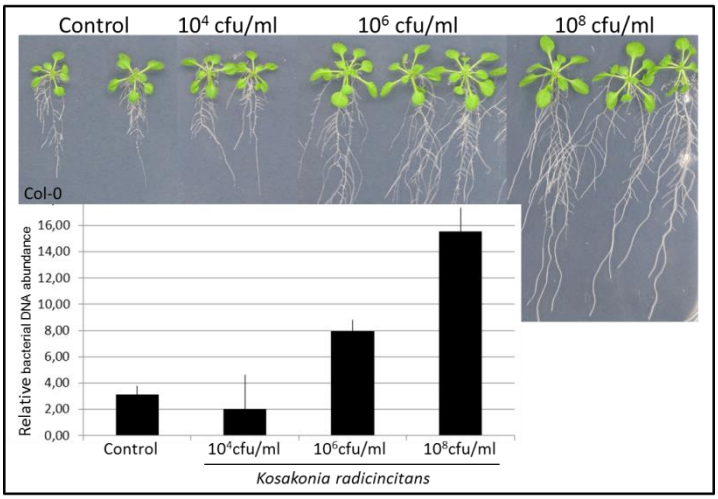

Fig.9: Growth-promotion of *A. thaliana* upon the colonisation with *K. radicincitans* is dependent on the bacterial density used for inoculation. Content of bacterial DNA in plant roots determined by qPCR and normalized to the abundance of two plant reference genes.

A model organism for beneficial plant-microbe-interaction at the IGZ is *K. radicinitans* and central efforts in course of the project were devoted to investigate the molecular basis of plant growth promotion and the impact root exudation. The strain was sequenced and the draft genome sequence presented a genome size of 6.04 Mb. Genome annotation revealed a total of 6,124 proteincoding genes, 69 tRNAs, and 9 rRNAs, which equals ca. 88 % of the genome sequence. The main proposed mode of plant growth promotion is the enhanced nutritional status through atmospheric nitrogen fixation. Two genes coding for nitrogenase (nifH) were present in the genome of *K. radicincitans* sharing a sequence identity of 60 %. Gene-specific primers were generated and quantitative real-time PCR showed that both genes are active when bacteria are grown in nitrogen-free liquid media and two-dimensional immunoblot analysis using an antiNifH antibody revealed the expression of both proteins. The genome sequence indicates phytohormone production with genes for indole-3-acetaldehyde synthesis and an auxin efflux carrier. All 19 MAGIC parent accessions were analysed for induced growth promotion by *K. radicincitans* when plants were grown on soil, sand and in vitro. The screen identified accessions with different levels of growth promotion.

The accession Oy-0 was selected for further analysis due to the most reproducible positive effect on biomass production. Gene expression analysis of control and inoculated roots and gene ontology enrichment analysis of significantly changed transcripts revealed an over-representation of functional categories related to development, primary and secondary plant metabolism. Belonging to the latter group, carotenoids are known for their antioxidant properties and their functional role in plant-arbuscular mycorrhiza interaction. For the interaction between *K. radicincitans* and *A. thaliana*, an effect on gene transcription, metabolite accumulation and a stimulating response on bacterial growth were found and indicate that carotenoids might play a role in bacterial interactions with plants (Fig. 11). Microarray analysis also revealed an under-representation of GO terms related to transport processes in roots of inoculated plants and therefore, an untargeted analysis of root exudates was performed, together with the research group of D. Scheel. Fifteen compounds decreased and eleven increased upon bacterial inoculation. These candidates were not among the 98 mass-retention time pairs that are altered in abundance due to the protocol of exudate collection. Sugars, amino acids, organic acids, glucosinolate precursor amino acids, glucosinolate degradation products and phenylpropanoids were lower abundant, while fatty acid derivatives increased in root exudates. Alteration in the abundance of glucosinolate-related metabolites in exudates was reflected by a decrease in specific glucosinolates in roots of inoculated plants, and similar results were found for phenylpropanoids.
The soil-borne biotrophic fungus *Verticillium spp*. is a principal pathogen of numerous economically important crops. Primary metabolite profiling of *A. thaliana* and *Solanum lycopersicum* in response to pathogen colonization indicated that abundance of amino acids is influenced by the pathogen (together with research groups D. Scheel and S. Abel). At the level of secondary plant metabolites, the group of glucosinolates (GLS) is also involved in the plant's response. First, a screening of the 19 parent accession of the MAGIC population was performed to characterize the natural variation in GLS patterns (Fig. 12A). HPLC-based analysis of leaves and roots led to the detection of 20 different GLS. Among those, the indole GLS glucobrassicin, 4-methoxy-glucobrassicin and neoglucobrassicin were found in all genotypes, while alkenyl GLS sinigrin, gluconapin and glucobrassicanapin were detected in definite lines. The GLS patterns of roots resembled those of leaves, while the total concentration was about 5-10 fold higher in the latter. The expression of modifying enzymes governs the formation of GLS breakdown products, which defines the biological activity of GLS. Therefore, the expression of the epithiospecifier protein (ESP, kindly provided by R. Kissen), controlling the formation of epithionitriles and nitriles, was tested in the 19 accessions and revealed a high level of plasticity (Fig. 12B). To investigate the influence of the GLS profile on the interaction with *V. longisporum*, four *A. thaliana* accessions were selected based on their GLS patterns and their breakdown products. Bur-0 and Hi-0 are rich in alkenyl GLS, but ESP expression was found in Bur-0, but not in Hi-0, meaning that Bur-0 produced mostly epithionitriles and Hi-0 isothiocyanates. Ler-0 and Kn-0 accumulate hydroxyalkenyl GLS and expression of ESP was detectd in Ler-0 and not in Kn-0. Hence, Ler-0 accumulates mainly nitriles and Kn-0 isothiocyanates. In response to fungal colonization, levels of GLS increased in roots of all four accessions, while the quantity of breakdown products decreased. In order to test the effect of GLS breakdown products on *V. longisporum* growth, a biofumigation assay was developed to identify volatile metabolites deterring fungal growth (Fig. 12C). The antifungal activity of volatile GLS breakdown products, mainly isothiocyanates, are assumed as driving compounds in biofumigation where crop residues (particularly those of Brassica spp.) with high glucosinolate content are incorporated into the soil for control of soil-borne pathogens. Those *A. thaliana* accessions that accumulate high levels of alkenyl GLS were most restrictive to *V. longisporum* growth and 2-propenyl GLS was isolated as active substance.

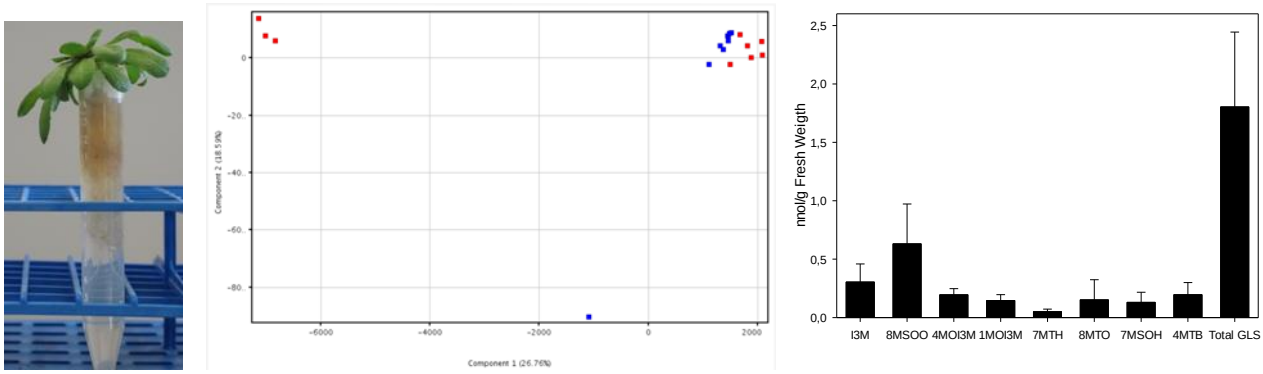

Fig. 10: Collection and analysis of root exudates from *A. thaliana*. Plants were grown in sand and watered with nutrient solution (A). Hierarchical clustering revealed a minimal effect of exudate collection procedure on the overall root metabolic pattern (B). The glucosinolate profile in root exudates of the Col-0 accession (C).

A

Transcript ID	Gene Title	Fold change root
At1g06820	carotenoid isomerase	1,039 down
At3g10230	lycopene beta cyclase	1,306 up
At3g04870	carotene 7,8-desaturase	1,082 up
At4g25700	carotene beta-ring hydroxylase	1,016 down
At3g53130	epsilon hydroxylase/ oxygen binding	1,232 down
At3g53140	epsilon hydroxylase/ oxygen binding	1,019 up
At3g63520	9-cis-epoxycarotenoid dioxygenase	1,258 up
At5g52570	carotene beta-ring hydroxylase	1,642 up
At5g57030	lycopene epsilon cyclase	1,635 up

B

	Leaves		Roots	
	Control	Inoculated	Control	Inoculated
α-Carotene	3.7±0.3	3.9±1.6	n.d.	
Lutein	42.7±0.4	51.0±7.8	0.03±0.009*	0.05±0.003*
β-Carotene	161.2±26.8*	197.2±16.6*	0.01±0.003*	0.02±0.001*
Zeaxanthin	3.9±0.3	4.4±0.4	n.d.	
Violaxanthin	11.1±3.3	18.9±5.5	n.d.	
Neoxanthin	27.3±0.6	33.9±0.2	n.d.	

C

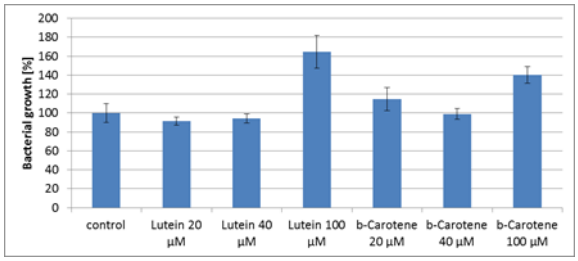

Fig. 11: Influence of K. radicincitans on carotenoid metabolism of *A. thaliana* accession Oy-0. Expression profiling revealed an induction of several genes related to carotenoid biosynthesis and catabolism in roots of inoculated plants (A). Carotenoid profiling [µg g-1 dry weight] detected an increased abundance of lutein and β-carotene in the same tissue (*p < 0.05, B). Supplementation of both metabolites to *K. radicincitans* augmented biomass production in pure culture (C).

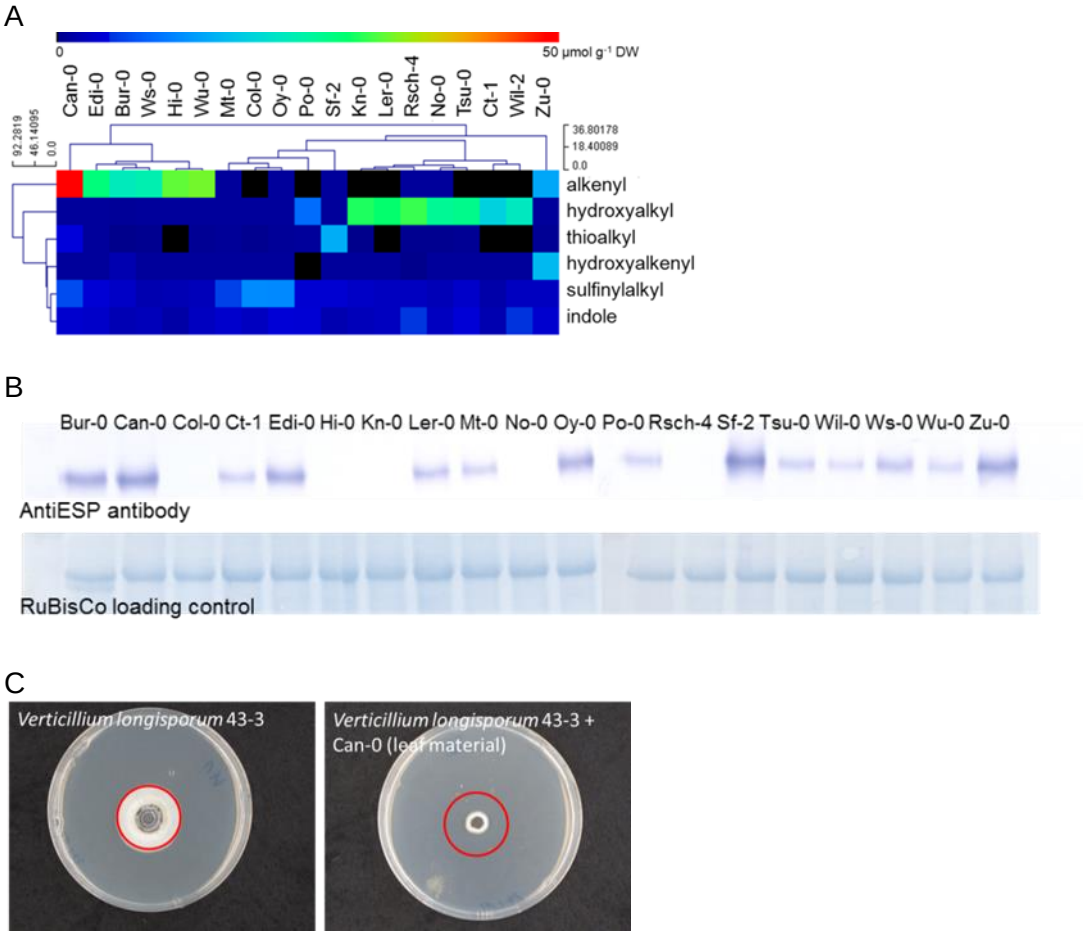

Fig. 12: Natural variation in *A. thaliana* glucosinolate patterns and influence of glucosinolate breakdown products on *V. longisporum* growth. The leaf glucosinolate patterns of MAGIC population parents (A). Expression of ESP in leaves of the same accessions (B). Effect of volatile glucosinolate breakdown products released from Can-0 on *in vitro growth* of *V. longisporum* (C).

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Publications from the project

Strehmel, N., Böttcher, C., Schmidt, S., Scheel, D. (2014) Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. Phytochemistry, 108C, 35-46.

Witzel, K., Neugart, S., Ruppel, S., Schreiner, M., Wiesner, M., Baldermann, S. (2015). Recent progress in the use of 'omics technologies in brassicaceous vegetables. Frontiers in Plant Science 6.

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Several further publications are submitted or in preparation. Two PhD theses (Dorothée Klemann, Carla Porges) will be published from this project.

Data Storage and Availability

All data will be stored at the institutes servers and made available upon publication through appropriate public repositories. All data will be published.