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Root cap is an important determinant of rhizosphere microbiome assembly

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[Correction added on 25 July 2023 after first online publication: Michael Bonkowski was designated as corresponding author].

Summary

- Plants impact the development of their rhizosphere microbial communities. It is yet unclear to what extent the root cap and specific root zones contribute to microbial community assembly.
- To test the roles of root caps and root hairs in the establishment of microbiomes along maize roots (*Zea mays*), we compared the composition of prokaryote (archaea and bacteria) and protist (Cercozoa and Endomyxa) microbiomes of intact or decapped primary roots of maize inbred line B73 with its isogenic root hairless (rth3) mutant. In addition, we tracked gene expression along the root axis to identify molecular control points for an active microbiome assembly by roots.
- Absence of root caps had stronger effects on microbiome composition than the absence of root hairs and affected microbial community composition also at older root zones and at higher trophic levels (protists). Specific bacterial and cercozoan taxa correlated with root genes involved in immune response.
- Our results indicate a central role of root caps in microbiome assembly with ripple-on effects affecting higher trophic levels and microbiome composition on older root zones.

Introduction

Root microbiomes are critical to plant health and productivity (Giri *et al.*, 2018; Cantó *et al.*, 2020; Trivedi *et al.*, 2020). As a root grows into the bulk soil, the assembly of its rhizosphere microbiome begins at the root tip and proceeds toward the older root zones (Zelenev *et al.*, 2005; Dupuy & Silk, 2016; Rüger *et al.*, 2021). Differences in rhizodeposition at both the tip (Humphris *et al.*, 2005; Benizri *et al.*, 2007) and the root hair zone appear crucial in the microbial assembly process (Robertson-Albertyn *et al.*, 2017; Kelly *et al.*, 2018; Korenblum *et al.*, 2020). The root tip sheds root border cells and actively secretes mucilage, a water-soluble, high-molecular-weight polysaccharide and protein matrix to lubricate root movement and to protect the apical root meristem (Iijima *et al.*, 2000; Nguyen, 2003). Microbial selection occurs mainly through feeding on mucilage (Iijima *et al.*, 2000) and the antimicrobial and signaling molecules it contains (Gu *et al.*, 2016; Hu *et al.*, 2018; Driouich *et al.*, 2021). For example, antimicrobial compounds act against pathogens (Driouich *et al.*, 2013), while microorganisms with glycosyl hydrolases to degrade mucilage are attracted (Amicucci *et al.*, 2019). Also, ethylene (Hahn *et al.*, 2008) and other secondary metabolites likely play active roles in the microbial community assembly process (Haichar *et al.*, 2014; Hawes *et al.*, 2016; Ravanbakhsh *et al.*, 2018; Zhou *et al.*, 2019; Chen *et al.*, 2020). However, the vast majority of primary metabolites, especially sugars, are thought to be passively released directly behind the root cap, where phloem and xylem vessels of the root central cylinder are not yet closed (Farrar *et al.*, 2003; Dennis *et al.*, 2010). This may lead to a massive microbial proliferation in the root hair zone (Rüger *et al.*, 2021) and contribute to its role as a hotspot of microbial activity (Blagodatskaya & Kuzyakov, 2015; Zhang *et al.*, 2020). Root hairs play important roles for the uptake of poorly accessible nutrients such as phosphorus and iron. Facilitated by exudation of organic acids and metal-chelating compounds (Yan *et al.*, 2004; Marschner *et al.*, 2011), this activity further modifies the composition of the rhizosphere microbial community. The root hair zone is a region of intensive crosstalk with beneficial and pathogenic microorganisms (Peleg-Grossman *et al.*, 2009; Libault *et al.*, 2010; Poitout *et al.*, 2017). Therefore, it is not surprising that a great

number of specific molecules, including flavonoids, coumarins, phenolics, indoles, amino acids, and proteins are released in this area (Bertin *et al.*, 2003; Badri & Vivanco, 2009; Hassan & Mathesius, 2012; Stringlis *et al.*, 2018) and were found to feedback on microbiome assembly and function (Gochnauer *et al.*, 1989; Korenblum *et al.*, 2020; Gebauer *et al.*, 2021). In maize, root development is characterized by gradual merging of the root hair zone with the zone of lateral root emergence (Fig. 1). Cracks around the breakage sites of emerging lateral roots release metabolites that might fuel microbial growth (Jaeger III *et al.*, 1999) and may be vulnerable to pathogen invasion and host infection (Gopalaswamy *et al.*, 2000; Lagopodi *et al.*, 2002; Sprague *et al.*, 2007). Here, lectins and benzoxazinoids around freshly emerged lateral roots were shown to counteract infections and to modify microbiome composition (Sicker *et al.*, 2000; Park *et al.*, 2004; Péret *et al.*, 2009; Cotton *et al.*, 2019).

Beyond root-driven processes, bacterivore protists control the community composition of prokaryotic microbiomes in the rhizosphere (Gao *et al.*, 2019; Dumack *et al.*, 2022). For example, certain phyla of bacterivorous protists are specifically enriched in the maize rhizosphere (Rüger *et al.*, 2021; Taerum *et al.*, 2022), and exert significant top-down control on microbiome assembly and function. Selective feeding by protists strongly shapes rhizosphere bacterial community composition (Jousset *et al.*, 2008; Rosenberg *et al.*, 2009; Jousset & Bonkowski, 2010) and imposes a selection pressure leading to the alteration of microbial functional traits (Jousset *et al.*, 2006; Flues *et al.*, 2017; Xiong *et al.*, 2018; Gao *et al.*, 2019; Amacker *et al.*, 2020; Bahroun *et al.*, 2021). This can reduce deleterious effects of phytopathogens on plant growth (Weidner *et al.*, 2017; Amacker *et al.*, 2020), while the function of mutualists, such as mycorrhizal fungi, can be significantly enhanced (Herdler *et al.*, 2008; Koller *et al.*, 2013; Rozmoš *et al.*, 2021). Overall, protistan feedbacks were shown to alter root metabolite profiles and plant stress responses (Kuppardt *et al.*, 2018). They may even change root architecture (Kreuzer *et al.*, 2006), not only by altering bacterial communities, but also by remobilization of nutrients or direct plant-protist interactions (see Bonkowski, 2004).

The assumption that plants actively orchestrate the microbial assembly process (Haichar *et al.*, 2014) implies specific upstream control points of plant gene regulation underlying the crosstalk between plants and microbes (Phillips *et al.*, 2003). Gene expression patterns differ strongly between root zones along the root axis. Generally, transcripts related to growth are enriched in the maize root tips and decline toward the zone of lateral root emergence, where defense-related genes are upregulated (Cesco *et al.*, 2010; Stelpflug *et al.*, 2016; Yu *et al.*, 2021). In particular, the plant immune system is highly regulated and is assumed to play a crucial role as a molecular control point for microbiome assembly (reviewed in Segonzac & Zipfel, 2011; Hacquard *et al.*, 2015). As a first line of defense, immunoreceptors targeting microbial-associated molecular patterns (MAMP) on the root cell surface trigger defenses against specific classes of microorganisms (MAMP-Triggered Immunity; Bittel & Robatzek, 2007). A second line of defense targets microbial effector molecules (Ceulemans *et al.*, 2021) via pathogen recognition (PR) receptor proteins (effector-triggered immunity (ETI), Hacquard *et al.*, 2017). It is in this context that the phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play critical roles in regulating microbial root colonization (Van Loon *et al.*, 2006; Hause & Schaarschmidt, 2009; Lebeis *et al.*, 2015; Chen *et al.*, 2020). Rhizosphere microorganisms can activate plant defense pathways both locally (Hartmann & Schikora, 2012; Brotman *et al.*, 2013) and systemically (Schuhegger *et al.*, 2006; Van Loon *et al.*, 2006; Henkes *et al.*, 2011; Kurth *et al.*, 2014; Pieterse *et al.*, 2014; Verbon *et al.*, 2017) with direct

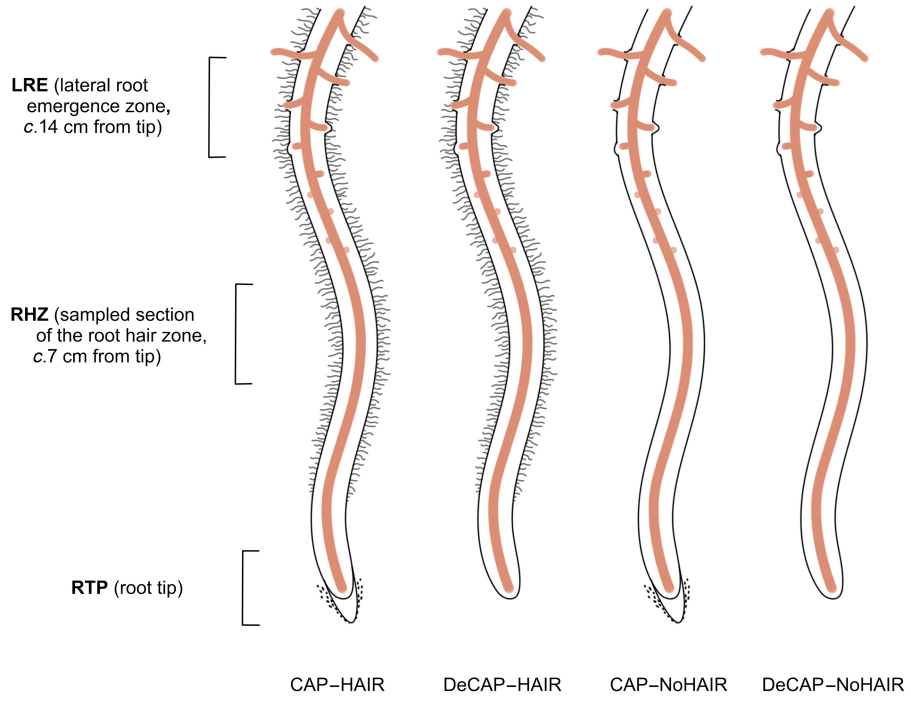

Fig. 1 Four tested treatment combinations and three sampled zones of the root (*Zea mays*). The treatment combinations included roots with intact (CAP) or removed root caps (DeCAP) and roots with typical root hairs (HAIR) or without root hairs (NoHAIR) in the root hair-deficient mutant rth3. The three sampled zones included root tip (RTP) comprising the first cm from the root tip, the root hair zone (RHZ) 2 cm of the region below the emergence of the first lateral root, and the region of lateral root emergence (LRE), that is, 2 cm of the region above the first visible lateral root. Root zones are indicated by brackets.

and indirect feedbacks on microbiome assembly (Pieterse & Ton, 2009; Lebeis *et al.*, 2015; Tanaka *et al.*, 2015; Ravanbakhsh *et al.*, 2018; Jones *et al.*, 2019; Chen *et al.*, 2020). Direct effects involve the activation of reactive oxygen species (ROS) and biosynthesis of secondary metabolites (Jacoby *et al.*, 2020; Schütz *et al.*, 2021), such as phenylpropanoids and their derivatives like flavonoids and antimicrobial phytoalexins (Shaw *et al.*, 2006; Steinkellner *et al.*, 2007). Indirectly, systemic responses affect source–sink relationships in plants (Liu *et al.*, 2010; Schultz *et al.*, 2013), with immediate consequences for root C allocation and microbiome assembly (Henkes *et al.*, 2008, 2018).

In view of the complexity of microbiome assembly, our first aim was to determine which of the specific structures (root cap or root hairs) exerts a stronger effect on microbiome assembly along the longitudinal root axis, and whether the absence of root cap or root hair region mutually influences each other's effect on the microbiome. We hypothesized that decapping of the mucilage-releasing root tips and the absence of root hairs in root hair-deficient mutant maize impact prokaryote communities, which in turn affect their protist consumers through feedback mechanisms. After characterizing the main contributors to microbiome assembly along the root axis of maize, we hypothesized that if plants actively orchestrated microbiome assembly, it should be possible to identify potential molecular rhizosphere control points by correlations of microbial taxa with the expression patterns of plant (defense) genes. Finally, we hypothesized that specific trophic relationships between prokaryotes and protists are especially relevant for the rhizosphere microbiome structure, and that co-occurrence networks between prokaryotes and protists indicate such relationships.

Materials and Methods

Experimental setup

The experiment was carried out in a two-factorial design. Wild-type *Zea mays* L. (inbred line B73) plants with intact root caps or with manipulated root caps (CAP vs DeCAP) were compared with root hair-deficient mutants (*root hairless 3*, *rth3*; HAIR vs NoHAIR) with root caps intact or removed, resulting in four (2 × 2) treatment combinations (Fig. 1). The *rth3* mutant is a highly homozygous line (Hochholdinger *et al.*, 2008), exhibiting impaired root hair elongation. Root caps were removed under a dissection microscope with a sterile scalpel as in Humphris *et al.* (2005). Each cap junction was checked and only roots whose caps came off cleanly at the first attempt were used for further experimentation. Prokaryote (bacteria and archaea) and protist (Rhizaria: Cercozoa and Endomyxa) community composition and plant gene expression were analyzed in different root zones. The experiment was set up in rhizoboxes with 36 replicates per treatment combination. Six additional replicates were set up per treatment combination for measurements of root length and diameter to characterize the effect of decapping on root growth. Maize plants were planted in rhizoboxes filled with an agricultural loam soil with a sand : silt : clay, 33 : 48 : 19, harboring its original microbial community (Supporting information Fig. S1; Methods S1). Plants were grown for 6 d in a climate chamber at 12 h : 12 h, day : night (350 µmol m^{-2} s^{-1} photosynthetically active radiation) at 24°C : 18°C and 65% humidity, until the first roots reached the bottom of rhizoboxes.

Sampling

Three zones along the primary root of each plant were sampled: the root tip (RTP), the root hair zone (RHZ), and the region of lateral root emergence (LRE; Fig. 1). For microbial DNA extraction and subsequent amplicon sequencing of 16S and 18S rRNA gene fragments and quantification of prokaryotes, 0.25 g soil was collected with a sterile spatula from the direct vicinity of each of the three root zones of each plant. Rhizosphere samples were pooled from two plants to ensure better comparability with the pooled root transcriptome data, resulting in 18 replicates for each treatment combination. For root RNA extractions, corresponding root zones were cut out with a sterile scalpel, vortexed in 0.3% NaCl to remove adhering soil, frozen in liquid nitrogen, and stored at −80°C. Roots were pooled per rhizobox, resulting in three replicates per treatment before RNA extraction. Two DNA samples from the prokaryote dataset and one RNA sample were excluded from further analysis due to quality issues. Total root systems were scanned (Epson Perfection V700) and primary root length, the length of the primary root including laterals, and average root diameter was analyzed, by WinRHIZO (v.5.0; Regent Instruments, Quebec City, QC, Canada).

Microbial quantification, amplicon sequencing, plant transcriptome sequencing, and data processing

For soil DNA extraction and purification, the FastDNA SPIN Kit for soil and the GeneClean Spin Kit (MP Biomedicals, Santa Ana, CA, USA) were used, following the manufacturer's instructions. Prokaryote community abundance was determined in extracted DNA by quantitative polymerase chain reaction (qPCR) using the forward primer Eub 338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer Eub518 (5′-ATTACCGCGGCTGCTGG-3′; Methods S2).

For sequencing, an *c.* 250-bp long fragment of the prokaryotic V4 region of the 16S rRNA gene was amplified with the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′; Caporaso *et al.*, 2011) and the reverse primer 806R (5′-GGACTACNVGGGTWTCTAAT-3′; Apprill *et al.*, 2015). The amplicons were double-indexed with Nextera XT indexes to provide unique index combinations per sample. Concentrations of amplicons were measured using Picogreen fluorescence assay before an equimolar mixture was sequenced on a 2x300 MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA) at the Joint Microbiome Facility, Vienna University, Austria. For sequence processing forward and reverse sequence, reads were first paired, quality checked, and filtered. Sequences were then clustered into OTUs at 97% similarity level, identified, resampled to 26 000 sequences per sample, and clustered again. OTUs represented by < 55 reads were discarded.

To amplify a circa 350-bp long fragment of the cercozoan and endomyxan V4 region of the SSU/18S rRNA, a two-step PCR was conducted (Fiore-Donno *et al.*, 2020). In a first PCR, the forward primers S615F_Cerco (5′-GTTAAAAAGCTCGT AGTTG-3′) and S615F_Phyt (5′-GTTAAAARGCTCGTAGT CG-3′) and the reverse primer S963R_Phyt (5′-CAACTTT CGTTCTTGATTAAA-3′) were used. In a subsequent semi-nested PCR, using the forward primer S615F_Cer (5′-GTT AAAARGCTCGTAGTYG-3′) and the reverse primer S947R_Cer (5′-AAGARGAYATCCTTGGTG-3′), both barcoded, samples were indexed (Table S1). The primers specifically target cercozoan and endomyxan DNA as these groups make up a major part of bacterivorous protists in soils (Bates *et al.*, 2012; Burki & Keeling, 2014; Geisen *et al.*, 2015). General Eukaryote primers were not suitable for this study as they have been shown to exclude a significant part of diversity, are highly biased, and amplify a substantial proportion of multicellular organisms (Aslani *et al.*, 2022; Vaulot *et al.*, 2022), and see Lentendu *et al.* (2014) and discussion in (Fiore-Donno *et al.*, 2018). The PCR products were purified and normalized using SequalPrep Normalization Plate Kit (Invitrogen) and sequenced on a 2x300 Illumina MiSeq platform at the Cologne Center for Genomics (Cologne, Germany). As for prokaryotes, forward and reverse reads were paired, quality checked, filtered, and clustered at 97% similarity into OTUs. Those represented by < 1000 reads were removed, and remaining OTUs were assigned to taxa. Chimeras were removed, and samples were resampled to 5290 sequences.

For plant transcriptome sequencing, frozen root samples were homogenized in liquid nitrogen using mortar and pestle. Total RNA was isolated using the NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and treated with RNase-free DNase (Qiagen). RNA quantity and integrity were determined spectrophotometrically using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer (Agilent RNA 6000 Nano kit, Santa Clara, CA, USA). All samples passed quality control with RIN (RNA integrity number) values > 8. Library preparation and sequencing of three independent biological replicates were conducted by Genewiz (Leipzig, Germany) with a strand-specific paired-end 2 × 150-bp design on the Illumina NovaSeq platform. After adapter removal and quality trimming, reads were aligned to maize B73_RefGen_v4 genome and assigned to genes.

From prokaryote and Cercozoa sequencing data, rarefaction curves were calculated, to confirm sufficient sequencing depth. OUT richness, Pielou evenness, Shannon diversity, and prokaryote abundance were compared by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD). Permutational multivariate analysis of variance (PERMANOVA), variance partitioning, nonmetric multidimensional scaling (NMDS), and comparison of group dispersion were used to analyze beta diversity. In order to assess how much variance in beta diversity of prokaryotes could be explained by diversity of their protistan predators, the first two axes of a principal coordinates analysis (PCoA) of cercozoan Bray–Curtis dissimilarities were extracted and used as explanatory variables for Bray–Curtis dissimilarities of prokaryote data in a db-RDA. Plant gene expression data were checked for differentially expressed genes; a gene set enrichment analysis was conducted, as well as a variance partitioning analysis (see Methods S3, S4 for detailed descriptions of sequencing data processing and statistical analyses).

Network analysis

Co-occurrence network analysis was performed with FlashWeave (v.0.18.0, Tackmann *et al.*, 2019) implemented in Julia (v.1.5.3, Bezanson *et al.*, 2012) to identify patterns in the associations between prokaryotes, protists, and expression levels of 55 significantly differentially expressed plant genes. Gene expression data, root zones, root cap status, and root hair status were integrated as metadata, with root zones included as ordinal scaled factors (increasing from root tip to lateral roots). To reduce spurious edges, rare taxa that only occurred in < 1/3 of all samples were combined into one pseudo taxon (Röttjers & Faust, 2018; Faust, 2021). To account for compositionality of individual datasets (e.g. prokaryotes and protists), datasets were individually normalized by centered log-ratio transformation before networks were calculated. The networks were visualized in Cytoscape (v.3.8.0, Shannon *et al.*, 2003). To cross-compare the abundances of prokaryote and protist OTUs with the gene expression levels in maize roots, the data were combined into one co-occurrence network, which was split into sub-networks including only microbe-microbe interactions or microbe-metadata and metadata-metadata interactions.

Results

Influence of decapping on plant transcriptome and root architecture

Low numbers of differentially expressed genes (DEG) and the induction of only few stress response genes in the DEG palette when comparing DeCAP and CAP treatments at RTP indicated successful decapping without harming the root tip (Table S2). Furthermore, decapping caused no obvious differences in root development and had neither a significant effect on primary root length, nor on the total root length including lateral roots. Only the average root diameter increased in DeCAP–HAIR compared with other treatment combinations (Fig. S2; Table S3).

Microbial diversity

Prokaryote and protist OTU richness reached saturation and showed excellent coverage of samples (Fig. S3). Their respective local (alpha) diversities were best explained by root zonation, the root cap status only affected prokaryotes, but the interaction of root zonation and the root cap status affected both (Table S4). Prokaryote OTU richness, evenness, and Shannon diversity were lower but highly variable at RTP, and higher but less variable at RHZ and LRE zones (Fig. 2a). Removal of the root cap changed prokaryote Shannon diversity along the root axis, especially in NoHAIR (Table S5). The impact of the tested variables on prokaryote abundance was negligible (Fig. 2b; Tables S6, S7).

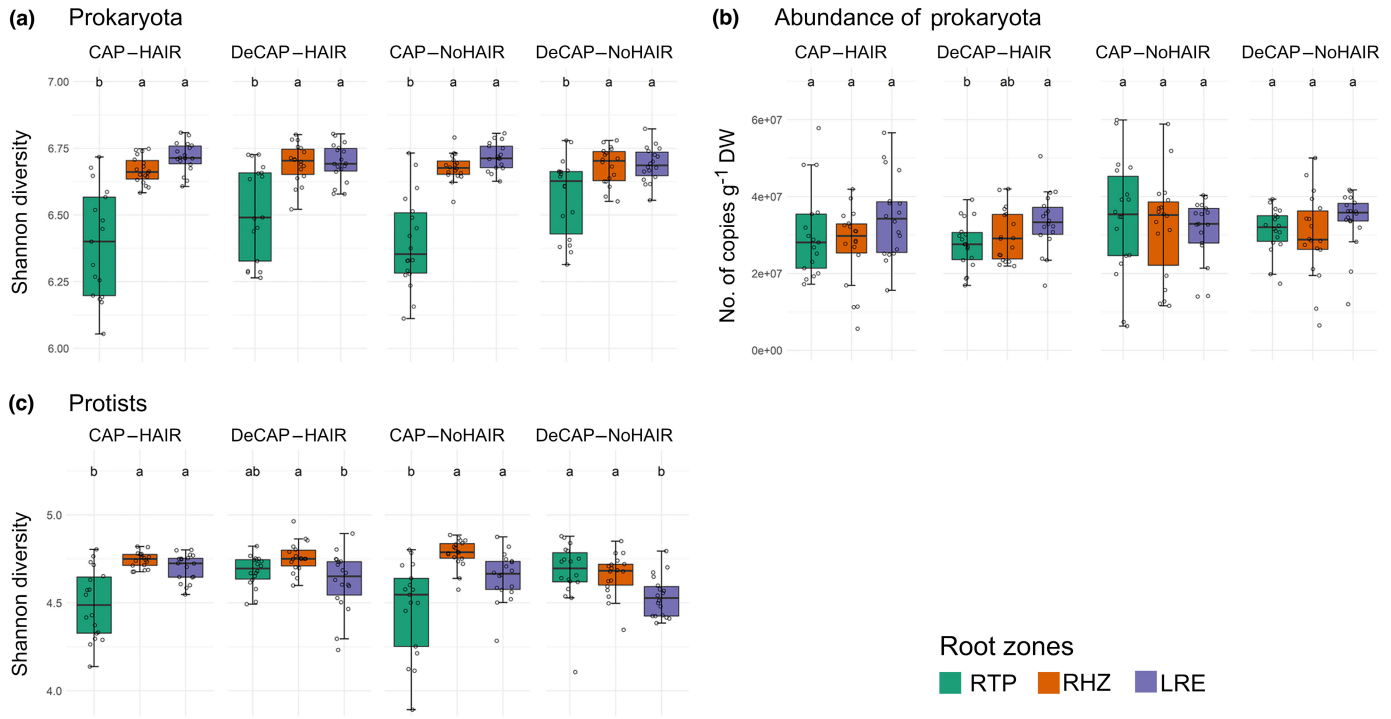

Fig. 2 Boxplots illustrating Shannon diversity of (a) prokaryote (bacteria and archaea) and (c) protist (Cercozoa and Endomyxa) communities and (b) prokaryote abundance measured by qPCR (no. of copies g^{-1} soil dry weight) in the rhizosphere of *Zea mays* in the four treatment combinations with and without root caps (CAP vs DeCAP) and with and without root hairs (HAIR vs NoHAIR) at three root zones: root tip (RTP, green), root hair zone (RHZ, red), and lateral root emergence (LRE, blue). The horizontal line within each box represents the median. Whiskers extend to the lowest and highest scores within 1.5 times the interquartile range from the box. Points represent individual datapoints. Letters indicate significant differences between means (Tukey's HSD).

Alpha diversity of protists on roots with intact root caps corresponded to patterns in prokaryotes, with the highest variability at RTP (Fig. 2c). Decapping strongly reduced variability of Shannon diversity at RTP and especially NoHAIR had a higher Shannon diversity at RTP compared with LRE (Fig. 2c), because protist evenness decreased at LRE (Fig. S4b).

Irrespective of experimental treatments, prokaryote and protist beta diversity clearly shifted from the root tip toward older root zones (Fig. 3a,b), and variance partitioning explained 7.49% and 8.36% of variation in prokaryote and protist beta diversity between root zones, respectively. The cap explained by far more variance of beta diversity compared with the hair status with 0.76% vs 0.22% in prokaryotes and 1.84% vs 0.39% in protists, respectively (Table S8). In general, the communities of both prokaryotes and protists of root tips (RTP) were clearly distinct from RHZ and LRE (Fig. S5), but the effect size was dependent on root cap status (Table S8). Beta diversity dispersal, comparing the differences of community composition among individual root zones, was always higher for protists than prokaryotes and generally highest on root tips, exception the DeCAP–HAIR treatment for prokaryotes (Fig. S5a,b; Table S8). Beta diversity of Cercozoa (first two axes of PCoA) explained 5.8% of prokaryote beta diversity in db-RDA, indicating a significant influence of predation on community structure of bacteria, because when all treatment factors (Root zones, CAP, HAIR) were further included in db-RDA, in total, 10.3% variance of prokaryote beta diversity could be explained.

Plant gene expression patterns

The three different root zones along the primary root showed clearly distinct patterns of gene expression as shown by PCA, variance partitioning (Fig. S6a,b), and PERMANOVA ($R^2 = 0.765$, $P < 0.001$). The presence or absence of root cap or root hairs had a more subtle influence on gene expression patterns, which were not significant on a global level (PERMANOVA, $P = 0.071$ and $P = 0.126$, respectively).

A highly dynamic transition of gene expression related to growth and development, stress response, metabolism, signaling, and transport characterized the three root zones (Fig. S7; Tables S9, S10). The largest number of differentially expressed genes (DEG) were found between RTP and RHZ (9237, $P < 0.01$, $|LFC| > 1$), followed by RTP vs LRE (6784) and RHZ vs LRE (3173; Table S10). Genes with a specific upregulation at the RTP reflected the functional organization of root tip growth with an enrichment of Gene Ontology (GO) terms 'cell tip growth' and 'plant-type cell wall organization' (Table S9). However, genes indicative of stress responses were more highly expressed in RTP than in other zones, reflected by enriched GO terms 'response to karrikin' and 'response to water deprivation'. The RHZ was characterized by higher expression of genes related to exudation, nitrate, and water transport ('nicotianamine biosynthetic process', 'nitrate transport', and 'water transport'), as well as a range of genes involved in defense response and signaling ('response to jasmonic acid', 'phenylpropanoid biosynthetic

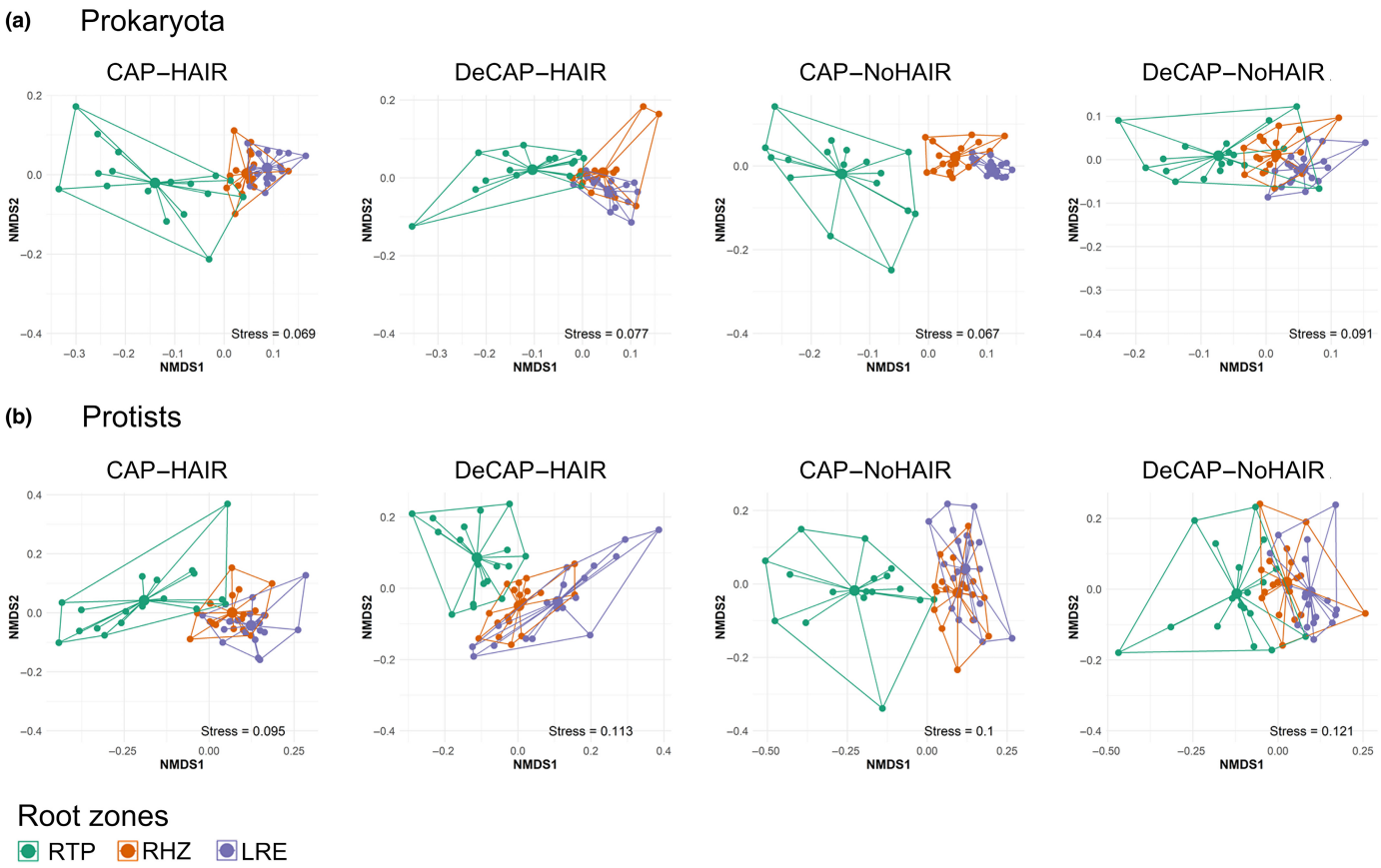

Fig. 3 Nonmetric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities of (a) prokaryote (bacteria and archaea) and (b) protist (Cercozoa and Endomyxa) communities in the rhizosphere of *Zea mays* in the four treatment combinations with and without root caps (CAP vs DeCAP) and with and without root hairs (HAIR vs NoHAIR) at three root zones: root tip (RTP, green), root hair zone (RHZ, red), and lateral root emergence (LRE, blue). Each point represents a microbial community sample, and the color of the point indicates the sample source. Group centroids are marked with a larger dot. The two axes (NMDS1 and NMDS2) represent the two most significant dimensions of variation among the samples.

process', and 'response to wounding'). In the LRE zone beta-glucosidases, expansins, and cellulases were upregulated, indicative of cell wall-structuring functions, as well as several *NRT1/PTR* transporters and nicotianamine transporters *YSL2*, pointing toward transport and exudation.

For root gene expression data, the effect of root cap removal (DeCAP vs CAP) was moderate in the RTP, very low in the RHZ and absent in the LRE (Table S2). The DEG in the RTP were related to transport and exudation (Fig. 4a), such as the GO terms 'nitrate transport', 'nicotianamine biosynthetic process', 'sucrose transport', and 'water transport', but also to stress and defense, such as 'systemic acquired resistance' and 'response to jasmonic acid'.

Differences between the NoHAIR and the HAIR treatment were moderate in the RTP with enriched GO terms 'suberin biosynthetic process', 'nitrate transport', 'systemic acquired resistance', and 'positive regulation of flavonoid biosynthetic process' (Fig. 4b), but again very low in the RHZ and LRE (Table S10).

Generally, the differences in gene expression between the root zones were less pronounced in the NoHAIR compared with the HAIR plants, but nevertheless, a large part (60–70%) of the genes that were differentially expressed between root zones were the same for HAIR and NoHAIR maize roots (Fig. S8).

Co-occurrence of prokaryotes and protists and their associations with DEGs

To cross-compare the abundances of prokaryote and protist OTUs with the gene expression levels in maize roots, the data were combined into a co-occurrence network (1910 edges). For better visualization, subnetworks including only microbe–microbe interactions (Fig. 5a) and correlations of microbes, metadata, and root gene expression (Fig. 5b) were separated. The microbe–microbe association network (Fig. 5a) was characterized by a particularly high number of edges among prokaryotes (1294) and between prokaryotes and protists (397), indicating potential interactions due to competition and predation. Especially, bacterivore cercozoan taxa in the class Filosa-Sarcomonadea showed negative associations with various prokaryote taxa. Among these protists, the Sandonidae (Cercozoa) showed an explicitly high proportion of negative associations with different bacterial genera (positive: 49, negative: 31), especially with Massilia (edge weight = 3). By contrast, among prokaryotes, the number of positive edges exceeded negative ones almost fivefold (positive: 1061, negative: 233). Links between the treatment factors and plant gene expression levels or microbes were scarce (18).

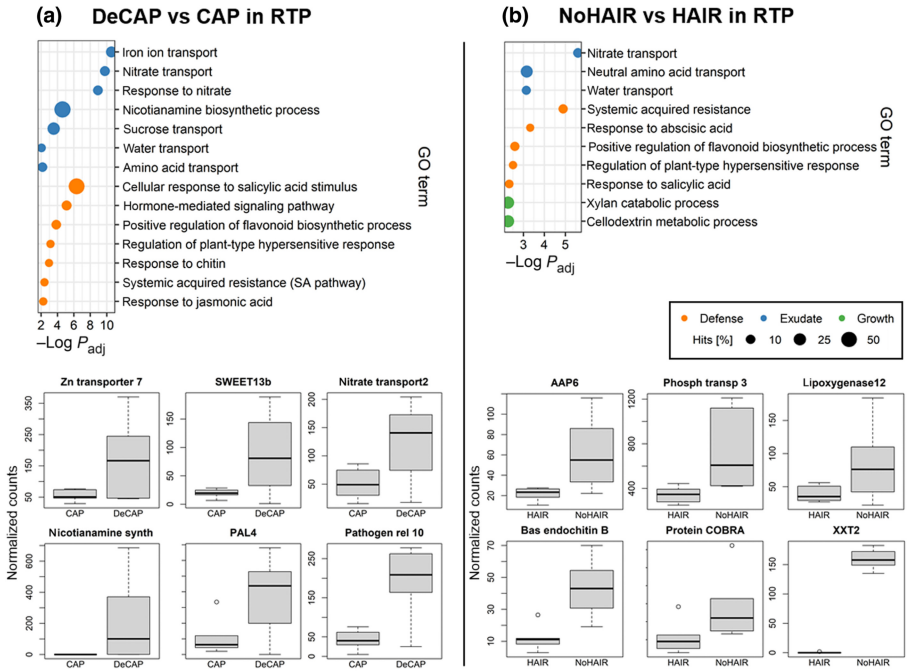

Fig. 4 Functional annotation analysis of differentially expressed genes in the root tip of *Zea mays*, showing the effects of (a) root cap removal or (b) lack of root hairs. Upper panels show selected enriched Gene Ontology terms for upregulated genes in the DeCAP/NoHAIR treatment, relating to processes of microbial defense (orange), transport (blue), and growth (green). Point size encodes 'hits per term' (percentage of enriched genes per total number of annotated genes in the GO term). Lower panels show boxplots of a selection of differentially expressed genes with DESeq-normalized counts. The horizontal line within each box represents the median. Whiskers extend to the lowest and highest scores within 1.5 times the interquartile range from the box. Data beyond this range are represented by individual points.

Among prokaryotes, 32 genera were associated with the expression level of specific genes. For example, the highly abundant genus *Massilia* (Oxalobacteraceae, Burkholderiales, and Betaproteobacteria) with a degree centrality (DC) of 88 was positively linked with the expression of *Cys-rich PK* (Cysteine-rich protein kinase, that is, MAMP-Triggered Immunity signaling). Another genus of the Burkholderiales was positively associated with the expression of JA ind (encoding jasmonate-induced protein, immune response). The genus *Lysobacter* (Xanthomonadales, Gammaproteobacteria) with a DC of 44 showed negative associations with the expression of *HCT2* and *JA ind* (encoding hydroxycinnamoyltransferase 2 and jasmonate-induced protein, immune response), and a genus of the Gaiellales (Actinobacteria) with a high DC (52) was positively linked with the expression of *JA reg 21* (jasmonate-regulated 21, immune response). Within Cercozoa, genera of five families were linked to the expression levels of specific genes. The Sandonidae (DC of 112), marked by the highest number of reads, were associated with the expression of various plant genes. For example, a negative association was found with the expression of ERF 55 (Ethylene-responsive transcription factor 55, signaling), and positive associations with expression levels of genes encoding *JA ind* and *e_glucanase 1* (jasmonate-induced protein and endoglucanase 1, immune response). The Allapsidae (DC of 54) showed positive associations to plant genes involved in growth and development, such as *LRP 1* (lateral root primordium 1), and especially strong (edge weight = 2) with *Pectinesterase*.

Discussion

We explored the roles of root border cells and mucilage secretion (CAP vs DeCAP) and root hairs (HAIR vs NoHAIR) on the assembly of rhizosphere microbial communities along the primary root axis of maize. At the same time, we examined associations of microbial community composition with root gene expression, indicative of rhizosphere control points (sensu Phillips & Strong, 2003). Finally, we investigated the effects of a dominant group of protistan predators (Cercozoa and Endomyxa) on the community structure of the prokaryote microbiomes and the effect of prokaryotes on protists. As hypothesized, root cap removal affected microbiome assembly patterns, particularly at root tips. Despite CAP and HAIR treatments, rhizosphere microbiome composition was still mainly driven by the microbial succession from root tips to older root zones. In accordance with Rüger *et al.* (2021), variability of local communities (alpha diversity) and communities between individual roots (beta diversity) of prokaryotes and of their protistan predators was significantly higher at root tips compared with older root zones (Figs 2, S5), assuming randomness through priority effects of early colonizers that initially leads to dominance of different taxa on different root tips (Chase, 2003; Fukami, 2015; Attia *et al.*, 2022). The reduced variability of all components of alpha diversity (i.e. OTU richness, evenness, and Shannon index) at RHZ and LRE is a strong indication of the fast formation of a distinct microbiome along the root axis and is further corroborated by reduced dispersal of beta diversity from RTP to RHZ and LRE (Fig. S5). The increased Shannon diversity at RHZ and LRE was due to both, increased numbers of different taxa (i.e. OTU richness), but with reduced dominance of single taxa (i.e. enhanced evenness; Fig. S4). At first sight, this pattern appears counterintuitive, as one would expect fewer, specialized taxa gaining dominance during the assembly process if plants favor certain taxa over others. Instead, increased evenness and taxon richness resemble typical outcomes of predation, where the fastest growing, dominant taxa are preferentially consumed

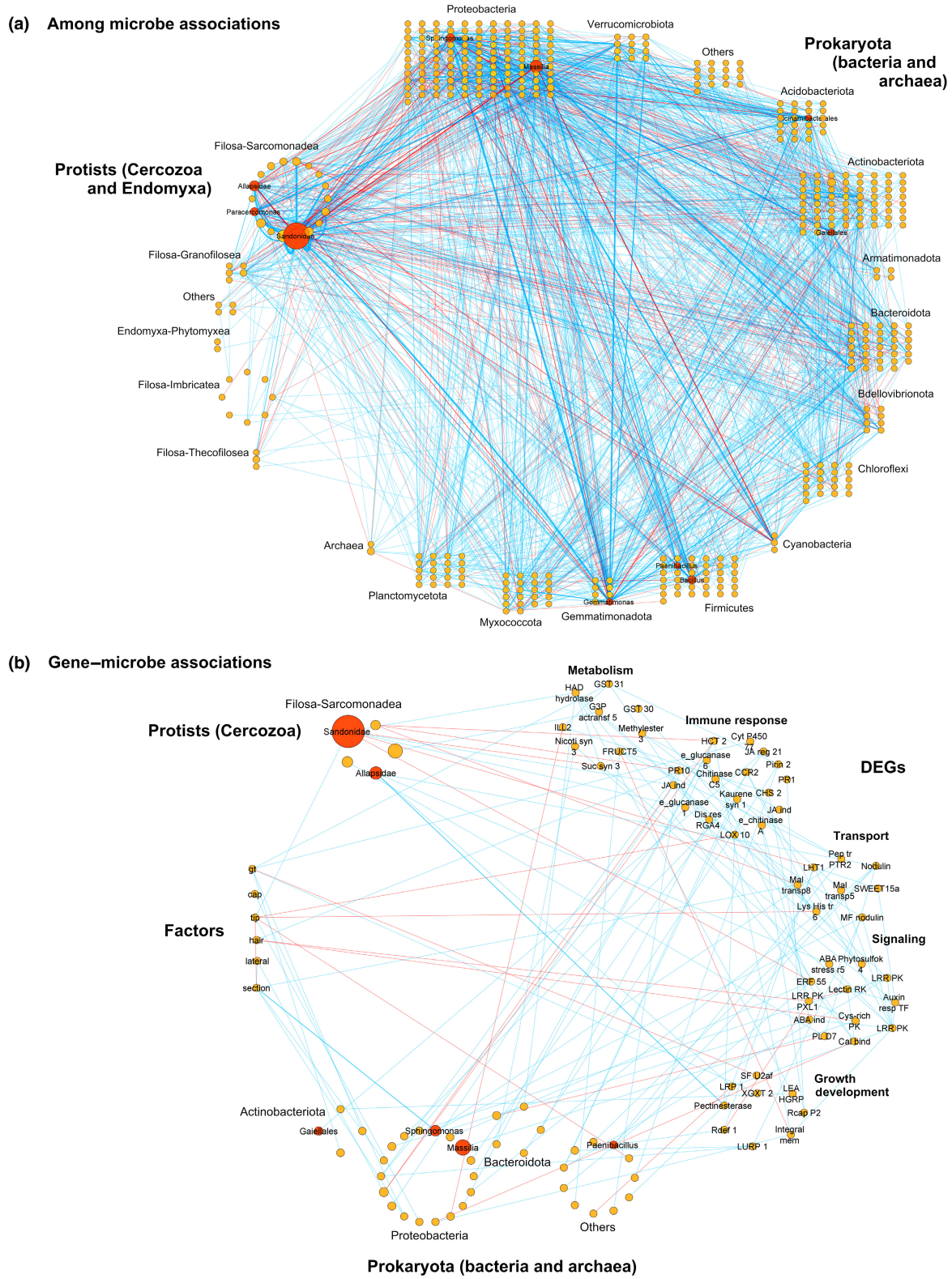

Fig. 5 Co-occurrence networks illustrating (a) associations among and between prokaryotes (bacteria and archaea) and protists (Cercozoa and Endomyxa), and (b) between the microorganisms, 55 selected DEGs of *Zea mays* and the factors genotype (gt; i.e. HAIR vs NoHAIR), and cap (i.e. CAP vs DeCAP) and root zones (section) with root tip (tip), root hair zone (hair), and lateral root zone (lateral). Nodes represent taxa summed at genus level and grouped at class (protists) or phylum level (prokaryota) for better visualization. Nodes representing genes are grouped into five categories regarding their function. Nodes with a degree centrality ≥ 50 are highlighted (orange). The size of nodes indicates the number of reads (normalized). The color of edges indicates whether an association is positive (blue) or negative (red). The edge width reflects the edge weight, that is, the numbers of associations between taxa or metadata.

and greater numbers of competitive subordinate species can coexist (Rosenberg *et al.*, 2009; Karakoç *et al.*, 2020). The structuring impact of predation on the prokaryote microbiome was supported by a high proportion of bacterial beta diversity explained by cercozoa in a constrained (db-RDA) analysis.

CAP and HAIR treatments modified microbiome assembly in distinct ways. The absence of root caps had far stronger effects than the absence of root hairs, which would imply that root caps are more important than root hairs for microbiome assembly. Microbial communities of DeCAP–HAIR differed from other treatments first of all by reduced abundance of prokaryotes at root tips, likely a result of decreased availability of mucilage for microbial growth (Benizri *et al.*, 2007). Taxon richness, evenness, and Shannon diversity of prokaryotes, however, appeared rather unaffected. Instead, the DeCAP–HAIR treatment affected more strongly the diversity of protists. Apparently, the reduced availability of prey via decapping reduced the variability of protist alpha diversity at RTP. It furthermore caused ripple effects that were still noticeable at LRE: Reduced evenness and decreased Shannon diversity, which resulted in far higher variability of beta diversity at LRE than on roots with caps (Fig. S5). The absence of root hairs led to less distinct differences in beta diversity between root tips and older root zones in communities of both prokaryotes and protists, and only when the root caps had been removed (DeCAP–NoHAIR, Fig. 3). This suggests that the minor differences in bacterial community composition that have been associated with NoHAIR maize (Gebauer *et al.*, 2021) or barley (Robertson-Albertyn *et al.*, 2017) are amplified by the absence of root cap, supporting the root cap's importance as the first selector and amplifier of rhizosphere compatible taxa. Rhizosphere community assembly processes at the tip feedback on root hair zones.

For root gene expression profiles, root cap removal also attenuated patterning of root zonation, evident by smaller numbers of differentially expressed genes between root zones. In accordance with the patterns of microbiome assembly, the changes in root gene expression after cap removal were most prominent in the root tip. Genes relating to pathways of water/exudation (e.g. *ABA stress ripening 5*, *nicotianamine synthases*) and defense response, especially of the phenylpropanoid-flavonoid biosynthesis pathway, were upregulated after decapping. Plant-derived flavonoids are known to mediate interactions between plant host and both symbiotic as well as pathogenic microorganisms (Treutter, 2005; Mierziak *et al.*, 2014; Block *et al.*, 2018). For example, Yu *et al.* (2021) showed how flavone production promotes the enrichment of bacteria from Oxalobacteraceae, like *Massilia* in the maize rhizosphere, and their data suggest that this led to stimulation of maize growth and enhanced nitrogen acquisition.

Similarly, like flavonoids, coumarins (phenylpropanoid pathway) were found to alter microbiome composition through strain-specific antimicrobial effects (Stringlis *et al.*, 2018; Voges *et al.*, 2019). Specifically, benzoxazinoids play an acknowledged role in fine-tuning microbial communities of maize roots (Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Schütz *et al.*, 2021). Other phenylpropanoids such as chlorogenic acid were shown to improve plant resistance against herbivores and pathogens (Leiss *et al.*, 2009). Furthermore, gene functions relating to plant hormone signaling via ABA, SA, and JA, which also play a role in microbe assembly (Jacobsen *et al.*, 2021), were enriched after decapping. The upregulation of such genes – related to defense and immunity – has been associated with basal root zones (Stelpflug *et al.*, 2016). At the root tip, this could assist to reduce the initial random assembly by more deterministic processes, similar to those known for older root zones, thus strengthening microbiome assembly at subsequent root zones. In addition to the observed expression patterns of defense and immunity-related genes, enhanced gene expression of nicotianamine synthases and mineral nutrient transporters indicates a change in root exudation. Nicotianamine synthases are involved in the biosynthesis of phytosiderophores (Mizuno *et al.*, 2003) to facilitate iron and zinc mobilization and plant uptake (Wirén *et al.*, 1996). The iron nutritional status of the plant is connected to root colonization of beneficial rhizobacteria, such as *Paenibacillus polyxyma* and *Bacillus subtilis* (Zhang *et al.*, 2009; Zhou *et al.*, 2016), which enhance plant resistance to microbial pathogens by competing for iron (Verbon *et al.*, 2017). Consequently, subtle defense responses at the root tip in concert with changes in exudate supply and composition appear to have a considerable influence on the assembly of the rhizosphere microbiome, even at more distant root zones. Changes in gene expression were not indicative of heightened stress or accompanied by a stark reduction in growth; therefore, we expect that the influence of root cap removal has a direct effect on the microbial community assembly.

Presence of root hairs had a surprisingly marginal effect on the root zonal distribution of the rhizosphere microbiome as well as on root gene expression. The absence of associations between prokaryotes or protists with the factor 'hair' in the network (Fig. 5b) together with the marginal effects of the NoHAIR treatment on microbiome assembly sustain the assumption of Rüger *et al.* (2021) that the root hair zone might play a rather small regulatory role in the assembly of the microbiome in maize. This is surprising, as root hairs were shown to significantly enhance the carbon input into the rhizosphere (Holz *et al.*, 2018), but the lack of root hairs did not reduce prokaryote abundance in our experiment (Fig. 2b). Potentially, the carbon input by root hairs is mainly used as substrate to fuel enzyme production for microbial nutrient mining as proposed by Zhang *et al.* (2020) and has little selective effect because all fast-growing copiotrophic rhizobacteria are stimulated simultaneously (Rüger *et al.*, 2021). At the level of root gene expression, the minor impact of *rth3* mutants – exerted mostly at the level of cell wall biosynthesis and organization-related gene expression – was confirmed for maize root systems at four-leaf stadium (Ganther *et al.*, 2021). The significant upregulation of genes involved in nitrate transport and signaling in RHZ and LRE, such as *NRT1* together with *sugar transporter 1*, might indicate a stronger role of these root zones in guiding sink–source allocation patterns (Remans *et al.*, 2006; Krouk *et al.*, 2010; Schultz *et al.*, 2013; Wang *et al.*, 2019). This sink–source allocation may be linked to trophic relationships between protists and prokaryotes (Kuikman & Van Veen, 1989; Kuikman *et al.*, 1991), considering the constant release of nitrogen by protists from consumed microbial biomass (Clarholm, 1985), their stimulation of bacterial

turnover, and nitrification in the rhizosphere (Bonkowski *et al.*, 2000; Bonkowski, 2004), as well as on auxin balance in roots (Krome *et al.*, 2010).

Only in the DeCAP–NoHAIR treatment, Shannon diversity of protists decreased significantly from root tip to base, yet in the DeCAP–HAIR treatment, almost similar patterns were found. The low alpha diversity at older root zones resulted from a decrease in evenness (Fig. S4), indicating enhanced dominance of specific cercozoan taxa. Apparently, the structuring effect that the removal of the root cap had on protist communities, was enhanced by the lack of root hairs, but primarily exerted at the region of LRE.

Such an interaction between root cap and root hairs was not found for plant gene expression. The NoHAIR treatment did not show any globally enriched GO terms, but induced rootzone-specific effects at the RTP, which was unexpected considering that root hair formation occurs from the root elongation zone upward (Bibikova & Gilroy, 2003). Root hairs increase the depletion zone for immobile nutrients such as P and K (Jungk, 2001) and facilitate the spatial diffusion of exudates such as organic acids, siderophores, or exoenzymes (Jungk, 2001; Marschner *et al.*, 2011; Bilyera *et al.*, 2022). In line with this, genes related to nutrient uptake, predominantly iron, and nitrate were upregulated in NoHAIR-RTP, suggesting nutrient depletion at root tips. How processes in the root hair zone are expressed in the root tip remains unclear at present. But, reactive oxygen species (ROS)-associated signaling has been implicated in regulation between the developmental zones of the root (Yamada *et al.*, 2020), and interestingly, NoHAIR caused in RTP a limited but significant induction of genes related to hypersensitive response, which is associated with ROS formation (Hacquard *et al.*, 2015). Taken together, the initial microbiome assembly at the root tip is highly variable and prone to priority effects (see Rüger *et al.*, 2021). Therefore, it would be most advantageous if the plants would gain better control over the community assembly process already at root tips. Prime candidates of molecular rhizosphere control points are genes involved in plant defense and stress responses. Plant gene expression indicates roles of jasmonate and ethylene signals triggered by immune response and suggests pleiotropic effects on microbiome assembly through the expression of genes related to ROS signaling and flavonoid production. As the absence of root hairs caused an extremely low impact on root gene expression, in this experiment as well as in earlier studies (Ganther *et al.*, 2021, 2022), and the plants were not grown under nutrient deficiency that could potentially enhance their dependency on root hairs (Bienert *et al.*, 2021), pleiotrophic effects by the rth3 mutation appear unlikely.

Network analysis confirmed significant correlations of root gene expression with microbial taxa that could be indicative of molecular control points in plant roots. Feedbacks between bacterial and plant signaling through systemic changes in root defense and exudation may significantly contribute to microbiome assembly in the rhizosphere. For example, bacterial quorum-sensing molecules were shown to activate systemic induced resistance in tomato through the induction of the SA- and ET-dependent defense genes, PR1 and chitinases (Schuhegger *et al.*, 2006).

Lysobacter, a ubiquitous bacterial genus, of which several species carry potential plant protective biocontrol traits (Hayward *et al.*, 2010), was negatively linked with the gene expression of *jasmonate-induced protein and hydroxycinnamoyl transferase 2* (Sullivan & Zarnowski, 2011). Both plant genes are associated with immune responses. A high abundance of *Lysobacter* might lead to a reduced expression of specific genes involved in defense (negative correlation) possibly through antimicrobial, plant-beneficial effects or, on the contrary, the abundance of *Lysobacter* might be controlled by the plant immune response.

A bacterial taxon of the common plant-associated Burkholderiales (Estrada-De Los Santos *et al.*, 2001), that contain beneficial and plant-pathogenic species (Compant *et al.*, 2008; Suarez-Moreno *et al.*, 2012), showed a positive association with gene expression of *jasmonate-induced protein*, potentially inducing a plant immune response.

Further bacterial and also cercozoan taxa correlated to genes with functions associated with transport, signaling, metabolism, development, and growth. Remarkably, there was a negative association between a cercozoan taxon in Sandonidae and the expression level of *ethylene-responsive transcription factor 55*. ET response factors play regulatory roles in stress signaling with likely feedbacks on microbiome assembly (Müller & Munné-Bosch, 2015; Ravanbakhsh *et al.*, 2018; Chen *et al.*, 2020). Another negative association occurred in DeCAP treatments between a protist in the genus *Cercomonas* and *pathogenesis-related protein 10* (PR10) that plays an important role in ETI host resistance (Chen *et al.*, 2010). These results corroborate findings of Kuppardt *et al.* (2018) who demonstrated that protists decreased typical plant stress responses, such as metabolites connected to the phenolic metabolism in the maize rhizosphere.

A protist taxon in the Allapsidae was positively linked to the expression levels of *Pectinesterase* and *lateral root primordium 1*, both genes involved in plant growth and development. Beneficial effects of protists on root growth have been repeatedly reported and are thought to work directly through remobilization of nutrients, as well as indirectly by changing the bacterial community composition (Bonkowski, 2004; Krome *et al.*, 2009; Weidner *et al.*, 2017). As the respective Allapsid was also highly associated with various prokaryote genera, direct effects on root gene expressions are as likely as indirect effects via changes in bacterial microbiome composition. Overall, our data indicate cross-communication between plants and microbes at different trophic levels in the rhizosphere.

Besides plant–microbe interactions, interactions among microbes significantly drive the self-organization of the rhizosphere microbiome (Bonkowski *et al.*, 2021) – this was indicated by comparable assembly patterns of prokaryotes and their protistan predators along the longitudinal root axis and the particularly high number of associations among microorganisms in co-occurrence networks. Such interactions can be facilitative or mutualistic, especially in the presence of predators at high resource supply from rhizodeposition (Nakajima & Kurihara, 1994; Leibold, 1996), because predator preferences are

directed toward less defended bacterial taxa and benefit their grazing-resistant competitors (Jousset *et al.*, 2010; Flues *et al.*, 2017). The large proportion of negative edges between Cercozoa, especially Sandonidae, and specific prokaryotes are likely indicative of consumer relationships, and the negative edges among prokaryotic taxa emphasized the role of competition in community assembly.

Concluding remarks

Overall, the absence of root caps had by far stronger effects on microbiome composition than the absence of root hairs. Decapping affected microbial community composition at older root zones (bacteria and archaea) as well as higher trophic levels (protists). The absence of root hairs had surprisingly small effects on microbiome assembly, which corresponded well with the low levels of differentially expressed genes in NoHAIR treatments. The bacterial genus *Massilia* (Oxalobacteraceae, Burkholderiales) held a central position in the microbiota network, and Burkholderiales together with other bacterial genera like *Lysobacter* showed associations with plant immune response genes. Community structure of cercozoan protists explained almost as much variation of prokaryote community turnover as the influence of different root zones and manipulations of the root cap and the root hair region. However, the role of heterotrophic protists in shaping the plant microbiome may not only be limited to predator–prey interactions, as specific cercozoan taxa were directly associated with plant immune responses. Overall, our results indicate a central role of root tips in microbiome assembly with ripple-on effects affecting higher trophic levels as well as microbial succession on older root zones.

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Competing interests

None declared.

Author contributions

MB, MTT, MG and LR conceived the study and planned the experiment. MG and LR performed the experiments and analyzed the data together with JF and AH-B. Analysis of pokaryota was conducted by JJ. All authors wrote the manuscript. MG and LR contributed equally to this work.

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Data availability

Raw RNA-Seq reads and protist and prokaryotic sequences were deposited at the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA77586.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Assembled rhizobox filled with loam and planted with *Zea mays*.

Fig. S2 Effects of the root cap treatment on root growth.

Fig. S3 Rarefaction curves showing the sequencing depth for protists and prokaryota.

Fig. S4 OTU richness and Pielou evenness of prokaryote and protist communities at the three root zones, with HAIR or NoHAIR, and CAP or DeCAP treatment.

Fig. S5 Group dispersion of beta diversity in prokaryote and protist communities at the three root zones, with HAIR or NoHAIR, and CAP or DeCAP treatment.

Fig. S6 Principal component analysis and variance partitioning analysis of RNA-Seq root gene expression data.

Fig. S7 Root zone-specific genes and enriched Gene Ontology terms.

Fig. S8 Venn diagrams of root zone differences in either HAIR or NoHAIR samples.

Methods S1 Rhizobox construction and preparation of maize seedlings.

Methods S2 Quantification of prokaryota by qPCR.

Methods S3 Processing of prokaryotic, protist, and root gene expression sequencing data.

Methods S4 Statistical analysis.

Table S1 Primer tag sequences used for barcoding protist sequences.

Table S2 Differentially expressed genes by root zonation, root cap, and root hair treatment.

Table S3 One-factor analysis of variance in root growth, between roots with HAIR and NoHAIR, and CAP and DeCAP treatments.

Table S4 Three-factor analysis of variance in protist and prokaryote OTU richness, Shannon diversity, Pielou evenness, and beta dispersal.

Table S5 One-factor analysis of variance in prokaryote abundance, protist and prokaryote OTU richness, Shannon diversity, Pielou evenness, and beta dispersal.

Table S6 Three-factor analysis of variance in prokaryote abundance.

Table S7 Prokaryote abundances measured by qPCR.

Table S8 Permutational multivariate analysis of variance and variance partitioning analysis of beta diversity of prokaryota and protists.

Table S9 Enriched Gene Ontology terms for root zone comparisons and root zone-specific gene expression.

Table S10 Number of differentially expressed genes between root zones, root hair treatments, and cap treatments.

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