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Review

C4 cereal and biofuel crop microbiomes

Xiaoyu Zai[1,2,3,4], Viviane Cordovez[5,*], Feng Zhu[6], Meicheng Zhao[6,7], Xianmin Diao[7], Fusuo Zhang[1,2,3,4], Jos M. Raaijmakers[5,8], and Chunxu Song[1,2,3,4,*]

Microbiomes provide multiple life-support functions for plants, including nutrient acquisition and tolerance to abiotic and biotic stresses. Considering the importance of C4 cereal and biofuel crops for food security under climate change conditions, more attention has been given recently to C4 plant microbiome assembly and functions. Here, we review the current status of C4 cereal and biofuel crop microbiome research with a focus on beneficial microbial traits for crop growth and health. We highlight the importance of environmental factors and plant genetics in C4 crop microbiome assembly and pinpoint current knowledge gaps. Finally, we discuss the potential of foxtail millet as a C4 model species and outline future perspectives of C4 plant microbiome research.

Highlights

Microbiomes play a crucial role in plant health, aiding nutrient acquisition and enhancing stress tolerance in C3 and C4 plants. While past microbiome research focused on the C3 model plant Arabidopsis (*Arabidopsis thaliana*), recent attention has shifted towards C4 food and biofuel crops such as maize, millet, sorghum, switchgrass, and miscanthus.

Similar to C3 plant microbiome assembly, C4 plant microbiome assembly is influenced by environmental factors, such as drought and nutrient availability, as well as plant genetics.

Root and shoot microbiomes of C4 cereal plants can extend diverse plant phenotypes of agronomic importance, including tolerance to drought and nitrogen stress, and enhanced yield.

Foxtail millet, a climate-resilient crop, holds promise as a model for C4 plant microbiome research due to its small, well-annotated genome and versatile genetic resources.

C4 plant adaptations to environmental challenges

C4 plants represent a specialized group of plants with unique photosynthetic adaptations that confer advantages in certain environmental conditions [1,2]. Maize, sorghum and millet are the most important C4 cereal crops due to their nutritional value and potential health benefits, such as the prevention of diabetes mellitus and cancer [3]. Next to these cereal crops, switchgrass (*Panicum virgatum L.*) and miscanthus (*Miscanthus* x *giganteus*) have received considerable attention as C4 biofuel crops due to their high growth rates, resilience to environmental stresses [4–8] and high photosynthesis rates (Box 1). Increasing the cultivation area of C4 crops, integrating the C4 photosynthetic pathway into C3 crops, as well as harnessing the functional potential of plant-associated microbiomes have been proposed as new sustainable strategies to secure and enhance crop productivity while reducing the greenhouse gas CO_2 [9,10].

Due to different biochemical and anatomical characteristics to concentrate CO_2, C4 plants have significantly higher photosynthesis rates and can survive in environments that are too harsh for most C3 plants (Box 1; [1,2]). A recent study indicated that C4 photosynthesis drives immediate plant population demography shifts, leading to expanded geographic ranges and increased population sizes compared to non-C4 populations of *Alloteropsis semialata* [11]. Metabolomic differences between C3 and C4 plants, such as *Flaveria robusta* and *Flaveria trinervia*, respectively, likely result from their divergent photosynthesis pathway [12]. Indeed, root exudates of C4 plants can exhibit higher concentrations of amino acids and organic acids than exudates of C3 plants [13,14]. C4 plants (i.e., *Digitaria eriantha*, *Themeda triandra*, *Chloris Gayana*) typically display larger mean root diameters, higher tissue densities, and starch concentrations, whereas C3 plants (i.e., *Festuca arundinacea*, *Phalaris aquatica*, *Lolium perenne*, and *Rytidosperma caespitosum*) have greater specific root lengths, nitrogen content, and soluble sugar concentrations [15]. These metabolic differences confer various advantages to C4 plants, including enhanced potential for polycyclic aromatic hydrocarbon degradation due to elevated concentrations of succinic, aconitic, and citric acids [13,16]. Collectively, these traits have provided C4 plants with enhanced resilience to environmental stressors such as high temperature, water scarcity and drought, salinity and nutrient deficiencies. Yet, modern 'omics methodologies are still underutilized in examining the bidirectional impact between C3 and C4 photosynthesis

[1]State Key Laboratory of Nutrient Use and Management, College of Resources and Environmental Sciences, China Agricultural University, Beijing, China
[2]National Academy of Agriculture Green Development, China Agricultural University, Beijing, China
[3]Key Laboratory of Plant–Soil Interactions, Ministry of Education, China Agricultural University, 100193 Beijing, China
[4]National Observation and Research Station of Agriculture Green Development, 057250 Quzhou, Hebei, China
[5]Department of Microbial Ecology, Netherlands Institute of Ecology, Wageningen, The Netherlands
[6]Key Laboratory of Agricultural Water Resources, Hebei Laboratory of Agricultural Water-Saving, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, 050021 Shijiazhuang, China

Box 1. Differences in photosynthesis between C4 and C3 plants

C4 plants contribute to approximately a quarter of global primary biomass production whereas they represent only 3% of all land plant species [1]. Photosynthesis plays an essential role in biomass production and in mitigation of global warning via consumption of the greenhouse gas CO_2. C4 plants have different mechanisms of carbon fixation than C3 plants, leading to nearly 50% higher photosynthetic efficiency [1]. The pathways associated with photosynthesis take place between mesophyll and bundle sheath cells in C4 plants instead of only in mesophyll cell of C3 plants [107,108]. The higher affinity for CO_2 by phosphoenolpyruvate carboxylase and the unique 'Kranz' anatomy (a small number of mesophyll cells surround the bundle sheath cells in concentric manner) enhances carbon fixation by the carboxylation of photosynthetic enzyme ribulose-1,5 bisphosphate carboxylase oxygenase (Rubisco) while inhibiting the oxygenation of Rubisco [107–109]. The reduced oxygenation of Rubisco results from elevated CO_2 levels in bundle-sheath cells subsequent to the decarboxylation of aspartate or malate, leading to reduced photorespiratory losses and enhancing photosynthetic efficiency in C4 plants [109,110]. 'Kranz' anatomy also causes shorter interveinal distances and an increased vein density, facilitating C4 biochemistry, thereby ensuring enhanced water use efficiency and photosynthesis rates [2,108]. The stomatal structure with dumbbell-shaped guard cells, instead of elliptical-shaped guard cells, allows faster responses to dynamic environmental factors, such as light, temperature and water [111]. Combined, these biochemical and anatomical traits allow a higher water use efficiency, nitrogen use, drought tolerance and photosynthesis rate of C4 plants.

[7]Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, 100081 Beijing, China
[8]Institute of Biology, Leiden University, Leiden, The Netherlands

*Correspondence:
v.cordovez@nioo.knaw.nl (V. Cordovez) and chunxu.song@cau.edu.cn (C. Song).

and microbial communities and functions. Culture-dependent and -independent approaches have further suggested that divergent traits of C4 plants, as compared to C3 plants, also have impacted on the root microbiome, with increased responsiveness to mycorrhizal inoculation [17], as well as the enrichment of Actinobacteria and Firmicutes [18,19].

Functional importance of C4 cereal and biofuel crop microbiomes

The **plant microbiome** (see Glossary), also referred to as the plants' second genome, impacts on plant growth, development, and tolerance to diverse biotic and abiotic stresses [20–22]. In recent years, several reviews and perspective papers have highlighted the progress in microbiome research of the C3 model plant Arabidopsis (*Arabidopsis thaliana*) and of C3 cereal crops, in particular rice and wheat (see Table S1 in the supplemental information online) [23–27]. Here, we review for the first time the current status of microbiome research on C4 cereal and biofuel crops. More specifically, we discuss the roles of the microbiome in growth and health of C4 cereal and biofuel crops, and the importance of environmental factors and plant genetics for microbiome assembly. In parallel, we highlight the similarities and differences with C3 plant microbiome research to identify knowledge gaps. Finally, we pinpoint the lessons learned from the C3 model plant species *A. thaliana* and discuss future directions of C4 plant microbiome research including the pros and cons of foxtail millet as a C4 model species.

Microbiome assembly is governed by dynamic temporal and spatial processes which are driven, in part, by environmental factors and plant host genetics [28,29]. Most studies on C4 plant microbiome assembly have focused on economically important cereal crops, such as maize, millet and sorghum, and the biofuel crops switchgrass and miscanthus (Figure 1 and see Table S1 in the supplemental information online). We now present the current insights into the different factors driving microbiome assembly and functions of C4 crops.

Environmental stresses

Some of the major environmental stresses faced by C4 plants include high temperatures, water scarcity and drought, high light intensity, salinity and nutrient limitation. Microbiome studies have started to shed light on the impact of plant-associated microorganisms on plant resilience to these stresses [30,31] as well as the reciprocal effects of these stresses on plant microbiome assembly. For example, drought stress profoundly impacted the composition of the sorghum rhizosphere microbiome [28], adversely affected symbiosis with arbuscular mycorrhizal fungi (AMF) [32] and enriched for Gram-positive bacteria, in particular *Streptomyces* [33]. Xu *et al*. [34] employed metagenomics to assemble 55 **metagenome-assembled genomes** (**MAGs**) of the

sorghum root microbiome, in which drought-enriched actinobacterial MAGs were identified. Comparative genomics further revealed that bacterial genes involved in iron transport and metabolism were more abundant in drought-enriched actinobacterial MAGs. Subsequent sorghum root transcriptome analysis indicated that the decreased expression of phytosiderophore iron transporter genes under drought conditions was associated with the abundance of drought-enriched Actinobacteria. This result was further supported in experiments with the maize mutant *tom1*, disrupted in the phytosiderophore transporter TOM1 and with reduced iron uptake, where Actinobacteria were enriched under drought stress. Exogenous addition of iron not only diminished the abundance of drought-enriched Actinobacteria but also eliminated their plant growth-promoting effects under drought stress [34]. Drought conditions caused perturbed gene expression related to iron metabolism in Arabidopsis [35] and barley [36]; however, the intricate interplay among drought, iron metabolism, and the plant root microbiome remains unresolved in these plants. Meta-analysis of mycorrhizal symbiotic relationship between C3 and C4 plants under salt stress showed that C4 plants had a more significant increase in phosphorus uptake efficiency compared with C3 plants. Furthermore, C4 plants, especially maize, exhibit significantly improved potassium uptake in response to mycorrhizal inoculation compared to other plant species [37]. Liu *et al*. [38] comprehensively reviewed the mechanisms by which plant-associated microorganisms can mitigate salt stress-induced negative effects on plants.

Another example of how environmental factors can impact C4 plant microbiome assembly was shown for maize, where low nutrient conditions affected maize root traits (e.g., root-to-shoot ratio, root dry weight, total root length, axial and lateral root length) and concomitantly impacted microbiome composition [39]. Network analyses, transcriptome, and microbiome data showed an enrichment of members of the Oxalobacteraceae family (i.e., *Massilia* species) on roots of maize plants grown under nitrogen-deficient conditions. Secretion of flavonoids, regulating plant–microbe interactions in distinct plant species [40–43], was associated with the enrichment of members of this bacterial family and the formation of lateral roots and nitrogen uptake [44]. Also, benzoxazinoids (BXs), released in root exudates, were shown to mediate microbiome assembly of the maize rhizosphere under controlled conditions and in the field [45–47]. The composition of the root-associated microbiome of wild-type maize differed markedly from the near-isogenic BX mutant deficient in 6-methoxy-benzoxazolin-2-one (MBOA) [45]. Moreover, soil conditioned with wild-type maize reduced plant growth in subsequent cultivation cycles and enhanced plant defenses upon herbivore attack as compared to the BX mutant. This so-called 'BX-legacy' affected resistance of cereals (maize, wheat) to the fungal pathogen *Zymoseptoria tritici* and the insects *Spodoptera frugiperda* and *Spodoptera littoralis* in a soil- and genotype-dependent manner [46]. Regarding the effects of the maize root microbiome on biotic stress tolerance, an earlier study by Niu *et al*. [48] elegantly showed that a simplified bacterial consortium can provide protection against the fungal pathogen *Fusarium verticillioides*. Although the underlying mechanisms involved in this microbiome-mediated protection of maize were not investigated, the work by Niu *et al.* illustrated that, similarly to the work of Finkel *et al*. [49] on *Variovorax* and *A. thaliana*, specific **microbiome-associated phenotypes** (**MAPs**) can be linked to only a few specific members of the root microbiome.

The microbiome of C4 biofuel crops, in particular switchgrass (*P. virgatum L*.) and miscanthus (*M.* x *giganteus*) [5,7], has great potential to enhance productivity and resilience to environmental stresses. **Core bacterial taxa** were identified for switchgrass and miscanthus, and included Proteobacteria (*Methylobacterium*, *Sphingomonas*, *Pseudomonas*) and Bacteroidetes (*Hymenobacter*) [5]. Significant correlations were found between **leaf epiphytic fungi** and leaf nitrogen concentration, leaf dry matter content, and height of switchgrass plants [6]. Furthermore, inoculation of switchgrass seedlings with **leaf endophytic fungi** improved plant performance

Glossary

Core bacterial taxa: a group of bacterial species that are consistently present in a specific host or environment, regardless of temporal or spatial variations. They are usually defined by a certain threshold of prevalence or abundance across samples from the same host or environment.

F1 hybrids: also known as filial 1 hybrids, are the first filial generation of offspring of distinctly different parental types. The benefits of F1 hybrids include higher yield, better quality, more uniformity, and greater resistance to stress.

Hybrid lines: the outcome of plant breeding approaches involving the deliberate mating of genetically diverse parent plants to produce offspring with improved characteristics, a common approach to enhance desired traits in crops.

Inbred lines: the result of prolonged self-fertilization or controlled cross-fertilization within a plant population over several generations, resulting in genetically uniform individuals sharing similar genetic information.

Keystone taxa: microbial taxa that are highly connected and influential in a microbial community, regardless of their abundance. They play critical roles in the structure, function, and stability of the microbiome. Keystone taxa are often identified by network analysis, which reveals the interactions and dependencies among different taxa in the community.

Leaf endophytic fungi: fungi colonizing inside leaf tissues.

Leaf epiphytic fungi: fungi colonizing leaf surfaces.

Metagenome-assembled genomes (MAGs): microbial genomes reconstructed from metagenome data, providing insights into the genomic makeup and functional traits of the microbiome present in a specific environment.

Microbiome-associated phenotypes (MAPs): the host's health and fitness attributes directly or indirectly influenced by interactions with the microbiome.

Microbiome genome-wide association studies (mGWAS): an approach employing variants in the host plant genome to identify associations between the plant genetic variation and the microbial composition or functions.

(survival rate, shoot biomass) depending on water availability. These strains displayed higher osmotic stress tolerance and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity and better growth on carboxylic acid substrates [4]. In this context, Giauque *et al*. [4] hypothesized that fungal production of the stress-related compound ACC and osmolytes may have contributed to the enhanced plant drought stress tolerance. Also, other members of the switchgrass microbiome, such as fungal endophytes (*Pleosporales*, *Hypoxylon*, *Meyerozyma guilliermondii*), potential beneficial members of bacteria (*Paenibacillus*, *Rahnella*, *Serratia*, *Pseudomonas*) as well as N-fixing bacteria (*Hyphomicrobium*, *Geobacter*, *Polaromonas*, *Methylocella*), were shown to improve plant growth and nitrogen acquisition [7,50,51]. To begin to understand the underlying mechanisms of these MAPs, metagenome and metatranscriptome analyses of the leaf microbiomes of switchgrass and miscanthus revealed the enrichment of genes associated with carbohydrate and amino acid metabolism, respiration, protein metabolism and stress response [8]. Collectively, these recent findings highlighted the large but yet untapped potential of the root and shoot microbiomes to improve productivity of C4 cereal and biofuel crops and their resilience to environmental stresses.

Plant domestication: the process of adapting wild plants for human use via selecting, improving and breeding plants that have human desirable traits, such as high yield, quality, resistance, and diversity.
Plant microbiome: the collective communities of plant-associated microorganisms and their genomic traits.

Host genetics

Plant genetics plays an important role in the assembly and functioning of the plant microbiome (Figure 2) [52]. The heritability of the root microbiome has been the focus of several C4 plant microbiome studies. For maize, root microbiome analysis of 27 **inbred lines** revealed that the maize genotype explained 19.1% of the variation in operational taxonomic unit (OTU) richness and a smaller yet significant fraction of the β-diversity (5.0–7.7%) [53]. Subsequent analyses

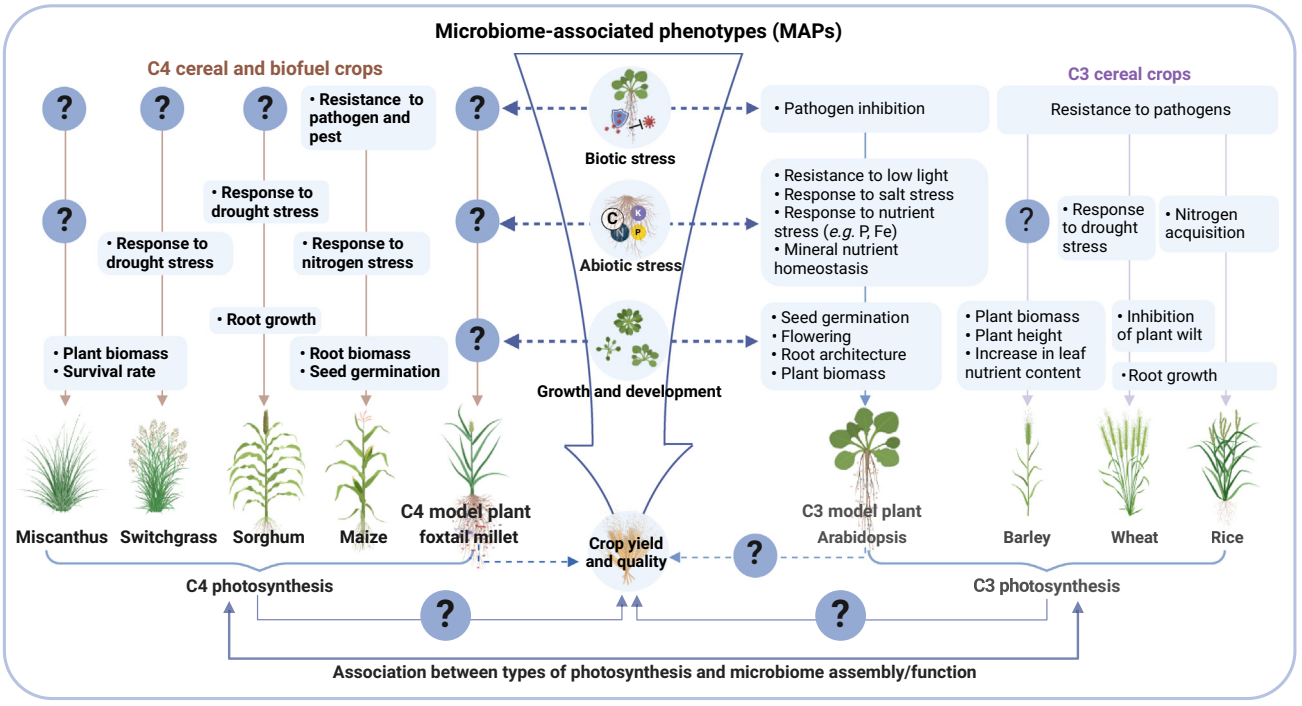

Figure 1. Overview of microbiome-associated phenotypes (MAPs) of C4 and C3 cereal and biofuel crops and the model plants foxtail millet and Arabidopsis. MAPs identified in C4 and C3 plant microbiome research include plant growth and development, nutrient acquisition and (a)biotic stress tolerance. Current knowledge gaps are indicated by question marks.

showed that the genetic distance of these 27 maize inbred lines was associated with the relative abundance of specific bacterial taxa in the root microbiome [54]. Wagner *et al*. [55] extended the work on maize leaf and root microbiome heritability and showed that **F1 hybrids** have higher microbiome diversity and higher abundance of specific taxa (*Moesziomyces*, *Pseudonocardia*, *Sphingomonas, Acinetobacter*) than their inbred parental lines. Wagner *et al.* [56] further demonstrated experimentally that soil microbes can differentially impact seedling growth of maize inbred and **hybrid lines**. The heterosis phenotypes (i.e., traits of hybrids exceeding that of their parents, such as higher root fresh weight and seed germination rate) were reduced under sterile soil conditions and could be restored by inoculation of a previously established synthetic community (SynCom) of seven bacterial strains [48] or of a soil (microbial) extract [56]. Previous studies have shown that heterosis largely results from different processes, including the biosynthesis of secondary metabolites, photosynthesis and stress response [56,57], but if and how the microbiome affects these processes or vice versa is still to be resolved.

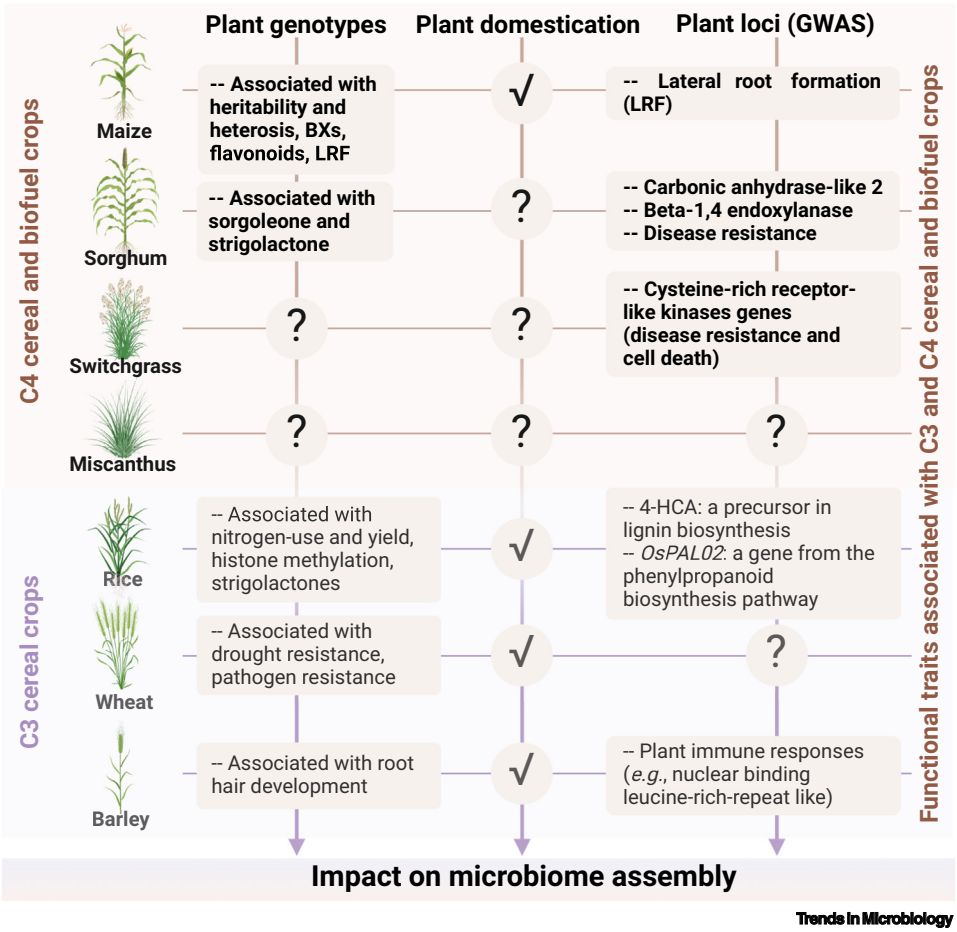

Figure 2. Impact of plant genetics on microbiome assembly of C4 and C3 cereal and biofuel crops. Plant genetics is categorized into three distinct classes concerning their impact on microbiome assembly: plant genotypes (encompassing cultivars, wild types, and mutants), plant domestication (encompassing wild relatives and their cultivated descendants), and plant loci unveiled through genome-wide association studies (GWAS), potentially involving mechanistic insights. Abbreviations: BXs, benzoxazinoids; LRF, lateral root formation; 4-HCA, 4-hydroxycinnamic acid.

Mutant lines in maize, associated with the biosynthesis of BXs and flavonoids [44–47,58], and in sorghum, linked to sorgoleone and strigolactones [59,60], along with those involved in lateral root formation [44], have been identified as key determinants shaping the microbiome assembly in these crops. To further identify host genomic regions that are associated with microbiome assembly, **microbiome genome-wide association studies (mGWAS)** by Meier *et al.* [61] showed that 622 plant loci of more than 200 maize genotypes were associated with more than 100 rhizobacterial groups [clustered from 3626 amplicon sequence variants (ASVs)]. Work by Deng *et al*. [62] showed that the genetic distance of 200 sorghum genotypes correlated significantly with root microbiome composition. Furthermore, sorghum loci on chromosomes 4 and 6 were significantly associated with the abundance of members of the Burkholderiales. The sorghum locus on chromosome 4 was 1.15 Mb in size and harbored genes encoding various traits including gamma carbonic anhydrase-like 2, a putative β-1,4 endoxylanase, and disease-resistance protein RGA2. The candidate locus on chromosome 6 was found to be associated with a distinct set of microbes that differed from those associated with the chromosome 4 locus. This suggests that different mechanisms drive the abundance, and presumably also the activity, of distinct groups of microorganisms associated with sorghum roots. Also for switchgrass, specific associations between plant genetics and root endosphere microbiome assembly were found. More specifically, the abundance of members of the Sphingomonadaceae correlated with plant loci that harbor genes involved in the immune response, signaling and secondary metabolism. For maize, He *et al.* [29] investigated the genetic mechanisms and adaptive traits of microbiome assembly of 129 accessions grown under nitrogen-, phosphorus- and water-limited conditions. Their results showed that the maize genotype and native environment were predictive of the rhizosphere microbiome composition. Interestingly, the impact of maize genotype on the rhizosphere bacterial community composition increased under stress. Maize genetic loci associated with changes in the microbiome composition included a gene putatively associated with the control of lateral root formation. Subsequent random forest models predicted *Massilia* as a **keystone taxon** linked to growth promotion and the heterosis phenotype (i.e., biomass). Isolation and reintroduction of *Massilia* ASV37 on maize roots showed induced lateral root formation in both inbred lines and hybrids under nitrogen-poor conditions, thereby experimentally supporting the results of the mGWAS analysis [29].

Moving aboveground, Wallace *et al.* [63] found that while most of the leaf microbiome composition of 300 maize lines is driven by environmental factors, a subset of bacterial taxa and inferred metabolic functions was significantly impacted by maize genetics. Only a few bacterial clades and diversity metrics were significantly heritable, while a much larger number of inferred metabolic functions were identified, including short-chain carbon metabolism, secretion, and nitrotoluene degradation, among others. VanWallendael *et al*. [64] further showed that environmental factors and host genetics impacted the successional fungal community of switchgrass leaves. By coupling mGWAS and RNA sequencing of the fungal microbiome of switchgrass leaves, they identified three cysteine-rich receptor-like kinases genes associated with the microbiome structure and postulated that host immunity is a key factor controlling leaf microbiome assembly, alike root microbiome assembly [25,62].

Domestication impact

Plant domestication, the process of selecting and breeding plants for human use, has led to several changes in plant morphology, including size and shape of fruits and roots, as well as functional traits, such as tolerance to (a)biotic stresses [65]. Recent studies have shown the impact of these changes on plant microbiome compositions and functions. Compared to C3 plants, there has been limited research on the impact of **plant domestication** on microbiomes of C4 cereal and biofuel crops (Figure 2 and Box 2). Adopting the ‘back-to-the-roots’ approach

Box 2. Impact of domestication of C3 cereal crops on microbiome assembly

Various studies examining the microbiomes of rice, wheat, and barley have highlighted the potential effects of domestication (see Figure 2 in main text and Table S1 in the supplemental information online). Compared with domesticated rice, specific bacterial and fungal taxa were enriched in the rhizosphere of wild rice [112,113]. Genes involved in carbon, amino acid and methane metabolism [114] and bacterial chemotaxis [115] were more abundant in the wild rice rhizosphere microbiome, while genes involved in nitrogen and lipid metabolism were enriched in the rhizosphere of domesticated rice [114]. Under biotic stress, genes involved in lignin and phenylpropanoid biosynthesis, and di-terpenoid metabolism were enriched in wild rice, whereas genes involved in fatty acid elongation, phenylpropanoid metabolism, wax, cutin and flavone syntheses were enriched in modern rice [116]. For wheat, a recent study by Yue *et al.* [117] further revealed that wild wheat harbors higher functional diversity of the microbiome, particularly in N transformation and P mineralization pathways. Studies by Bulgarelli *et al.* [118] showed significant differences in root-associated bacterial community composition between wild and domesticated barley. Nitrogen deficiency and high salt levels were associated with the alterations in microbiome composition [119,120], whereas differences in the rhizosphere microbiome composition correlated to difference in root exudate constituents [121]. *Pseudomonas* isolates, enriched in the rhizosphere of the modern cultivar, preferred hexose sugars, exudated with a greater content in the modern compared to the landrace. Studies also showed reduced levels of indole-alkaloids gramine in cultivated varieties as compared to wild barley [122,123]. The external application of indole-alkaloids gramine resulted in the enrichment of 18 bacterial genera in the barley rhizosphere, irrespective of the plant genotype [124]. To further disentangle the association between barley genetics on the microbiome, Escudero-Martinez *et al*. [125] performed quantitative trait loci (QTL) mapping and identified specific loci and candidate genes associated with rhizobacterial community assembly. The locus *QRMC-3HS* was identified as a significant determinant of microbiome assembly. Furthermore, by using comparative RNA-sequencing of the root tissue, they identified three candidate genes, including a nucleotide-binding-leucine-rich-repeat (NLR). How domestication has changed root exudation, and how putative changes in exudation affect the microbiome, is largely unknown. If and how root exudates affect the expression of specific functional traits in the root microbiome will be an exciting next step towards understanding the genetics and chemistry of domestication and microbiome assembly (see Figure 2 in main text).

proposed by Perez *et al.* [66], Abera *et al.* [67] recently analyzed the bacterial diversity in 48 agricultural fields sampled across the sorghum belt of Ethiopia, the center of origin of sorghum. By coupling DArT-SNP genotyping of 12 sorghum accessions with different domestication stages and phenotypic traits (selected from of a total of 59 sorghum accessions) and root microbiome analysis, they revealed that differentially abundant bacterial ASVs, in particular members of the Pseudomonadaceae, were associated with specific sorghum genotypes, in particular stay-green, drought-tolerant, and wild sorghum accessions. Also studies on wild and domesticated maize cultivars suggested that domestication and breeding affected the abundance of a small subset of the microbial community, with 3.7% of prokaryotic community members and 4.9% of fungal community members significantly associated with a particular maize genetic group (two teosinte, three inbred maize lines, five modern maize hybrids). Network analysis further revealed that microbial co-occurrence patterns in the rhizosphere of inbred maize lines were more similar to those of teosintes than of modern hybrids [68]. These findings suggested that domestication and breeding had a significant impact on the composition and assembly of rhizosphere microbial communities. A comparable investigation involving nine maize accessions (three teosintes, three landraces, three modern inbred lines) yielded similar findings via high-throughput 16S rRNA sequencing, thereby extending the observation that domestication and genetic improvement led to a greater diversity of the rhizobacterial community of maize [69]. Although not experimentally validated, Huang *et al.* [69] postulated that this increased diversity may enhance maize resilience to biotic stresses and promote nutrient acquisition. In line with this, Raaijmakers and Kiers [70] proposed a research roadmap for rewilding plant microbiomes to improve plant health and performance through identification and transplantation of ancestral microbiota to seeds, seedlings or adult plants of domesticated crop accessions.

Although exciting steps are being made towards disentangling the genetic and chemical basis of C4 plant microbiome assembly, validation of the causal relationship between the genetic basis, plant phenotypes and microbiome assembly and functions remain to be performed for

most putative associations reported to date. In future research, exploring a broader range of germplasm resources spanning various domestication stages or utilizing near-isogenic lines for mGWAS studies and validation experiments could provide valuable insights into the impact of domestication and breeding on the plant microbiome [71,72].

Importance of model species for C4 plant microbiome research

The model plant *A. thaliana* has been highly instrumental in resolving the molecular mechanisms of microbiome assembly and the underlying chemical dialogues (Figure 1 and see Table S1 in the supplemental information online). Also from a translational perspective, Arabidopsis microbiome research has served as a conceptual framework for the microbiome studies of other C3 crops – going from correlation to causation [23]. Most studies of the microbiome of C4 plants to date focused on maize, sorghum, switchgrass and miscanthus. These studies have provided a wealth of information on the heritability, genetics and functional potential of the microbiome. However, relying on a single model species to fully capture the spectrum of plant diversity, given the diverse phylogenetic, environmental, and physiological differences across species, especially between C3 and C4 species, will lead to unsuccessful application of microbial strains ([17–19,73]; Box 1). While direct evidence linking the divergent C4 photosynthesis to microbiome assembly remains elusive, numerous studies have shown the impact of plant genetics on microbiome composition and function (Figure 2 and see Table S1 in the supplemental information online). Moreover, the plant microbiome, especially endophytes, holds significant potential for future strategies to enhance plant photosynthesis [74,75].

A key constraint of using C4 cereal and biofuel crops as model organisms is their large genome sizes (maize: ~2.1 Gb; sorghum: ~800 Mb; switchgrass: ~1.2 Gb; miscanthus: ~7 Gb) [76–80], large plant sizes and relatively long life cycles, which limit the adoption of genetic transformation [81]. Given the greater efficacy of microbiota transplantation in promoting plant growth among closely related hosts [82], foxtail millet (*Setaria italica*) may serve as a C4 model plant for investigating microbiomes in other C4 plants. Foxtail millet is receiving increased interest as a model plant due to its relatively small and well annotated genome (~490 Mb) [83–85], available databases [86–89] and genetic transformation systems [90–92]. Foxtail millet is regarded as a C4 model plant for both fundamental and applied research. Comparative genome analysis showed that foxtail millet 'Zhang gu' shares 69.5%, 65.2%, and 32.1% homology with rice, sorghum, and maize, respectively [84]. Foxtail millet genes responsible for various agronomically important traits have been successfully tested and expressed in other crops, including maize, tobacco, and rice, to enhance tolerance to abiotic stresses, photosynthetic efficiency and anthocyanin biosynthesis. For instance, *SiATG8a* [93], *SiMYB3* [94] and *SiMYB19* [95] were expressed in rice to withstand nitrogen starvation and salt stress, while *SiATG8a* [93], *SiWLIM2b* [96], *SiMYB56* [97], and *SiMYB19* [95] improved drought tolerance in rice under laboratory or field conditions. Furthermore, foxtail millet genes associated with the C4 photosynthetic pathway, for example, *SiPPDK* encoding pyruvate orthophosphate dikinase and *SiME* encoding NADP-malic enzyme, were successfully introduced into rice and resulted in increased photosynthetic efficiency, growth and yield [98]. The introduction of the acetyl-coenzyme A carboxylase (ACCase) gene into maize increased resistance to the herbicide sethoxydim and boosted the oil content of maize seeds [99]. Coexpression of *PPLS1* and *SiMYB85* from foxtail millet in tobacco leaves increased the level of anthocyanin, which plays a key role in protection against pathogens and abiotic stress [100,101]. These examples highlight the potential of foxtail millet as a genomic resource to improve agronomic traits of both C4 and C3 crops.

The foxtail millet microbiome, its assembly and functioning have not been extensively studied (Figure 1 and see Table S1 in the supplemental information online; [102–105]). Initial foxtail

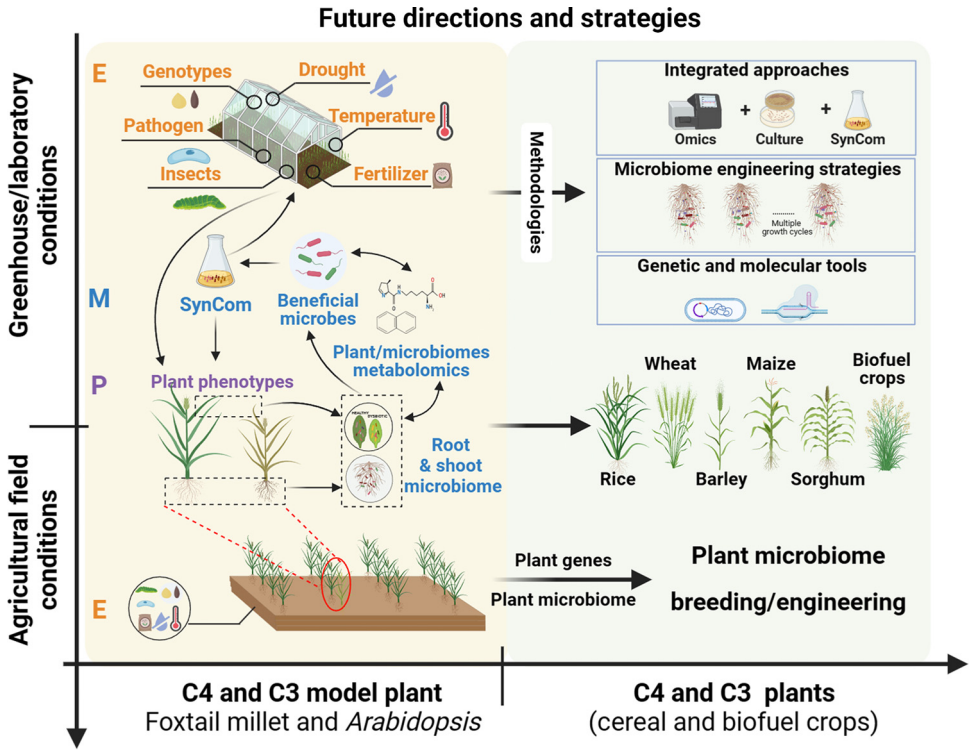

Figure 3. Future directions for microbiome research of C4 plants. Future plant breeding strategies can be guided via coupling the mechanistic understanding of the interactions between environment (E), plant (P) and microbiome (M) under laboratory conditions with results under greenhouse and field conditions. Diverse approaches, such high-throughput sequencing, metagenomics, metatranscriptomics, proteomics, metabolomics and microbiome genome-wide association study (mGWAS), microbiome engineering strategies (e.g., experimental evolution, metabolic engineering and synthetic biology) and genetic and molecular tools, such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing and genetic transformation, can be integrated, with the use of the model plants foxtail millet and Arabidopsis, to help design future microbiome-based strategies for sustainable production of C4 and C3 cereal and biofuel crops.

millet microbiome studies showed an enrichment of Actinobacteria in the rhizosphere upon leaf infection with the fungal pathogen *Ustilago crameri* [102]. Jin *et al*. [103] showed that foxtail millet yield was negatively correlated with Actinobacteria (Pseudonocardiaceae, Streptomycetacea, Nocardioidaceae and Gaiellaceae) but positively correlated with the abundances of Firmicutes (mainly Bacillaceae). Niu *et al*. [104] further showed that inoculation of foxtail millet seeds with *Pseudomonas fluorescens*, *Enterobacter hormaechei*, and *Pseudomonas migulae* promoted seed germination and growth under drought stress. Recently, Wang *et al*. [105] employed GWAS to explore the relationship between plant genotypic and phenotypic traits (e.g., agronomic traits) and the root microbiome. Candidate genes related to plant growth and development (e.g., autophagy-related protein 8C, ethylene-responsive transcription factor), abiotic stress response (e.g., phosphatidylinositol-specific phospholipase C, serine/threonine-protein kinase SAPK9) as well as plant immune responsive genes and pathogen defense genes were identified. The effects of ten representative isolated strains on the growth of foxtail millet were validated in bioassays. Wang *et al*. [105] also demonstrated that foxtail millet shares similarities in heritable microbiome members (microbes with relatively higher heritability, that is, the extent to which their traits are

transferred through successive generations) with sorghum and maize, especially in seven bacterial orders: Bacillales, Actinomycetales, Burkholderiales, Rhizobiales, Myxococcales, Sphingobacteriales, and Xanthomonadales. This suggests a similar plant–microbiome coevolution pattern across C4 cereal and biofuel crops, supporting further exploration of foxtail millet as a model for C4 microbiome research. Despite these exciting yet fragmentary results, more mechanistic understanding of the functional importance of the microbiome for the various C4 plant agronomic phenotypes is needed (Figure 3).

Concluding remarks and future perspectives

Future C4 microbiome research requires an integrated approach to obtain a deeper understanding of the link between plant genetic factors and microbiome composition and functions (Figure 3). Also, establishing genomically and phenotypically well-characterized microbial repositories (e.g., the '*At* microbial collection' for Arabidopsis [106]), in combination with field trials, will be important to understand the mechanisms underlying host-to-microbe, microbe-to-host and microbe-to-microbe interactions. Furthermore, the availability of germplasm resources of wild progenitors and modern cultivars as well as soils from their native habitats from the centers of origin can be exploited to decipher the impact of domestication on C4 crop microbiome assembly and its impact on plant phenotypes. In addition, it will be intriguing to investigate if and how the differences in photosynthesis with C3 plants influence the composition and function of plant microbiomes, and in turn how these microbes contribute to productivity of C4 crops. These will provide valuable and exciting prospects for further research into the relationship between C4 plant genetics and microbiome assembly and functioning. To achieve a comprehensive understanding of C4 plant microbiome assembly and functions, foxtail millet can be instrumental as a model plant species in parallel with C4 crops that are of high importance for enhancing sustainable food and energy supply under climate change conditions (see Outstanding questions).

Outstanding questions

How does the composition of microbiomes in C4 and C3 plants differ, especially concerning the impact of environmental factors and plant genetics? Do the different photosynthetic pathways of C3 and C4 plants affect microbiome assembly and functions? How do microbial adaptations to the specific biochemical and anatomical characteristics of C4 photosynthetic pathways affect plant–microbe interactions and the broader environmental adaptability of C4 crops?

What is the taxonomic and functional diversity of understudied members of the C4 plant microbiome, such as fungi, oomycetes, viruses, protozoa and archaea? How do these cross-kingdom microorganisms interact and respond when C4 plants are exposed to stress conditions?

How can microbiome-associated plant phenotypes (MAPs) identified for C4 crops be utilized in breeding programs to improve crop resilience and productivity, particularly in the context of climate change? What are the molecular and chemical mechanisms underlying MAPs?

How can cultivation of climate-resilient crops, such as millet, sorghum and biofuel crops, in synergy with beneficial members of their microbiomes, address global food security challenges, enhance agricultural sustainability in arid regions, and contribute to the mitigation of greenhouse gas emissions?

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Declaration of interests

The authors declare no competing interests.

Supplemental information

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