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Ecotype-Dependent Response of Bacterial Communities Associated with *Arabidopsis* to Cold Acclimation

Mohammad Etemadi, Institute of Environmental Biotechnology, Graz University of Technology Graz, Austria; **Ellen Zuther**, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476, Potsdam, Germany; **Henry Müller**, Institute of Environmental Biotechnology, Graz University of Technology Graz, Austria; **Dirk K. Hinch**, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476, Potsdam, Germany; and **Gabriele Berg**,[†] Institute of Environmental Biotechnology, Graz University of Technology Graz, Austria

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ABSTRACT

Low temperature is a primary factor limiting geographical distribution of plants and crop yield in large areas of the world. Plant-associated microbial communities have rarely been acknowledged as possible determinants of cold acclimation, the process leading to freezing tolerance in plants. Here we studied the impact of cold acclimation on leaf-associated bacteria by analyzing 10 different *Arabidopsis* ecotypes differing widely in their freezing tolerance. In climate chamber experiments, we found that leaves of all *Arabidopsis* accessions were colonized by highly diverse bacterial taxa (852 operational taxonomic units) mainly from *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. During cold acclimation, in all ecotypes the bacterial community composition

and diversity, and especially the core species composition changed drastically. However, the response of the bacterial communities was highly ecotype-dependent; sensitive and tolerant accessions shared only eight responders. Unique responders in tolerant accessions were identified as putative indicators of freezing tolerance. Thus, leaf bacteria appear to be genotype-dependent associated with cold acclimation, which suggests an additional function of plant-microbe interaction and may open new possibilities for biotechnological applications.

Additional keywords: endophytes, ecotype specificity, microbiome function, phyllosphere.

Periods of low and freezing temperatures are common major environmental challenges that plants from temperate regions face during their life cycle. They are a primary factor limiting the geographical distribution of native plant species but also the growth and yield of crops in many areas of the world (Xin and Browse 2000). In a process termed cold acclimation, plants from temperate climates increase their freezing tolerance during exposure to low, nonfreezing temperatures (Steponkus 1984). Natural variation of freezing tolerance along latitudinal climate gradients provides an excellent tool to study the potential importance of metabolic and physiological differences among accessions of a species for variation in freezing tolerance. *Arabidopsis thaliana* L. is a model species for temperate plants that is widely distributed on the Northern hemisphere. Natural accessions show different levels of freezing tolerance (Mckhann et al. 2008; Zhen and Ungerer 2008), and in a study with 54 accessions,

evidence for latitudinal and longitudinal clines in nonacclimated and acclimated freezing tolerance were presented (Zuther et al. 2012). Massive changes in gene expression and content of primary metabolites (Guy et al. 2008; Hinch et al. 2012) as well as lipids and flavonoids (Degenkolbe 2012; Schulz et al. 2015) were identified during cold acclimation. As an example, expression of some CBF-regulated *COR* genes and sugar content was correlated with freezing tolerance in the acclimated but not nonacclimated state in an analysis with 54 *Arabidopsis* accessions (Zuther et al. 2012). However, it is unclear whether these changes can quantitatively account for the observed differences in freezing tolerance among the accessions. Here, we have tested the hypothesis that the bacterial communities associated with *Arabidopsis* leaves are influenced by cold acclimation and differ between freezing tolerant and sensitive accessions. The microbiome of this plant species is well studied (Bai et al. 2015; Schlaeppli et al. 2014) and has previously been used to explore the role of microbiomes in various other processes, e.g., in the early flowering effect (Panke-Buisse et al. 2017).

The plant microbiota has been known as one of the key determinants of plant growth and health since more than 100 years (Hartmann et al. 2007; Weller et al. 2002). Recently, much deeper insights into the function of plant-associated microbial communities

[†]Corresponding author: G. Berg; E-mail: gabriele.berg@tugraz.at

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were gained by omics technologies (Bulgarelli et al. 2012; Mendes et al. 2011). The most recent research has shown that the microbiome is not only involved in coping with biotic stress, but is also involved in the reaction of plants to abiotic stresses (Bragina et al. 2014) including high salinity and drought (Rolli et al. 2015; Yang et al. 2009). Recently, Castrillo et al. (2017) identified a genetic network controlling the phosphate stress response and influences the structure of the root microbiome community. Plant microbiota also have an influence on the composition of plant secondary metabolites (Schmidt et al. 2014; Verginer et al. 2010). Peñuelas et al. (2014) showed that the removal of the floral microbiota of *Sam-bucus nigra* resulted in reduced floral terpene emission, which plays a key role in pollination and consequently in fruit and seed production. Recent studies also revealed the direct impact of the root microbiome on plant phenology (Panke-Buisse et al. 2014; Wagner et al. 2014). Additional essential roles of the plant microbiome for phenotypic and epigenetic plasticity as well as the evolution of plants were already suggested by Partida-Martínez and Heil (2011). The relevance of microorganisms for cold adaptation was shown for particular bacterial species, such as ice nucleation active bacteria that induce ice crystallization in leaves (Gurian-Sherman et al. 1993). In addition, plant-associated *Pseudomonas* and *Burkholderia* strains can induce chilling tolerance (Fernandez et al. 2012; Subramanian et al. 2015). However, the role of the whole plant microbiome in cold acclimation is still unexplored. The objective of this study was therefore to determine and compare the composition of the leaf-associated bacteria of different *Arabidopsis* accessions that were previously classified as freezing tolerant or sensitive (Zuther et al. 2012) both before and after 14 days of cold acclimation at 4°C.

MATERIALS AND METHODS

Plant material and experimental design. Samples of 10 previously characterized *Arabidopsis* accessions were used for the analysis of the bacterial communities. Four accessions with high freezing tolerance (tolerant) were selected (N14, Ms-0, N6, and Kn-0), five accessions with low freezing tolerance (sensitive) (Can-0, Alc-0, C24, Ita-0, and Ran-0), and the accession Col-0 with an intermediate freezing tolerance as the reference. The samples originated from a large study with 54 accessions performed in several separate experiments with three accessions each over a time period of 1 year described in Zuther et al. (2012) (Table 1). In this study, accessions were completely randomized with respect to their geographic origin. Plants were grown in a greenhouse with 16 h day,

200 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity, and a temperature of 20/18°C day/night until 42 days after sowing (nonacclimated conditions, NA). For cold acclimation (ACC), plants were transferred to a growth chamber at 4°C, 16 h day length with 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ at 60% humidity for an additional period of 14 days. Three biological replicates per accession and condition were collected with each replicate containing two fully developed rosette leaves each from a different plant.

Extraction of bacterial DNA. Samples were lyophilized and ground in a ball mill prior to isolation of total genomic DNA. DNA was extracted from 100 mg of plant material using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol and stored at -21°C.

16S rRNA amplicon sample preparation, sequencing, and analysis. An amplicon sequencing using Illumina's MiSeq platform was applied to study the taxonomic structure of the bacterial communities using three biological replicates per studied accession and condition. 16S rRNA gene sequences containing the hyper-variable region (V4) were amplified by the region-specific primer pair 515f and 806r (Caporaso et al. 2011) that carried Illumina cell flow adaptors and sample-specific barcodes. The polymerase chain reaction (PCR) involved specific PNA (peptide nucleic acid) oligos to preclude the amplification of plant-derived plastid DNA according to the adjusted protocol described by Lundberg et al. (2013). The PCR mixture (30 μl) contained 1 $\times$ Taq&Go (MP Biomedicals, Illkirch, France), 0.2 μM of each primer, 0.75 μM of each mPNA and pPNA oligos, and 2 μl of template DNA (96°C for 6 min, 30 cycles at 96°C for 1 min, 78°C for 5 s, 54°C for 1 min, 74°C for 1 min, and final elongation at 74°C for 10 min). Three independent PCR runs were performed for each sample and pooled prior to purification by applying the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). After spectrophotometric estimation of DNA concentrations (Nanodrop 2000, Thermo Scientific, Wilmington, DE), equimolar aliquots of all samples were combined for amplicon sequencing using Illumina MiSeq chemistry v3 (250 bp paired-end) conducted by Eurofins Genomics (Ebersberg, Germany). Raw sequencing paired-end reads were assembled with default settings of PandaSeq software, version 2.8 (Masella et al. 2012). Low-quality reads defined as reads with an average quality score below 25, with more than one ambiguous base and a length <250 and >260 were removed using the Prinseq program, version 0.20.4 (Schmieder and Edwards 2011). In addition, barcodes and primers were trimmed by Prinseq software. Resulting sequence data sets are deposited at NCBI's Sequence Read Archive under the BioSample accession numbers SAMN07542821 to SAMN07542840.

TABLE 1
List of 10 accessions of *Arabidopsis thaliana* with information about their geographic origin, INRA ID, and freezing tolerance according to Zuther et al. (2012)

Abb.	Name	Origin	Latitude of origin	Longitude of origin	INRA accession ID	Category
N6	Karelia	RUS	62.00	34.00	262 AV	Tolerant
N14	Sampo Mountain	RUS	61.10	34.50	267 AV	Tolerant
Ms-0	Moscow	RUS	55.75	37.35	93 AV	Tolerant
Kn-0	Kaunas	LIT	54.90	23.54	70 AV	Tolerant
Col-0	Columbia	POL	52.73	15.15	186 AV	Intermediate
Can-0	Canary Island	ESP	28.00	-15.30	163 AV	Sensitive
Ita-0	Ibel Tazekka	MAR	34.10	-4.12	157 AV	Sensitive
C24	—	POR	40.00	-8.25	N.A.	Sensitive
Alc-0	Alcala de Henares	ESP	40.48	-3.22	178 AV	Sensitive
Ran	Randan	FRA	40.50	-2.00	21 AV	Sensitive

The processing of filtered reads to operational taxonomic units (OTUs) was done with the LotuS program using usearch at 97% similarity (Hildebrand et al. 2014). OTUs assigned to plant-derived chloroplasts and mitochondria were filtered out from the dataset by QIIME 1.9.0 (Caporaso et al. 2010). Since the applied primer pair 515f/806r (Caporaso et al. 2011) was reported to be biased toward archaeal 16S rRNA gene sequences, we decided to exclude those reads from the data set prior to further analysis. Bacterial 16S rRNA sequence data analysis and visualization were done by Phyloseq and Shiny-phyloseq packages of R software (McMurdie and Holmes 2013, 2014; R Development Core Team 2011). To account for heterogeneity in read counts among samples, we employed the DESeq2 statistical package according to Love et al. (2014) to assess differential OTU abundances (Thorsen et al. 2016; Weiss et al. 2017). Statistical significance of differences regarding the relative taxonomic composition between tolerance categories was tested using analysis of variance (ANOVA). Alpha and beta diversity were analyzed using OTU table at an even read depth of 20,000. To test the significance of differences of alpha diversity metrics, we applied nonparametric *t* test using Monte Carlo permutations (number of permutations: 999) and false discovery rate correction. Multidimensional scaling was performed based on a Bray-Curtis dissimilarity distance matrix. The statistical significance of groupings was tested by applying analysis of similarities (ANOSIM).

RESULTS

The structure of the bacterial community associated with *Arabidopsis* leaves changes during cold acclimation. The bacterial communities of the 10 *Arabidopsis* accessions that are either freezing tolerant (N6, N14, Ms-0, and Kn-0), intermediate (Col-0), or sensitive (Ran, Ita-0, Can-0, C24, Alc-0) according to Zuther et al. (2012), were highly diverse under both nonacclimated (NA) and cold acclimated (ACC) conditions. Overall, after removing sequences assigned to chloroplasts, mitochondria, and archaeal 16S rRNA, 5,472 bacterial OTUs (16S rDNA at 97% similarity cut-off) were identified in the final dataset comprising 1.479 million reads with a median of 20,110 reads per sample. After removing single- and doubleton OTUs, a total of 852 OTUs were detected in the 60 samples. The composition of bacterial communities at phylum level revealed *Proteobacteria* (40.7 to 56.8%), *Actinobacteria* (19.7 to 27.5%), *Firmicutes* (0.4 to 19.5%), and *Bacteroidetes* (5.0 to 15.5%), which dominated with more than 80% abundance in the three *Arabidopsis* freezing tolerance categories (Fig. 1A, Supplementary Table S1). At class level, *Alpha*-, *Beta*-, and *Gammaproteobacteria* (7 to 13.36%, 7 to 38.86%, 9 to 28.91%), *Actinobacteria* (19.34 to 27.16%), *Bacilli* (0.4 to 19.42%), *Flavobacteria* (2.06 to 7.73%), *Sphingobacteria* (1.87 to 5.57%), and *Saprospirae* (0.9 to 4.21%) were the major representative taxa. Noticeably, the genus *Buchnera*, known as an obligate intracellular symbiont of aphid species, represents an average of 4.7% of the bacterial communities. The assignment to *Buchnera*, was confirmed by stringent alignment of all reads contributing to the respective OTUs. All reads show high nucleotide similarities (99%) with 16S rRNA gene reference sequences of *Buchnera*. Although most of the bacterial groups were found among all tolerance categories under both growth conditions, some classes such as TK17 and Ellin6529 from the *Chloroflexi* phylum were only present after cold acclimation and TM7-3 from TM7 was only present in the sensitive category (Fig. 1B). No statistically significant differences of relative abundances were observed for the dominant taxa at phylum and class level between acclimated and nonacclimated plants within accession ($P \leq 0.05$). Hence, to detect the impact of cold conditions on dominant members within the bacterial community, we studied the 100 most abundant among the

852 identified OTUs in depth. They were assigned to nine classes of *Alpha*-, *Beta*-, *Gammaproteobacteria*, *Actinobacteria*, *Bacilli*, *Flavobacteriia*, *Saprospirae*, *Sphingobacteriia*, and *Verrucomicrobiae*. The distribution and composition of these top 100 OTUs varied among accessions (Fig. 2). In general, OTUs belonging to *Gammaproteobacteria* and *Bacilli* in tolerant and *Bacilli* and *Actinobacteria* in sensitive accessions and to *Betaproteobacteria* and *Actinobacteria* in Col-0 were found to dominate the respective bacterial communities (Fig. 2). Moreover, the relative abundance of particular OTUs within the microbial communities in all tolerance categories was altered during cold acclimation, e.g., the abundance of OTUs assigned to *Gammaproteobacteria* was higher in the tolerant category under cold acclimated than under nonacclimated conditions (Fig. 2).

Cold acclimation was accompanied by a shift of bacterial diversity. The number of observed OTUs at a depth of 20,000 reads varied from 243 to 718 in the 60 samples (Supplementary Fig. S1). In the course of cold acclimation, the number of observed OTUs decreased within respective tolerance categories; however, a statistically significant decrease (740 to 530 bacterial species) was only detected between nonacclimated and cold acclimated plants of the accession Col-0 (Fig. 3A). In addition, the evenness indicated by the Shannon index was not significantly different between tolerance categories or within tolerance categories and both conditions except for a decrease in cold acclimated Col-0 compared with nonacclimated plants of the same accession (5.6 to 3.9) (Fig. 3B). The richness and evenness of bacterial species among the individual accessions showed a wide variation (Supplementary Fig. S2). The bacterial communities of the accessions N6 and N14 (tolerant) and Col-0 (intermediate) had the highest richness and Alc-0 (sensitive), Col-0 (intermediate), and Ms-0 (tolerant) had the highest Shannon index under nonacclimated condition compared with all other accessions. Under acclimated conditions, the highest bacterial richness was found in N6 (tolerant), Alc-0 (sensitive), N14 (tolerant), and RAN (sensitive) and the highest evenness in N6 (tolerant), C24, and Alc-0 (sensitive).

Multidimensional scaling with the proportional normalized OTU tables and the distance matrix calculated using Bray-Curtis showed a clear separation into two groups under nonacclimated conditions (ANOSIM, $P = 0.05$). One group contains three sensitive (Alc-0, C24, and Ran) and the other group contained all tolerant accessions and two sensitive accessions (Ita-0 and Can-0) together with Col-0 (Fig. 4A). Except for the accessions N6 (tolerant) and Ita-0 (sensitive), cold acclimation caused a statistically significant shift of bacterial communities within each *Arabidopsis* accessions (Supplementary Table S2, $P \leq 0.05$) and the differentiation of the bacterial community structure of the different accessions are even more distinct (Fig. 4B). The results revealed a separation into a tolerant group (N6, N14, Ms-0, and Kn-0), a sensitive group I (C24, Alc-0, and Ran) and a sensitive group II (Ita-0 and Can-0) which also contains Col-0 (ANOSIM, $P = 0.002$). The stronger separation of the bacterial community structure under cold acclimated than under nonacclimated conditions is also visualized by interactive network graphics (0.32 as Bray-Curtis distance threshold) based on the bacterial community of each accession (Supplementary Fig. S3).

Cold temperature affected the bacterial core. Since core species are critical to the function of a microbial community, we identified bacterial OTUs representing the core in the leaves of each *A. thaliana* accession under both conditions. For that, the OTUs present in at least 80% of samples in each tolerance category were chosen as a representation of the bacterial core (Supplementary Table S3). By visualizing the shared OTUs between freezing tolerance categories under both growth conditions, Venn diagrams clearly showed the increase of specific core bacterial species in the sensitive group (I) and the tolerant group during cold acclimation compared with non-acclimated samples (Fig. 5A and B). In contrast, this was not the case for the

Fig. 1. Taxonomic composition of bacterial communities in the phyllosphere of different *Arabidopsis* accessions grown under nonacclimated (NA) or cold acclimated (ACC) conditions. **A**, Relative composition at phylum level analyzed by Illumina MiSEquation 16S rRNA gene amplicon sequencing. The color code of bacterial classes can be found in **B**. Ten *Arabidopsis* accessions were grouped according to their freezing tolerance into a tolerant (N14, Ms-0, N6, and Kn-0) and a sensitive (Can-0, Alc-0, C24, Ita-0, and Ran-0) group, and Col-0 is shown as reference. No statistically significant differences of relative abundances were observed for the dominant taxa at phylum and class level between acclimated and nonacclimated plants within accession ($P \leq 0.05$). **B**, Microbial classes detected in the three differently tolerant categories. The exclusive presence of the respective microbial class within a freezing tolerance group is indicated by asterisks.

sensitive group (II) and Col-0, where a significant reduction of bacterial richness in response to cold was detected.

Common responses of bacterial communities to cold. We performed an additional statistical analysis (Love et al. 2014; McMurdie and Holmes 2014) between the sensitive and tolerant

categories under nonacclimated and cold acclimated conditions to investigate whether there were common microbial responses among the sensitive and tolerant accessions during cold acclimation. Among the 852 OTUs, 53 and 46 OTUs were significantly different ($P < 0.05$) after cold acclimation compared with nonacclimated

Fig. 2. Percentage of sequences representing the 100 most abundant operational taxonomic units (OTUs) in the leaves of different *Arabidopsis* accessions under nonacclimated (NA) or cold acclimated (ACC) conditions. The distribution of the 100 most abundant OTUs among 852 identified OTUs in the dataset is shown for each accession and condition within the respective tolerance category and additionally averaged for the tolerant and sensitive category for both conditions separately.

samples in the sensitive and tolerant category, respectively (Supplementary Table S4). Among these, eight OTUs overlapped between the tolerant and sensitive categories. The OTU_2839 (genus *Truepera*), OTU_2099 and OTU_2390 (*Nevskia*), OTU_2453, and OTU_5856 (genus unknown, phylum *Chloroflexi*) showed significantly ($P < 0.05$) higher abundance under cold acclimated compared with nonacclimated conditions in both sensitive and tolerant accessions, while OTU_779 (*Sphingopyxis*), OTU_1903 (*Luteibacter*), and OTU_1237 (genus: unknown, phylum: Bacteroidetes) showed significantly ($P < 0.05$) lower abundance (Fig. 6). This indicates that part of the microbial community of *A. thaliana* leaves responded similarly to cold in all investigated genotypes.

The main bacterial responders in tolerant *Arabidopsis* accessions. To identify the OTUs separating the tolerant from the sensitive accessions, we performed a pairwise statistical analysis between the tolerant and the sensitive category. In comparisons

between the tolerant versus sensitive group (I) and sensitive group (II) and Col-0, 261, 224, and 122 OTUs, respectively, were significantly different ($P < 0.05$) (Supplementary Table S5). Among these different OTUs, 29 were shared between all three comparisons. These common OTUs separate the tolerant category significantly from all others under cold acclimated conditions (Table 2). To visualize, whether these 29 OTUs separated the tolerant from the sensitive accessions and Col-0, we performed a nonmetric multidimensional scaling plot with the replicate average for each accession (Supplementary Fig. S4). A separation of tolerance categories by these OTUs could be verified. In the next step, we used ANOVA to analyze whether these 29 OTUs were distributed equally among the tolerant accessions (Supplementary Table S6). OTU_1795, OTU_2099, OTU_2390, OTU_270, OTU_2769, OTU_3360, OTU_4007, OTU_4288, OTU_4622, OTU_4915, OTU_67, and OTU_878 were significantly ($P < 0.05$) different

Fig. 3. Alpha diversity of bacterial communities associated with *Arabidopsis* accessions of different tolerance categories under nonacclimated (NA) or cold acclimated (ACC) conditions. **A**, Number of observed operational taxonomic units (OTUs) at 97% similarity and even depth of 20,000 reads for *Arabidopsis* accessions grouped in tolerance categories (Col-0, $n = 3$ per condition; sensitive, $n = 15$ per condition; and tolerant, $n = 12$ per condition). Comparing nonacclimated and cold acclimated plants of the same tolerance category, a nonsignificant decrease in the number of observed OTUs after cold acclimation was observed for all categories, except for Col-0, which significantly decreased. **B**, The Shannon diversity index was calculated based on the distribution of values across all tolerance categories. Error bars (**A** and **B**) represent the standard deviation (Col-0, $n = 3$ per condition; sensitive, $n = 15$ per condition; and tolerant, $n = 12$ per condition). Except for Col-0, no statistically significant differences between nonacclimated and cold acclimated plants of the same tolerance category were observed.

Fig. 4. Bray-Curtis-based multidimensional scaling of bacterial communities under **A**, nonacclimated (NA) and **B**, cold acclimated (ACC) conditions.

between tolerant accessions (Table 2). Some of them were part of core bacterial species such as OTU_2099, OTU_2390, OTU_270, OUT_4007, OTU_4622, OTU_4915, and OTU_67, suggesting accession-specific responses of bacterial communities to cold (Table 2). The abundance of these 29 OTUs in the different accessions was

visualized in a heatmap (Fig. 7). Interestingly OTU_4869 was only present in tolerant accessions and Col-0, while OTU_4070 was only detected in tolerant accessions after cold acclimation. In contrast, OTU_2206 was only present in sensitive accessions and Col-0.

Fig. 5. Venn diagrams of the core bacterial operational taxonomic units (OTUs) of *Arabidopsis* accessions from three different tolerance categories. Each number refers to an OTU that constitute the core of different tolerance categories classified under **A**, nonacclimated (NA) or **B**, cold acclimated (ACC) conditions. The sensitive tolerance category was split into two groups, group I and group II, according to the network analysis.

Fig. 6. Fold change (log2) of common operational taxonomic units (OTUs) in bacterial communities of *Arabidopsis* accessions of the sensitive and tolerant categories between nonacclimated and cold acclimated conditions. The unknown genera are either from the phylum *Chloroflexi* and represented by OTU_2453 and OTU_5856 or from the phylum *Bacteroidetes* and represented by OTU_1237. *Luteibacter* is represented by OTU_1903, *Nevskia* by OTU_2099 and OTU_2390, *Sphingopyxis* by OTU_779, and *Truepera* by OTU_2839.

DISCUSSION

The natural genetic variation among *Arabidopsis* accessions results in different levels of tolerance under various environmental conditions (Weigel 2012). Here, we show that the response of bacterial community composition to cold acclimation significantly correlates with freezing tolerance levels of different *Arabidopsis* accessions, which has previously been related to differences in gene expression and metabolite content (Hincha et al. 2012).

In all *Arabidopsis* accessions, a significant change of bacterial community structure and diversity was observed following the cold acclimation period at 4°C for 14 days. Our study included bacteria associated with leaves comprising the phyllosphere as well as the endosphere. While it has been reported that the plant phyllosphere is a dynamic environment subjected to large variation in temperature, moisture, and radiation throughout the day and night (Bai et al. 2015; Turner et al. 2013; Vorholt 2012), the endosphere harbors a much more stable microbial community (Hardoim et al. 2015). Even though in *Arabidopsis* the species compositions in both microenvironments were reported to be similar (Bodenhausen et al.

2013). Under nonacclimated conditions, the composition of bacterial communities associated with leaves showed a genotype-specific signature as already reported for *Arabidopsis* (Bai et al. 2015) and for several crop species (Berg and Smalla 2009; Bulgarelli et al. 2015; Cardinale et al. 2014). All these studies describe the impact of diversification and especially breeding on the microbiome composition but the functional relevance remains largely unexplored. Interestingly, in our study, the genotype specificity of bacterial communities after cold acclimation was even more distinct. This confirms our hypothesis that the bacterial communities associated with *Arabidopsis* leaves are influenced by cold acclimation and differs between freezing tolerant and sensitive accessions. Moreover, the identified relationship between the level of freezing tolerance and the bacterial responses to cold acclimation represents a first functional indication. In addition to the changed structure in response to the cold, we observed the tendency that bacterial diversity decreased. Bacterial diversity is a crucial factor for ecosystem function and plant health (Berg et al. 2017; Laforest-Lapointe et al. 2017). However, in general, all samples showed an unexpected high diversity and 852 OTUs were identified.

TABLE 2
Twenty-nine selected operational taxonomic units (OTUs) that separate tolerant *Arabidopsis* accessions from the sensitive accessions or Col-0 under cold acclimated conditions^a

OTU	Taxonomy							Analysis of variance	Core microbiome (tolerant)
	Kingdom	Phylum	Class	Order	Family	Genus	Species		
OTU_1016	Bacteria	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	?	?		C
OTU_1406	Bacteria	Proteobacteria	Alohaproteobacteria	Caulobacterales	Caulobacteraceae	Arthrosira	fusiformis		
OTU_1539	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	?		C
OTU_164	Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Flavobacterium	?		C
OTU_1745	Bacteria	Verrucomicrobia	Spartobacteria	Chthoniobacterales	Chthoniobacteraceae	heteroC45_4W	?		C
OTU_1795	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	?	?	?	*	
OTU_1951	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	Promicromonospora	?		C
OTU_1968	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	plantarum		
OTU_2099	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Nevskia	ramosa	*	C
OTU_2206	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	?	?		
OTU_2390	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Nevskia	ramosa	*	C
OTU_270	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buchnera	?	*	C
OTU_2769	Bacteria	Verrucomicrobia	Methylacidiphilae	Methylacidiphilales	LD19	?	?	*	
OTU_3072	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	?	?		
OTU_3360	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	?	?	?	*	
OTU_3470	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Phaeospirillum	fulvum		C
OTU_4007	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	?	?	*	C
OTU_4070	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	wittichii		C
OTU_4177	Bacteria	TM6	SJA-4	?	?	?	?		
OTU_4183	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	?		
OTU_4223	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Rathayibacter	caricis		C
OTU_4288	Bacteria	TM6	SJA-4	?	?	?	?	*	
OTU_4622	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	?	?	*	C
OTU_4869	Bacteria	Verrucomicrobia	Methylacidiphilae	Methylacidiphilales	LD19	?	?		
OTU_4915	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea	genosp.	*	C
OTU_5871	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Phaeospirillum	fulvum		C
OTU_67	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buchnera	?	*	C
OTU_878	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	plantarum	*	
OTU_991	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	?		C

^a The abundance of these OTUs was significantly ($P < 0.05$) different in *Arabidopsis* accessions of the tolerant category compared with the sensitive category or Col-0 under cold acclimated conditions. An analysis of variance indicated a significantly different abundance of several OTUs within tolerant accessions (shown by asterisk, $P < 0.05$). Seventeen shared OTUs (more than 80% abundance in each category), representing the core microbiome within tolerant accessions, are indicated by C.

Comparing sensitive and tolerant *Arabidopsis* accessions, we found category-specific differences. Some members of the bacterial community such as OTU_270 and OTU_67 (*Buchnera* spp.) were more abundant in tolerant than in sensitive accessions. Moreover, their abundance increased in tolerant accessions after cold acclimation. Although *Buchnera* spp. are known as obligate intracellular symbiont of aphid species (Wilson and Duncan 2015), members of the genus were frequently detected in plant microbiomes (Ottesen et al. 2013). The occurrence of these species could, however, also originate from aphids associated with leaves. In contrast, OTU_3072 and OTU_2206 (*Sphingobacteriaceae*) were mostly present in sensitive accessions and not in the tolerant group. These discriminating bacterial species could potentially contribute to temperature adaptation and affect the tolerance/sensitivity of *Arabidopsis* accessions to freezing. However, this hypothesis must be tested experimentally by isolation and application of single bacterial strains.

Cold acclimation affected the relative composition of leaf-associated bacterial communities in all *Arabidopsis* genotypes. We identified eight common OTUs responding to the adaptation process in both freezing tolerance categories. Five OTUs comprising the genera *Nevskia*, *Truepera*, and an unknown genus within the phylum *Chloroflexi* were observed to be enriched in cold

acclimated plants. In contrast, three OTUs assigned to *Luteibacter*, *Sphingopyxis*, and an unknown genus within the phylum *Bacteroidetes* appears to be depleted. Although all responding taxa were frequently ascribed to plant microbiota, their specific role in the association with plants subjected to abiotic stresses is unclear. In rice plants, the relative abundance of several OTUs, including OTUs assigned to *Chloroflexi*, increased in response to drought stress (Santos-Medellín et al. 2017). The enrichment of particular bacteria was assumed to be mediated by the accumulation of compatible solutes, a similar protective mechanism that is induced by cold stress in *Arabidopsis* (Smallwood and Bowles 2002). A major determinant of epiphytic colonization is the availability of carbon-containing nutrients (Lindow and Brandl 2003). Sugars can be washed off from the surface of leaves (Mercier and Lindow 2000), but sugar distribution over the leaf area is heterogenous, probably due to localized leakage or transport from underlying cells (Leveau and Lindow 2001). Studies on bean leaves with bacterial biosensors consisting of *Erwinia herbicola* harboring a sucrose and fructose-responsive *scrY* promoter fused to a *gfp* or *inaZ* (ice nucleating protein) reporter gene, showed GFP fluorescence and ice nucleation in a sugar-dependent manner (Miller et al. 2001). During cold acclimation, the content of compatible solutes such as sugars

Fig. 7. Heat map depicting the relative bacterial diversity abundance in tolerant and sensitive accessions and Col-0 under nonacclimated (NA) or cold acclimated (ACC) conditions. The colored lines around the accessions indicate the treatments (green = nonacclimated [NA], blue = cold acclimated [ACC]). The colors in the heat map indicate the abundance of each operational taxonomic unit (OTU) within the samples. An absence of the specific bacterial community is shown in white. All presented OTUs are referred to in Table 2.

and amino acids is strongly increased in *Arabidopsis* leaves where the content of glucose, fructose, sucrose, and raffinose is strongly correlated with freezing tolerance across diverse accessions (Korn et al. 2008; Rohde et al. 2004; Zuther et al. 2012). Investigating 54 *Arabidopsis* accessions, we showed that sensitive accessions accumulated lower amounts of sugars in rosette leaves after cold acclimation compared with tolerant accessions in rosette leaves (Zuther et al. 2012). Whether this difference contributes to the different distribution of OTUs in the bacterial community of differing tolerant accessions is not known and has to be investigated in the future. To our knowledge there is currently no information available on how leaf sugar content and composition affect the bacterial leaf microbiome at all. However, protection of bacteria during freezing involves the accumulation of compatible solutes such as sugars, polyols, amino acids, polyamines (Margesin 2011), and raffinose, which is highly correlated with freezing tolerance in *Arabidopsis* accessions, was found in *Pseudomonads* isolated from the rhizosphere of Himalayan plants grown at 4°C (Bisht et al. 2013). *Burkholderia phytofirmans* PsJN, a plant growth-promoting rhizobacterium, improved the tolerance of grapevine to cold by modulating plant carbohydrate metabolism in inoculated nonacclimated plants (Fernandez et al. 2012). Furthermore, psychrotolerant strains of *Pseudomonas* alleviated cold stress symptoms in wheat seedlings and induced chilling resistance in tomato by inducing the expression of the cold regulated transcription factors LeCBF1 and LeCBF3 (Subramanian et al. 2015). Our hypothesis is also supported by plant metagenome analysis, which showed a high proportion of bacterial genes present for tolerance against abiotic stress in the *Sphagnum* microbiome (Bragina et al. 2014).

Altogether, protection of plants against abiotic stress by bacteria opens the window for interesting biotechnological applications (Berg et al. 2016). Our study showed a novel example that metabolism and morphology of plants and their associated bacteria are intensively connected with each other, and an intense interplay of both maintains the functioning of the holobiont. Due to this importance, the microbiome should be considered in experimental botany.

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