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Foundational Research Towards Plant Microbiome Engineering Capability

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by

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Committee in charge:

Doctor Peggy G. Lemaux, Co-Chair
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Professor Devin Coleman-Derr
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Abstract

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With the threat of climate change becoming more apparent in recent years, it is clear that the increased severity and regularity of extreme weather events will inevitably impact our ability to maintain agricultural productivity in the future. One relatively unexplored avenue towards increasing plant health and resiliency has also recently emerged, however, as the microbes which live in and around plant roots have been shown to influence biomass and yield, as well as resistance to both biotic and abiotic stresses. Therefore, an ability to manipulate and engineer these microbial communities, referred to as the plant microbiome, has the potential to help ensure food security amidst environmental turmoil.

Unfortunately, this dream of an engineered, beneficial plant microbiome has yet to be realized. This is in large part due simply to the nascency of the field of research as a whole, with large gaps in our basic understanding still needing to be addressed. Work must be done to generate a more complete understanding of the microbes themselves and the processes by which they are regulated by plant hosts, but also towards developing more robust and efficient plant engineering capabilities in order to realize the potential real-world benefits of an engineered microbiome. The research presented in this dissertation was undertaken in an effort to address these current shortcomings, and hopefully contribute to future efforts aimed at harnessing and utilizing the plant microbiome for societal gain.

First, this work investigates factors influencing the fungal microbiome assembly in a forest tree, the coast redwood *Sequoia sempervirens*. Because most research has been performed in model or crop species in agricultural or fully artificial conditions, investigating patterns of microbiome structure in a gymnosperm growing in a natural forest setting will allow us to assess the universality of previously reported phenomena related to the plant microbiome. We found that, as expected, soil chemistry and pH in particular is a strong determinant of root-associated fungal communities. We also demonstrated that forest continuity and heterogeneity influence the soil microbiome, which shows that principles of landscape ecology can indeed be useful in deepening our understanding of microbial ecology. Finally, we discovered that redwoods have

specialized organs which function to morphologically compartmentalize symbiotic arbuscular mycorrhizal fungi, which serves to highlight the need for holistic assessment of factors influencing the plant root microbiome rather than a narrow focus on, for instance, singular -omic techniques.

To build upon the theme of holistic rather than narrow microbiome investigations, this research goes on to summarize an ambitious holo-omic characterization of how sorghum and its microbiome interact with drought stress. Termed the EPICON Project, this undertaking serves as an exemplary case-study of using multiple -omic techniques, termed holo-omics, in order to better understand complex interactions governing microbiome assembly and development. Specifically, this work highlighted the correlation between a key metabolite, glycerol-3-phosphate (G3P), as well as transcriptional response to reactive oxygen stress (ROS) mitigation, with reproducible shifts in the sorghum root microbiome under drought. These findings serve as testaments to the power of holo-omic studies in progressing the field of plant microbiome research towards the ultimate goal of being able to engineer desired phenotypes relevant to world agriculture as it faces climate change-induced challenges.

Finally, we used the lessons learned through holo-omic investigation of the sorghum root microbiome in conjunction with novel plant transformation techniques and the CRISPR-Cas9 gene editing system in order to provide a framework for future efforts aimed at microbiome engineering. Although significant progress was made in improving overall transformation efficiency in sorghum through the use of the morphogenic genes Babyboom (BBM) and Wuschel (WUS), the successful editing of our target genes remained elusive. Nonetheless, we hope that this work has helped lay the groundwork for future efforts towards realizing an engineered plant root microbiome.

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Chapter 1

Introduction

Motivation

Unless significant changes and technological advancements are made, the current food system could well collapse by 2050. Several intersecting emergencies are due to converge right around the middle of this century: fishery collapse (Worm et al. 2006; Holsman et al. 2020), mineral phosphorus exhaustion (Smil 2000; Steen 1998; Cordell, Drangert, and White 2009), groundwater depletion (de Graaf et al. 2019), topsoil erosion (Dubois and Others 2011), and of course the greatest threat of all, rapid anthropogenic climate change (Rosenzweig and Parry 1994; Asseng et al. 2015; Hasegawa et al. 2018; Ray et al. 2019). As terrifying as this is, fretting over the future of societal food supply is not a 21st century invention. Over two hundred years ago Thomas Malthus was the first to envision a possibility where exponential population growth outstrips arithmetic increases in agricultural production (Malthus 1809). Thus far humanity has managed to avoid this fate thanks to scientific and technological advances, most notably in the 1960s when classical breeding methods led to the development of high yielding grain varieties during what is now referred to as the Green Revolution (Khush 1999). The most important cultivars developed during this time are the semi-dwarf varieties of rice and maize, which have been shown to possess loss-of-function mutations in their gibberellic acid transduction pathways, leading to their characteristically short stature and improved harvest index (Hedden 2003).

Although the breeding of these semi-dwarf high yielding varieties was undoubtedly a landmark achievement in plant biology as well as a humanitarian triumph, the process of finding noteworthy mutants and breeding them repetitively into agricultural cultivars is a slow and laborious process. Today, these exact same mutants could be generated much more rapidly with the application of genetic engineering, which includes both transgene insertion and CRISPR/Cas9 editing. Sequence-specific targeted gene editing utilizing CRISPR/Cas9 endonuclease technology allows for the deletion of specified regions of the genome (Jinek et al. 2012), and can be used to cause frame-shift knockouts in genes of interest. This means that the semi-dwarf phenotype of the Green Revolution can be replicated with ease in a fraction of the time it took to breed it into cultivars. The ability to edit genes has also expanded our ability to investigate genes of unknown function, as they can be selectively knocked out in order to observe phenotypic changes and provide the basic science groundwork for future genetic engineering and breeding projects. This exploration is pivotal, as new engineering technologies also require new engineering targets. Excitingly, the recent breakthroughs in genetic engineering have coincided with advances in DNA sequencing technology which allow for the investigation of a relatively unexplored yet increasingly prominent aspect of plant biology: the microbiome (Caporaso et al. 2012).

Outside of the laboratory, no plant lives in isolation. Every individual plant in both natural and agricultural systems is in constant contact and communication with a diverse community of microbes - bacteria, fungi, archaea, viruses, and protists. This community of plant-associated microbes is known collectively as the microbiome, and it works in concert with the host plant to provide what is essentially a pool of additional genomic possibilities. Because of the sheer diversity of soil microbes and their participation in nutrient acquisition (Salas-González et al. 2020; Jacoby et al. 2017), the

root microbiome presents a particularly dynamic and consequential interface of plant-microbe interaction, often being compared to the human gut microbiome in terms of both function and importance. Research has shown that the root microbiome has a significant effect on plant health and productivity, being capable of protecting plants against biotic stressors like pathogens (Compant et al. 2019; Saikkonen, Nissinen, and Helander 2020) as well as abiotic stressors such as drought (Rodriguez et al. 2019; Xu et al. 2018). Changes to the root microbiome can also directly modulate plant development by influencing biomass accumulation (Santoyo et al. 2016; Orozco-Mosqueda et al. 2018) and even flowering time (Panke-Buisse, Lee, and Kao-Kniffin 2017). Therefore, the ability to engineer the microbiome, either through genetic modification of the plant host or manipulation of the microbes themselves, has the potential for multiple real-world applications, especially in agriculture. However, due to technical limitations in addition to a dearth of basic biological knowledge, the engineering of a microbiome to produce repeatable, desirable plant phenotypes has remained elusive. During my thesis work, I attempted to move the field forward in three areas I believe are foundationally crucial to both plant and microbiome engineering that must be addressed before this lofty goal can be achieved. These are: 1) understanding of the microbiome and the abiotic processes influencing it, 2) understanding how the plant exerts control over its microbiome, and 3) developing tools to allow us to genetically engineer more species of plants more easily in order to develop a more informed understanding of the plant-microbiome relationship.

Understanding the Microbiome

The first knowledge gap that must be overcome on the path towards an engineered microbiome is straightforward: we need a better understanding of the microbiome itself. In particular, researchers need a more complete view of the microbial taxa present in the microbiome as well as the elucidation of abiotic forces shaping their community structure. To date, most plant microbiome research has been more focused on discovering who is there, leveraging molecular techniques such as 16S and ITS sequencing to determine which microbial taxa live with certain plants or in certain places (Brunel et al. 2020; Griffiths et al. 2011; Terrat et al. 2017). Even nascent, purely descriptive studies have already revolutionized plant biology and microbiology, revealing an intricate ecosystem with thousands of taxa, many of which were completely unknown until the advent of community metagenomics. We have learned that the soil harbors the most complex microbial ecosystem on earth, and a multitude of these microbes interact with and oftentimes live inside plants in the environment (Compant et al. 2019; Fitzpatrick et al. 2020).

Recently, there has been more focus shifted towards investigating why those taxa are present in particular environments, and how abiotic factors might influence microbial community structure. For instance, (Xu et al. 2018) showed that the root microbiome of sorghum (*Sorghum bicolor* (L.) Moench) undergoes predictable shifts in bacterial community structure when the plant is subjected to drought stress, with monoderm bacteria like Actinobacteria becoming more abundant while diderm bacteria become less so. However, the fact that the discovery of such a dramatic effect was

groundbreaking reveals that there is still a huge amount of basic research yet to be done in order to expand our limited knowledge about the plant microbiome.

Nonetheless, cutting edge molecular techniques have begun to be applied to plant microbiome research in order to more fully characterize and dissect the interactions therein. In addition to their presence and abundance (Antunes et al. 2016), metatranscriptomics, for instance, has allowed researchers to investigate the transcriptional activity of microbes in an environment . This has led to the discovery of novel regulatory mechanisms involved in microbial recruitment, such as iron homeostasis (Xu, Dong, et al. 2021). Additionally, techniques have been developed to extract complete microbial genomes from environmental samples, which should in the future give researchers a better idea of the functions these organisms are performing (Chen et al. 2020).

However, because plant microbiome research has been built on the foundation of basic genetic research in plants, many pre-established biases have appeared in this emerging field. For example, while model and agriculturally important crops have been relatively well studied in artificial and manipulatable environments, only a few studies have investigated the microbial communities interacting with long-lived plants in the wild. Although drought-induced Actinobacterial enrichment has now been shown in a diverse array of angiosperm species such as sorghum, rice, millet and arabidopsis as well as chicory, amaranth, cinquefoil, asparagus, and other non-model organisms (Fitzpatrick et al. 2018; Naylor et al. 2017), this pattern has only very recently been reported in a gymnosperm species along a natural precipitation gradient at the ecosystem scale (Willing et al. 2020). This study supports the idea that this phenomenon of Actinobacterial enrichment under drought is a conserved response universal to the entire plant kingdom, not just unique to angiosperms living in artificially irrigated conditions. It also shows that while the lack of focus on long-lived and wild-grown plants is excusable in plant molecular genetic research due to technical limitations, many of these limitations do not actually apply to microbiome studies. Generation time is immaterial, for example, since cross-breeding is not necessary. Plant height is no longer an issue if the organisms are not being grown in greenhouses or growth chambers. Because microbiome studies can be performed on any kind of sample, I would argue that one of their greatest strengths is the fact that researchers are now able to apply molecular genetic techniques to plant species previously inaccessible to such studies, particularly large forest trees in their natural habitats.

Expanding the scope of plant microbiome research beyond easily manipulatable artificial habitats will also allow the field to begin to address important unanswered questions in plant-microbe interactions that simply do not apply to agricultural systems. For instance, how does habitat loss and fragmentation impact the microbiome present across an ecosystem? While an answer to this question will most likely not result in direct agricultural yield increases, it might help ensure the health and functionality of the world's forests, which act as a carbon sink to help mitigate global climate change (Sohngen and Mendelsohn 2003) while also being particularly vulnerable to the detrimental effects of said climate change (Gauthier et al. 2015).

Clearly, a better understanding of how microbiomes interact with abiotic forces has implications for agriculture as well as conservation and climate change mitigation

and response, but another important question to be addressed is the amount of control the plant exerts on its own microbiome structure. Any patterns of abiotic influence over the microbiome will be out of context without being able to assess their interplay with the physiology and genetics of the plants acting as hosts and carbon sources, so a better understanding of the plants themselves is invariably required as well.

Understanding the Plant

As with any symbiosis, plant-microbiome interactions are bidirectional. The microbial community can elicit phenotypic changes in the plant while the plant also exerts some control over the microbes inhabiting the soil surrounding the root system as well as the microbes living inside the roots themselves. While some obvious microbe-induced phenotypes have been described, especially with regards to growth promotion (Glick et al. 1999; Olanrewaju, Glick, and Babalola 2017; Santoyo et al. 2016) and disease suppression (Compant et al. 2005; Pascale et al. 2019), relatively little is known about the mechanistic causes of these observed phenotypes. A handful of high-profile plant-microbe interactions are well characterized at a molecular level, such as with legume root nodule-inducing *Rhizobia spp.* (Oldroyd 2013; Qi Wang, Liu, and Zhu 2018). Additionally, some speculation has been made about the role of plant hormones either induced or produced by microbes influencing plant growth and vitality (Foo et al. 2019; Spaepen 2015), but this phenomenon is neither fully understood nor universally applicable. A better understanding of the microbial processes which elicit desirable plant phenotypes would therefore facilitate more reproducible and consistent elicitation of these phenotypes through human intervention, with the application of engineered synthetic communities for example. However, because the plant-microbiome symbiosis is bidirectional, it is equally important to investigate the ways in which plant biochemistry, physiology, and morphology influence and exert control over the composition of the microbiome.

With regards to biochemistry, the ability of plants to shape their microbiome through the production and exudation of certain molecules has in fact been studied in depth. This phenomenon is referred to as rhizodeposition, and it encompasses the release of both primary and secondary metabolites into the surrounding soil. Carbohydrates are a major component of these deposited compounds, taking the form of sugars either exuded from the roots (Kawasaki et al. 2016; Traoré et al. 2000) or traded endophytically with specific microbes like arbuscular mycorrhizal fungi (AMF) in exchange for mineral nutrients (Roth and Paszkowski 2017), as well as cell wall debris sloughed off of the root cap as it penetrates the soil matrix (Dennis, Miller, and Hirsch 2010). The carbohydrates released from plants in these manners are not insignificant, with some estimating up to 20% of a plant's fixed carbon being exuded into the soil (Sasse, Martinoia, and Northen 2018). Plants also exude amino acids (Moe 2013), organic acids (Qin, Hirano, and Brunner 2007), fatty acids (Jiang et al. 2017), and other secondary metabolites from their roots as well (Badri et al. 2008; Korenblum et al. 2020), all of which have the potential to influence plant microbiome composition and function. Some of these secondary metabolites, particularly strigolactones, are even known to be signalling molecules specifically used to attract beneficial microbes and

establish symbioses (Lanfranco et al. 2018; Rehman et al. 2018; Oldroyd 2013). Despite being relatively well studied, the biochemical influence on root microbiome structure remains too convoluted to be fully understood, itself being influenced by plant genotype but also abiotic factors such as drought as well as plant age, development, and physiology.

Therefore, plant physiology and development are also becoming increasingly recognized as important regulators of microbiome structure in their own right. Research has shown for instance that the microbiome develops in a predictable way throughout the life of a plant, but that this directional development is interrupted by the onset of drought (Xu et al. 2018). Studies have also suggested that root architecture (Saleem et al. 2018), the thickness of the suberized layer of the endodermis (Salas-González et al. 2020), and the nutritional status of the plant (Caddell, Deng, and Coleman-Derr 2019) can all influence the root microbiome community composition. Interestingly, all of these factors are also heavily influenced by abiotic factors such as drought (Redillas et al. 2012; Franke and Schreiber 2007; Y. Hu and Schmidhalter 2005), adding another layer of complexity to the poorly understood confluence between plant physiology and the microbiome.

In addition to biochemical and physiological factors shaping the root microbiome, plant morphology has long been known to exert especially precise control over endophytic microbes in particular. For instance, it is well understood that certain plants produce specialized organs in their root systems called nodules which foster symbiotic endophytes. Such root nodules have been shown to house *Rhizobium spp.* in legumes (Oldroyd and Downie 2008), *Frankia spp.* in alders (Baker, Torrey, and Kidd 1979), cyanobacteria in certain cycads (Gutiérrez-García et al. 2019), and AMF in southern hemisphere conifers (Dickie and Holdaway 2011; Padamsee et al. 2016). Some microbe-related morphological changes are even more subtle than these, such as the transition to dichotomous root branching induced by ectomycorrhizal fungi (EMF) in *Pinus spp.* (Heller et al. 2012). Additionally, some plant pathogens are known to elicit macro-scale morphological changes in their hosts, one example being the induction of pseudoflowers in *Arabis spp.* by the rust fungus *Puccinia monoica* (Roy 1993). With the exception of root nodules in legumes, the underlying genetics behind these myriad microbe-induced morphological changes are very poorly understood, as are their contributions to overall plant health. What is more, because these morphological microbiome-filtering mechanisms are often subtle and underground yet pervasive across the plant kingdom, while encompassing microbial partners across multiple kingdoms, it stands to reason that more of these specialized structures exist and are waiting to be discovered.

Truly, the ability to interrogate and understand the genetic bases underlying the biochemical, physiological, and morphological strategies plants use to shape their microbiomes is of utmost importance, as this will allow researchers the ability to engineer microbiomes by engineering the plants. Considering what we know about the importance of the microbiome to plant health, many agricultural applications of this technology already exist, as well as await discovery. Unfortunately however, an oftentimes extreme genotype dependency halters current plant transformation capabilities, with even important crop species such as maize having only a few specific

cultivars which can be transformed (Yadava et al. 2016). Because many agriculturally relevant cultivars are therefore unable to be transformed, expanding the number of genotypes amenable to transformation is necessary to engineer plant microbiomes which are themselves agriculturally relevant.

Ability to modify difficult-to-engineer plants

As stated previously, genetic engineering through plant transformation is a technology that can be used to develop new cultivars with agriculturally relevant phenotypes, but this technology is also equally impactful in terms of studying unknown gene function in order to lay the groundwork for future crop improvements. For instance, *Agrobacterium tumefaciens*-induced gene transformation has been utilized to develop the most complete T-DNA knockout library available to the plant biology community in the model dicot plant, *Arabidopsis thaliana* (Berardini et al. 2015). This resource has allowed researchers the ability to investigate the function of every single gene in the Arabidopsis genome in order to make hypotheses about gene function across the plant kingdom. This monumental contribution to the field was made possible in part by the fact that Arabidopsis is among the easiest plant species to transform, thanks to its amenability to the floral dip method (Clough and Bent 1998). Unfortunately, the vast majority of plant species have proven infinitely more difficult to genetically engineer, especially when considering monocot species such as cereal crops (Cheng et al. 2004). This is due at least in part to Agrobacterium's native range as a plant pathogen being limited almost exclusively to eudicots (De Cleene and De Ley 1976). However, additional factors, such as the strain of Agrobacterium, the choice of selection methods, the appropriate explant type, the composition of culture media, and the availability of amenable plant genotypes, have caused Agrobacterium-mediated gene transformation success in monocots to lag far behind its application in eudicots (Hiei, Ishida, and Komari 2014).

Moreover, because the monocot genotypes actually amenable to transformation have until recently not been the elite lines grown agriculturally, any engineered phenotypes must be repetitively backcrossed into those lines in order to achieve any real-world impact. This significantly dampens the benefit of genetic engineering in terms of accelerating the production of new agriculturally relevant cultivars. Nevertheless, a recent breakthrough in transformation technology incorporating the developmental regulators Babyboom (BBM) and Wuschel (WUS) has the potential to greatly expand the range of monocot genotypes amenable to transformation, with the added benefit of greatly decreasing the amount of time required to generate engineered lines (Lowe et al. 2016).

Both BBM and WUS are transcription factor master regulators of cell proliferation. BBM controls cell proliferation during embryogenesis (Boutilier et al. 2002), while WUS is integral to the maintenance of a reservoir of pluripotent stem cells within the shoot apical meristem (Laux et al. 1996). The tightly regulated overexpression of these two genes in maize led to the spontaneous generation of somatic embryos growing directly from cells on the outer surface of the explant tissue (Lowe et al. 2016). This strategy not only obviated the need for a lengthy tissue culture process progressing

through a callus phase that often leads to somaclonal variation (Bregitzer, Halbert, and Lemaux 1998), it has also allowed for the transformation of maize and sorghum genotypes which were previously recalcitrant (Mookkan et al. 2017; Che et al. 2018; Lowe et al. 2018). The BBM/WUS-assisted strategy of plant transformation therefore has revolutionized plant genetic engineering as well as gene function investigations, which are crucial for understanding the large number of genes of unknown function. Consider the fact that sorghum has numerous sequenced genomes, but around half of its genes lack any kind of functional annotation whatsoever. The ability to manipulate and study its genes, outside of the constraints of genotype dependence in transformation, would pave the way for future basic research as well as improved cultivars for agriculture.

Although the tools used to study the microbiome are plant genotype agnostic, the microbiome itself is undoubtedly influenced by plant species and cultivar differences (Simonin et al. 2020; Stopnisek and Shade 2021; Cregger et al. 2018)). The ability to study and engineer plants, therefore, needs to be possible regardless of plant genotype if researchers are to be able to engineer the plant microbiome in the pursuit of food security. Overcoming limitations to the plant transformation process therefore truly represents one of the last hurdles in the way of engineering the root microbiome.

Conclusion

The confluence of a panoply of dire threats to humankind's food system in the 21st century requires a coordinated effort by the scientific community to imagine creative solutions aimed at mitigating their effects in order to ensure the survival of our species and society. In less than a decade, the emerging field of plant microbiome study has shown exceptional potential for the discovery of novel avenues towards increased food security. Considering what we already know about the impact the microbiome can have on plant health and fitness, the ability to engineer reproducible microbial community phenotypes would help translate theoretical understandings into agriculturally relevant solutions. Presently however, three distinct aspects of plant and microbial biology still need further investigation before engineered microbiomes can become a reality. Put simply, we need to know what microbial taxa comprise the community, determine what biotic and abiotic signals determine their presence, and also acquire the technical ability to modulate these signals and affect desired plant phenotypes. My thesis work addresses all three of these avenues aimed towards an engineered microbiome. Specifically, investigating abiotic influences on the coast redwood (*Sequoia sempervirens*) fungal microbiome reveals correlations between forest connectivity and microbiome community structure. However, the characterization of a previously unknown nodule-like structure housing symbiotic fungi found along redwood roots also reminds us that plants have multiple mechanisms to select for beneficial microbes and buffer against abiotic factors influencing their microbiome structure (Chapter 2). The use of multiple -omic techniques to identify genes and metabolites potentially involved in microbiome structuring in sorghum illustrates the utility of holo-omics and the necessity to renew focus on integrating various investigatory platforms to develop more complete and evidence-based hypotheses around plants'

control over their microbiomes, particularly under adverse conditions such as drought (Chapter 3). Finally, development of the BBM/WUS developmental gene transformation method in sorghum to radically enhance transformation speed and efficiency paves the way for being able to genetically transform a much wider spectrum of plant genotypes and facilitate future exploratory studies aimed at gene function as well as attempts to engineer microbiome-relevant plant phenotypes (Chapter 4).

Chapter 2
Keep your friends close: Host compartmentalization of microbial communities facilitates decoupling from patterns of island biogeography

Contributions

The contents of this chapter are derived from a collaboration with Dr. Claire Willing, a former graduate student in the lab of Dr. Todd Dawson. The entire chapter represents a manuscript currently in review that I am listed as co-first author with Dr. Claire Willing. The full citation for said manuscript is as follows: Willing, C. E.*, Pierroz, G.*, Guzman, A., Anderegg, L.D.L., Gao, C., Coleman-Derr, D., Taylor, J, Bruns, T.D, and Dawson, T. E.. Keep your friends close: Host compartmentalization of microbial communities facilitates decoupling from patterns of island biogeography. In revision at Ecology Letters; ("*"indicates co-first authorship). The contributions of each co-author are as follows: C.E.W., G.P., T.D.B., and T.E.D. planned and designed the research. C.G., D.C.D., and J.W.T all contributed substantially to the design of molecular methods and A.G. and L.D.L.A contributed substantially to statistical design and spatial methods used to assess landscape heterogeneity. C.E.W. conducted field and lab work for the biogeography aspects of the study and C.E.W. and G.P. performed field and lab work for the rhizonode component of the study. C.E.W. analyzed and interpreted the data with important contributions from G.P., A.G., L.D.L.A, and C.G.. The manuscript was written by C.E.W. and all coauthors provided important contributions and critical revisions. All authors approve of the final version of this manuscript. In the next section, I more fully outline in narrative style my specific contributions to this work.

The California coast redwood (*Sequoia sempervirens*) is an iconic, relictual tree species which represents an economically important timber species that is also listed as endangered by the International Union for the Conservation of Nature (Farjon and Schmid 2017). For these two reasons, ensuring the health and productivity of redwood forests, especially in the face of rapid anthropogenic climate change, is of utmost importance. A growing body of research has revealed that the plant root microbiome is a critical determinant of plant health, being shown to promote growth in the face of biotic (Compant et al. 2019; Saikkonen, Nissinen, and Helander 2020) as well as abiotic stress (Rodriguez et al. 2019; Xu et al. 2018). Therefore, in order to characterize the redwood root microbiome, my collaborator Dr. Claire Willing sampled root and soil samples from eight sites encompassing the entirety of the redwood's natural range, and she extracted microbial DNA from these samples. She used this DNA to amplify and sequence an ITS2 amplicon sequencing library to assess the fungal component of the microbiome, while I used the same DNA samples to amplify and sequence a 16S amplicon sequencing library to assess the bacterial component of the microbiome. Initial analysis of the 16S amplicon data revealed that a *Bradyrhizobium sp.* was the most abundant taxon in the community. Because some *Bradyrhizobium spp.* are known to nodulate certain legume species, we decided to investigate the nodule-like structures, hereafter referred to as "rhizonodes", present on redwood roots in greater detail.

Upon learning from my collaborator Dr. Willing that redwood roots harbor these rhizonodes, I set out to determine whether they were in fact morphologically distinct. I collected tissue samples from redwood trees growing outside the Plant Gene Expression Center (PGEC) in Albany, CA and imaged them under a dissecting microscope. Because the rhizonodes did indeed appear distinct from true lateral roots

even at low magnification (Fig. 4a), I collected additional samples and washed, embedded, fixed, sectioned, stained, and imaged them on a compound microscope to analyze anatomical differences. This analysis revealed that the rhizonodes lacked a root cap and appeared determinate, i.e. all of the cells were differentiated and fully expanded and there was no remaining meristematic tissue at the rhizonode tips (Fig 4b-c). Microscopic investigation also revealed an abundance of fungal hyphae within the cortices of the rhizonodes in addition to Glomeromycotina-type spores, both of which were lacking from any root samples that were imaged (Fig. 4d-f). These initial observations provided rationale for designing metagenomic experiments to assess potential differences in the fungal microbiome composition between rhizonodes and true roots.

A pilot program was carried out in which I collected three samples from three redwood trees again outside the PGEC in Albany. These tissue samples were washed, and the rhizonodes were manually separated from the roots. DNA was extracted from the roots and separated rhizonodes, and both 16S and ITS2 amplicon sequencing was carried out. Although the 16S sequencing did not yield any exciting results, the ITS2 results demonstrated that rhizonodes contained a much higher proportion of reads assigned to the Glomeromycotina, and indeed nearly all of the reads assigned to the Glomeromycotina were exclusively present in rhizonode samples. We therefore hypothesized that the rhizonodes function as morphologically distinct domiciles of symbiotic arbuscular mycorrhizal fungi (AMF).

In order to test this hypothesis using a more robust dataset from a natural, redwood-dominated forest, Dr. Willing and I returned to a site she had sampled previously, the Big Creek UC Reserve in Big Sur, CA. There we collected four root, rhizonode, and soil samples from six different trees. These samples were subsequently processed, amplified, and sequenced to provide ITS2 amplicon sequencing results for the roots, rhizonodes, and soil. This final dataset reflected the previous results, but with much higher statistical and methodological certainty. Once again, the rhizondes contained a much higher proportion of AMF-assigned DNA reads and over 90% of all of the AMF reads from the entire study were found in rhizonode samples (Fig. 6a). All of this evidence led us to the conclusion that rhizonodes are indeed morphologically distinct structures which have evolved to house symbiotic AMF. They may even function to compartmentalize these important fungi and provide a specialized environment to facilitate symbiotic nutrient exchange between the tree and the fungus, analogous to the well-characterized rhizobium-associated nodules of various legume species in the Fabaceae.

Abstract

Root-associated fungal communities modify the climatic niches and even competitive ability of their hosts, yet how the different components of the root microbiome are modified by habitat loss remains a key knowledge gap. Using principles of landscape ecology, we tested how free-living versus host-associated microbes might differ in their response to landscape heterogeneity. Further, we explore how compartmentalization of microbes into specialized root structures might filter for key fungal symbionts. Our study demonstrates that free-living fungal community structure correlates with landscape heterogeneity, but that host-associated fungal communities depart from these patterns. Specifically, biotic filtering in roots, especially via compartmentalization within specialized root structures, decouples the biogeographic patterns of host-associated fungal communities from the soil community. In this way, even as habitat loss and fragmentation threaten fungal diversity in the soils, plant hosts exert biotic controls to ensure associations with critical mutualists, helping to preserve the root mycobiome.

Introduction

Island biogeography theory (IBT) has greatly enhanced our understanding of fungal ecology in recent decades. Seminal work has demonstrated the importance of forest “island” size in predicting the number of fungal species observed (Peay et al. 2007). However, while IBT introduces ideas of area and isolation as important factors in determining diversity, global change (including deforestation) necessitates that we move beyond IBT to consider the “quality” of islands (Laurance 2008). Few studies have explicitly considered the role of landscape fragmentation or host-availability in the structure of microbial communities, nor whether host-associated versus free-living microbial communities respond to the same landscape-level drivers (Vannette, Leopold, and Fukami 2016; Mony, Brunellière, et al. 2020). Root microbiomes in particular play a critical role in expanding host climatic niche and biogeographic distribution (e.g. the mutualistic niche; (Afkhami, McIntyre, and Strauss 2014; Peay 2016), and the different components of microbiomes (e.g. saprotrophs, symbiotrophs) may be governed by different drivers of community assembly and have different functional consequences for plants. Incorporating landscape heterogeneity into fungal ecology therefore provides an opportunity to advance our understanding of both fungal and plant ecology (Mony, Vandenkoornhuyse, et al. 2020).

Some fungi exist as free-living or facultative associates with plant hosts whereas others are obligately host-associated, depending on hosts for growth and reproduction. Therefore, free-living fungi are likely filtered by the abiotic soil environment (e.g. nutrient availability), while host-associated fungi occupy a somewhat buffered habitat where biotic filtering (e.g. host selection) plays an additional role in structuring these communities (Nuccio et al. 2016, 2020; Coleman-Derr et al. 2016; Goldmann et al. 2016). As a result, we predict that host-associated symbiotrophic fungal communities demonstrate some degree of decoupling from biogeographic patterns observed for

free-living fungal communities. However, few studies have tested whether these filtering processes for free-living versus host-associated fungi are ultimately shaped by changes in the surrounding landscape, such as habitat loss and fragmentation (Vannette, Leopold, and Fukami 2016; Mony, Brunellière, et al. 2020). For plant hosts, processes that result in the loss of fungal diversity, especially among fungal mutualists, may have negative consequences for their competitive ability (Peay 2018).

Strong biotic selection, including through processes such as compartmentalization, or the evolution of morphological structures that isolate mutualists, may result in different patterns of fungal biogeography (Chomicki et al. 2020). By facilitating further discrimination among partners, the theory of compartmentalization predicts that microbial communities sequestered into specialized structures experience decreased competition and enhanced reproduction, which might alter biogeographic patterns of microbial associates. For instance, the N-fixing bacteria found in compartments (nodules) of *Alnus* species do not demonstrate classic biogeographic patterns despite the broad geographic range of this host (Higgins and Kennedy 2012). Additionally, in the presence of strong competition in the rhizosphere, compartments may play a crucial role in ensuring colonization of hosts by particular microbial mutualists. A number of tree species associated with symbiotic, root-associated arbuscular mycorrhizal fungi (AMF) form nodule-like outgrowths on their roots (Grand 1969; Beslow, Hacskaylo, and Melhuish 1970; Dickie and Holdaway 2011; Schwendemann et al. 2011; Padamsee et al. 2016; Nunes et al. 2020), possibly representing an additional example of compartmentalization, but their role in fungal community assembly and biogeography has yet to be investigated.

The coast redwood forest represents a critical forest system threatened by habitat loss, fragmentation, and climate change, where fungal community ecology remains largely unstudied (Mejstrik, Kelley, and Others 1979; Noss 1999; Johnstone and Dawson 2010; Van Pelt et al. 2016). As a foundational tree species, the coast redwood (*Sequoia sempervirens,* D. Don) currently occupies its narrowest range in the past 140 million years and logging has resulted in a 95% decline in redwood habitat (Noss 1999). The present study tests how changes in the coast redwood habitat structures fungal community composition in these forests (Mejstrik, Kelley, and Others 1979; Adams et al. 1990; Afek et al. 1994). In addition, we report for the first time that swollen, nodule-like structures (herein “rhizonodes”) are present on redwood roots, raising questions about their role in compartmentalization of fungal mutualists in the face of habitat loss and fragmentation. Specifically, we address three hypotheses: (H1) that the extent of redwood habitat cover (percent redwood cover) and habitat fragmentation (edge density, EDG) will drive structure and richness of soil fungal communities; (H2) that fungal community structure associated with redwood roots will be decoupled from biogeographic patterns found for soil fungi as a result of strong biotic filtering; and (H3) that compartmentalization will further facilitate biotic filtering of the fungal community by enriching for symbiotrophic taxa in rhizonodes. Additionally, we test if rhizonodes explicitly compartmentalize AMF, akin to a mycorrhizal version of an N-fixing nodule.

Materials and Methods

Site descriptions

The geographic range of *S. sempervirens* extends from the central California coast, USA to just north of the Oregon border (36.08°N, 121.60°W to 41.77°N, 124.11°W) in a narrow band characterized by mild temperatures and coastal fog water inputs. We sampled a transect of 8 sites spanning this range based on an established plot network (Van Pelt et al. 2016). Further environmental information can be found in the Supplemental Information.

Sampling description

We collected soil and root samples for the biogeographic component of this study in fall of 2015 as previously described (Willing et al. 2020). Briefly, 16 soil cores (15 cm deep and 8 cm diameter) were collected at each site between September and October 2015. Soil cores were collected in a nested design from four trees within each 100 m by 100 m plot and samples were taken in the four cardinal directions around each tree at 3 m from diameter at breast height (DBH) (see Fig. 1). Details, including our assessment of soil chemistry, can be found in the Supplemental Materials. After repeatedly discovering rhizonodes on root samples during initial sampling, we collected additional samples for both morphological and molecular analysis of fungal communities. For morphological characterization, root and rhizonode samples were collected from mature redwood trees near Albany, CA in April 2018. Roots with attached rhizonodes were immediately sonicated in epiphyte removal buffer (Simmons et al. 2018), then washed 3 times in sterile water. A subset of tissue was imaged immediately but most was embedded in 7% agarose gel, fixed in formalin-aceto-alcohol (FAA) overnight, then rehydrated the next day. To compare the fungal community between the different components of the symbiotrophic system (root, rhizonodes, and soil), we collected samples from Site 8 (Landels-Hill Big Creek Reserve) in November 2019 in an identical manner as in the biogeographic component of the study except that the sampling was expanded to include a total of 6 trees.

Landscape metrics

Several landscape metrics were calculated to quantify the availability of redwood habitat around the sampling sites and the heterogeneity or patchiness of their spatial distribution. To do this, 10 km radius buffer zones were calculated around the midpoint of each plot in R version 4.0.0 (R Core Team 2020) based on the scale at which dispersal limitation becomes important for fungi (Peay, Kennedy, and Talbot 2016). Geodesic buffers were calculated using the geosphere (Hijmans 2019) and sp (Pebesma and Bivand 2005)(Pebesma & Bivand 2005) packages in R. Landcover data were then extracted for each of these buffer zones based on the National Land Cover Dataset for California (National Terrestrial Ecosystems 2011 imagery), courtesy of the U.S. Geological Survey. Metrics of landscape heterogeneity were calculated at 30 m by 30 m resolution using the landscapemeterics package in R (Hesselbarth et al. 2019). Habitat cover was calculated as the percentage of redwood cover for each buffer area to capture total redwood habitat available in the distribution zone, irrespective of spatial

distribution. Edge density, the total length of habitat edges over the total area (perimeter to area ratio), was used as a metric of habitat fragmentation for each buffer zone. Largest patch index, the size of the largest patch comprised by a single habitat type, was included as a measure of dominance. As the diversity of landcover types might correspond to shifts in fungal diversity, landcover diversity indices (Shannon's diversity, Simpson's diversity, landcover richness density) were calculated.

DNA extractions

Soil cores were sieved to 2 mm and 0.25 g of soil was collected into PowerSoil buffer (MoBio, Carlsbad, CA, USA, now Qiagen). "Rhizosphere soil" generally refers to the soil that is adhered to the outside of roots and "bulk soil" refers to soil proximate to roots (e.g. (Nuccio et al. 2020)), thus our soil samples are considered bulk soils despite their proximity to an abundance of roots in these soil cores. Soils were immediately processed according to manufacturer's instructions and soil DNA was stored at -80°C. For the biogeographic component of this study, we collected fine roots (which included rhizonodes) from the same core as soils and 0.70 g of roots were washed in running distilled water 5 times to remove particles adhered to the root surface. Root samples from the biogeographic component of the study contained both root and rhizonode tissue (due to the abundance of these structures on redwood roots); we herein refer to these samples as "root systems." These samples were frozen at -80°C prior to DNA extractions. We ground root systems in liquid nitrogen using mortars and pestles and transferred them into PowerSoil buffer. For the rhizonode component of the study, we manually separated rhizonodes from roots with sterilized forceps, then placed them into 1.5 mL tubes and flash-froze in liquid nitrogen. Rhizonode tissue was more limited due to their small size, therefore we collected 0.25 g of soil, 0.25 g of root tissue and 0.10 g of rhizonode tissue for comparisons of the fungal community across these samples. Extractions were carried out according to manufacturer's instructions. The concentration of extracted DNA was quantified using a Nanodrop One/One (Thermo Fisher Scientific, Waltham, MA, USA). Samples were randomized in 96-well plates (including negative PCR controls and DNA extraction controls) and DNA was diluted to 10 ng/μL for the biogeographic component of the study and 3.3 ng/μL for the rhizonode component of the study (due to more limited tissue availability).

Molecular identification of species

The PCR primers used in this study target the ITS2 rRNA region with a fragment length between 267-511 base pairs (BP) (Taylor et al. 2016); we specifically selected these primers as they have successfully been used to study AMF communities (Gao et al. 2019). In our study, the 5.8S-Fun and ITS4-Fun primers included heterogenicity spacers (0-8BP) to improve the sequencing quality and contained 12 BP barcodes, linkers, and pads for single step PCR (Lundberg et al. 2013). For PCR conditions, please refer to the Supplementary Materials. Bioanalyzer traces were used to determine products were of the correct length and purity. Paired-end (2 x 300 bp) sequencing was performed on the Illumina Miseq V3 platform (Illumina, San Diego, CA, USA) at The California Institute for Quantitative Biosciences (QB3) at the University of California, Berkeley.

Sequencing data were processed using the AMPtk pipeline (Jusino et al. 2019). Briefly, we merged reads, trimmed primers, and removed PhiX. Reads were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity and filtered to remove chimeric reads, singletons, and index bleed at a 0.005% rate as per AMPtk standards. We assigned taxonomy using AMPtk's hybrid approach, which uses Bayesian methods to select consensus lineages between Global Alignment, UTAX, and SINTAX (Jusino et al. 2019). To correct for over-splitting (especially important for AMF taxa using the ITS2 region), the rhizonode data were then processed using the LULU algorithm within the AMPtk pipeline (Frøslev et al. 2017). OTUs were parsed into ecological guilds using FUNguild, with 49.3% of OTUs assigned to a guild (Nguyen et al. 2016). Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under Accession no. PRJNA530796.

Statistics

All data were analyzed in R version 4.0.0 (R Core Team 2020). Phyloseq was used to import sequencing data (McMurdie and Holmes 2013). Non-fungal reads (e.g. plant DNA) were removed by filtering for reads that matched to Kingdom Fungi. After filtering, we identified 4,334 OTUs in the biogeography portion and 2,420 OTUs in the rhizonode portion of the study. Data were rarefied to 1000 sequencing reads per sample for the biogeography study and 2000 reads per sample for the rhizonode study, which allowed us to retain approximately 90% of our samples across the study. After rarefaction, the biogeography component of the study retained 111 of 128 root system samples and 118 of 128 soil samples. Two of 72 samples were dropped from the rhizonode portion of the study after rarefaction.

To test our hypothesis (H1) that total habitat cover and fragmentation correlate with community structure, we used generalized-dissimilarity modeling (GDM) to test the strength of the relationship between fungal community structure and habitat cover and fragmentation compared to biogeographic, climatic, and soil chemistry data (Manion et al. 2017). GDM accounts for non-linear fits of community dissimilarity (Bray-Curtis dissimilarity was used here) across individual environmental and geographic gradients within a single model (Glassman, Wang, and Bruns 2017; Daws et al. 2020; Willing et al. 2020). The full models included geographic distance, redwood cover (percent of redwood cover in buffer zone), habitat fragmentation (edge density; EDG), actual evapotranspiration (AET), minimum mean winter temperature (DJF) and maximum mean summer temperature (JJA), soil pH, cation exchange capacity (CEC), C:N ratios, phosphorus concentrations in soils, and climatic controls on decomposition rates (k); see Supplementary Materials for a further description of soil chemistry determination. Backwards elimination of GDM predictor variables was performed with the "gdm.varImp" function (Manion et al. 2017) and the most parsimonious models are reported. Non-metric multidimensional scaling (NMDS) was used to visualize differences in community structure using rarefied data and Bray-Curtis distances. Nested PERMANOVA using site of origin, sample type, and interaction between those factors was used to test for differences in fungal community structure using the "adonis" function in vegan (Oksanen et al., n.d.). FUNguild assignments were used to parse the different fungi by trophic mode (saprotrophs, pathogens, and symbiotrophs) and we

extracted the AMF component by filtering for fungal taxa that keyed to the Phylum Glomeromycota (now Subphylum Glomeromycotina). GDMs were also constructed for the different fungal trophic modes and for AMF specifically. The AMF community found in the soil was at very low abundance, so we did not report these values as the GDMs constructed were based on very sparse data.

To investigate patterns of fungal beta-diversity, we used the “beta.pair” function with Jaccard dissimilarity from the betapart package, which partitions beta-diversity into components of turnover (species replacement) and nestedness (species losses) (Baselga 2012; Baselga et al. 2017). Shifts in beta-diversity are typically driven by either species replacement (turnover) or predictable loss/gain of species (nestedness), where species at less diverse sites are a consistent subset of the species found at sites with greater diversity; the relative importance of these different processes has implications for management and restoration practices (Socolar et al. 2016). Mantel tests compared turnover and nestedness across shifts in redwood cover and geographic distance (the two parameters that were retained in the GDM model) using the ecodist package in R (Goslee and Urban 2017).

Where habitat fragmentation was greater, we predicted more variation in fungal community structure (beta-dispersion) (Anderson et al. 2011). We calculated beta-dispersion using the “betadiver” function in vegan (Oksanen et al., n.d.). Linear-mixed effects models were used to test which parameters corresponded to shifts in beta-dispersion using the lme4 package in R (Bates 2010). First, full models were compared using climatic controls on decomposition (k), EDG, redwood cover, and latitude, including interactions of these variables with sample type (root systems versus soils) and a random effect of site. Both Akaike information criterion (AIC) and Bayesian information criterion (BIC) scores were compared for candidate models fit using the full maximum likelihood. The most parsimonious models (those with the lowest AIC and BIC scores) were selected and the full model was compared to reduced models using the “anova” function in R. We then refit the most parsimonious model using restricted maximum likelihood. Metrics of alpha diversity were calculated in phyloseq using the “estimate_richness” function. Linear mixed effects models were used as described above to compare patterns for both observed richness and Shannon diversity with EDG, redwood cover, k, and soil pH.

To test our second hypothesis (H2) that fungal community structure associated with redwood roots is more decoupled from biogeographic patterns found for soil fungi, we used the same analyses (e.g. GDM and mixed effect models with landscape and environmental predictors) as described for soil fungi but applied them to the root system communities.

For our third hypothesis (H3), that compartmentalization will further facilitate the biotic filtering of the fungal community by enriching for symbiotrophic taxa, we compared the fungal community structure between soil, root, and rhizonode samples. We visualized these data using NMDS based on Bray-Curtis dissimilarity. We then used PERMANOVA as above with sample type (root, soil, rhizonode) and tree individual as fixed effects. A post-hoc test was performed using the “pairwise.adonis2” function from the pairwiseAdonis package in R (Arbizu n.d.). Species richness between these sample types was compared as above. To test if rhizonodes explicitly compartmentalize AMF,

the proportion of AMF between these different sample types was compared using an ANOVA with the “aov” function. Differences across samples were assessed with a post-hoc Tukey test. Lastly, we ran indicator species analyses using the IndVal function from the labdsv package (Roberts and Others 2007). All reported statistics and figures are based on rarefied data and Bray-Curtis dissimilarity, unless otherwise noted.

Results

Habitat cover and fragmentation drive soil fungal community structure

Throughout the redwood range, we identified 2304 fungal OTUs in soils. The fungal community in soils differed significantly by site (*PERMANOVA;* $F_{7,110}$=5.2339, R^2=0.250, P<0.001; Fig. S1). After rarefaction to 1000 reads per sample, we found an average of 77 OTUs per rhizosphere soil sample. In support of our first hypothesis (H1), we found that, in addition to geographic distance, redwood cover in the 10 km buffer zones was an important predictor of soil fungal community structure (Fig. 2a, Table S2). Additionally, habitat fragmentation (edge density; EDG) best predicted both observed fungal richness (linear mixed effects; marginal R^2= 0.069, slope=-0.190, P=0.071) and the Shannon index of fungal diversity (linear mixed effects; marginal R^2= 0.115, slope=-0.001, P=0.002; Fig. 2b, Table S4).

After partitioning beta-diversity into components of turnover and nestedness, we found that differences in fungal community structure were comprised in large part by species replacement (turnover) across geographic distance and redwood cover (Mantel R=0.491; p=0.001 and Mantel R=0.406; p=0.001, respectively). Species losses (nestedness) accounted for a smaller but still significant portion of the differences in community structure across shifts in redwood cover (Mantel R=-0.200, p=0.001) and geographic distance (Mantel R=-0.233, p=0.001). While the saprotrophic community showed strong geographic patterns of turnover (Fig. S2a; Table S5), the symbiotrophic component of the soil community accounted for patterns of turnover associated with shifts in redwood cover and EDG (Fig. 2a; Table S6). Particularly sharp rates of turnover in the symbiotrophic community were observed both as redwood cover dropped below 20% and as redwood cover exceeded 50% in the 10 km buffer zones around each plot. Interestingly, turnover in the pathogenic component of the community did not correspond to either shifts in redwood cover or geographic distance, but rather with habitat fragmentation (EDG) and annual precipitation (PPT) (Fig. S2b; Table S7).

Counter to our prediction, patterns of beta-dispersion were best predicted by climatic controls on decomposition (k), where lower rates of decomposition corresponded to higher rates of dispersion (linear mixed effects; marginal R^2= 0.117, slope=-0.200 P<0.001; Fig. 2c). This indicated that where rates of decomposition were slowest, the structure of the soil fungal community was most variable.

Fungal communities in root systems do not follow patterns of habitat cover or fragmentation

In root systems throughout the redwood range, we observed 1484 fungal OTUs. After rarefaction to 1000 reads per sample, root samples averaged 54 OTUs per sample. Root systems differed significantly by site (*PERMANOVA;* $F_{7,103}$=3.678, R^2=0.200, P<0.001; Fig. S1, Table S8). However, in support of our second hypothesis

(H2), patterns of fungal community structure in redwood root systems were not predicted by metrics of landscape heterogeneity, but geographic distance was an important predictor of shifts in root system community structure. While percent redwood cover was an important predictor of fungal community in the soil, soil pH (but not percentage redwood cover) emerged as an important predictor of root system community structure (Fig. 3a, Tables S9-S10). The AMF component of the root systems followed similar overall patterns to those observed for the total fungal community found in roots (Fig. 3a). The difference in fungal community structure in root systems across soil pH and geographic distance was driven by both species turnover (Mantel R=0.3764; p=0.001 and Mantel R=0.3827; p=0.001, respectively) and, to a lesser degree, nestedness (Mantel R=0.1942, p=0.001 and Mantel R=0.2150, p=0.001, respectively).

While habitat fragmentation was an important predictor of patterns of observed species richness for soil fungi, neither landscape fragmentation (EDG) (*P*=0.984; Table S11) nor soil pH (*P*=0.287) corresponded to patterns of fungal richness for root systems (Fig. 3b, Table S3). The observed number of species and Shannon richness did differ by site ($F_{7,103}$=3.586, *P*=0.002 and $F_{7,103}$= 4.916, *P*<0.001, respectively), however these differences did not correspond to any of our predictor variables. As was observed in soils, k best predicted patterns of beta-dispersion for root systems, where lower rates of decomposition corresponded to higher rates of dispersion (linear mixed effects; marginal R^2= 0.117, slope=-0.200 *P*=0.002; Fig. 3c).

Compartmentalization facilitates biotic filtering and enriches for symbiotrophic AMF taxa

Rhizonode compartments were determined to be morphologically distinct from adjacent root tissue (Figs. 4a, S5a). Ranging from ovoid to conical and 1 to 5 mm in length, rhizonodes were lighter in color than the adjacent root tissue. Microscopic analysis revealed that rhizonode structures emerge from the endodermis and contain a vascular cylinder like roots, but lack a root cap or root apical meristem (Fig. 4a), two features apparent in true roots (Fig. 4b). Cortical cells in rhizonodes appear to contain coils of fungal hyphae (Fig. 4c) as well as fungal spores (Fig. 4e) indicative of a Paris-type AMF symbiosis (Dickson 2004). Interestingly, we did not observe these typical attributes of AMF colonization in other components of the redwood root system (Fig. 4b).

Host filtering and compartmentalization play important roles in structuring fungal communities associated with root systems, which may explain the relative conservation of fungal communities in root systems, even across habitat fragmentation and shifts in host availability. Roots showed clear patterns of biotic filtering from the soil environment (*Pairwise-PERMANOVA* R^2= 0.131, *P*<0.001; Fig. 5; Table S12) and rhizonodes hosted distinct fungal communities from both adjacent root tissue (*Pairwise-PERMANOVA*; $F_{1,5}$=3.042, *R2*= 0.056, *P*<0.001) and the surrounding soil environment (*Pairwise-PERMANOVA*; $F_{1,5}$=11.472, R^2= 0.177, *P*<0.001). Rhizonodes exhibited marginally higher species richness than root communities based on Shannon (Fig. 6b, *Tukey P adj*=0.065; Table S13) and Simpson (*Tukey P adj P*=0.070) indices, however, this trend did not hold for observed species counts (*Tukey P adj P*=0.392).

Analysis of variance (ANOVA) demonstrated that there were significant

differences in AMF relative abundance between sample types ($F_{2,68}$ = 31.67, P<0.001; Table S14). A post hoc Tukey test showed that compartmentalization into rhizonode structures resulted in 20-fold higher relative enrichment of AMF in rhizonodes compared to adjacent root tissue (Fig. 5-6; P<0.001). Interestingly, biotic filtering alone did not result in a significantly greater proportion of AMF in roots compared to soils (P=0.957). These data demonstrated that, while AMF were certainly present in other root tissues, compartmentalization specifically enriched for AMF. Approximately 60% of indicator taxa for rhizonodes were identified as AMF and all AMF indicator taxa associated with rhizonode samples identified to the family level belonged to the Glomeraceae (Fig. S4; Table S15). By comparison, no AMF were identified as indicator species of root or soil samples.

Discussion

In extending beyond IBT to principles of landscape ecology (Laurance 2008), our study indicates that habitat fragmentation and habitat loss correspond to a decline in fungal richness and shifts in fungal community structure in soils. While both bacteria and fungi display strong species-area relationships (Peay et al. 2007; S.-P. Li et al. 2020), previous research has demonstrated that the underlying mechanisms determining these patterns differ for both groups, where water-availability influences bacterial diversity and biogeography is often more correlated with patterns of fungal diversity (S.-P. Li et al. 2020). In a previous study across these sites, we found that water-availability was a key factor in determining bacterial diversity and community composition (Willing et al. 2020). In contrast, here we show that biogeographic factors, including habitat fragmentation and redwood cover, are important determinants of soil fungal community structure and diversity, especially for symbiotrophic fungi, which could have important feedbacks for redwood cover or potential transitions to ectomycorrhizal-dominated forest (Peay 2018; Steidinger et al. 2019).

Interestingly, despite predicting that greater habitat fragmentation would correspond to increased beta-dispersion, we found climatic controls on decomposition were the best predictor of beta-dispersion for both soils and root systems. As inferred rates of decomposition decreased (calculated from the Yasso07 by (Tuomi et al. 2009), fungal community composition became more variable. We posit that as rates of decomposition decline, the soil matrix becomes more heterogenous as detritus, such as dead roots, remains for longer periods resulting in more spatially variable niches in the soil profile. Temperatures are somewhat buffered in the coastal habitat of the redwood forest where we observe a 2.2°C variation throughout the entire range in the warmest quarter. However, rainfall at the northernmost edge of the redwood range is nearly 6 times that in the southernmost extent of the redwood forest. Consequently, we predict that while shifts in water-availability were not found to directly correspond to patterns of fungal community composition, they may indirectly influence fungal community structure.

Although fungal communities in redwood soils were predicted by metrics of landscape heterogeneity, there was no relationship between landscape heterogeneity and fungal community structure in root systems. Plant organs typically display more conserved community structure compared to soils (Coleman-Derr et al. 2016;

Goldmann et al. 2016; Barajas et al. 2020) and our data demonstrate that these patterns are also consistent for a long-lived gymnosperm species. Root microbiomes are critical centers of nutrient acquisition and metabolism thus they are likely under strong selection (Ramírez-Puebla et al. 2013).

Soil pH emerged as an important predictor of host-associated fungal communities. In part, this follows previous studies that have shown pH an important predictor of host-associated fungal communities, and therefore, key in predicting the realized niche of these fungi (Glassman, Wang, and Bruns 2017; Davison et al. 2021). Surprisingly, soil pH did not emerge in our models as one of the primary correlates with fungal community structure in soils. We predict that habitat fragmentation and host cover had a strong influence on fungal dispersal and that the soil community is therefore more representative of the larger species pool prior to strong biotic filtering. Further research should emphasize the discrepancies between the available species pool in soils and the abiotic factors that drive colonization of roots (Barajas et al. 2020).

A number of fungi known to form other types of mycorrhizal associations were found associated with redwood roots, even after vigorous washing in DI water. We identified 357 potential ectomycorrhizal fungi (EMF) and 25 ericoid mycorrhizal OTUs throughout this study based on FUNGUILD delineations of “highly probable” and “probable” matches (Nguyen et al. 2016) and of these, 206 (approximately 58%) EMF OTUs were found in association with roots at low, but consistent relative abundance (Fig. S3). Similar reports have been recorded in other studies where EMF are found associated with non-host species (Dawkins and Esiobu 2017; Carey et al. 2020; Schneider-Maunoury et al. 2020) and the “waiting-room” hypothesis, where the ectomycorrhizal habit evolved from fungal endophytes, has been proposed as an explanation (Selosse, Dubois, and Alvarez 2009; Selosse, Schneider-Maunoury, and Martos 2018; Smith et al. 2017).

Compartmentalization may also explain the modification of biogeographic patterns observed within the symbiotrophic system. Given that fungal richness in the root systems did not shift with habitat fragmentation, we find evidence that root systems exhibit some degree of resistance to habitat fragmentation at the scales measured. While it remains to be tested if these core microbes play a role in host fitness or resistance/resilience, this will be an important area of research in the era of global change.

Rhizonodes, which appear to derive from the protoxylem poles (Nunes et al. 2020)), are morphologically distinct from adjacent root tissue and were very abundant throughout the redwood range. Our investigations revealed that rhizonodes act as domiciles for AMF, akin to a mycorrhizal version of a N-fixing root nodule. The theory of compartmentalization predicts that the evolution of distinct morphological structures allows hosts to isolate symbionts, control their reproduction, govern rewards or punishments, and reduce competition (Chomicki et al. 2020). Previous descriptions of similar nodule-like structures in podocarps indicated that these structures can double root cortex volume, allowing for greater AMF interaction (Dickie and Holdaway 2011). In this way, the development of specialized structures may enable hosts to attract more beneficial partners by spatially partitioning “rewards” (e.g. carbon) while building less costly roots (due to the reduced cell wall construction required for rhizonodes) (Dickie

and Holdaway 2011; Kiers et al. 2011). AMF have particularly slow rates of recovery from forest disturbance, even after the employment of restoration practices (Wall et al. 2020), thus compartmentalization may play a critical role in the maintenance of redwood forests. Further, the common presence of similar nodule-like structures on both extinct and extant AMF-associated tree species (Fig. S5) provides an exciting opportunity to explore these structures from both ecological and evolutionary perspectives (Duhoux et al. 2001; Grand 1969; Beslow, Hacskaylo, and Melhuish 1970; Russell, Bidartondo, and Butterfield 2002; Dickie and Holdaway 2011).

Biotic interactions, especially interactions with microbial symbionts which may expand plant niches (e.g. the “mutualistic niche; (Afkhami, McIntyre, and Strauss 2014; Peay 2016), are important for our understanding of biogeography in the Anthropocene. We demonstrate how habitat loss and fragmentation predict patterns of free-living fungal community composition. Interestingly, we also find that *Sequoia* exhibits mechanisms of preserving critical components of their mycobiomes, but note that there may be important thresholds for shifts in landscape heterogeneity.

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Figures and Tables

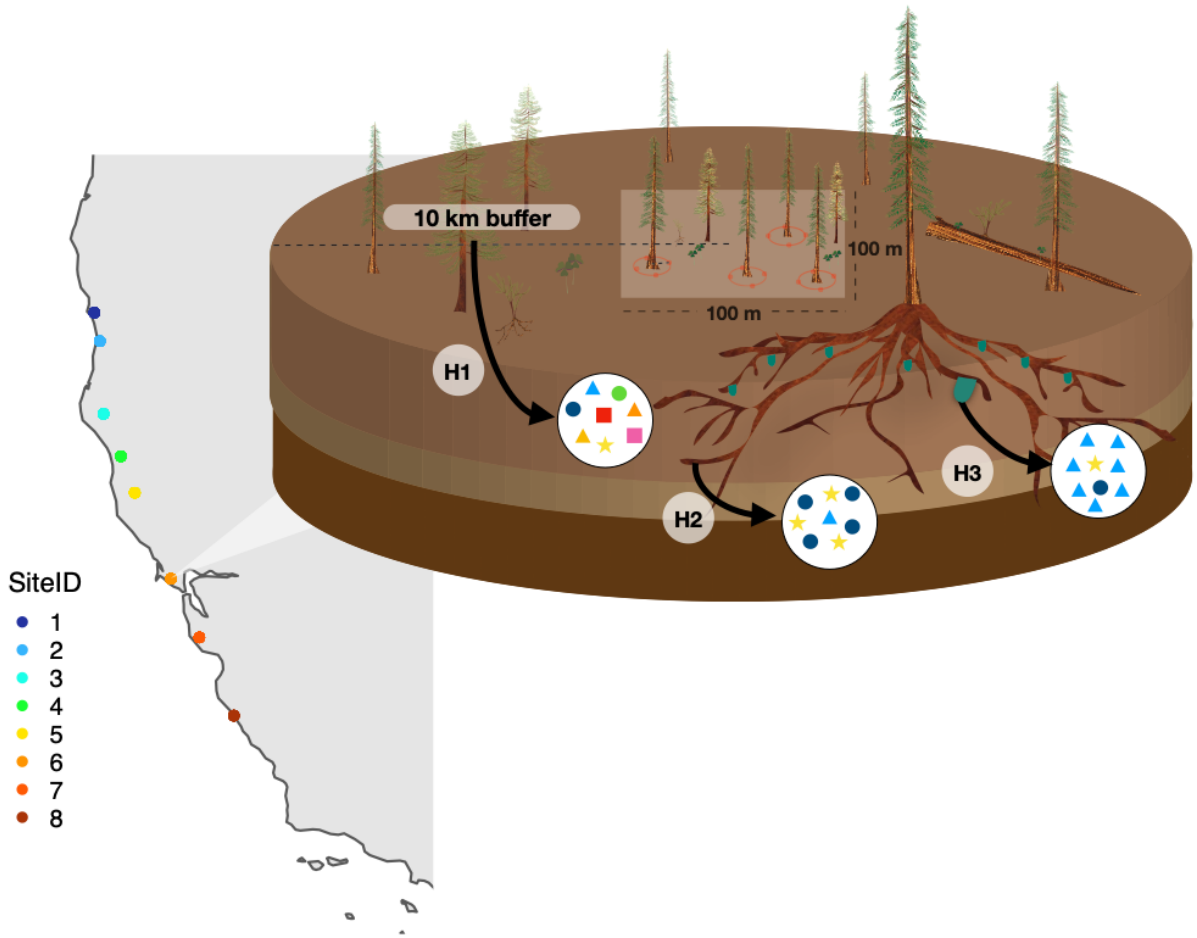

Figure 1. Conceptual figure of the study design and associated hypotheses. The plots in this study span the approximately 800 km distance of the entire redwood range in Northern California, USA. Plots were located throughout the range and four trees were sampled in a 100 by 100 m plot. Trees were sampled at each plot where red dots denote sampling locations three meters from diameter at breast height (DBH) of tree in the cardinal directions. A 10 km buffer zone was determined around the midpoint of each plot and metrics of landscape heterogeneity were calculated for these buffer zones. Our hypotheses were that H1) total habitat cover and habitat fragmentation (in buffer zones) drive community structure and richness of soil fungal communities; (H2) fungal community structure associated with redwood roots will be more decoupled from biogeographic patterns found for soil fungi as a result of strong biotic filtering; and (H3) different components of the symbiotrophic system will differ in their capacity for discrimination of the fungal community, where rhizonodes will compartmentalize key fungi, even across fragmented landscapes and habitat decline.

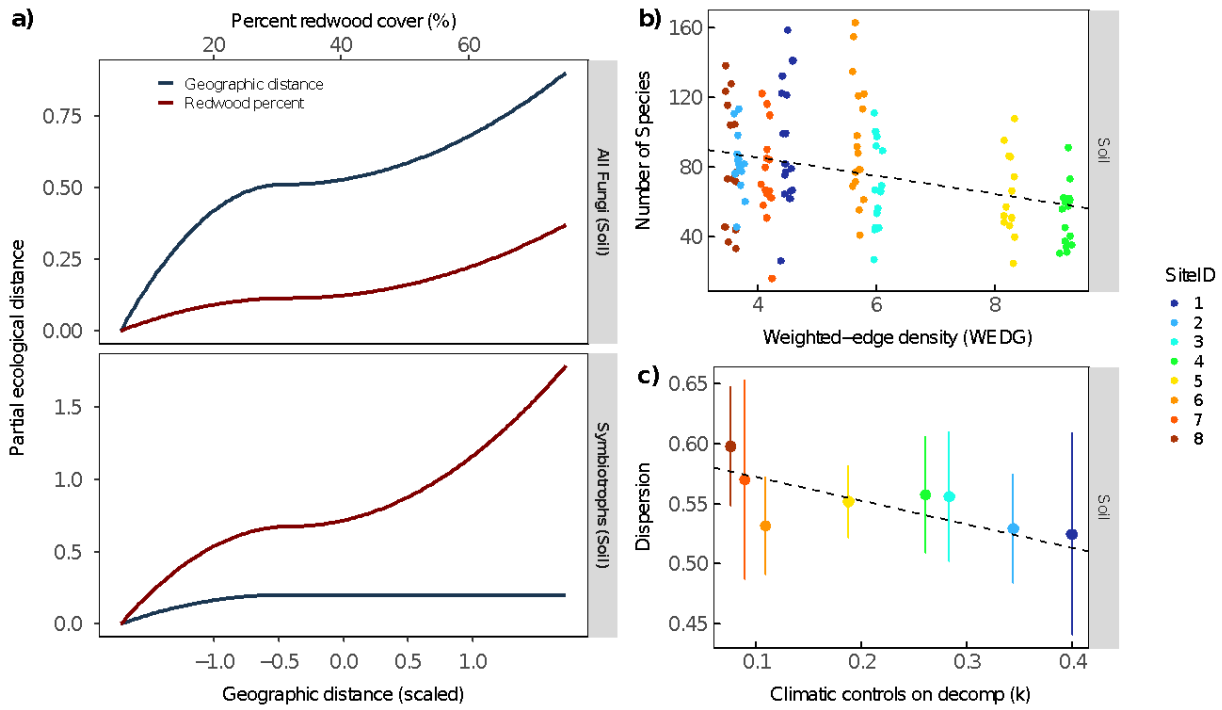

Figure 2. (a) Generalized dissimilarity modeling (GDM) of total fungal community in soils (standardized across predictor variables). GDM-fitted I-splines for each environmental covariate and geographic distance. The maximum height of each curve indicates the total amount of turnover in fungal communities associated with that variable (the relative importance of that variable in explaining changes in fungal community structure) holding all other variables constant. The shape of each function indicates how the rate of change varies along the gradient. (b) GDM of the symbiotrophic component of the soil fungal community. (c) Observed number of fungal species in soils versus landscape heterogeneity calculated as weighted-edge density ('WEDG'), where larger WEDG values correspond to more fragmented landscapes; regression line from best fit linear mixed-effects model. (d) Beta-dispersion (distance from centroid) of soil communities versus calculated values for climatic controls on decomposition (k); regression line from best fit linear mixed-effects model.

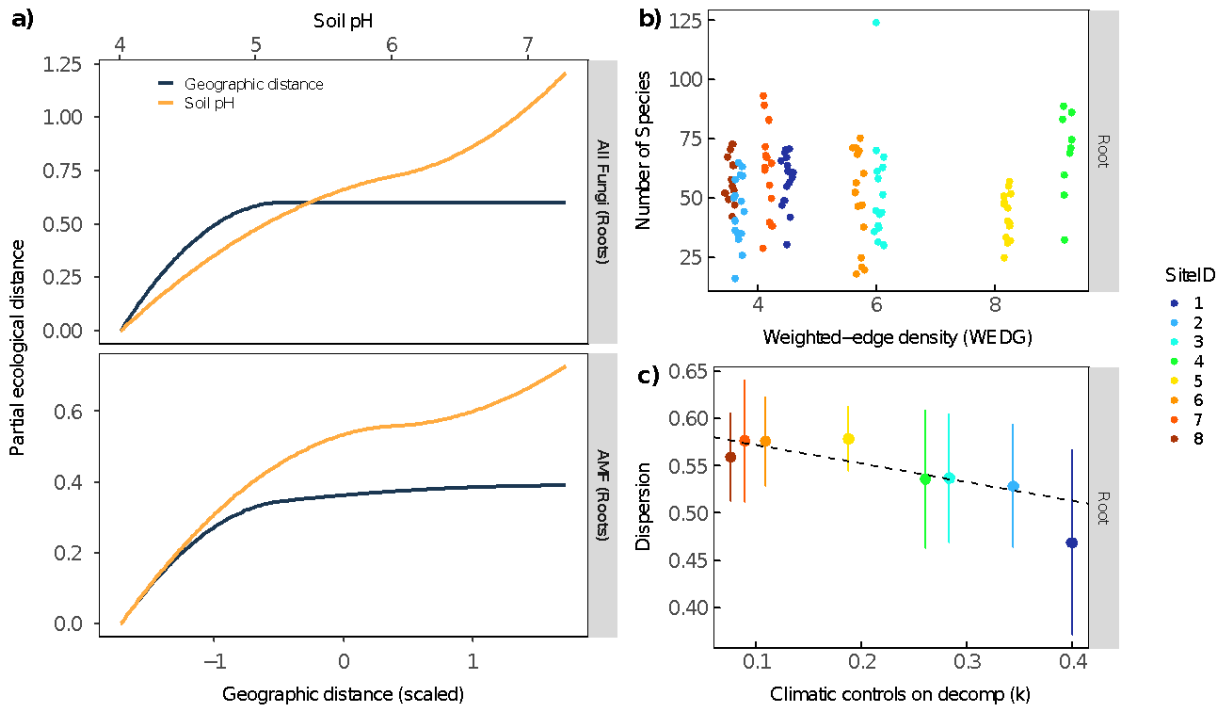

Figure 3. (a) GDM of total fungal community in roots (standardized across predictor variables). (b) GDM of the AMF component of the root fungal community. (c) Observed number of fungal species in roots versus landscape heterogeneity calculated as weighted-edge density ('WEDG'); regression line from best fit linear mixed-effects model. (d) Beta-dispersion (distance from centroid) of root communities versus calculated values for climatic controls on decomposition (k); regression line from best fit linear mixed-effects model.

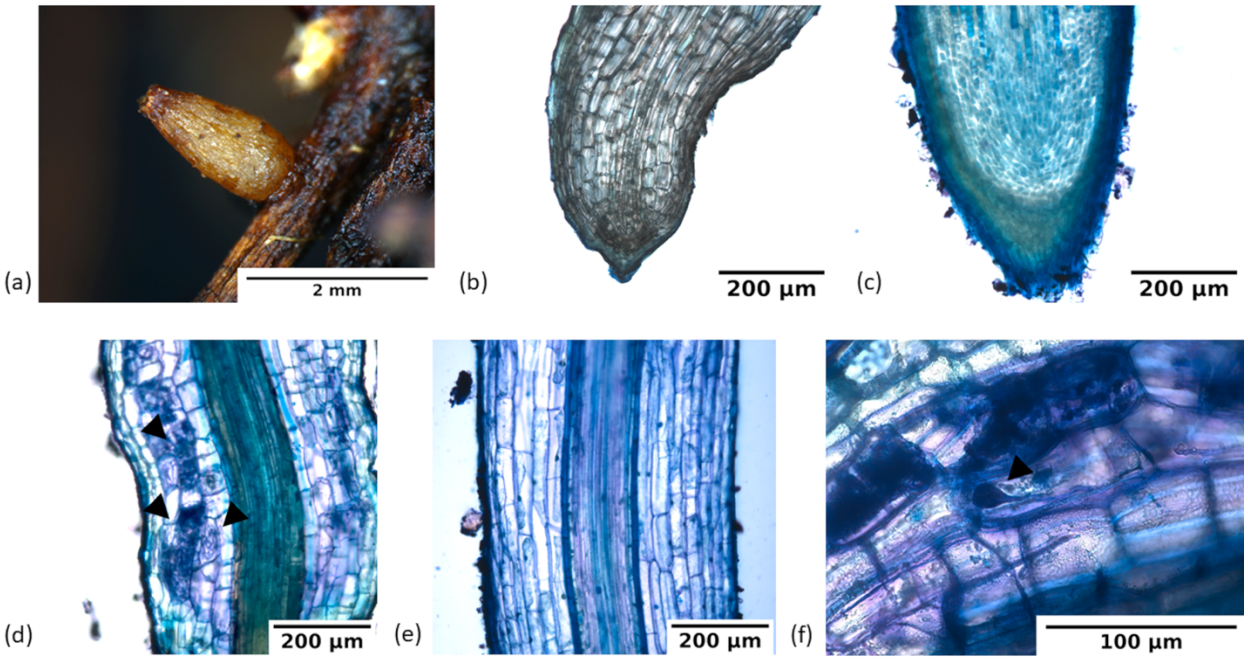

Figure 4. Rhizonode structures on fine roots of *Sequoia sempervirens*. (a) Macroscope view of rhizonode structure on redwood root. (b) Cross section of the tip of redwood rhizonode, showing fully expanded and developed cells and no root cap. (c) Cross section of the tip of a true root from the same plant, with a clearly defined meristematic center and root cap. (d) Dark masses of coiled fungal hyphae in the cortex of redwood root nodules, indicative of a Paris-type AMF association. (e) Similar structures were absent from cortices of true roots. (f) AMF vegetative spore in the cortex of a root nodule.

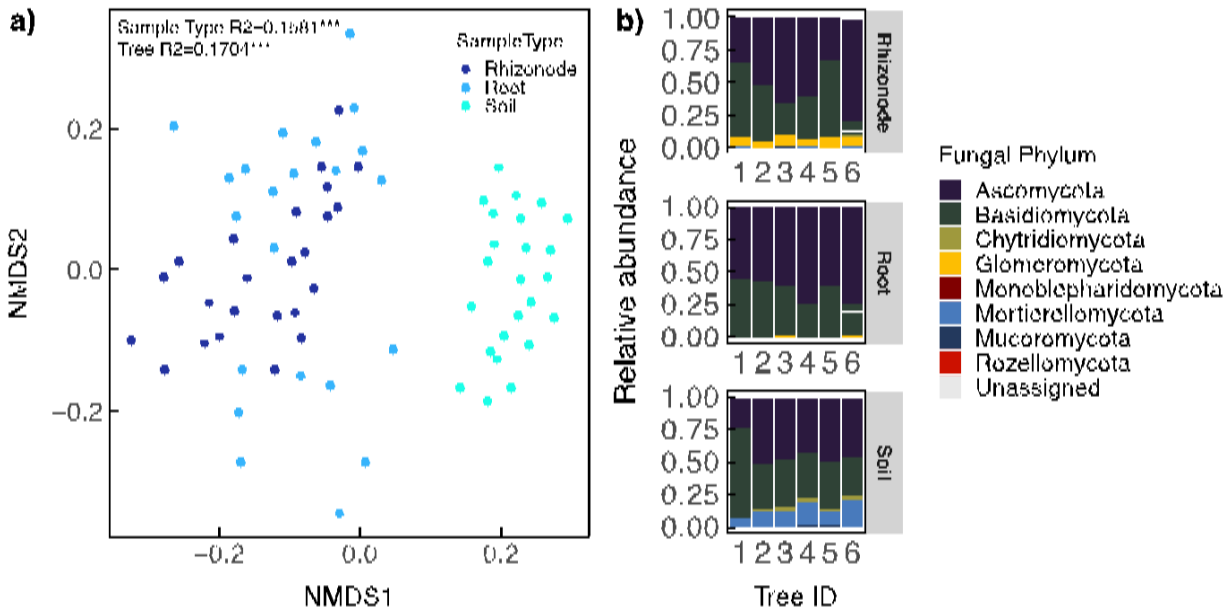

Figure 5. (a) Non-metric multidimensional scaling (NMDS) of the different sample types (rhizonodes, roots, and soil). The total fungal community differed significantly by sample type. (b) Barplot of the top 500 fungal phyla found in each sample type at the Phylum level. Glomeromycota (the monophyletic group to which arbuscular mycorrhizal fungi belong; shown in yellow) are significantly enriched in rhizonodes compared to roots or soils.

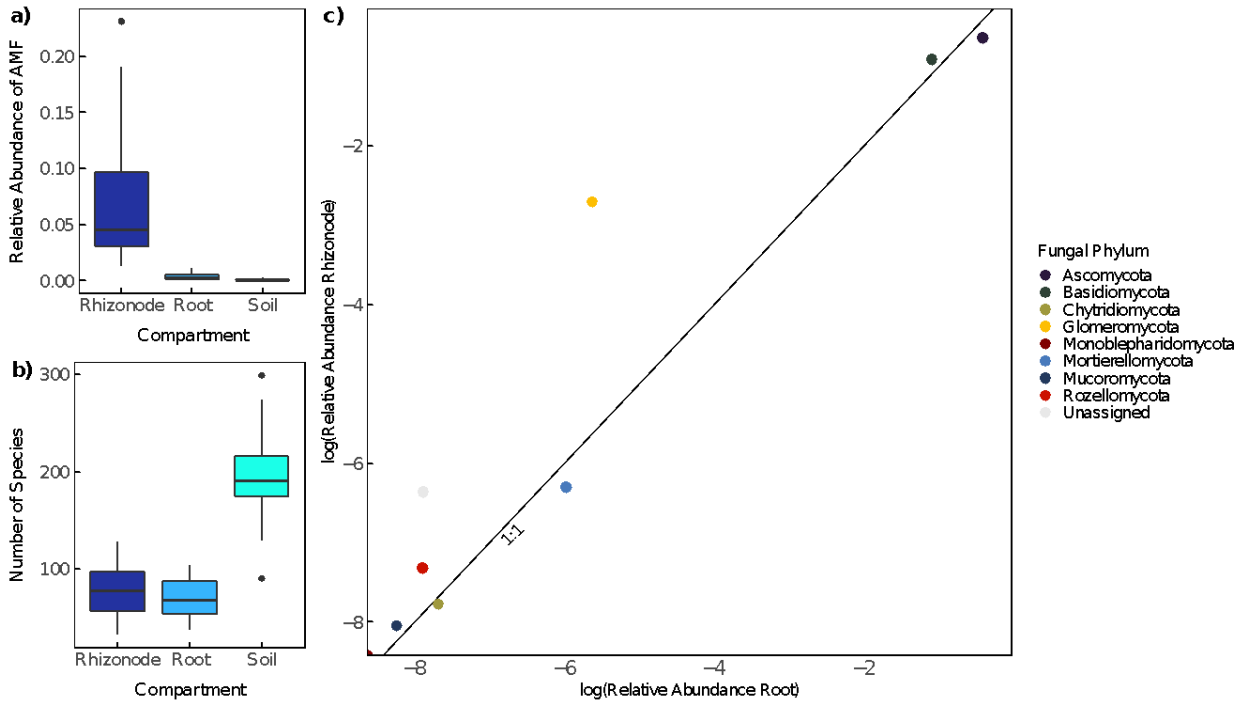

Figure 6. (a) Relative abundance of AMF across sample types (b) Shannon diversity across sample types (c) a log-log plot of the different fungal phyla found in root versus rhizonode samples. Anything falling off of the 1:1 line is interpreted as being more or less represented in the corresponding sample type (either root or rhizonode). Glomeromycota (arbuscular mycorrhizal fungi) is the only phylum that appear to fall far off the 1:1 line, indicating that arbuscular mycorrhizal fungi are found in much higher relative proportion in rhizonodes compared to roots.

Soil fungal community: Best model Predictor	***Deviance explained=31.9994*** **Coefficients**
Geographic distance	0.9090
Redwood cover	0.4744
Root fungal community: Best model Predictor	***Deviance explained=26.1710*** **Coefficients**
Geographic distance	0.5996
Soil pH	1.2191

Table 1. Results from generalized dissimilarity modeling (GDM) of the fungal community for redwood roots and rhizosphere soils based on Bray-Curtis dissimilarity. Results represent the most parsimonious models determined through backwards elimination model selection.

Supplementary Information

Methods:

Sampling description

Sixteen soil cores (15 cm deep and 8 cm diameter) were collected at each site between September and October 2015. Soil cores were collected in a nested design from four trees within a 100 m by 100 m plot and samples were taken in the four cardinal directions around each tree at 3 m from DBH (see Fig. 1). In sampling, the duff layer was physically removed and cores were taken from the top 15 cm of soil; redwood roots were abundant in cores as this species has no tap root and employs expansive shallow rooting systems for stabilization (Noss 1999). Cores were collected into freezer-safe bags, transported from the field, and kept on ice until returning back to the lab. They were then kept at 4°C until processing, which occurred within 3-5 days of returning to the lab (and no more than 1-week post-collection).

PCR conditions and sequencing

The primers used in the present study were adapted to Illumina platform for dual-indexed, single-step PCR based on best-practices for high-throughput amplicon sequencing, including heterospacers to help increase base-pair diversity thereby increasing accuracy (Lundberg *et al.*, 2013). 25 µl reactions were set up for PCR using 2.5 µl of forward primer, 2.5 µl of reverse primer, 2 µl of DNA, 7 µl of water, 10 µl of Kapa HiFi ready-mix (Kapa Biosystems, Boston, MA, USA) and 1 µl of BSA. PCR conditions were: 96°C for 2 min, 34 cycles of 94°C for 30 sec, 58°C for 40 sec, 72°C for 2 min, and 72°C for 10 min before 4°C hold. PCR products were run on a gel to verify the product and then quantified via Qubit dsDNA HS kit. Samples that did not initially produce PCR products were amplified again and these were used for subsequent sequencing. Samples were then pooled at equimolar concentrations (100 ng PCR product per sample) and Pippin Prep (Sage Science, Beverly, MA, USA) was used to purify for PCR products between 200-800 bp (as PCR products included hetero-spacers and Illumina adapters). Due to low DNA concentration of amplified products for the comparison of roots, rhizonodes, and soils, the sample PCR protocol was followed as per above, however, samples were amplified again with the following conditions: 96° C for 2 min, 10 cycles of 94 °C for 30 sec, 58 °C for 40 sec, 72 °C for 2 min, and 72 °C for 10 min before 4 °C hold. DNA concentrations were re-quantified using the Qubit dsDNA HS kit, then pooled at equimolar concentrations (50 ng total DNA per sample) and cleaned with Ampure XP (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions.

Imaging

Fresh tissue was imaged on a Leica MZ16F dissecting microscope with a Qimaging MicroPublisher 6 USB 3.0 CCD camera. Rehydrated fixed samples were sectioned to 70 µm on a Leica VT1000 S Vibrating blade microtome. They were then stained in 0.05% aniline blue and destained with sterile water. Stained sections were

imaged on a Leica DM4000 compound microscope with a Leica DFC7000 USB 3.0 CCD camera.

	Observed			Shannon		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	106.29	83.50 – 129.08	**<0.001**	3.23	2.81 – 3.65	**<0.001**
WEDG	-5.24	-9.07 – -1.41	**0.007**	-0.17	-0.24 – -0.09	**<0.001**
Random Effects						
σ^2	804.27			0.59		
τ_{00}	63.92 $_{SiteID}$			0.00 $_{SiteID}$		
ICC	0.07					
N	8 $_{SiteID}$			8 $_{SiteID}$		
Observations	118			118		
Marginal R^2 / Conditional R^2	0.109 / 0.175			0.151 / NA		

Table S1. Weighted-edge density models for observed species richness and Shannon index of diversity for soil fungi (linear-mixed effects models)

	Simpson			Shannon		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.76	0.69 – 0.82	**<0.001**	2.26	2.00 – 2.52	**<0.001**
scale(pH)	0.03	-0.02 – 0.07	0.209	0.11	-0.06 – 0.29	0.212
Random Effects						
σ^2	0.03			0.37		
τ_{00}	0.01 $_{SiteID}$			0.12 $_{SiteID}$		
ICC	0.19			0.24		
N	8 $_{SiteID}$			8 $_{SiteID}$		
Observations	111			111		
Marginal R^2 / Conditional R^2	0.025 / 0.210			0.025 / 0.254		

Table S2. Best-fit models for alpha-diversity of root fungi based on linear-mixed effects models.

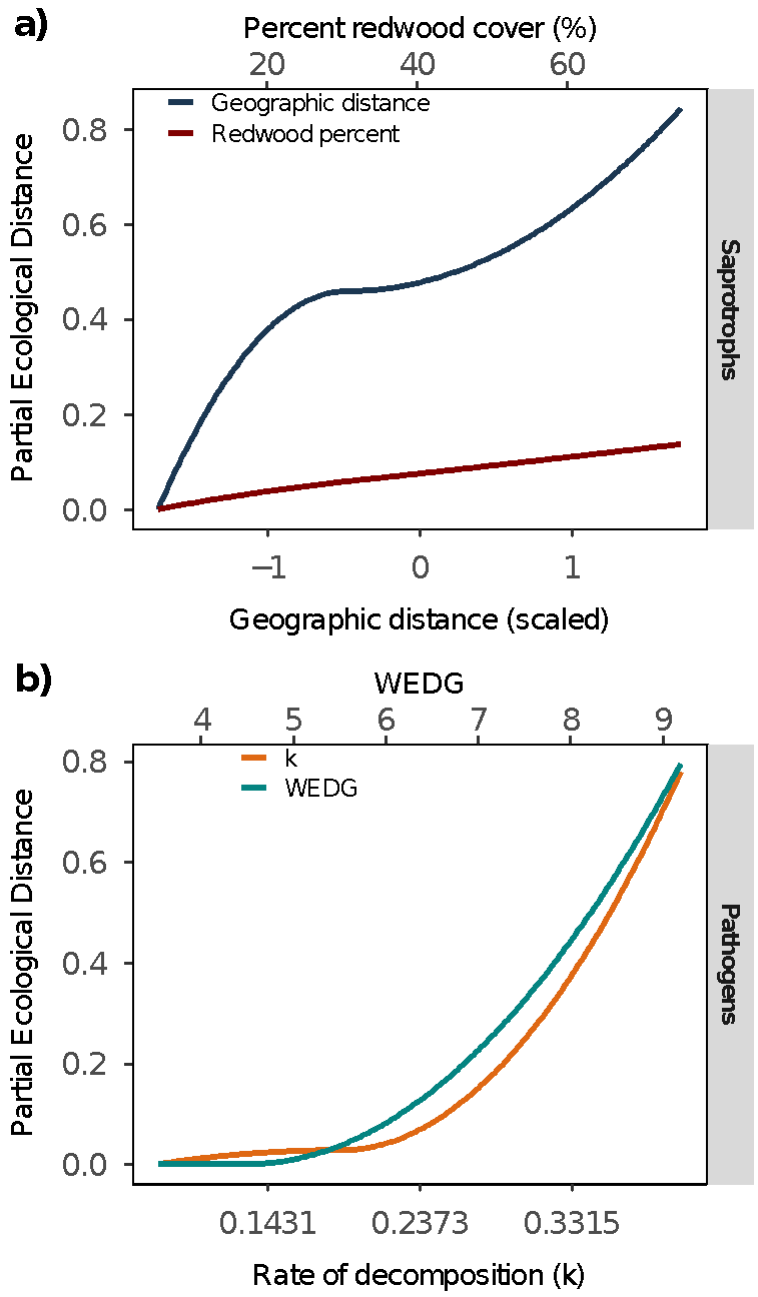

Figure S1. Generalized dissimilarity modeling (GDM) of saprotrophic (a) and pathogenic (b) components of the fungal community in soils (standardized across predictor variables). GDM-fitted I-splines for each environmental covariate and geographic distance. The maximum height of each curve indicates the total amount of turnover in fungal communities associated with that variable (the relative importance of that variable in explaining changes in fungal community structure) holding all other variables constant. The shape of each function indicates how the rate of change varies along the gradient.

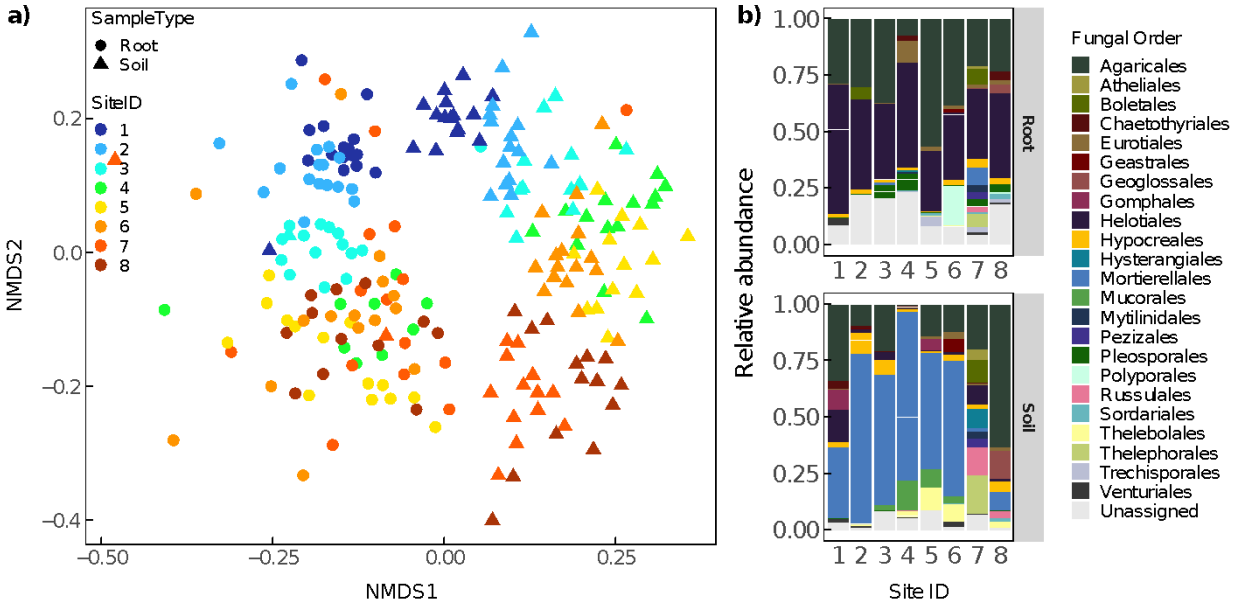

Figure S2. Non-metric multidimensional scaling (NMDS) across the redwood range for both roots and soils. (b) Barplot of the top 100 taxa by Fungal Order found across the geographic range of redwoods found in roots and soils. Fungal communities differed significantly by site and sample type (root versus soils).

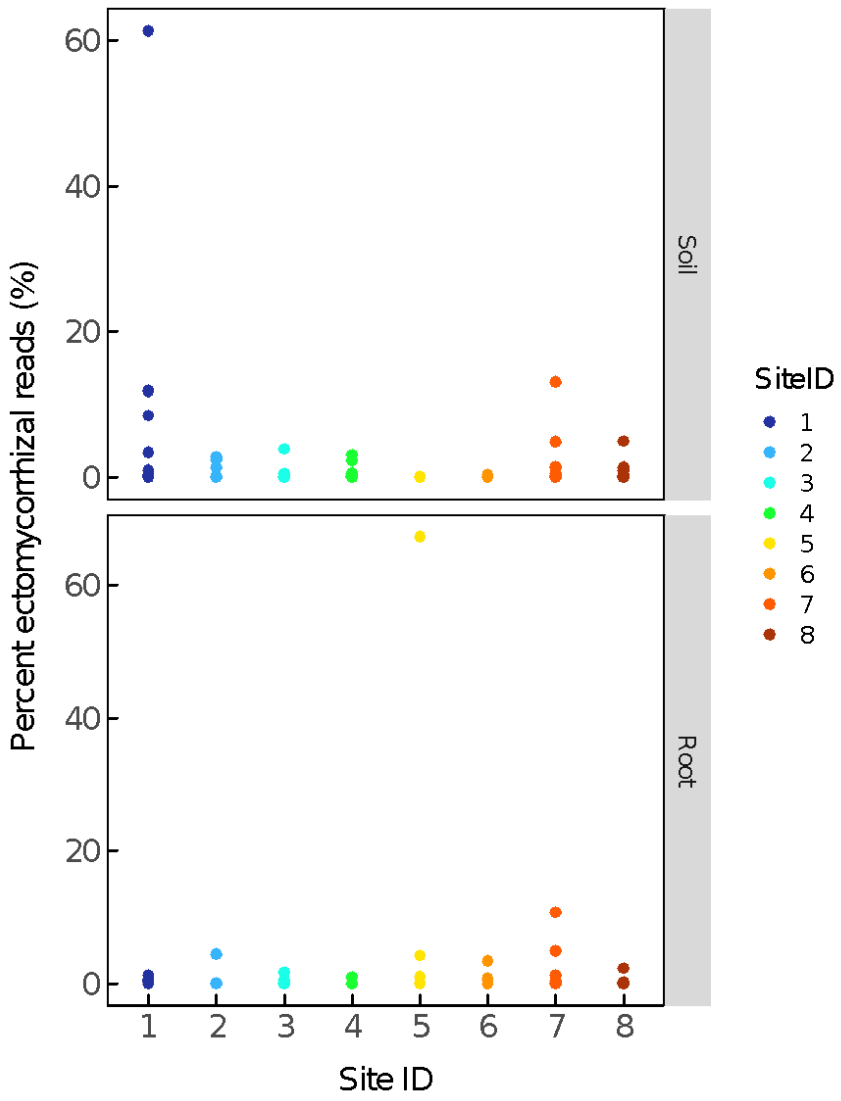

Figure S3. Relative proportion of ectomycorrhizal fungal reads found associated with coast redwood root versus soil samples based on FUNGUILD delineations from rarified samples. A relatively small, but consistent proportion of fungal read depth in roots belong to taxa which were identified as ectomycorrhizal.

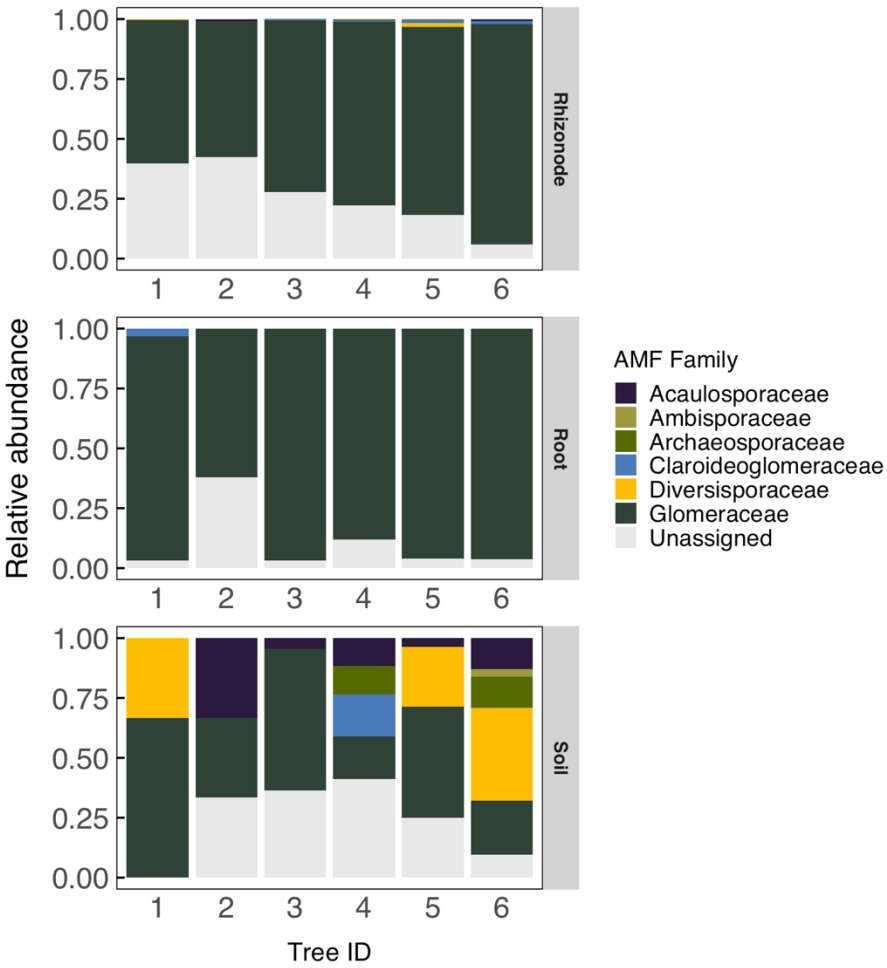

Figure S4. Barplot of different arbuscular mycorrhizal fungal families found across the soil, root, and rhizonode environments in the top 500 taxa.

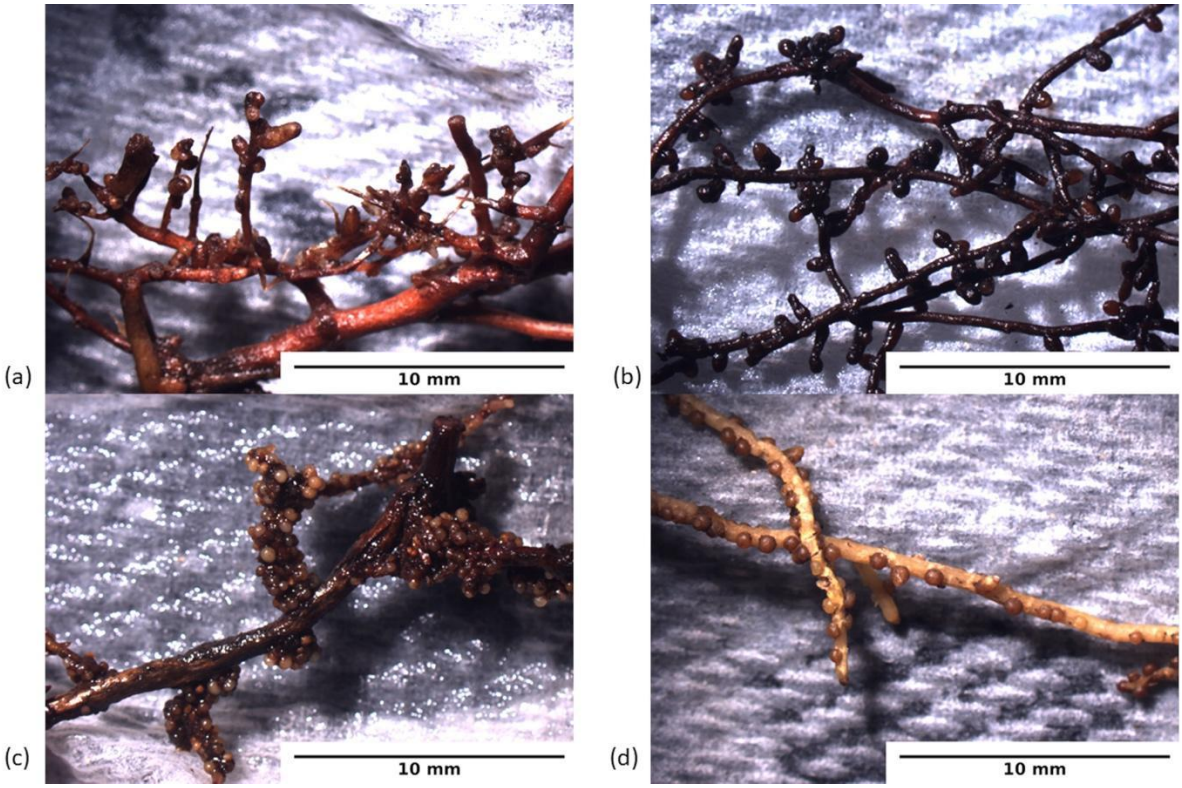

Figure S5. Similar nodule-like rhizonode structures on roots of other conifers associated with arbuscular mycorrhizal fungi. (a) *Sequoia sempervirens*, the coast redwood, Cupressaceae, (b) *Sciadopitys verticillata*, the Japanese umbrella tree, Sciadopityaceae, (c) *Dacrydium cupressinum*, rimu, Podocarpaceae, and (d) *Agathis ovata*, the mountain kauri, Araucariaceae, all collected from the UC Botanical Garden, Berkeley, CA.

Chapter 3
Using Holo-omics to Identify Target Genes for CRISPR/Cas9 Editing

Contributions

The contents of this chapter are derived from a collaboration with the EPICON Project. The chapter represents work from three separate manuscripts on which I am a co-author. Their full citations are as follows:

Xu, Ling, Dan Naylor, Zhaobin Dong, Tuesday Simmons, Grady Pierroz, Kim K. Hixson, Young-Mo Kim, Erika M. Zink, Kristin M. Engbrecht, Yi Wang, Cheng Gao, Stephanie DeGraaf, Mary A. Madera, Julie A. Sievert, Joy Hollingsworth, Devon Birdseye, Henrik V. Scheller, Robert Hutmacher, Jeffery Dahlberg, Christer Jansson, John W. Taylor, Peggy G. Lemaux, and Devin Coleman-Derr. 2018. "Drought Delays Development of the Sorghum Root Microbiome and Enriches for Monoderm Bacteria." Proceedings of the National Academy of Sciences of the United States of America 115 (18): E4284–93.

Varoquaux, Nelle, Benjamin Cole, Cheng Gao, Grady Pierroz, Christopher R. Baker, Dhruv Patel, Mary Madera, Tim Jeffers, Joy Hollingsworth, Julie Sievert, Yuko Yoshinaga, Judith A. Owiti, Vasanth R. Singan, Stephanie DeGraaf, Ling Xu, Matthew J. Blow, Maria J. Harrison, Axel Visel, Christer Jansson, Krishna K. Niyogi, Robert Hutmacher, Devin Coleman-Derr, Ronan C. O'Malley, John W. Taylor, Jeffery Dahlberg, John P. Vogel, Peggy G. Lemaux, and Elizabeth Purdom 2019. "Transcriptomic Analysis of Field-Droughted Sorghum from Seedling to Maturity Reveals Biotic and Metabolic Responses." Proceedings of the National Academy of Sciences of the United States of America, December. https://doi.org/10.1073/pnas.1907500116.

Xu, Ling, Grady Pierroz, Heidi M-L Wipf, Cheng Gao, John W. Taylor, Peggy G. Lemaux, and Devin Coleman-Derr. 2021. "Holo-Omics for Deciphering Plant-Microbiome Interactions." Microbiome 9 (1): 69.

Sorghum bicolor is the fifth most important grain crop in the world, grown primarily in China and sub-Saharan Africa (Mace et al. 2013). Sorghum has become increasingly popular in the United States as well, in large part due to its phenomenal drought tolerance, which is an important trait for ensuring food security in the face of anthropogenic climate change (Duff et al. 2019; Mundia et al. 2019). In order to gain more insight into the molecular underpinnings of its drought tolerance, nine different Principal Investigators (PIs) from multiple institutions participated in an ambitious study on two sorghum genotypes grown in an agricultural field under two different drought regimes, known as the EPICON Project. This project incorporated data across multiple omic levels from both hosts and their microbiomes, and therefore falls into the newly designated category of "holo-omics" (Nyholm et al. 2020).

Because both of my faculty advisors were on the EPICON project, and one was in fact the lead PI for the project, I was in a unique position to participate in the investigation in a very broad capacity. As a member of the Lemaux Lab, I travelled to the Kearney Agricultural Research and Extension (KARE) Center on multiple occasions to participate directly in sampling from the field and transporting the samples back to UC Berkeley for processing. I also participated in monthly EPICON meetings and the yearly

retreats, and helped shape the directions of experimentation as well as determine the most appropriate methods for such an ambitious holo-omic study.

I was also heavily involved in analyzing the RNA-sequencing data processed by Dr. Nelle Varoquaux. Using data visualizations she produced, I assessed functional categories, Gene Ontology (GO) enrichment terms, and custom *a priori* gene lists based on the literature for observable differential expression patterns either under drought or between genotypes. Of the many possible avenues queried, we eventually decided to focus our story on transcriptional changes in photosynthesis, ROS, and AMF-related genes.

As a member of the Coleman-Derr lab, I helped build upon and validate Dr. Ling Xu's 16S amplicon sequencing results as well. In order to build further support for the hypothesis that elevated G3P levels in sorghum roots may be involved in shaping microbiome structure, I designed qPCR primers for a suite of G3P-related genes highlighted in the transcriptomic study. I then analyzed the expression of these genes and developed a model for our hypothesis, namely that intracellular utilization of G3P decreases during drought while export of G3P to the apoplast increases, which is summarized in Fig. 4 of this chapter.

Finally, in large part because I was involved so broadly in EPICON, I helped write and edit a review paper about the advantages and challenges of performing large holo-omic studies, specifically in plants as a means of investigating causal mechanisms behind microbiome community structure. This review extolled the virtues of the EPICON project, and served as a summation or capstone to the project as a whole. The EPICON Project was truly an exemplary holo-omic study in plant biology, and was very successful in generating evidence-based hypotheses about factors that might be involved in sorghum's extreme drought tolerance. The following chapter is taken in large part from three manuscripts on which I am a co-author that were written as part of the EPICON project (Varoquaux et al. 2019; Xu et al. 2018; Xu, Pierroz, et al. 2021).

Introduction

Although it is well understood that the root microbiome is critical for maintaining plant health (Compant et al. 2019; Saikkonen, Nissinen, and Helander 2020); (Rodriguez et al. 2019; Xu et al. 2018) and that the plant host can exert some level of control over the composition of its microbiome via compartmentalization and biological filtering (Chomicki et al. 2020; Maciá-Vicente, Nam, and Thines 2020), the exact genetic basis behind these observations has remained a mystery. Due to the inherent complexity of the root microbiome and interplay between host-microbe, microbe-microbe, host-environment, and microbe-environment interactions, the identification of single genes determining root microbiome structure and function have been elusive, particularly since classical forward genetic screens are all-but impossible due to the difficulty of discerning discrete phenotypes at low levels of replication. CRISPR/Cas9 gene editing technology, however, has made the application of reverse genetic screens for determining gene function much more feasible.

The advent of CRISPR/Cas9 targeted gene editing truly has the potential to revolutionize reverse genetic screens in plants. The ability to selectively knock out almost any gene in a variety of species will allow researchers to investigate gene function in non-model organisms with unparalleled ease and precision. However, when working in non-model species or on complex traits such as microbiome structure, a new problem presents itself: Where to begin? A dearth of foundational experimentation and resultant poorly annotated genomes makes identifying interesting candidate genes for Cas9 editing difficult. Determinations based on sequence homology in model systems become less relevant with increased genetic distance and precludes the discovery of anything truly novel, i.e. not already well characterized in a model species. This issue becomes even more difficult when considering emerging fields in plant biology, such as the root microbiome, which are poorly understood even in model systems.

Thus, in order to investigate plant genetics coupled with simultaneous microbiome phenotypic changes, researchers have begun to pair host-centered omic strategies, such as transcriptomics, metabolomics, epigenomics, and proteo-mics, with the more commonly used microbe-focused techniques, such as amplicon sequencing, shotgun meta-genomics, metatranscriptomics, and exo-metabolomics. Nyholm et al. recently coined the phrase “holo-omics” to describe such experiments that incorporate data across multiple omic levels from both host and microbiota domains (Nyholm et al. 2020). Such holo-omic studies have the power to resolve the functionality of a plant microbiome ecosystem by generating an image of what is being expressed, translated, and produced during plant-microbiome interactions. This multifaceted image can help winnow results obtained from each individual dataset to meaningful biological signals, and help build support for specific hypotheses with data gathered through orthogonal approaches. These data-driven hypotheses can then be investigated and validated directly through the use of CRISPR/Cas9 gene editing.

In this chapter I will present a large, collaborative holo-omic project called EPICON as a case study on the applicability of plant holo-omics in general as a means to determine CRISPR/Cas9 gene editing targets in non-model species, specifically with regards to investigating genetic control over microbiome structure under drought stress.

I will briefly discuss the extensive results obtained from EPICON, while detailing the methods and rationale used to determine gene editing targets for validation. I hope this work can act as a road map for other researchers wishing to investigate and validate the multifaceted phenomenon of the plants' genetic control over the structure of their root microbiomes.

Materials and Methods

Overview and Rationale

The EPICON Project was originally conceived as an investigation of the effects of epigenetics on drought tolerance in sorghum (*Sorghum bicolor*), hence the name being derived from EPIgenetic CONtrol of Drought. Sorghum was chosen as the system to investigate because of its well documented drought tolerance (Ngara and Ndimba 2014) as well as its status as a staple food for millions of people (Mace et al. 2013) and its phylogenetic relatedness to both maize (*Zea mays*) and sugar cane (*Saccharum officinarum*) (Nair et al. 2005). In brief, two sorghum genotypes were grown under three watering conditions (no irrigation until anthesis i.e. pre-flowering drought, no irrigation after anthesis i.e. postl-flowering drought, and a fully watered control). Leaf, root, rhizosphere, and soil samples were collected once a week for the entire growing season (seventeen weeks in total). These samples were flash frozen in the field and subsequently processed and investigated via transcriptomic, metabolomic, metagenomic, epigenomic, and metatranscriptomic analysis pipelines. This provided us with a massive, synchronous, holo-omic, time-course dataset reflecting sorghum's response to drought across genotypes and watering treatments. The synthesis of these datasets was subsequently used to develop and test hypotheses, as well as to determine genes of interest for CRISPR/Cas9 gene editing for functional validation.

Field experimental design

The main field experiments were conducted in Parlier, CA (36.6008°N, 119.5109°W); historical monthly averages of rainfall in this region during the summer growth season (May to September) are approximately zero and soils are characterized as sandy loam soils with a silky substratum and pH 7.37. We planted two different sorghum cultivars (RTx430 and BTx642) in the summer of 2016 within a randomized block design that accounted for treatment, genotype and replication, with three replicate blocks in total. BTx642 (formerly B35) and RTx430 were chosen for testing the drought response in early and late plant developmental stages based on their contrasting drought tolerances and similar flowering times. BTx642 is described as a pre-flowering drought susceptible, and post-flowering drought-tolerant variety, while RTx430 is described as pre-flowering drought-tolerant, post-flowering drought-susceptible. A simplified sampling diagram as well as images comparing the two cultivars are presented in Fig 1A-E.

Irrigation management

Pre-flowering drought treatment was imposed on one third of the blocks and consisted of a complete lack of irrigation until rewetting, at the ninth week after planting

(WAP). In contrast, the post-flowering drought treatment was induced on another third of the blocks by stopping watering only after over 50% of sorghum plants reached flowering (anthesis), at the 10th WAP. Control blocks, which account for the remaining third of the blocks, received 80% of calculated evapotranspiration each week for the entire growing season. A drip system was utilized to apply all irrigation water during the growing season, consisting of drip lines placed on the surface of each furrow (0.76 m row spacing), with 0.3 m emitter spacing and 2 L/h emitter output. The pre-flowering period stress treatment was imposed by providing no irrigation during the period from seedling emergence (June 3) until the flowering growth stage, with the first within-season irrigations started on July 29 for that treatment. Irrigations continue after that date at timings and amounts that matched the non-stressed control treatments. In contrast, the post-flowering drought treatment was irrigated at 7-day intervals during the period from June 21 through July 29 on the same dates and amounts as the control treatment. The July 29 date corresponded with the time at which approximately 50-55% of sorghum plants were flowering on primary heads, at the 10th WAP. Measured rainfall during the June 3 to September 29 period was less than 1 mm. To avoid redundancy in sampling, pre-flowering treatment blocks were collected from TP3 to TP17 and post-flowering treatment blocks were collected from TP8 to TP17, as TP1-TP2 of pre-flowering drought, and TP1-TP7 of post-flowering drought were identical in treatment to the control samples.

Sample collection and processing

Plant samples were collected by manually extracting whole plants with root systems using a shovel to a depth of approximately 30cm. We collected rhizosphere samples (soil tightly adhering to the sorghum root surface) by collecting and pooling roots severed with sterile blades from 10 plants per genotype and treatment type at each time point. Roots were vortexed in epiphyte removal buffer (0.75% KH_2PO_4, 0.95% K_2HPO_4, 1% Triton X-100 in ddH_2O; filter sterilized at 0.2 μM) for 5 minutes and centrifuged to pellet the resulting rhizosphere soil at 3500rpm for 5 minutes after removal of the root tissue. Root endosphere samples were obtained by washing the vortexed roots twice in fresh sterile buffer and quick-frozen in liquid nitrogen. Bulk soil samples were collected approximately 12 inches away from the sample plants we collected using a 6” soil corer. All samples were collected weekly at the same time of day (between 10am and 1pm) and the same day of the week for seventeen weeks following seedling emergence. To aid in the DNA extraction process, roots samples were first ground in a cryogenic Freezer Mill (SPEX SamplePrep 6875D, Metuchen, NJ, USA).

16S library preparation

Root endophyte DNA, as well as rhizosphere and soil-associated microbe DNA, were extracted from processed samples using the MoBio Power Soil DNA isolation kit (Catalog No. 12888-100; MoBio, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA concentrations were measured with a Qubit 3 Fluorometer and samples were diluted to 5ng/ μl to help ensure approximately equal template amounts. Samples were amplified using a dual-indexed 16s rRNA Illumina iTags primer set

specific to the V3-V4 region (341F (5'-CCTACGGGNBGCASCAG-3') and 785R (5'-GACTACNVGGGTATCTAATCC-3') as described in (Takahashi et al. 2014) using 5-Prime Hot Master Mix (catalog No. 2200410). Reactions included 11.12 µl DNase-free sterile H2O, 0.4 µg BSA, 10.0 µl 5-Prime Hot Master Mix, 2 µl template, and 0.75 µM of each of two peptide nucleic acids (PNAs) (Sakai and Ikenaga 2013) designed to target host-derived amplicons from chloroplast and mitochondria 16S rRNA sequences. PCR reactions were performed in triplicate in three individual thermocyclers with the following conditions: initial 3 min cycle at 94°C, then 30 cycles of 45 s at 94°C, 10 s at 78°C, 1 min at 50°C, and 1.5 min at 72°C, followed by a final cycle of 10 min at 72°C. Triplicates were then pooled (192 samples per library) and DNA concentration for each sample was quantified using Qubit 3 Fluorometer. Pools of amplicons were constructed using 100 ng for each PCR product. Before submitting for sequencing, pooled samples were cleaned up with 1.0 X volume Agencourt AMPureXP (Beckman-Coulter, West Sacramento, CA) beads according to the manufacturer's directions, except for the modifications of using 1.0 X rather than 1.6 X volume beads per sample, dispensing 1500 µl 70% EtOH to each well rather than 200 µl, and eluting in 100 µl DNase-free H2O rather than 40 µl. An aliquot of the pooled amplicons was diluted to 10 nM in 30 µl total volume before submitting to the QB3 facility at UC Berkeley for sequencing using Miseq 300bp pair-end with v3 chemistry. The resulting amplicon libraries produced on average approximately 45259, 41582 and 34649 reads per sample for soils, rhizospheres, and roots respectively. The use of PNAs reduced non-bacterial read contaminants to <3% in all sample types. All of our sequencing was performed by QB3-Berkeley Functional Genomics Laboratory (http://qb3.berkeley.edu/fgl/).

Amplicon sequence data processing

Raw reads were processed using the iTagger pipeline (Tremblay et al. 2015) developed at the Joint Genome Institute. In brief, after filtering 16S rRNA raw reads for known contaminants), primer sequences were trimmed from the 5' ends of both forward and reverse reads. Low-quality bases were trimmed from the 3' ends prior to assembly of forward and reverse reads with FLASH (Magoc and Salzberg 2011). The remaining high-quality merged reads were clustered with simultaneous chimera removal using UPARSE (Edgar 2013). Taxonomies were assigned to each Operational Taxonomic Unit (OTU) using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier (Qiong Wang et al. 2007) with custom reference databases. After taxonomies were assigned to each OTU, we discarded 1) all OTUs that were not assigned a Kingdom-level RDP classification score of at least 0.5, and 2) all OTUs that were not assigned to Kingdom Bacteria. To remove low abundance OTUs, we removed OTUs without at least 5 reads in at least 3 samples. We also removed samples having less than 10,000 reads. All samples were then rarefied to 13,000 reads per sample for specific analyses, or alternatively, by dividing the reads per OTU in a sample by the sum of usable reads in that sample, resulting in a table of relative abundance frequencies; OTUs which were reduced to less than one read per OTU after rarefaction were also discarded. For differentially expressed OTU analysis, we inputted raw read counts to the DESeq package and processed with a negative binomial model. In total, 249, 251, and 246 soil, rhizosphere, and root samples, respectively, were included in downstream analysis. All

scripts used for the statistical analysis of data in this manuscript are included as part of a public repository on github (https://github.com/dcolemanderr/EPICON210 Drought-Study). All raw reads from the 16S rRNA analysis are available to the public, and were submitted to the NCBI sequence read archive (PRJNA435634). More detailed methods describing 16S amplicon library creation, sequencing, and data processing can be found in (Xu et al. 2018).

Quantitative PCR

For the qPCR experiments, plant root total RNA was extracted using the RNeasy Mini Kit (Qiagen Catalog No. 74104). A sample of 2 µg of total RNA was treated with DNase I (Invitrogen catalog No. 18068015), and reverse transcription was conducted using Superscript III First-Strand Synthesis System (Invitrogen Catalog No. 18080051) with oligo (dT) 18 primers. The quality of the cDNA was tested by qPCR with an internal control gene GAPDH (Sudhakar Reddy et al. 2016). By the Ct (cycle threshold) value of the test run, we were able to normalize the amount of cDNA between samples.

Quantitative reverse transcription-PCR was performed using QX200 EvaGreen Supermix (Bio Rad, Catalog No. 1864034). Three biological replicates were performed for each reverse transcription-PCR experiment. Sorghum CYP, PP2A and GAPDH genes were used as the internal reference to normalize the expression data. Relative expression levels were calculated according to the 2-2ΔΔCT 423 (cycle threshold) method, and the standard deviation was calculated among the three biological replicates. The starting DNA template amount for taxa-specific qPCR is 10ng per sample.

Metatrascriptome sequencing

Microbial RNA for metatranscriptomic analysis was extracted from soil and rhizosphere samples using the PowerMax soil DNA isolation kit for RNA extraction with a modified protocol provided by MoBio. RNA was purified using an RNeasy PowerClean Pro Cleanup Kit (MoBio, Carlsbad, CA, USA). The concentration was assessed using a Qubit 3 Fluorometer (Invitrogen, Carlsbad, CA, USA) and quality was assessed using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

For preparation of the metatranscriptome libraries from soil, a DNase Max kit (Qiagen, Catalog No. 15200-50) was used to digest DNA from the total RNA. Next, we used the Ribo-Zero rRNA Removal Kit (Bacteria, Illumina, Catalog No. MRZB12424) to remove ribosomal RNA from bacteria by following the manufacturer's instruction. Subsequently, the TruSeq Stranded Total RNA Library Prep plant Kit (Illumina, Catalog No. 20020610) was used according to the manufacturer’s instructions to make 300-500bp fragment libraries for sequencing on a HiSeq 2500 platform 150bp paired-end. All of our sequencing were performed by QB3- Berkeley Functional Genomics Laboratory (http://qb3.berkeley.edu/fgl/).

RNA-sequencing, read processing, and quality control

Plant RNA for transcriptomic analysis was extracted using the QIAGEN miRNeasy Mini Kit (Catalog No. AM217004, QIAGEN, Redwood City, CA, USA) with slight modifications.
DNA contamination was removed from each extracted RNA sample using the TURBO DNA-free kit, (Cat. #AM1907, Invitrogen) according to the manufacturer's recommendations. Stranded cDNA libraries were generated using the Illumina Truseq Stranded RNA LT kit. mRNA was purified from 1 µg of total RNA using magnetic beads containing poly-T oligos. mRNA was
fragmented and reverse transcribed using random hexamers and SSII (Invitrogen) followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles of PCR. qPCR was used to determine the concentration of the libraries. Libraries were sequenced on the Illumina HiSeq.

To increase read mapping rates for transcriptomic data, we generated custom SNP corrected reference genome sequences for the two strains used in this study. Then, raw fastq file reads were filtered and trimmed using the JGI QC pipeline resulting in the filtered fastq file (*.filter-RNA.gz files). Using BBDuk (bbd), raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed. Filtered reads from each library were aligned to the reference genome (phytozome v3.1, supplemented with RTx430 and BTx642 SNP information) using HISAT version 2.1.0 (D. Kim, Langmead, and Salzberg 2015) (BAMs/ directory). FeatureCounts (Liao, Smyth, and Shi 2014) was used to generate the raw gene counts (counts.txt) file using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p –primary options). We then performed quality analysis on the raw data and removed one sample of low-quality (low correlation to its replicates). Next, we applied the following normalization procedure: we filtered out low expressed genes and retained genes containing at least 20 reads in at least 3 samples and then applied upper-quartile normalization on the resulting set of genes to normalize using EDA-Seq (Bullard et al. 2010; Risso et al. 2011).

Differential expression analysis

First, we identified genes differentially expressed across the time-course experiment. Then, for each genotype and drought regime, we tested if functional gene expression was different between the watered and droughted conditions. Next, we compared the functional gene expression for the two genotypes under watered conditions to assess constitutive genotype differences. We also assessed genotype-specific drought responses by testing whether the change in gene expression due to drought is the same in the two genotypes for each drought regime. Finally, we evaluated the number of genes differentially expressed in the first weeks of pre-flowering drought, and we considered an additional contrast to identify genes whose expression abruptly changes between watering changes (either water resumption for pre-flowering or commencement of drought for post-flowering).

We then applied a clustering strategy to group genes on their gene expression patterns. We performed this clustering routine separately for the four different combinations of sample types (leaf/root) and drought conditions (pre- versus post-flowering droughted plants). First, we calculated the Fisher's combined probability (Fisher 1992) based on the unadjusted p-values from three of the across-time DE analyses (drought versus control in RTx430, drought versus control in BTx642, and genotype-specific drought response) as well as the unadjusted p-value from the "water change" analysis. We then ranked the genes by their Fisher's combined probability. We selected the 5000 genes with the lowest Fisher's p-value, and that had a log-fold change of at least 2 in at least a week. We then fitted a split-spline function to each gene and each condition. Finally, we then applied k-means to the resulting fitted values, with the number of clusters (K) set to 20 for pre-flowering drought stress, and 10 for post-flowering drought stress. This results in 60 centroid vectors.

To facilitate interpretation of clusters, we performed KEGG pathway enrichment analysis and GO term enrichment analysis on the set of "good-matched" genes from each cluster independently from one another. To perform KEGG pathway enrichment analysis, we applied the R package KEGGprofile. To detect GO terms highly enriched in each cluster, we applied gene set enrichment analysis (GSEA) (Subramanian et al. 2005). We then corrected for multiple tests using Benjamini-Hochberg. More detailed methods regarding the RNA-sequencing and differential expression analysis can be found in (Varoquaux et al. 2019).

Metabolic data collection

The flash frozen leaf and root tissues were mechanically ground separately using a cryogenic freezer mill (SPEX, Metuchen, NJ). Then MPLEx extraction was applied to samples after being weighed to 1 g (Y.-M. Kim et al. 2015). For the analysis of metabolites, 5% of metabolite extract was transferred to a glass vial and 10 µL of 13C-labeled levoglucosan (1 mg/mL) was added as an internal standard. Subsequently, the samples were completely dried under a speed vacuum concentrator and the dried metabolites were analyzed by gas chromatography-mass spectrometry (GC/MS) at the Pacific Northwest National Laboratory, Richland, WA, as reported previously (Hiller et al. 2009). Briefly, dried samples were derivatized by adding 20 µL of methoxyamine solution (30 mg/mL in pyridine) and were incubated at 37 °C for 90 min to protect the carbonyl groups and reduce carbohydrate isoforms. Then, 80 µL of N-methyl-N-(trimethylsilyl)- trifluoroacetamide with 1% trimethylchlorosilane was added to each sample and incubated for 30 minutes as a minimum. The derivatized samples were analyzed by GC/MS within 24 hours after the derivatization. Data collected by GC/MS were processed using the Metabolite Detector software, version 2.5 beta (Kind et al. 2009).

Metabolic data analysis

Retention indices of detected metabolites were calculated based on analysis of the fatty acid methyl esters mixture (C8 - C28), followed by chromatographic alignment across all analyses after de-convolution. The intensity values of selected three fragmented ions after deconvolution were integrated for a peak value of the metabolite.

Metabolites were initially identified by matching experimental spectra to a PNNL-augmented version of the Agilent Fiehn Metabolomics Library containing spectra and validated retention indices for almost 900 metabolites (Richter-Heitmann et al. 2016) and additionally cross-checked by matching with NIST14 GC-MS Spectral Library (https://www.nist.gov/srd/nist-standard-reference-database-1a-v17). All metabolite identifications were manually validated to minimize deconvolution and identification error during the automated data processing. Prior to further statistical analysis to determine significantly enriched metabolites, the data were normalized through a log2 transformation and then mean centered across the log2 distribution. Differentially expressed metabolites were analyzed by edgeR with quasi-likelihood F-tests. The cutoff was set as fold change > 2, and p-value < =0.05.

HPLC Quantification of G3P

For G3P quantification, approximately 1 g of fresh root tissue per sample was frozen in liquid nitrogen and homogenized in 25 ml 80% (v/v) ethanol containing 20 µM 2-Deoxy D-glucose as an internal standard. The samples were heated in a boiling water bath for 10 minutes and centrifuged at 3000 x g for 20 minutes. 1 mL of supernatant was collected from each sample into a new tube and dried by vacuum centrifugation. Extracts were resuspended in 0.5 ml water and filtered through 0.45 µm centrifugal filters. Samples were analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on an ICS-3000 (Thermo Fisher Scientific) fitted with a CarboPac PA1 (2 x 250 mm) column.

Results

Drought induces enrichment of monoderm lineages in root endosphere communities

To determine how drought affects the observed developmental dynamics of the root microbiome, we compared the phylum-level compositional profile of pre-flowering drought- and control-treated samples. In roots grown under pre-flowering drought treatment, we observed an enrichment for Actinobacteria and Firmicutes that is consistent with recently published reports (Naylor et al. 2017; Santos-Medellín et al. 2017; Fitzpatrick et al. 2018). This enrichment progressed over the course of drought treatment (6 wk) until watering resumed (Fig. 2A). Strikingly, within 1 wk following rewatering, the root microbiome of previously drought-treated plants rapidly returned to a pattern of community progression observed in younger control roots (Fig. 2A-C). This rapid rewatering-induced shift in root endosphere composition is driven by a more than 200% increase in the relative abundance of the diderm lineages Proteobacteria and Bacteroidetes ($P < 0.001$) and a similarly large decrease in the monoderm phyla Actinobacteria ($P = 0.012$) (Fig. 2A). As the relative enrichment in Actinobacteria could be the result of an absolute increase in their abundance, or an absolute decrease in other dominant taxa, we measured the absolute abundance of several lineages in drought and control root samples through qPCR using lineage-specific primers. These results demonstrate that in this field experiment, drought treatment leads to an overall decrease in total bacterial abundance, but that Actinobacteria and Firmicutes show

significantly greater resistance to these shifts compared with Proteobacteria (Fig. 2D-E). Additionally, we observed that the decreases were greater for all community members at the peak of drought (TP8; Fig. 2D) compared with earlier in the drought treatment (TP4; Fig. 2E), which suggests that the absolute decrease in abundance is correlated with the length of drought treatment. Taken together, these results suggest that the root microbiome composition of field-grown sorghum is sensitive to drought perturbation early in plant development, and that this perturbation results in decreased abundance of the total bacterial community and a phylum-level relative enrichment of select monoderm bacterial lineages.

Drought Increases Actinobacterial Transcription Related to Metabolite Transport.

We next sought to establish if the drought-induced shifts in our rhizosphere metatranscriptomes were correlated with changes in the expression of specific bacterial functions. Within the rhizosphere, we observed a significant increase at the peak of drought (TP8) in transcripts associated with carbohydrate transport and metabolism, amino acid transport and metabolism, and secondary metabolite biosynthesis (Fig. 3A); by contrast, soils exhibited a relative decrease in all three of these categories and an enrichment for a distinct set of functions, including energy production, transcription, and posttranslational modification (Fig. 3A). Additionally, we observed that gene categories exhibiting enrichment under drought changed after recovery (TP9) for both the rhizosphere and soil (Fig. 3B). A finer resolution analysis of functional subcategories influenced by drought in the rhizosphere at TP8 revealed that a significant number of the most enriched gene subfunctions were related to resource transport, including those for both amino acids (Fisher's exact test: $P < 0.039$; Table 1) and carbohydrates (Fisher's exact test: $P < 0.0005$; Table 2), although it is worth noting that ATP-binding cassette (ABC)–type transporters are three-component systems, which may be artificially inflating this observed enrichment. In accordance with our taxonomic analysis of the metatranscriptome data, these data also revealed that the majority of differentially expressed genes in the rhizosphere (~90%) in all functional categories belong to Actinobacteria (Fig. 3C), compared with ~50% in the soil community (Fig. 3C). Finally, to determine if the enrichment in carbohydrate and amino acid transport and metabolism GO categories is merely a consequence of the increased relative abundance of Actinobacteria or, alternatively, a shift in Actinobacterial function, we performed an analysis of the GO functional category assignments of all actinobacterial transcripts (Fig. 3D). This analysis revealed that the drought-induced shifts in rhizosphere function are driven by significant changes in gene expression within the actinobacterial lineage in almost all GO functional categories, by carbohydrate and amino acid transport and metabolism, and by increased expression of ABC transporters (Fig. 3E). Taken together, these data suggest that drought has a significant effect on the transcriptional activity of the root-associated microbiome, that rhizosphere genes associated with carbohydrate and amino acid metabolism and transport show increased expression under drought, and that the altered transcriptional activity in the rhizosphere microbiome during drought is largely due to shifts in actinobacterial activity and function.

Drought-Induced Shifts in Root Metabolism Correlate with Altered Rhizosphere Transcriptional Activity.

To investigate whether drought-induced shifts in the rhizosphere microbiome transcriptional activity, specifically the increased expression of transporters of carbohydrates and amino acids, are correlated with shifts in sorghum root metabolism, we performed untargeted metabolomics on sorghum roots using gas chromatography–mass spectrometry (GC-MS) at the peak of pre-flowering drought (TP8) and after rewatering (TP9). Through comparative analyses across treatments, we identified a large number of identifiable drought-enriched root metabolites (n = 114), including a variety of carbohydrates and amino acids (Table 3). The most significantly enriched metabolite is glycerol-3-phosphate (G3P), which is 4.34 log10-fold more abundant in drought-treated than control roots. Interestingly, among the significantly drought-enriched carbohydrate gene subcategories in the rhizosphere metatranscriptomes (Table 2), we observed a strong enrichment of ABC-type transporters of G3P, which had the largest number of up-regulated genes in the dataset (n = 191; P = 0.0001328).

As the observed increase in G3P within roots could potentially be produced by either the plant or the microbes in the system, we performed a high-performance liquid chromatography (HPLC) analysis of G3P levels in gnotobiotically grown sorghum seedling roots following drought treatment. These results indicate that in the absence of microbes, G3P is produced at levels two-fold and 100-fold higher in roots exposed to 2 and 4 wks of drought, respectively, compared with controls (Fig. 4A). To further demonstrate that the G3P enrichment is host-generated, we performed qPCR with primers designed to quantify expression of several genes in the G3P transport and catabolism pathway on field-grown root samples collected during the peak of drought (TP8). These results revealed significant increases in genes involved in G3P transport across the plant cell plasma membrane (*G3PP*; Table 4) and decreases in two genes [cytosolic glycerol-3-phosphate dehydrogenase (cGPDH) and glycerol-3-phosphate acyltransferase 6 (GPAT6)] responsible for converting G3P to other products [dihydroxyacetone phosphate (DHAP) and precursors of cutin biosynthesis]. These observations are consistent with a model in which G3P accumulates under drought within plant root tissues and is subsequently transported into the apoplast, perhaps to help reduce oxidative stress faced by the cell (Fig. 4B). Furthermore, the metabolomic data demonstrate that drought leads to the accumulation of a variety of other carbohydrates and amino acids within the root (Table 3), and that many of these have potentially related gene categories with significant enrichment in the rhizosphere, including ribose, asparagine, proline, maltose, glucose, and threonine (Ngumbi and Kloepper 2016) (Tables 1 and 2). Notably, many fewer metabolites were found to be differentially enriched between drought and control (n = 7) at the peak of post-flowering drought treatment (Table 5), and G3P is not among them. We also observed that the relative enrichment of Actinobacteria in post-flowering drought-treated roots at TP17 is roughly threefold lower than their enrichment in the pre-flowering-treated roots at TP8. Taken together, these results suggest that a variety of root metabolites that are enriched under pre-flowering drought stress may be imported and used by root-associated Actinobacteria, and that the relatively large drought-induced shifts in community

structure in early compared with late development are correlated with correspondingly larger shifts in the plant metabolome.

ROS Scavenging is up-regulted during drought

Limiting excess accumulation of reactive oxygen species (ROS) is a vital component of drought tolerance (Suzuki et al. 2012). Proline biosynthesis is a key regulator of ROS-dependent processes and osmoprotection. Sorghum transcriptomic analysis revealed constitutively higher mRNA expression of the rate-limiting enzyme of proline biosynthesis, *P5CS2* (Sobic.003G356000; Fig. 5A), in BTx642 compared to RTx430, consistent with the results of a greenhouse study of 45-d-old sorghum plants (Johnson et al. 2015). We also observed similar increases in *P5CS2* mRNA expression under drought conditions for both genotypes. While our gene-expression results supported higher capacity for proline biosynthesis in BTx642, we observed lower levels of drought-induced proline accumulation in BTx642 relative to RTx430 (Fig. 5B), and we did not see a constitutive difference in proline abundance. This indicates a reduced demand for proline as an osmoprotectant and regulator of ROS in field-grown BTx642 in post-flowering drought, despite an apparent higher capacity for proline biosynthesis.

Glutathione S-Transferases (GSTs) are also key regulators of ROS in plant cells (Fracasso, Trindade, and Amaducci 2016; Ding et al. 2017; Das and Roychoudhury 2014), and expression of GSTs is induced by drought (**18**). We observed both constitutive and drought-specific expression differences between the 2 genotypes in multiple individual *GST* genes, despite similar levels of total *GST* mRNA expression between the genotypes in both control and drought conditions. One such gene, *GST29* (Sobic.003G264400), was strongly up-regulated in both pre-flowering and post-flowering drought in BTx642, but was not differentially expressed in RTx430 (Fig. 5C). To further explore genotype-specific GST redox-scavenging activity, we measured total GST enzymatic activity in leaf extracts sampled during weeks 8, 9, and 11 (representing pre-flowering drought, recovery, and post-flowering drought, respectively). We detected similar levels of bulk GST enzymatic activity in both genotypes under control conditions, but observed that both pre-flowering and post-flowering drought led to significantly greater increases in GST activity in RTx430 compared to BTx642 (Fig. 5D). Taken together, the lower proline levels and lower bulk GST enzymatic activity in BTx642 relative to RTx430 during post-flowering drought both point toward lower levels of perceived drought-induced ROS stress in BTx642. This is consistent with the observable drought tolerance phenotypic differences between BTx642 and RTx430, and their characterizations as post-flowering- and pre-flowering-drought tolerant cultivars, respectively.

In addition to constitutive up-regulation of *GST29*, we observed genotype-specific differences in two other pathways that might enable a higher capacity for the control of ROS levels in BTx642. First, a chloroplast-targeted ferredoxin *FD3* (Sobic.003G364400; Fig. 5E), located close to STG1, was constitutively up-regulated in BTx642, consistent with an increase in the efficiency of ROS scavenging in chloroplasts (Lin et al. 2013). Second, we found that BTx642 maintained constitutively higher mitochondrial alternative oxidase (AOX) capacity (Fig. 5F), a feature that prevents excess ROS accumulation in plant mitochondria (Maxwell, Wang, and McIntosh 1999) and that might

be important for drought tolerance (Dahal and Vanlerberghe 2017). From fresh field-sampled leaves, we found that AOX-mediated electron transport capacity was significantly higher in BTx642 leaf tissue relative to RTx430 (Fig. 5F) (false discovery rate-adjusted *P* value <0.05), despite similar dark respiration rates. Thus, we hypothesized that BTx642's post-flowering drought tolerance may be due to enhanced redox balancing in the chloroplast and mitochondria. Given the observed relative expression and protein abundance of core photosynthetic machinery, BTx642 is better at retaining the proteins necessary to sustain photochemistry under post-flowering drought. This work provides an attractive dataset to identify candidate genes that improve productivity under drought in other agronomically relevant crops.

Discussion

Amplicon sequencing experiments performed as a part of the EPICON study lends further credence to a growing body of evidence that plants under drought stress have predictable shifts in their microbiome structure, specifically that monoderm bacteria like the Actinobacteria become enriched during drought. This has been shown in numerous agricultural species (Naylor et al. 2017; Santos-Medellín et al. 2017; Fitzpatrick et al. 2018), and recent work has indicated that this might also be the case in natural forest settings (Willing et al. 2020). Metabolomic and metatranscriptomic analyses further showed that G3P production increases in sorghum roots during drought, and bacterial G3P transport proteins become overexpressed concurrently. Additionally, transcriptomic experiments revealed that genes involved in ROS mitigation are important for drought tolerance, and may drive cultivar-specific differences in drought response. This revelation may also be linked to the observed monoderm enrichment under drought, as research indicates monoderms are less susceptible to ROS-induced cellular damage due to their thickened cell wall (Mai-Prochnow et al. 2016).

While the generation of these hypotheses addressing causal mechanisms behind observed microbiome rearrangement during drought are intriguing on their own, the true triumph of the EPICON project was the robust use of holo-omic strategies to arrive at these hypotheses. The cross-talk among multiple molecular layers, within and between both host and associated microbiome, cannot be properly assessed solely by a reductionist approach that analyzes individual omics layers in isolation. While holo-omics has the power to help unlock the molecular dynamics at play within the plant microbiome (Nyholm et al., 2020), it is worth noting that we anticipate the primary function of such large-scale holo-omics studies is to be the generation, rather than testing, of hypotheses about functional relationships in the plant microbiome. While it has been argued that null-hypothesis testing is actually an outdated method for performing ecology studies (Stephens et al. 2005), to reach a functional understanding of the molecular mechanisms at play in the plant microbiome, validation experiments that follow a traditional hypothesis-driven approach will be necessary (H. Liu et al. 2020). Fortunately, a wide variety of new technical approaches in both plant and microbial biology have been developed that are well suited to the purpose of hypothesis testing in the plant microbiome. The use of CRISPR/Cas9 engineering to create plant

hosts altered in core functions represents one such powerful approach that has been used for validation (Rubin et al. 2020), as will be presented in the following chapter.

In conclusion, to take the next steps forward in understanding the basic biology of plant-microbiome interactions in particular, richer holo-omics studies will be necessary for better understanding plant-associated microbiomes. We envision that the knowledge gained through these endeavors will enable researchers to better explain and predict plant-microbiome interactions. To pursue this path, we encourage microbial and plant biologists, as well as ecologists, statisticians, and computer scientists, to work together to develop unified experimental frameworks that integrate omics datasets from both the host and microbe, and to use the resulting data to build and test hypotheses about the molecular interactions at play in the plant microbiome.

Figures

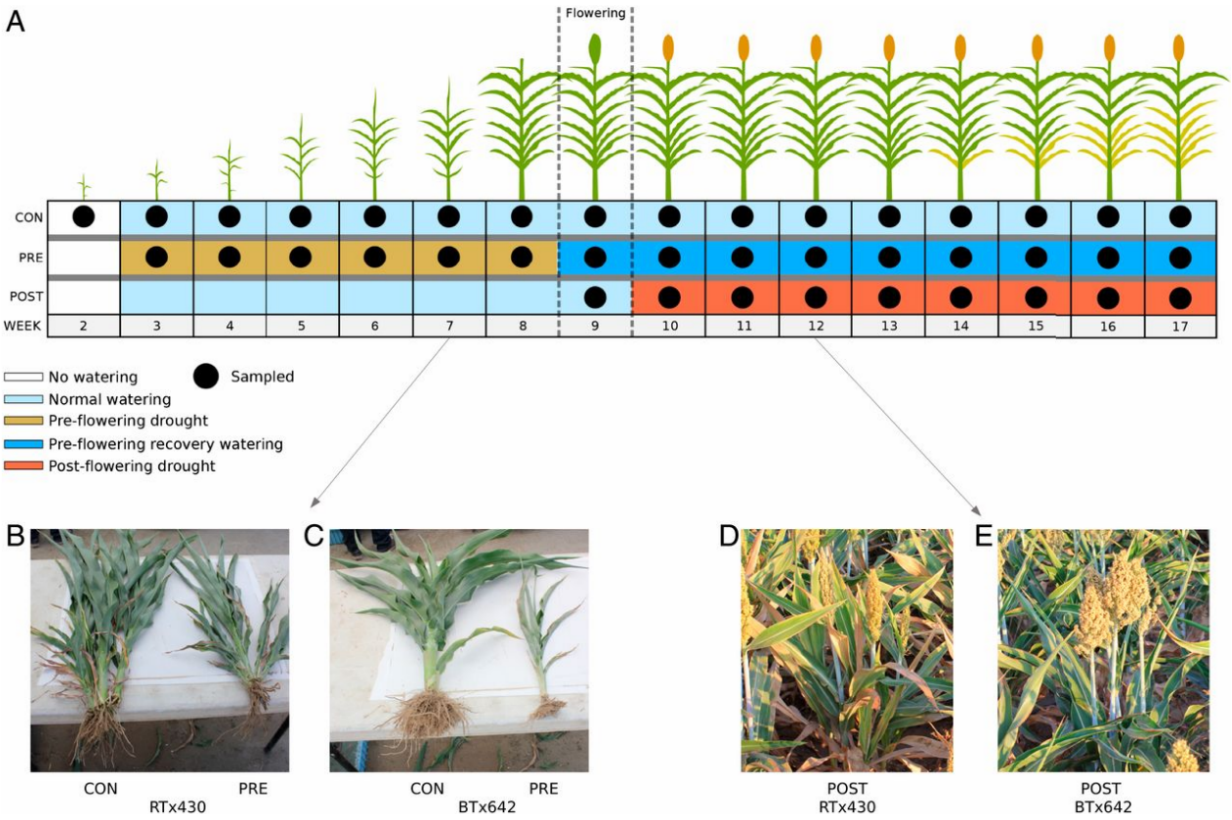

Figure 1. (A) Schematic overview of the experimental design for control (CON), pre-flowering (PRE), and post-flowering (POST) drought. Black dots represent whether plants were sampled for the specified treatment/week, and the color of the boxes reflects the irrigation status for the plants (light blue, watered; brown, pre-flowering drought; dark blue, watered, pre-flowering recovery; red, post-flowering drought); no plots were irrigated prior to week 3 (white boxes). All samples marked as “watered” were irrigated 5 d prior to sample collection. Samples from week 3 of pre-flowering drought and week 10 of post-flowering drought are considered the 1st samples of drought-exposed plants from the 2 drought regimes (i.e., the 1st samples experiencing different watering regimes from control; Materials and Methods). (B and C) Photos of side-by-side comparisons of control (CON; left) and pre-flowering droughted (PRE; right) plants at week 7 for RTx430 (B) and BTx642 (C). (D and E) Field picture at week 12 after 3 wk of post-flowering drought of RTx430 (D) and BTx642 (E), showing delayed senescence in this stay-green variety (Varoquaux et al. 2019).

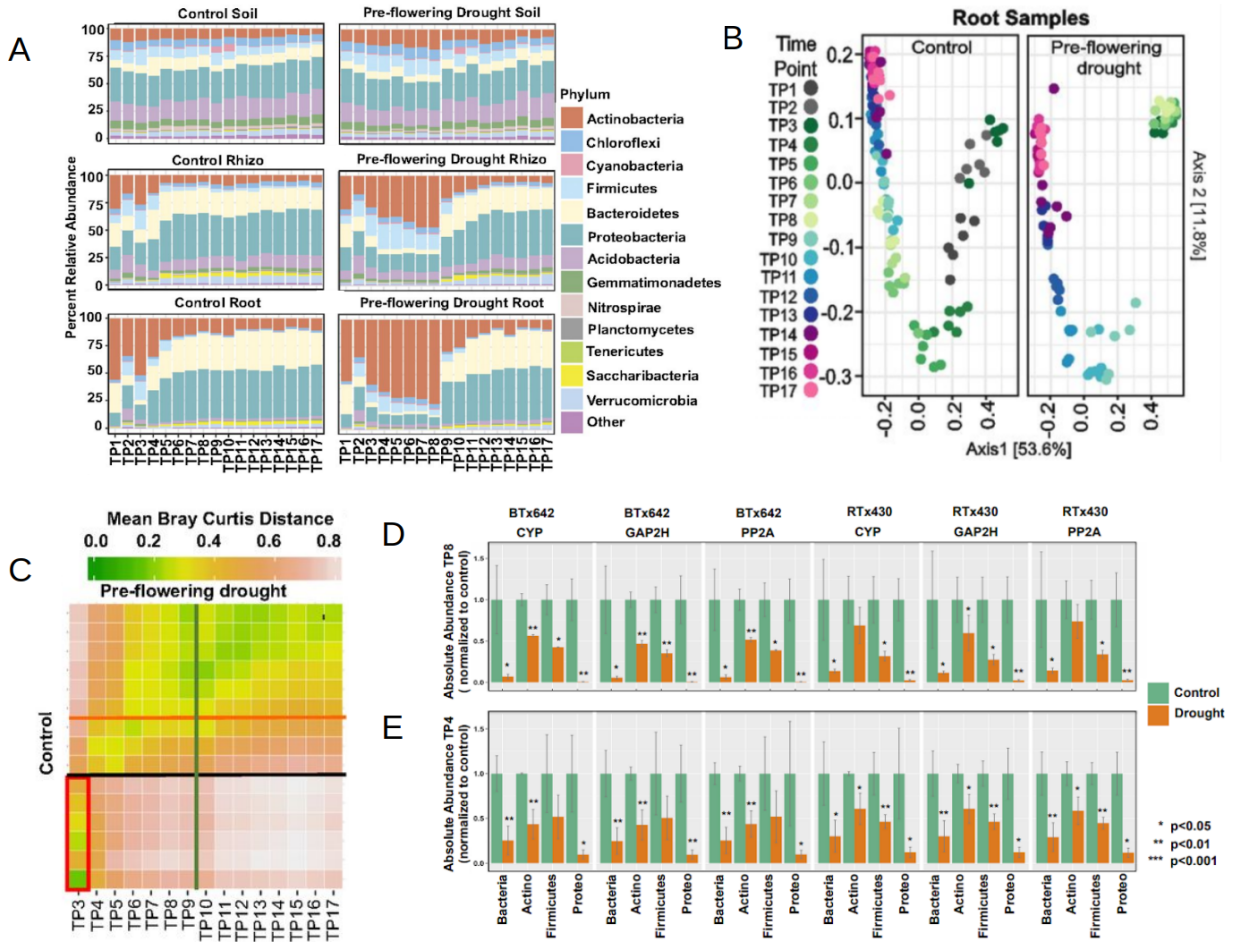

Figure 2. (A) Relative abundance for the most abundant bacterial phyla. Percent relative abundance of the top 13 most abundant phyla for control and drought treatments across soils), rhizospheres, and roots. All time points (TP1–TP17) are arranged in order along the x axis in each panel. (B) PCoA of Bray Curtis distances for all control and pre-flowering drought root samples colored by time point. Individual time points (TP1–TP17) are represented by distinct colors, with initial time points (TP1–TP2) shown as dark gray (control plot only), early time points (TP3–TP8) shown as shades of green, and late time points (TP9–TP17) shown as shades of blue and purple. (C) Heat map of the mean pairwise Bray Curtis dissimilarity comparing pre-flowering drought versus control samples. Shades of green and pink represent low and high Bray Curtis distances, respectively. The orange and green lines indicate the mean flowering times in drought and control treatments, respectively, while the black line represents the rewatering event at the end of drought treatment. The red rectangle highlights the strong similarity between drought-treated samples at TP3–TP8 and the control-treated samples belonging to TP3 (Xu et al. 2018).

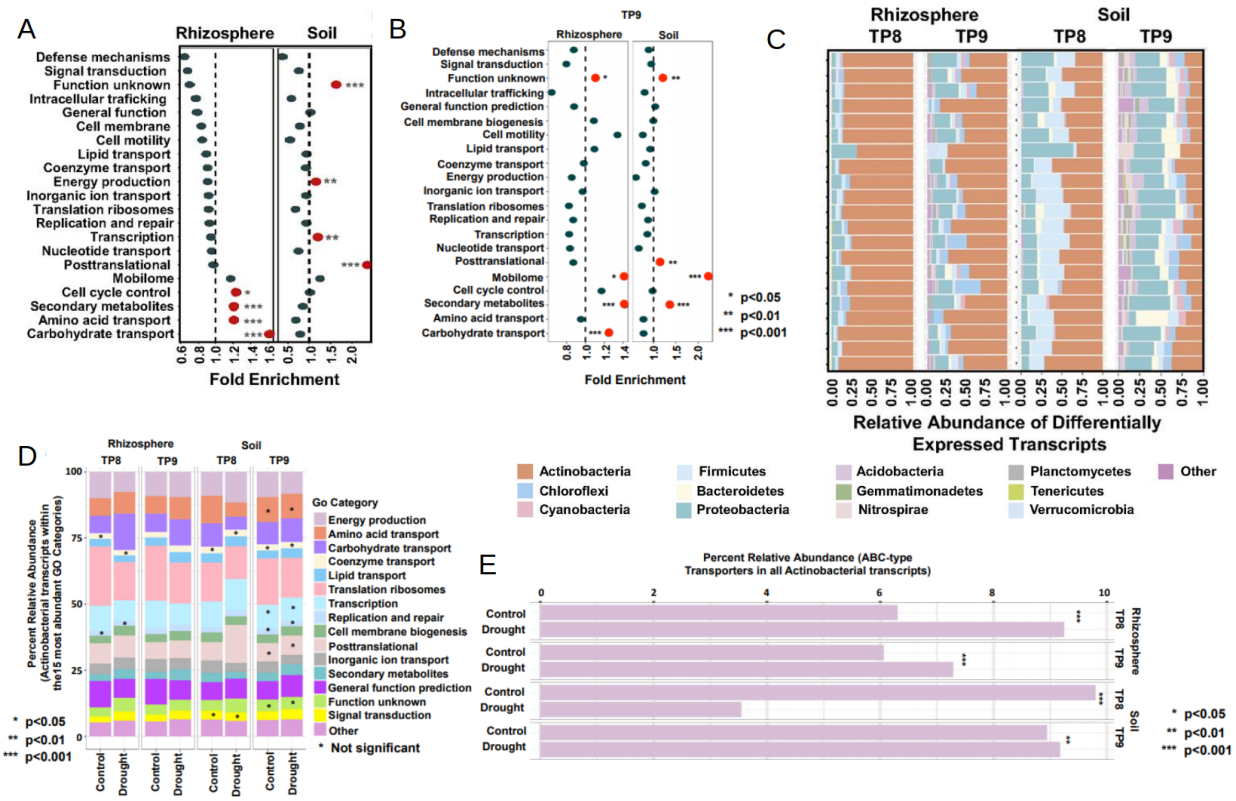

Figure 3. GO enrichment analysis for all genes showing enrichment under drought for both rhizospheres (Left) and soils (Right) at TP8 (A) and TP9 (B). The values on the x axis indicate the fold-enrichment ratio of the relative percentages of genes up-regulated under drought in each category relative to the total relative percentage of genes in the corresponding category within the entire dataset. The red circles indicate categories for which the enrichment had a P value of <0.05 in a hypergeometric test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) (C) Relative abundance across the top 13 phyla for all transcripts for which taxonomies could be assigned and which showed differential expression by treatment from rhizospheres (Left) and soils (Right) at TP8 and TP9, separated according to GO categories (y axis). The legend for colors used for each phylum is as in Fig. 2A (D) Relative abundance of the fifteen most abundant Gene Ontology (GO) categories for all transcripts belonging to Actinobacteria. All categories were observed to have significant changes ($p < 0.001$) by Fisher's Exact test between drought and control in each pairwise combination of time point and sample type, except where marked with an asterisk. All transcripts with unassigned function were omitted, and all transcripts assigned to other GO categories are grouped within "Other". (E) Relative abundance of the ABC transporter genes for all transcripts belonging to Actinobacteria. Significant changes ($p < 0.001$) by Fisher's Exact test between drought and control are indicated with asterisks (Xu et al. 2018).

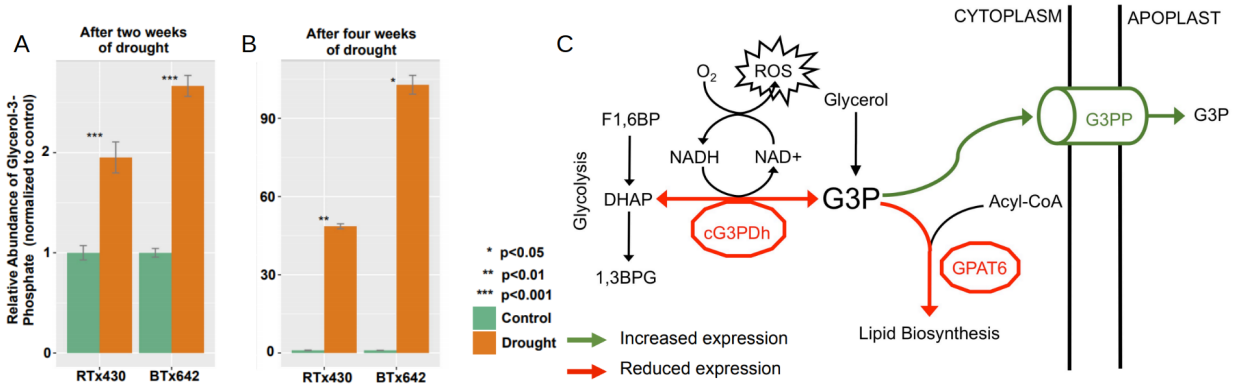

Figure 4. Relative abundance of glycerol-3-phosphate in sorghum root (Normalized to control). (A) The concentration was determined by HPLC in two and a half week-old sorghum roots of both genotypes grown gnotobiotically in sterile microboxes following control irrigation (green) or two weeks of drought (orange) . (B) The relative abundance of G3P under control (green) and drought (orange) treatment in five week old sorghum roots following control irrigation or four weeks of drought (normalized to control). Significance between control and drought is indicated by asterisks above each bar (* represents p<=0.05, ** represents p<=0.01, *** represents p<=0.001). The horizontal bars above and below each circle represents one standard error above and below the mean. (C) A model representing misregulation of host root transcripts in the G3P catabolism and transport pathways during drought. qPCR was performed on pre-flowering and control-treated root samples taken at the peak of drought (TP8) using primers designed to amplify genes involved in conversion of G3P to downstream products (cG3PDh and GPAT6) and G3P transport (G3PP). We observed significant increases in G3PP, which is involved in G3P transport across the plant cell plasma membrane, and decreases in two genes (cGPDH and GPAT6) responsible for converting G3P to DHAP and precursors of cutin biosynthesis, respectively. These observations are consistent with a model in which G3P accumulates under drought within plant root cells and is subsequently transported into the apoplast, perhaps to help reduce oxidative stress faced by the cell (Xu et al. 2018).

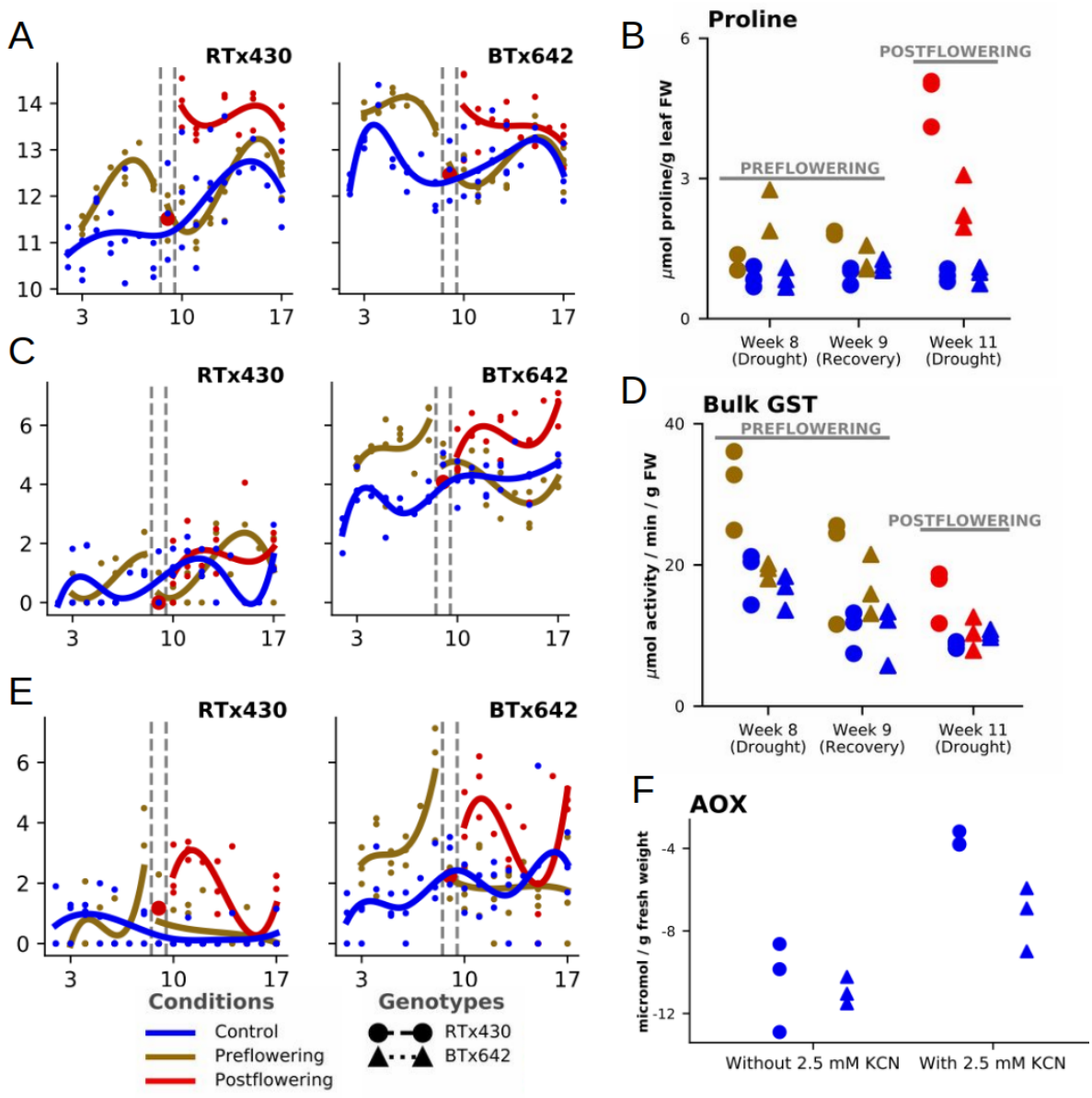

Figure 5. Log-2 gene expression values (y-axis) against time (x-axis) of (A) P5CS2 (Sobic.003G356000), (C) GST29 (Sobic.003G264400), and (E) FD3 (Sobic.003G364400) showing constitutive and drought-induced differences between RTx430 (left) and BTx642
(right). Individual values are shown by dots, with smooth split-splines showing the estimated functional forms. (B) Proline levels determined on fresh weight (FW) leaf samples via a ninhydrin spectrophotometric assay for both drought conditions and genotypes. (D) Bulk GST enzymatic activity in leaf extracts (y axis) at 3 timepoints (x axis). (E) Oxygraph measurements of respiration in week 5 for normally watered plants with and without treatment of 2.5 mM KCN show BTx642 maintaining AOX capacity better than RTx430 (Varoquaux et al. 2019).

COG ID	Annotation	Total DEGs	Total Genes	p value⁻
COG0591	Na_proline-symporter	15	111	0.000001
COG1126	ABC-type-polar-amino-acid-transport-system.-ATPase-component *	17	153	0.000002
COG0765	ABC-type-amino-acid-transport-system.-permease-component *	17	157	0.000002
COG0160	4-aminobutyrate-aminotransferase-or-related-aminotransferase	22	252	0.000003
COG0498	Threonine-synthase	15	143	0.000014
COG0687	Spermidine_putrescine-binding-periplasmic-protein	3	598	0.00002
COG1104	Cysteine-sulfinate-desulfinase_cysteine-desulfurase-or-related-enzyme	1	363	0.000244
COG0527	Aspartokinase	15	191	0.000316
COG2113	ABC-type-proline_glycine-betaine-transport-system.-periplasmic-component *	11	134	0.001266
COG0174	Glutamine-synthetase	39	846	0.001286
COG1231	Monoamine-oxidase	6	46	0.001663
COG0137	Argininosuccinate-synthase	10	121	0.001928
COG0308	Aminopeptidase-N	12	178	0.003659
COG4177	ABC-type-branched-chain-amino-acid-transport-system.-permease-component *	12	184	0.004634
COG0140	Phosphoribosyl-ATP-pyrophosphohydrolase	5	42	0.005736
COG0440	Acetolactate-synthase.-small-subunit	8	104	0.007383
COG0665	Glycine_D-amino-acid-oxidase---deaminating--	7	528	0.008216
COG0019	Diaminopimelate-decarboxylase	1	227	0.008552
COG0747	ABC-type-transport-system.-periplasmic-component *	172	5385	0.011598
COG0620	Methionine-synthase-II---cobalamin-independent--	1	214	0.011832

Table 1. Twenty most significant GO subcategories with differential enrichment under drought within the GO category “Amino acid transport and metabolism”. Hypergeometric tests were used to calculate the enrichment of functional categories and subcategories ($p <= 0.05$) according to COG ID. Asterisks denote ABC-type transporters (Xu et al. 2018).

COG ID	Annotation	Total DEGs	Total Genes	p value
COG3507	Beta-xylosidase	23	116	0
COG3669	Alpha-L-fucosidase	13	45	0
COG4993	Glucose-dehydrogenase	11	956	0
COG3693	Endo-1.4-beta-xylanase.-GH35-family	27	251	0
COG1523	Pullulanase_glycogen-debranching-enzyme	2	423	0.000001
COG0296	1.4-alpha-glucan-branching-enzyme	2	410	0.000008
COG3940	Beta-xylosidase.-GH43-family	9	40	0.000012
COG1175	ABC-type-sugar-transport-system.-permease-component *	69	1087	0.000015
COG0395	ABC-type-glycerol-3-phosphate-transport-system.-permease-component *	62	964	0.000015
COG1638	TRAP-type-C4-dicarboxylate-transport-system.-periplasmic-component	3	428	0.000027
COG4209	ABC-type-polysaccharide-transport-system.-permease-component *	8	39	0.000038
COG1653	ABC-type-glycerol-3-phosphate-transport-system.-periplasmic-component *	191	3969	0.000086
COG0380	Trehalose-6-phosphate-synthase	1	287	0.000133
COG3325	Chitinase.-GH18-family	7	34	0.00014
COG4213	ABC-type-xylose-transport-system.-periplasmic-component *	28	380	0.00023
COG2730	Aryl-phospho-beta-D-glucosidase-BglC.-GH1-family	15	152	0.00046
COG3386	Sugar-lactone-lactonase-YvrE	2	296	0.000538
COG3250	Beta-galactosidase_beta-glucuronidase	13	126	0.000601
COG3866	Pectate-lyase	10	84	0.000802
COG4214	ABC-type-xylose-transport-system.-permease-component *	9	71	0.00106
COG1874	Beta-galactosidase-GanA	5	23	0.001194

Table 2. Twenty most significant GO subcategories with differential enrichment under drought within the GO category “Carbohydrate transport and metabolism”. Hypergeometric tests were used to calculate the enrichment of functional categories and subcategories ($p <= 0.05$) according to COG ID. Asterisks denote ABC-type transporters, while red asterisks denote ABC-type glycerol-3-phosphate transporters (Xu et al. 2018).

Metabolite name	log $_{10}$ Fold Change	p value
Glycerol 3 phosphate	4.34014856	0.0000197
ribose	3.128237744	0.0000236
L.asparagine	2.172806424	0.0000291
L.proline	3.57606939	0.0000375
uracil	2.408340342	0.0002203103581
allantoin	1.82424244	0.000266920365
cycloleucine	-2.172559137	0.0005606630607
L.serine	1.660279514	0.001386031563
N.methylalanine	21.58267018	0.001478075243
maltose	-2.049364813	0.003311019759
X1.4.D.xylobiose	-17.61533687	0.003709558074
D.glucose	-1.61078505	0.004494171482
DL.4.hydroxymandelic.acid	-2.367384618	0.006158325183
isomaltose	-1.595944191	0.006578909786
ethanolamine.	2.054219662	0.01046672411
nicotinic.acid	2.71800344	0.0112309708
L.valine	1.368150771	0.01271747473
L.threonine	1.835541694	0.01322720333
X1.3.diaminopropane	2.02545246	0.01603307691
myo.inositol	1.119231507	0.02872320678
D.threitol	1.791082348	0.04516645606

Table 3. List of all identifiable drought-enriched or depleted root metabolites at the peak of pre-flowering drought (TP8). Prior to further statistical analysis to determine significantly enriched metabolites, the metabolite data were normalized through a log2 transformation and then mean centered across the log2 distribution. Differentially expressed metabolites were analyzed by edgeR with quasi-likelihood F-tests. The cutoff was set as fold change > 2, and p value < =0.05 (Xu et al. 2018).

Gene Annotation	log_2FC	p value		Genotype	Reference gene	Gene ID	Primers
Cytosolic cG3PDh	-3.030906727	0.03795845603	*	RT430	CYP	Sobic.009G183300	G3P1
Cytosolic cG3PDh	-2.568619241	0.03903031078	*	BT642	CYP	Sobic.009G183300	G3P1
G3PP (transport)	9.906460756	0.0002631826723	***	RT430	CYP	Sobic.004G290900	G3P8
G3PP (transport)	10.72859319	0.000892585992	***	BT642	CYP	Sobic.004G290900	G3P8
gpat6	-3.387592145	0.01079334018	*	RT430	CYP	Sobic.001G250200	G3P30
gpat6	-4.090603027	0.001480628733	**	BT642	CYP	Sobic.001G250200	G3P30
gpat6	-7.265405823	0.02190877883	*	RT430	CYP	Sobic.001G099300	G3P37
gpat6	-5.941512165	0.02366292925	*	BT642	CYP	Sobic.001G099300	G3P37
Cytosolic G3Pdh	-1.953478621	0.03795845603	*	RT430	PP2A	Sobic.009G183300	G3P1
Cytosolic G3Pdh	-1.648591824	0.03903031078	*	BT642	PP2A	Sobic.009G183300	G3P1
G3PP (transport)	10.87706686	0.0002631826723	***	RT430	PP2A	Sobic.004G290900	G3P8
G3PP (transport)	11.57158419	0.000892585992	***	BT642	PP2A	Sobic.004G290900	G3P8
gpat6	-2.384530273	0.01079334018	*	RT430	PP2A	Sobic.001G250200	G3P30
gpat6	-2.039574396	0.001480628733	**	BT642	PP2A	Sobic.001G250200	G3P30
gpat6	-6.652912721	0.02190877883	*	RT430	PP2A	Sobic.001G099300	G3P37
gpat6	-7.469445894	0.02366292925	*	BT642	PP2A	Sobic.001G099300	G3P37

Table 4. qPCR results for genes involved in glycerol-3-phosphate transport and catabolism. Quantitative PCR with primers designed to quantify expression of several genes in the G3P transport and catabolism pathway on field-grown root samples collected during the peak of pre-flowering drought (TP8). Relative expression levels were calculated according to the 2-2ΔΔCT (cycle threshold) method, and the standard deviation was calculated among the three biological replicates. Data was normalized to the expression of one of two housekeeping genes (CYP and PP2A) (Xu et al. 2018).

Metabolite name	log $_{10}$ Fold Change	p value
L.proline	4.71688208	0.0001869936657
maltose	3.622265852	0.001295841797
succinic.anhydride.	-2.823328202	0.009388363705
D.gluconic.acid	2.722253933	0.01189925701
dimethoxy.4.hydroxycinnamic.acid..sinapic.acid.	-2.529402557	0.01851271014
maleic.acid	2.165326392	0.04093869713
xylitol	2.084055308	0.04848288932

Table 5. A list of all identifiable drought-enriched or depleted root metabolites at the peak of post-flowering drought (TP17). Prior to further statistical analysis to determine significantly enriched metabolites, the metabolite data were normalized through a log2 transformation and then mean centered across the log2 distribution. Differentially expressed metabolites were analyzed by edgeR with quasi-likelihood F-tests. The cutoff was set as fold change > 2, and p-value < =0.05 (Xu et al. 2018).

Chapter 4
Improving Sorghum Transformation Using Morphogenic Genes, Babyboom/Wuschel

Contributions

Aspects of this chapter are derived from a manuscript currently in review on which I am a co-author, as well as unpublished data I have generated. The full citation of the manuscript is Kiflom Aregawi, Jianqiang Shen, Grady Pierroz, Manoj K. Sharma, Jeffery Dahlberg, Judith Owiti, Peggy G. Lemaux. Pathway to Discovery of Gene Function in Key Bioenergy Crop, Sorghum bicolor. In review, *The Plant Biotechnology Journal*

From the beginning of the EPICON Project, the ultimate goal was to use holo-omics to uncover interesting gene targets for functional validation in sorghum. We knew that such an ambitious holo-omic study had the potential to generate a plethora of hypotheses and highlight numerous genes of interest (GOIs). So in order to prepare for this undertaking in which we probe gene function using engineering and editing approaches, we were determined to radically improve the speed and efficiency of sorghum transformation. To do so, we attempted a newly-developed transformation technique using the morphogenic genes BBM and WUS which had been shown to improve maize transformation.

After some initial success with transforming sorghum solely with the morphogenic genes and an exemplary GOI, I performed some of the first altruistic transformation experiments along with my labmate Kiflom Aregawi. Altruistic approaches consisted of co-transforming the morphogenic genes along with a GOI, in this case RFP for proof-of-concept validation. I performed every aspect of the transformation experiments with the GOI, from initial transformation through tissue and molecular genotyping to planting regenerated transformants in soil. After demonstrating significant improvements in transformation efficiency, we integrated CRISPR/Cas9 editing technology with the BBM/WUS transformation approach.

Having been involved very broadly in the EPICON Project as it evolved, I used what we had learned from those studies (Xu et al. 2018; Varoquaux et al. 2019) to decide on interesting gene targets for editing to explore plant-microbiome interactions. Using the combination of metagenomic, transcriptomic, and metabolomic data produced by EPICON researchers and reported in the previous chapter, we decided that ROS and G3P production would be our focus since both of these pathways showed clear transcriptomic and metabolic shifts under drought. Eventually we narrowed the options to three genes: a catalase involved in the detoxification of hydrogen peroxide (CAT2), as well as the two biosynthetic enzymes that produce G3P (GLI1 and GLY1). I designed sgRNAs targeting these genes, and carried out much of the molecular cloning involved in building our two constructs, with extensive help from both Dr. Manoj Sharma and Dr. Jianqiang Shen.

With the integrated BBM/WUS and CRISPR/Cas9 transformation cassettes, I carried out two replications of transformation with both constructs; I performed every step of the transformation process, from transformation through planting. Finally, I helped write and edit the manuscript the Lemaux lab recently submitted to *Plant Biotechnology Journal* in addition to the related pre-print published in BioRXiv (K. Aregawi et al. 2020), which detail the lab's progress towards high efficiency sorghum transformation with the use of BBM/WUS, as well as editing efforts and novel

genotyping methods to screen for high quality (single-copy) transformants at a higher throughput level.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a cereal crop with multiple abiotic stress tolerances (Paterson et al. 2009; Paterson 2008) which has recently realized new market demands worldwide because of its use for fuel, feed, forage, and food (Duff et al. 2019; Mundia et al. 2019). In order to further understand sorghum's abiotic stress tolerance, particularly in regards to drought tolerance, EPICON researchers carried out an ambitious holo-omic study of sorghum grown in field-relevant conditions (see Chapter 3). That data was used to generate evidence-based hypotheses on connections between drought, monoderm bacterial enrichment, reactive oxygen species, and key metabolites like glycerol-3-phosphate (Xu et al. 2018; Varoquaux et al. 2019). Despite those revelations, our large transcriptomic data sets for sorghum also revealed that although 44% of expressed genes were affected by drought, 43% of the transcriptome had not yet been annotated (Varoquaux et al. 2019). This revelation highlighted the need for renewed focus on validating gene function in sorghum.

Serendipitously, the advent of CRISPR/Cas9 gene editing offers a powerful tool to validate gene function through targeted knock-outs and subsequent reverse genetic screens (Kumlehn et al. 2018). Unfortunately, a lack of high-efficiency transformation methods in sorghum has hampered the effective delivery of CRISPR/Cas9 editing cassettes into the sorghum genome. Since the first plant transformation success in tobacco (Fraley et al. 1983; Herrera-Estrella et al. 1983; Bevan, Flavell, and Chilton 1983) resulting in stable expression of introduced bacterial genes [for review, (Biotechnology and Biological Sciences Research Council 2013)], much progress has been realized in crop plants. However, challenges remain (Altpeter et al. 2016), especially for cereal crops like sorghum, that are classically more recalcitrant to Agrobacterium-mediated transformation than dicots, largely due to its natural host being dicots (De Cleene and De Ley 1976). Many cereal crops also exhibit severe genotype dependence in transformation success, meaning only a select few cultivars are able to be transformed at all (Hiei, Ishida, and Komari 2014). Because these transformation-amenable cultivars are almost never elite lines grown commercially, extensive backcrossing of engineered traits must often still be performed before those traits are agriculturally relevant. Barriers to Agrobacterium-mediated transformation in select monocot species were eventually overcome, with rice being the first successful transformation target (Raineri et al. 1990). Progress was also made in maize (Songstad et al. 1996; Frame et al. 2002; Shrawat and Lörz 2006), with recent efforts in maize using specific morphogenic genes showing particular promise (Lowe et al. 2016). Those morphogenic genes were *Zea mays Baby Boom* (BBM), an AP2/ERF transcription factor that promotes cell proliferation during embryogenesis (Boutilier et al. 2002), and *Wuschel2* (WUS), a transcription factor needed to maintain a reservoir of stem cells in the shoot meristem (Laux et al. 1996). These advancements inspired us to attempt similar strategies in sorghum by employing a strategy known as altruistic transformation, in which a construct containing the morphogenic genes is simultaneously co-transformed into sorghum immature embryos (IEs) along with a gene of interest, in our case the fluorescent reporter gene RFP. The theoretical rationale for altruistic transformation posits that because Wuschel is a mobile signal, cells transformed with

WUS will export the gene product to surrounding cells to induce somatic embryogenesis “altruistically”. By selecting for the antibiotic resistance gene linked to our gene of interest, we hoped to regenerate plants containing RFP that do not contain the morphogenic genes but rather benefitted from neighboring cells which do contain the morphogenic genes. We report in this study that the incorporation of BBM and WUS allows for transformation efficiencies in sorghum of almost 30%, over ten-times greater than previous experiments without morphogenic genes [(Wu et al. 2014); Lemaux et al., unpublished].

Considering this marked improvement in transformation efficiency in sorghum, we utilized our extensive holo-omic dataset as well as the hypotheses generated through its analysis to select gene targets for CRISPR/Cas9 editing and functional validation. We decided to specifically investigate ROS and G3P, as mentioned above, as potential nexuses of sorghum drought tolerance and repeatable shifts in microbiome community structure towards monoderm taxa. Three target genes were selected for editing. The first is the catalase gene SbCAT2 (Sobic.001G517700), a key regulator of ROS stress involved in hydrogen peroxide detoxification. The second and third genes are SbGLI1 (Sobic.006G239800) and SbGLY1 (Sobic.002G081500), which represent the two biosynthetic enzymes immediately upstream of G3P production. Guide RNA were designed and incorporated into plasmids containing the CRISPR/Cas9 editing cassette as well as the BBM/WUS morphogenic cassette. Although high rates of transgene integration were achieved, successful knockouts remained elusive, likely due to suboptimal expression and function of the guide RNA and Cas9 protein. Thus, although great strides have been made towards successful transformation, gene editing, and functional validation of genes in sorghum, one final hurdle remains in the optimization of gene editing efficacy in this non-model, abiotic stress-tolerant species.

Materials and Methods

Plant materials

Seeds from sorghum variety RTx430 (Miller 1984) were from GRIN (United States Department of Agriculture, Agricultural Research Service n.d.), planted in three-gallon pots with SuperSoil 1 (Rod McClellan Co., South San Francisco, CA) and grown in the greenhouse at 28 oC with 16 h light/ 8 h dark photoperiod. Immature embryos (Es) from panicles were collected at 12-14 d post-anthesis.

Preparation of Agrobacterium

LBA4404 Thy- is an auxotrophic (THY-) *A. tumefaciens* strain (Anand et al. 2017) into which helper plasmid, pPHP71539 (Anand, A., Bass, S.H., Cho, H.-J., Klein, T.M., Lassner, M., McBridge, K.E. 2017; K. Aregawi et al. 2020), was introduced. For other constructs, strains with those constructs were streaked on YEP agar medium (per L: 10 g yeast extract, 10 g Bacto Peptone, 5 g NaCl, 15 g Bacto Agar, pH 7.0, 100 mg/L thymidine, 50 mg/L gentamicin) from stocks stored at -80 ^{o}C at 25% final concentration glycerol. After dark incubation for 3 d at 28 oC, 3-5 colonies were used to make overnight cultures on YEP; suspension cultures from those cultures were adjusted to

OD_{550} 0.7, using PHI-I medium (Wu et al. 2014) with 0.005 % silwet and 0.2 mM acetosyringone.

Altruistic Transformation Constructs

Altruistic transformation requires the use of two separate constructs simultaneously co-transformed into sorghum IEs, one containing the gene of interest (GOI) and one containing the morphogenic genes BBM and WUS. The GOI construct we used for altruistic transformation was pANIC10A (Mann, Lafayette, et al. 2012), which contains the visible marker red fluorescence protein (RFP) driven by the pvUBI1 promoter, as well as the selectable marker gene hygromycin phosphotransferase (hph) driven by the pvUBI2 promoter. The construct containing morphogenic genes was pGL190 (Figure 1A), which codes for BBM and WUS, both of which are driven by Zm-PLTPpro. pGL190 also contains the visible reporter ZsGreen. All plasmids were electroporated into LBA4404 Thy^- and confirmed by complete plasmid sequencing (Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston MA).

CRISPR/Cas9 sgRNA target determination and design

Our holo-omic investigation of the interplay between sorghum, drought, and the microbiome highlighted the importance and potential for further investigation of both G3P and ROS in shaping how the plant as well as its associated microbes respond to drought. In order to continue pursuing these two avenues, we set out to select potential candidate genes involved in these processes which could be edited with CRISPR/Cas9 in order to perform reverse genetic screens and validate their function in determining observed drought-related phenotypes.

A literature review revealed two separate pathways converge upon G3P as a biosynthesis product. On the one hand, dihydroxyacetone phosphate (DHAP) can be reduced by glycerol-3-phosphate dehydrogenase to produce G3P. Alternatively, glycerol can be directly phosphorylated by glycerol kinase. In *Arabidopsis thaliana*, glycerol-3-phosphate dehydrogenase is encoded by the *AtGLI1* gene, AT1G80460. Glycerol kinase is encoded by the *AtGLY1* gene, AT2G40690. Mutations in both *AtGLI1* and *AtGLY1* have been shown previously to reduce intracellular G3P content (Chanda et al. 2008), so these two genes were determined to be good candidates for editing in sorghum.

A homology search revealed that *AtGLY1* has a single homolog in sorghum, Sobic.002G081500 (SbGLY1), which showed 78% similarity at the protein level. *AtGLI1* also has only a single homolog in sorghum, Sobic.006G239800 (SbGLI1), which showed 85% similarity. Transcriptional data confirmed that both of these genes are transcribed in the roots during normal development (Figure 2A,B), and *SbGLY1* even shows elevated expression during drought (Figure 2A). Interestingly, *SbGLI1* shows reduced expression during drought (Figure 2B), but because GLI1 is a bidirectional enzyme, we hypothesized that once levels of G3P in the cell become high enough GLI1 might reduce total G3P through conversion to DHAP. With this in mind, we decided targeting both SbGLI1 and SbGLY1 for editing in sorghum was the best strategy for engineering a plant with constitutively altered intracellular G3P content.

When considering ROS stress, many more gene targets must be considered, including superoxide dismutases, alternative oxidases, glutathione reductases, and catalases (Dvořák et al. 2020; Das and Roychoudhury 2014). Turning to the literature, we learned of a catalase mutant in Arabidopsis, AtCAT2 (AT4G35090), which displays constitutively elevated levels of hydrogen peroxide (Y.-Q. Hu et al. 2010). Because of this easily quantifiable and theoretically impactful phenotype, we chose to focus on editing *SbCAT2* in sorghum. The closest homolog to *AtCAT2* in sorghum is Sobic.001G517700 (SbCAT2), which showed 94% homology at the protein level. Transcriptional analysis revealed that SbCAT2 is expressed in sorghum roots, and is even strongly up-regulated under drought (Figure 2C), so we determined to pursue editing *SbCAT2* in hopes of generating a sorghum mutant with constitutively elevated levels of intracellular hydrogen peroxide.

Once target genes were selected, sgRNA were designed using using CHOP-CHOP (Labun et al. 2019) and CRISPR-PLANT (Minkenberg et al. 2019) using the following criteria: both design programs must return high on-target scores and low off-target scores, both sgRNA for a single target gene should be approximately 200 bp apart to encourage large genomic lesions resulting from successful editing events, both sgRNA target exons, and both sgRNA are as close to the transcription start site as possible. The six sgRNA sequences selected following these guidelines are presented in Table 3, and their locations on the target gene are presented in Figures 2D-F.

Non-Altruistic CRISPR Constructs

Two different guide RNA (gRNA) polycistronic cassettes, one targeting SbCAT2 and one targeting both SbGLI1 and SbGLY1, were amplified from pGTR (Xie et al., 2015) according to the published protocol. The final plasmids were generated by recombining each gRNA-tRNA polycistronic cassette and maize codon-optimized SpCas9 with destination vector pPHP85425 (K. Aregawi et al. 2020) using the Gateway Recombination method (Walhout et al. 2000) to produce the plasmids pGL203 and pGL204 (Figure 1B). These final constructs contain the morphogenic genes WUS and BBM, both driven by the Zm-PLTP promoter, as well as the MO-CRE recombinase driven by the ZmHSP2p promoter, a heat stress-inducible heat shock promoter. The morphogenic genes as well as the CRE gene are located within LoxP sites to allow for excision and prevent aberrant phenotypes induced by constitutive expression of the morphogenic genes. The destination vector also contains the selectable marker gene ALS, which confers resistance to the herbicide imazapyr. All constructs were confirmed by Sanger sequencing and electroporated into Agrobacterium strain LBA4404 Thy$^-$. Plasmids were then re-extracted from Agrobacterium and confirmed by complete plasmid sequencing (Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston MA).

***A. tumefaciens*-mediated sorghum transformation**

All media was modified from (Jones et al. 2019). Immature seeds of RTx430 from greenhouse-grown plants were surface-sterilized twice for two mins with 75% ethanol, then 20% bleach plus 0.2% Tween20 for 20 mins. IEs (1.5-2 mm) were isolated and put into PHI-I liquid medium. When all IEs were isolated, PHI-I was removed, 1 ml of

Agrobacterium suspension was added and mixed 5 mins at medium shaker speed; suspension was removed by pipetting. For altruistic transformation, Agrobacterium LBA4404 Thy^-, containing either pGL190 or pANIC10A at OD_{550} 0.7, were mixed in a 9:1 (pANIC10A:pGL190) volume:volume ratio. IEs, placed on co-cultivation medium (CCM) scutellum side up, were kept in the dark for 7 d at 24 ºC. IEs were transferred to resting medium for 7 d to halt Agrobacterium growth.

For non-altruistic editing transformations, before moving to Embryo Maturation Medium (EMM), IEs on resting media were heat-shocked at 45 °C, 75% humidity, for 2 hrs to trigger CRE-mediated excision. IEs were then moved to EMM with 0.05 mg/L IMZ (Sigma Aldrich Chemicals, St. Louis MO) until shoots formed. Plantlets (2-3 cm) were moved to rooting media (RM) with 0.05 mg/L IMZ under 16-h photoperiod at 26 °C. For altruistic transformation, tissues were transferred to EMM containing 20 mg/L hygromycin (hyg; PhytoTechnology Laboratories, Lenexa KS) without heat treatment; no selection was used in RM.

Plants with well-established roots and shoots (10-12 cm) were moved to soil in growth chambers at 26 ºC with 16-h light and 8-h dark photoperiod for two weeks of conditioning before transferring to the greenhouse. Percent transformation efficiency was calculated based on the number of T_0 plants PCR-positive for the target gene, divided by the number of IEs used, times 100.

DNA extraction

Leaf samples, ~3 cm, frozen in liquid nitrogen and ground in an MM300 bead beater (Retsch GmbH, Haan Germany) for 1.5 mins at 25 cps, were refrozen and ground again. 700 ul of urea buffer (2M urea, 0.35 mM NaCl, 20 mM Tris-HCl pH 8, 20 mM EDTA, 1% sarkosyl) was added and vortexed for 30 secs. 10 ul of RNase A was added, incubated 10 mins at room temperature and 700 ul of phenol:chloroform:IAA (25:24:1) was added, vortexed 15 mins, and centrifuged 13,800 x g for 15 mins. 55 ul of 3M sodium acetate (pH 5.2) and 367 ul of isopropanol were added to 550 ul of the aqueous phase; to precipitate DNA, tubes were inverted at -20 ºC overnight or -80 ºC for 1 h. After centrifugation at 13,800 x g for 5 mins, supernatant was removed and pellet rinsed in 500 ul 70% ETOH. Samples were centrifuged 5 mins at 13,800 x g, supernatant removed and samples centrifuged 1 min. Excess ethanol was removed, pellets air-dried 5-10 mins in laminar flow hood, resuspended in sterile distilled water and stored at -20 ºC.

Genotyping transformed plants

Total gDNA was extracted from leaves of seedlings on RM. For altruistic transformation, PCR was performed using primers for RFP and BBM. To identify putative edited plants in the non-altruistic experiments, PCR was performed using primers for the gRNA-tRNA polycistronic cassette and BBM. The target gene fragments for SbCAT2, SbGLY1, and SbGLI1, including the two gRNA target sites for each gene, were also amplified to analyze by Sanger sequencing. Amplicons were visualized in 1% agarose, purified with QIAquick® PCR purification kit (QIAgen, Redwood City CA). CRISPR/Cas9 target gene amplicons were additionally prepared for Sanger sequencing to assess the presence of genomic edits (UC Berkeley DNA Sequencing Facility).

Sequencing results were analyzed using the Inference of CRISPR Edits (ICE) analysis tool (Hsiau et al. 2018). All primers used for genotyping can be found in Table 2.

Results

Altruistic *A. tumefaciens*-mediated transformation

Severe genotype dependence as well as general recalcitrance to transformation in sorghum prompted development of a new transformation strategy, modified from a method in maize, termed altruistic transformation (Hoerster et al. 2020). The altruistic approach requires IEs to be transformed simultaneously with two Agrobacterium strains. In our approach, the first contained pGL190 (Figure 1A), which had the morphogenic genes BBM and WUS. The second strain had pANIC10A (Mann, LaFayette, et al. 2012) with our exemplary GOI, encoding the red fluorescent protein (RFP), and a selection gene, hygromycin phosphotransferase (hph). The two strains were mixed 1:9 v:v, pGL190:pANIC10A: and applied simultaneously to sorghum IEs. Tissues were cultured on EMM plus hyg to select for hph expression from pANIC10A; no selection was used on RM. Only a small number of escapes were found despite lack of selection on RM.

Four altruistic transformation replications were conducted using a total of 379 RTx430 IEs (Table 1); PCR was used to detect RFP and BBM. Transformation efficiency, the number of T_0 plants regenerated that were positive only for *RFP, was* 29.8%. From the regenerated transgenic plants, 28.0 % of plants contained both RFP and BBM (Table 1). This transformation efficiency was markedly better than any achieved without the use of morphogenic genes, which had peaked at around 2.5% (Lemaux et al., unpublished). This success encouraged us to modify the strategy and attempt to incorporate CRISPR/Cas9 editing with morphogenic gene-assisted transformation to begin to validate gene function in sorghum.

Non-altruistic *A. tumefaciens*-mediated transformation for CRISPR/Cas9 gene editing

In previous reports of sorghum editing, the Agrobacterium-mediated CRISPR-Cas9 delivery system resulted in low efficiencies (G. Liu, Li, and Godwin 2019; Char et al. 2020; Che et al. 2018; A. Li et al. 2018). Given the highly efficient sorghum transformation methods described in our studies, we combined the use of morphogenic gene transformation methods with CRISPR/Cas9 editing to improve genome editing efficiency.

Both non-altruistic and altruistic transformation methods were considered to introduce the CRISPR/Cas9 cassette. The attempt described here focused on non-altruistic transformation due to ease of construct manipulation and ability for T-DNA segregation. After editing targets were selected, constructs were created which contained two sgRNA targeting their respective genes. One construct, named pGL204, targeted *SbCAT2* and therefore contained two sgRNA in total. The second construct, named pGL203, targeted both *SbGLI1* and *SbGLY1* and thus contained four total sgRNAs. These non-altruistic constructs also contained BBM, WUS and the ALS selection genes (Figure 1B). The constructs were introduced into RTx430 via *A. tumefaciens*-mediated transformation, and after two weeks transformed tissue was

heat-treated to trigger expression of heat-shock-inducible CRE, causing excision of morphogenic genes flanked by loxP sites. Two replications using 50 IEs were conducted for each construct. Transformation with pGL204 yielded 14 plants positive only for sgRNAs, a 14% transformation efficiency. However, 19 plants were positive for both the sgRNAs and the morphogenic genes, which still have the potential to be edited but also carry a chance of aberrant phenotypes later in development. Transformation with pGL203 yielded 19 plants positive for gRNAs, a 19% transformation efficiency. Of these, 11 plants were positive for both the sgRNAs and the morphogenic genes. Unfortunately, Sanger sequencing of the target genes from the transformed plants did not indicate that any of the regenerated plants contained edits.

Discussion

To accelerate gene function studies in important crops, improvements in transformation are needed (Altpeter et al. 2016) since the most direct paths to validating function are through genetic engineering and editing. The major impediments to such approaches in sorghum, when using conventional transformation methods, are genotype-dependence and the long time needed for selection, regeneration and generation advance. Efforts described in this chapter are aimed at addressing these issues.

Initial altruistic efforts with the BBM/WUS morphogenic gene-assisted transformation system in sorghum attempted to utilize a transformation approach in which BBM/WUS and the GOI are located on different plasmids in different Agrobacterium strains which are simultaneously co-transformed. This strategy has two specific benefits. One is the low probability of regenerated plants that still contain the morphogenic genes, which can lead to aberrant phenotypes and poor seed set. The other advantage is that no new plasmids need to be constructed. The BBM/WUS construct can be used in conjunction with any other plasmid containing a GOI, allowing researchers to immediately improve the transformation efficiency of any construct they may be currently using or have used in the past. As such, we were able to rapidly assess the efficacy of the BBM/WUS strategy using an RFP reporter construct that our lab had used previously, and we did indeed observe a marked improvement in transformation efficiency compared to non-morphogenic methods employed previously, even in our initial experiments. The average transformation efficiency in our lab increased over ten-fold, from ~2.5% to almost 30%, an efficiency which seemed unfathomable using previous transformation techniques.

After the success with altruistic editing, we tested whether the BBM/WUS system could be used in conjunction with a CRISPR/Cas9 editing cassette on a single plasmid. This was done in part to see if we could overcome the challenge of building, manipulating, and transforming with a very large plasmid, as integrating both of these components resulted in a final construct around 36 kb. Additionally, because the morphogenic genes in this cassette exist between two LoxP sites, this strategy presented the added challenge of utilizing the CRE-LOX recombination strategy to excise the morphogenic genes after somatic embryogenesis to prevent aberrant phenotypes in regenerated plants. However, our results show that successful transformation of sorghum plants using a very large plasmid containing the

morphogenic genes as well as the CRISPR/Cas9 editing cassette is indeed possible, although transformation efficiencies with these non-altruistic experiments were slightly lower than those achieved with the altruistic approach. Additionally, the number of regenerated plants containing the morphogenic genes as well as the GOI was indeed higher when using the non-altruistic approach, approaching 50% of all transformed plants. Nevertheless, using the non-altruistic strategy did deliver impressive results, achieving efficiencies well above any reported for sorghum transformation without the use of morphogenic genes.

Another substantial benefit from using the BBM/WUS transformation strategy is the fact that it significantly cuts down the amount of time needed between transformation and the regeneration of transgenic plants. A process that once took 18-21 weeks for the standard transformation genotype, Tx430, now only takes 10-12 weeks thanks solely to the use of BBM and WUS. This allows for much more rapid investigation of promising transgenes and higher throughput for experimentation, while also reducing material and labor costs as well as the chance of somaclonal variation associated with protracted time in culture.

Unfortunately, although we were able to regenerate high quantities of transformed plants, the apparent low-level expression of Cas9 activity resulted in a lack of successfully gene edited plants for my constructs to date. We hypothesize that this may be due to low expression of the Cas9 protein and/or sgRNAs during somatic embryogenesis and early development of the regenerated plants, which were genotyped as soon as they produced enough tissue for successful DNA extraction. Efforts are underway to remedy this by testing new promoter sequences to drive more strongly components of the gene editing cassette. Because our T_0 plants are stably transformed with the CRISPR/Cas9 cassette there still remains a chance that edits may occur later in plant development, leading to successfully edited T_1 seeds.

Despite this setback, this study still represents a huge leap forward in sorghum transformation. With the BBM/WUS strategy, we are closer than we ever were to validating gene function in sorghum and addressing the underlying mechanisms behind complex, polygenic traits such as drought tolerance and microbiome structuring. Once the CRISPR/Cas9 gene editing system is optimized in sorghum, the doors will be open to uncovering the secrets of this impressively abiotic stress-tolerant cereal crop.

Images

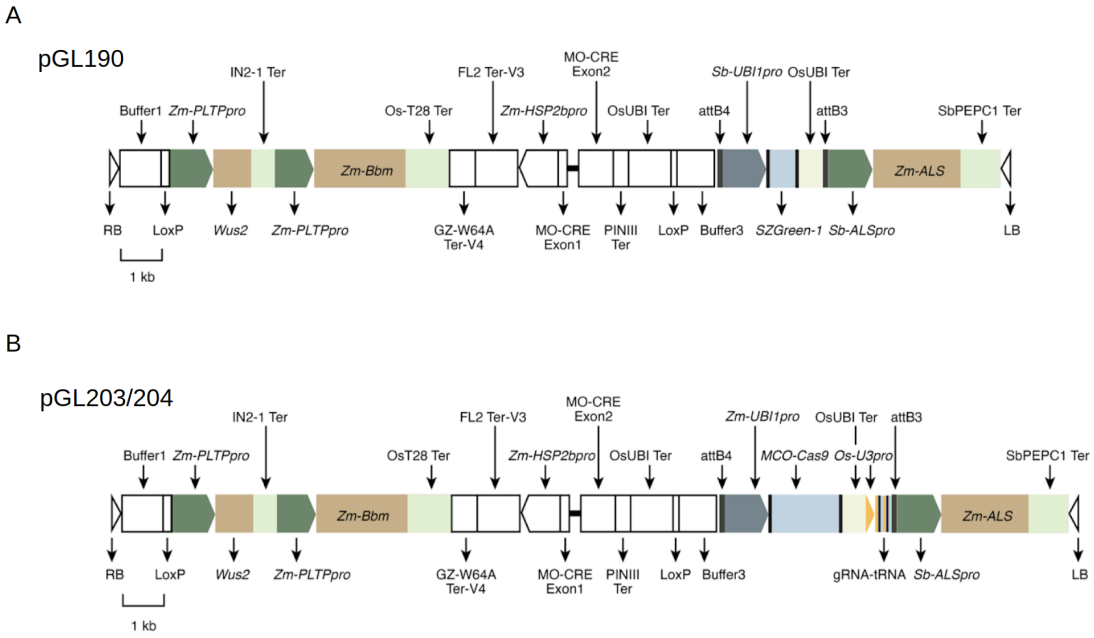

Figure 1. Constructs containing morphogenic genes BBM and WUS used in this study. Both contain WUS and BBM driven by the Zm-PLTP promoter, MO-CRE driven by the heat shock inducible Zm-HSP2b promoter. These three genes are all within the LoxP sites targeted by MO-CRE, which facilitates their excision after exposure to heat shock. Both constructs also contain the selectable marker gene ALS, which conveys resistance to imazapyr, behind the Sb-ALS promoter. The difference between these constructs is (A) pGL190 contains the visible reporter gene ZSGreen, while (B) pGL203 and pGL204 contain a CRISPR/Cas9 editing cassette. pGL203 and pGL204 are therefore identical except for their sgRNA sequences, which are presented in Table 2 (Kiflom Aregawi et al. 2021).

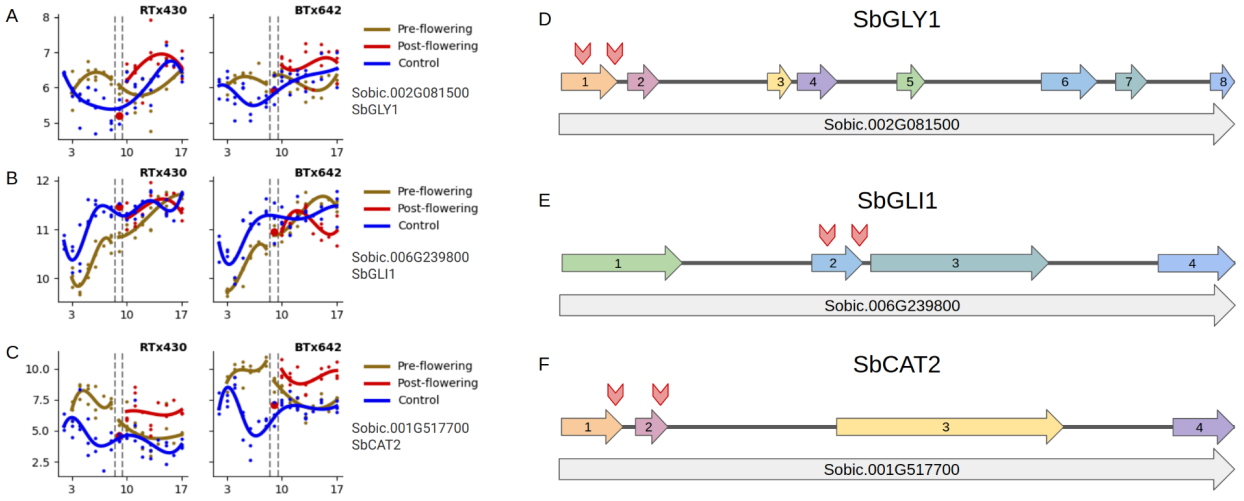

Figure 2. RNA-seq derived expression profiles for the three genes targeted for CRISPR/Cas9 editing: (A) SbGLY1, (B) SbGLI1, and (C) SbCAT2. RNA-seq data was generated as part of (Varoquaux et al. 2019). Plots show expression of each gene in two genotypes, RTx430 (left) and BTx642 (right). Plants were grown under three different watering conditions: fully watered control (blue), pre-flowering drought (brown), and post-flowering drought (red). Samples for RNA-seq were taken every week for 17 weeks, represented on the x-axes (Varoquaux et al. 2019). Simplified genomic maps for (D) SbGLY1, (E) SbGLI1, and (F) SbCAT2 showing exon structure and sgRNA target locations, represented by red chevrons.

Constructs	Replications	Total IEs	Positive for GOI only	Positive for GOI and ODP2	Positive for ODP2 only	Final Efficiency
pGL190 + pANIC10A	4	379	113	51	18	29.8%
pGL203	2	100	19	11	1	19.0%
pGL204	2	100	14	19	3	14.0%

Table 1. Transformation efficiency for both altruistic and non-altruistic experiments, calculated as the number of regenerated plants testing PCR positive for the gene of interest (GOI) but not the developmental genes divided by the number of initial immature embryos (IEs) transfected, represented as a percentage. GOI for pGL190+pANIC10A transformation refers to RFP, while the GOI for pGL203 and pGL204 transformations refers to the sgRNA region (Kiflom Aregawi et al. 2021).

Gene symbol	Gene name	sgRNA Sequences
SbCAT2	Sobic.001G517700	TACCTTGTACGGATCCATGG GGAGTTACCTCGTGCGCCGA
SbGLY1	Sobic.002G081500	GAGGACGAGGACTCCAGCGA ACGCGCAAGGTAGGACTCTC
SbGLI1	Sobic.006G239800	GGTAAATACAATGTGGTTGC GTTTGCAGGTCGTCTTGTTT

Table 2. sgRNA sequences for each of the genes targeted by the CRISPR/Cas9 gene editing system in this study.

Gene symbol	Protein name	Construct name	Primers (F/R) (5'-3')	Amplicon length (bp)	Tm (oC)
RFP	Red fluorescence protein	pANIC10A	GGCCATTATACGTGCGACTT GCATGTGCAATTTCTCGTTG	155	60
BBM	Baby boom	pGL190/ pGL203/ pGL204	GGTCGTCAAGTCTATTTAGGTGGCT AAGTAAAGATCCTTGTTCCCTGCAACT	299	63
gRNA unit	gRNA-tRNA polycistronic cassette	pGL203/ pGL204	CCTAGAAGGCCACCCAGCGGATAACAATTTCA CTGCAGGCATGCACGCGCTAAAAAC	1075 (pGL203) 579 (pGL204)	60
SbCAT2	Catalase 2	pGL204	CACACGTAGCACTAGAACGTC AAAGGTGTTACTTCGAAATTCTTCTAG	797	63
SbGLY1	Glycerol-3-phosphate dehydrogenase complex	pGL203	TCACTCGACTGGAATAGAAAAACA CCTAATAAAGTATCCTACGACATGGAT	714	63
SbGLI1	Glycerol kinase	pGL203	CCTAATTAGCTTGCTTTAAGCATGG GGCAAAATCTCAACAGGAACTC	926	63

Table 3. PCR primers used in this study to genotype potential transformants (Kiflom Aregawi et al. 2021).

Works Cited

Adams, D., T. Tidwell, J. Ritchey, H. Wells, and Others. 1990. “Effect of Nursery-Produced Endomycorrhizal Inoculum on Growth of Redwood Seedlings in Fumigated Soil.” *Tree Planters’ Notes* 41 (3): 7–11.

Afek, U., L. A. Lippet, D. Adams, J. A. Menge, and E. Pond. 1994. “Vesicular–arbuscular Mycorrhizae Colonization of Redwood and Incense Cedar Seedlings Following Storage.” *HortScience: A Publication of the American Society for Horticultural Science* 29 (11): 1362–65.

Afkhami, Michelle E., Patrick J. McIntyre, and Sharon Y. Strauss. 2014. “Mutualist-Mediated Effects on Species’ Range Limits across Large Geographic Scales.” *Ecology Letters* 17 (10): 1265–73.

Altpeter, Fredy, Nathan M. Springer, Laura E. Bartley, Ann E. Blechl, Thomas P. Brutnell, Vitaly Citovsky, Liza J. Conrad, et al. 2016. “Advancing Crop Transformation in the Era of Genome Editing.” *The Plant Cell* 28 (7): 1510–20.

Anand, A., Bass, S.H., Cho, H.-J., Klein, T.M., Lassner, M., McBridge, K.E. 2017. Methods and Compositions of Improved Plant Transformation. WIPO 2017/078836 Al. *World Patent*, filed August 26, 2016, and issued November 5, 2017.

Anand, Ajith, Maren L. Arling, Alexandre da Silva Conceicao, William James Gordon-Kamm, Craig E. Hastings, George M. Hoerster, Theodore Mitchell Klein, et al. 2017. Methods and compositions for rapid plant transformation. USPTO 20170121722:A1. *US Patent*, filed August 26, 2016, and issued May 4, 2017. https://patentimages.storage.googleapis.com/9a/69/72/9925b2e096f458/US20170121722A1.pdf.

Anderson, Marti J., Thomas O. Crist, Jonathan M. Chase, Mark Vellend, Brian D. Inouye, Amy L. Freestone, Nathan J. Sanders, et al. 2011. “Navigating the Multiple Meanings of β Diversity: A Roadmap for the Practicing Ecologist.” *Ecology Letters* 14 (1): 19–28.

Antunes, Luciana Principal, Layla Farage Martins, Roberta Verciano Pereira, Andrew Maltez Thomas, Deibs Barbosa, Leandro Nascimento Lemos, Gianluca Major Machado Silva, et al. 2016. “Microbial Community Structure and Dynamics in Thermophilic Composting Viewed through Metagenomics and Metatranscriptomics.” *Scientific Reports* 6 (December): 38915.

Arbizu, Pedro Martinez. n.d. *pairwiseAdonis*. Github. Accessed July 9, 2021. https://github.com/pmartinezarbizu/pairwiseAdonis.

Aregawi, Kiflom, Jianqiang Shen, Grady Pierroz, Manoj K. Sharma, Jeffery Dahlberg, Judith Judith Owiti, and Peggy G. Lemaux. 2021. “Pathway to Discovery of Gene Function in Key Bioenergy Crop, Sorghum Bicolor.” *In Review*.

Aregawi, K., J. Shen, G. Pierroz, C. Bucheli, M. Sharma, J. Dahlberg, J. Owiti, and P. G. Lemaux. 2020. “Pathway to Validate Gene Function in Key Bioenergy Crop, Sorghum Bicolor.” *bioRxiv*. https://doi.org/10.1101/2020.12.08.416347.

Asseng, S., F. Ewert, P. Martre, R. P. Rötter, D. B. Lobell, D. Cammarano, B. A. Kimball, et al. 2015. “Rising Temperatures Reduce Global Wheat Production.” *Nature Climate Change* 5 (2): 143–47.

Badri, Dayakar V., Victor M. Loyola-Vargas, Corey D. Broeckling, Clelia De-la-Peña,

Michal Jasinski, Diana Santelia, Enrico Martinoia, et al. 2008. “Altered Profile of Secondary Metabolites in the Root Exudates of Arabidopsis ATP-Binding Cassette Transporter Mutants.” *Plant Physiology* 146 (2): 762–71.

Baker, Dwight, John G. Torrey, and George H. Kidd. 1979. “Isolation by Sucrose-Density Fractionation and Cultivation in Vitro of Actinomycetes from Nitrogen-Fixing Root Nodules.” *Nature* 281 (5726): 76–78.

Barajas, Hugo R., Shamayim Martínez-Sánchez, Miguel F. Romero, Cristóbal Hernández Álvarez, Luis Servín-González, Mariana Peimbert, Rocío Cruz-Ortega, Felipe García-Oliva, and Luis D. Alcaraz. 2020. “Testing the Two-Step Model of Plant Root Microbiome Acquisition Under Multiple Plant Species and Soil Sources.” *Frontiers in Microbiology* 11 (October): 542742.

Baselga, Andrés. 2012. “The Relationship between Species Replacement, Dissimilarity Derived from Nestedness, and Nestedness.” *Global Ecology and Biogeography: A Journal of Macroecology* 21 (12): 1223–32.

Baselga, Andrés, David Orme, Sébastien Villeger, Julien De Bortoli, Fabien Leprieur, M. Logez, and R. Henriques-Silva. 2017. “Partitioning Beta Diversity into Turnover and Nestedness Components.” *Package Betapart, Version*, 1–4.

Bates, Douglas. 2010. “lme4: Linear Mixed-Effects Models Using S4 Classes. R Package Version 0.999375-33.” *http://CRAN. R-Project. Org/package= lme4*. https://ci.nii.ac.jp/naid/10030579855/.

Berardini, Tanya Z., Leonore Reiser, Donghui Li, Yarik Mezheritsky, Robert Muller, Emily Strait, and Eva Huala. 2015. “The Arabidopsis Information Resource: Making and Mining the ‘Gold Standard’ Annotated Reference Plant Genome.” *Genesis* 53 (8): 474–85.

Beslow, D. T., E. Hacskaylo, and J. H. Melhuish. 1970. “Effects of Environment on Beaded Root Development in Red Maple.” *Bulletin of the Torrey Botanical Club* 97 (5): 248–52.

Bevan, Michael W., Richard B. Flavell, and Mary-Dell Chilton. 1983. “A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation.” *Nature* 304 (5922): 184–87.

Biotechnology and Biological Sciences Research Council. 2013. “Thirty Years of Plant Transformation: A Case Study Exploring the Impact of Plant Transformation Technology on Plant Science Research and the Global Agricultural Biotechnology Industry.”

Boutilier, Kim, Remko Offringa, Vijay K. Sharma, Henk Kieft, Thérèse Ouellet, Lemin Zhang, Jiro Hattori, et al. 2002. “Ectopic Expression of BABY BOOM Triggers a Conversion from Vegetative to Embryonic Growth.” *The Plant Cell* 14 (8): 1737–49.

Bregitzer, P., S. E. Halbert, and P. G. Lemaux. 1998. “Somaclonal Variation in the Progeny of Transgenic Barley.” *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik* 96 (3-4): 421–25.

Brunel, Caroline, Robin Pouteau, Wayne Dawson, Michael Pester, Kelly S. Ramirez, and Mark van Kleunen. 2020. “Towards Unraveling Macroecological Patterns in Rhizosphere Microbiomes.” *Trends in Plant Science* 25 (10): 1017–29.

Bullard, James H., Elizabeth Purdom, Kasper D. Hansen, and Sandrine Dudoit. 2010. “Evaluation of Statistical Methods for Normalization and Differential Expression in

mRNA-Seq Experiments." *BMC Bioinformatics* 11 (February): 94.
Caddell, Daniel F., Siwen Deng, and Devin Coleman-Derr. 2019. "Role of the Plant Root Microbiome in Abiotic Stress Tolerance." In *Seed Endophytes: Biology and Biotechnology*, edited by Satish Kumar Verma, White, Jr, and James Francis, 273–311. Cham: Springer International Publishing.
Caporaso, J. Gregory, Christian L. Lauber, William A. Walters, Donna Berg-Lyons, James Huntley, Noah Fierer, Sarah M. Owens, et al. 2012. "Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms." *The ISME Journal* 6 (8): 1621–24.
Carey, Chelsea J., Sydney I. Glassman, Thomas D. Bruns, Emma L. Aronson, and Stephen C. Hart. 2020. "Soil Microbial Communities Associated with Giant Sequoia: How Does the World's Largest Tree Affect Some of the World's Smallest Organisms?" *Ecology and Evolution* 10 (13): 6593–6609.
Chanda, Bidisha, Srivathsa C. Venugopal, Saurabh Kulshrestha, Duroy A. Navarre, Bruce Downie, Lisa Vaillancourt, Aardra Kachroo, and Pradeep Kachroo. 2008. "Glycerol-3-Phosphate Levels Are Associated with Basal Resistance to the Hemibiotrophic Fungus Colletotrichum Higginsianum in Arabidopsis." *Plant Physiology* 147 (4): 2017–29.
Char, Si Nian, Jialu Wei, Qi Mu, Xianran Li, Zhanyuan J. Zhang, Jianming Yu, and Bing Yang. 2020. "An Agrobacterium-Delivered CRISPR/Cas9 System for Targeted Mutagenesis in Sorghum." *Plant Biotechnology Journal* 18 (2): 319–21.
Cheng, Ming, Brenda A. Lowe, T. Michael Spencer, Xudong Ye, and Charles L. Armstrong. 2004. "Factors Influencing Agrobacterium-Mediated Transformation of Monocotyledonous Species." *In Vitro Cellular & Developmental Biology-Plant* 40 (1): 31–45.
Chen, Lin-Xing, Karthik Anantharaman, Alon Shaiber, A. Murat Eren, and Jillian F. Banfield. 2020. "Accurate and Complete Genomes from Metagenomes." *Genome Research* 30 (3): 315–33.
Che, Ping, Ajith Anand, Emily Wu, Jeffry D. Sander, Marissa K. Simon, Weiwei Zhu, Amy L. Sigmund, et al. 2018. "Developing a Flexible, High-efficiencyAgrobacterium-Mediated Sorghum Transformation System with Broad Application." *Plant Biotechnology Journal* 16 (7): 1388–95.
Chomicki, Guillaume, Gijsbert D. A. Werner, Stuart A. West, and E. Toby Kiers. 2020. "Compartmentalization Drives the Evolution of Symbiotic Cooperation." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 375 (1808): 20190602.
Clough, S. J., and A. F. Bent. 1998. "Floral Dip: A Simplified Method for Agrobacterium ‐mediated Transformation of Arabidopsis Thaliana." *The Plant Journal: For Cell and Molecular Biology*. https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-313x.1998.00343.x.
Coleman-Derr, Devin, Damaris Desgarennes, Citlali Fonseca-Garcia, Stephen Gross, Scott Clingenpeel, Tanja Woyke, Gretchen North, Axel Visel, Laila P. Partida-Martinez, and Susannah G. Tringe. 2016. "Plant Compartment and Biogeography Affect Microbiome Composition in Cultivated and Native Agave Species." *The New Phytologist* 209 (2): 798–811.

Compant, Stéphane, Brion Duffy, Jerzy Nowak, Christophe Clément, and Essaïd Ait Barka. 2005. “Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects.” *Applied and Environmental Microbiology* 71 (9): 4951–59.

Compant, Stéphane, Abdul Samad, Hanna Faist, and Angela Sessitsch. 2019. “A Review on the Plant Microbiome: Ecology, Functions, and Emerging Trends in Microbial Application.” *Journal of Advertising Research* 19 (September): 29–37.

Cordell, Dana, Jan-Olof Drangert, and Stuart White. 2009. “The Story of Phosphorus: Global Food Security and Food for Thought.” *Global Environmental Change: Human and Policy Dimensions* 19 (2): 292–305.

Cregger, M. A., A. M. Veach, Z. K. Yang, M. J. Crouch, R. Vilgalys, G. A. Tuskan, and C. W. Schadt. 2018. “The Populus Holobiont: Dissecting the Effects of Plant Niches and Genotype on the Microbiome.” *Microbiome* 6 (1): 31.

Dahal, Keshav, and Greg C. Vanlerberghe. 2017. “Alternative Oxidase Respiration Maintains Both Mitochondrial and Chloroplast Function during Drought.” *The New Phytologist* 213 (2): 560–71.

Das, Kaushik, and Aryadeep Roychoudhury. 2014. “Reactive Oxygen Species (ROS) and Response of Antioxidants as ROS-Scavengers during Environmental Stress in Plants.” *Frontiers of Environmental Science & Engineering in China* 2: 53.

Davison, John, Mari Moora, Marina Semchenko, Sakeenah Binte Adenan, Talaat Ahmed, Asem A. Akhmetzhanova, Juha M. Alatalo, et al. 2021. “Temperature and pH Define the Realised Niche Space of Arbuscular Mycorrhizal Fungi.” *The New Phytologist* 231 (2): 763–76.

Dawkins, Karim, and Nwadiuto Esiobu. 2017. “Arbuscular and Ectomycorrhizal Fungi Associated with the Invasive Brazilian Pepper Tree (Schinus Terebinthifolius) and Two Native Plants in South Florida.” *Frontiers in Microbiology* 8 (April): 665.

Daws, S. Caroline, Lauren A. Cline, John Rotenberry, Michael J. Sadowsky, Christopher Staley, Brent Dalzell, and Peter G. Kennedy. 2020. “Do Shared Traits Create the Same Fates? Examining the Link between Morphological Type and the Biogeography of Fungal and Bacterial Communities.” *Fungal Ecology* 46 (August): 100948.

De Cleene, Marcel, and Jozef De Ley. 1976. “The Host Range of Crown Gall.” *The Botanical Review; Interpreting Botanical Progress* 42 (4): 389–466.

Dennis, Paul G., Anthony J. Miller, and Penny R. Hirsch. 2010. “Are Root Exudates More Important than Other Sources of Rhizodeposits in Structuring Rhizosphere Bacterial Communities?” *FEMS Microbiology Ecology* 72 (3): 313–27.

Dickie, Ian A., and Robert J. Holdaway. 2011. “Podocarp Roots, Mycorrhizas, and Nodules.” *Smithsonian Contributions to Botany*. https://repository.si.edu/bitstream/handle/10088/17182/SCB95_11_Final.pdf?sequence=1&isAllowed=y.

Dickson, S. 2004. “The Arum--Paris Continuum of Mycorrhizal Symbioses.” *The New Phytologist* 163 (1): 187–200.

Ding, Na, Aimin Wang, Xiaojun Zhang, Yunxiang Wu, Ruyuan Wang, Huihui Cui, Rulin Huang, and Yonghai Luo. 2017. “Identification and Analysis of Glutathione S-Transferase Gene Family in Sweet Potato Reveal Divergent GST-Mediated

Networks in Aboveground and Underground Tissues in Response to Abiotic Stresses." *BMC Plant Biology* 17 (1): 225.
Dubois, Olivier, and Others. 2011. *The State of the World's Land and Water Resources for Food and Agriculture: Managing Systems at Risk*. Earthscan.
Duff, John, Doug Bice, Ian Hoeffner, and Justin Weinheimer. 2019. "The Sorghum Industry and Its Market Perspective." In *Agronomy Monographs*, 503–14. Madison, WI, USA: Soil Science Society of America.
Duhoux, E., G. Rinaudo, H. G. Diem, F. Auguy, D. Fernandez, D. Bogusz, C. Franche, Y. Dommergues, and B. Huguenin. 2001. "Angiosperm Gymnostoma Trees Produce Root Nodules Colonized by Arbuscular Mycorrhizal Fungi Related to Glomus." *The New Phytologist* 149 (1): 115–25.
Dvořák, Petr, Yuliya Krasylenko, Adam Zeiner, Jozef Šamaj, and Tomáš Takáč. 2020. "Signaling toward Reactive Oxygen Species-Scavenging Enzymes in Plants." *Frontiers in Plant Science* 11: 618835.
Edgar, Robert C. 2013. "UPARSE: Highly Accurate OTU Sequences from Microbial Amplicon Reads." *Nature Methods* 10 (10): 996–98.
Farjon, A., and R. Schmid. 2017. "Sequoia Sempervirens. The IUCN Red List of Threatened Species 2013: E. T34051A2841558."
Fisher, R. A. 1992. "Statistical Methods for Research Workers." In *Breakthroughs in Statistics: Methodology and Distribution*, edited by Samuel Kotz and Norman L. Johnson, 66–70. New York, NY: Springer New York.
Fitzpatrick, Connor R., Julia Copeland, Pauline W. Wang, David S. Guttman, Peter M. Kotanen, and Marc T. J. Johnson. 2018. "Assembly and Ecological Function of the Root Microbiome across Angiosperm Plant Species." *Proceedings of the National Academy of Sciences of the United States of America* 115 (6): E1157–65.
Fitzpatrick, Connor R., Isai Salas-González, Jonathan M. Conway, Omri M. Finkel, Sarah Gilbert, Dor Russ, Paulo José Pereira Lima Teixeira, and Jeffery L. Dangl. 2020. "The Plant Microbiome: From Ecology to Reductionism and Beyond." *Annual Review of Microbiology* 74 (September): 81–100.
Foo, Eloise, Jonathan M. Plett, Juan Antonio Lopez-Raez, and Dugald Reid. 2019. "Editorial: The Role of Plant Hormones in Plant-Microbe Symbioses." *Frontiers in Plant Science* 10 (October): 1391.
Fracasso, Alessandra, Luisa M. Trindade, and Stefano Amaducci. 2016. "Drought Stress Tolerance Strategies Revealed by RNA-Seq in Two Sorghum Genotypes with Contrasting WUE." *BMC Plant Biology* 16 (1): 115.
Fraley, R. T., S. G. Rogers, R. B. Horsch, P. R. Sanders, J. S. Flick, S. P. Adams, M. L. Bittner, et al. 1983. "Expression of Bacterial Genes in Plant Cells." *Proceedings of the National Academy of Sciences of the United States of America* 80 (15): 4803–7.
Frame, Bronwyn R., Huixia Shou, Rachel K. Chikwamba, Zhanyuan Zhang, Chengbin Xiang, Tina M. Fonger, Sue Ellen K. Pegg, et al. 2002. "Agrobacterium Tumefaciens-Mediated Transformation of Maize Embryos Using a Standard Binary Vector System." *Plant Physiology* 129 (1): 13–22.
Franke, Rochus, and Lukas Schreiber. 2007. "Suberin--a Biopolyester Forming Apoplastic Plant Interfaces." *Current Opinion in Plant Biology* 10 (3): 252–59.
Frøslev, Tobias Guldberg, Rasmus Kjøller, Hans Henrik Bruun, Rasmus Ejrnæs, Ane

Kirstine Brunbjerg, Carlotta Pietroni, and Anders Johannes Hansen. 2017. “Algorithm for Post-Clustering Curation of DNA Amplicon Data Yields Reliable Biodiversity Estimates.” *Nature Communications* 8 (1): 1188.
Gao, Cheng, Liliam Montoya, Ling Xu, Mary Madera, Joy Hollingsworth, Elizabeth Purdom, Robert B. Hutmacher, et al. 2019. “Strong Succession in Arbuscular Mycorrhizal Fungal Communities.” *The ISME Journal* 13 (1): 214–26.
Gauthier, S., P. Bernier, T. Kuuluvainen, A. Z. Shvidenko, and D. G. Schepaschenko. 2015. “Boreal Forest Health and Global Change.” *Science* 349 (6250): 819–22.
Glassman, Sydney I., Ian J. Wang, and Thomas D. Bruns. 2017. “Environmental Filtering by pH and Soil Nutrients Drives Community Assembly in Fungi at Fine Spatial Scales.” *Molecular Ecology* 26 (24): 6960–73.
Glick, B. R., C. L. Patten, G. Holguin, and D. M. Penrose. 1999. “Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria.” https://doi.org/10.1142/p130.
Goldmann, Kezia, Kristina Schröter, Rodica Pena, Ingo Schöning, Marion Schrumpf, François Buscot, Andrea Polle, and Tesfaye Wubet. 2016. “Divergent Habitat Filtering of Root and Soil Fungal Communities in Temperate Beech Forests.” *Scientific Reports* 6 (August): 31439.
Goodstein, David M., Shengqiang Shu, Russell Howson, Rochak Neupane, Richard D. Hayes, Joni Fazo, Therese Mitros, et al. 2012. “Phytozome: A Comparative Platform for Green Plant Genomics.” *Nucleic Acids Research* 40 (Database issue): D1178–86.
Goslee, S., and D. Urban. 2017. “Ecodist: Dissimilarity-Based Functions for Ecological Analysis. R Package Version 2.0. 1.”
Graaf, Inge E. M. de, Tom Gleeson, L. P. H. Rens van Beek, Edwin H. Sutanudjaja, and Marc F. P. Bierkens. 2019. “Environmental Flow Limits to Global Groundwater Pumping.” *Nature* 574 (7776): 90–94.
Grand, Larry F. 1969. “A Beaded Endotrophic Mycorrhiza of Northern and Southern Red Oak.” *Mycologia* 61 (2): 408.
Griffiths, Robert I., Bruce C. Thomson, Phillip James, Thomas Bell, Mark Bailey, and Andrew S. Whiteley. 2011. “The Bacterial Biogeography of British Soils.” *Environmental Microbiology* 13 (6): 1642–54.
Gutiérrez-García, Karina, Edder D. Bustos-Díaz, José Antonio Corona-Gómez, Hilda E. Ramos-Aboites, Nelly Sélem-Mojica, Pablo Cruz-Morales, Miguel A. Pérez-Farrera, Francisco Barona-Gómez, and Angélica Cibrián-Jaramillo. 2019. “Cycad Coralloid Roots Contain Bacterial Communities Including Cyanobacteria and Caulobacter Spp. That Encode Niche-Specific Biosynthetic Gene Clusters.” *Genome Biology and Evolution* 11 (1): 319–34.
Hasegawa, Tomoko, Shinichiro Fujimori, Petr Havlík, Hugo Valin, Benjamin Leon Bodirsky, Jonathan C. Doelman, Thomas Fellmann, et al. 2018. “Risk of Increased Food Insecurity under Stringent Global Climate Change Mitigation Policy.” *Nature Climate Change* 8 (8): 699–703.
Hedden, Peter. 2003. “The Genes of the Green Revolution.” *Trends in Genetics: TIG* 19 (1): 5–9.
Heller, Gregory, Karl Lundén, Roger D. Finlay, Frederick O. Asiegbu, and Malin

Elfstrand. 2012. “Expression Analysis of Clavata1-like and Nodulin21-like Genes from Pinus Sylvestris during Ectomycorrhiza Formation.” *Mycorrhiza* 22 (4): 271–77.
Herrera-Estrella, Luis, Ann Depicker, Marc Van Montagu, and Jeff Schell. 1983. “Expression of Chimaeric Genes Transferred into Plant Cells Using a Ti-Plasmid-Derived Vector.” *Nature* 303 (5914): 209–13.
Hesselbarth, Maximilian H. K., Marco Sciaini, Kimberly A. With, Kerstin Wiegand, and Jakub Nowosad. 2019. “Landscapemetrics : An Open‐source R Tool to Calculate Landscape Metrics.” *Ecography* 42 (10): 1648–57.
Hiei, Yukoh, Yuji Ishida, and Toshihiko Komari. 2014. “Progress of Cereal Transformation Technology Mediated by Agrobacterium Tumefaciens.” *Frontiers in Plant Science* 5 (November): 628.
Higgins, Logan Massie, and Peter Gault Kennedy. 2012. “Symbiotic Frankia Bacteria in Alnus Forests in Mexico and the United States of America: Is Geographic Location a Good Predictor of Assemblage Structure?” *Botany* 90 (6): 423–31.
Hijmans, R. J. 2019. “Geosphere: Spherical Trigonometry. 2016. R Package Version 1.5--5.”
Hiller, Karsten, Jasper Hangebrauk, Christian Jäger, Jana Spura, Kerstin Schreiber, and Dietmar Schomburg. 2009. “MetaboliteDetector: Comprehensive Analysis Tool for Targeted and Nontargeted GC/MS Based Metabolome Analysis.” *Analytical Chemistry* 81 (9): 3429–39.
Hoerster, George, Ning Wang, Larisa Ryan, Emily Wu, Ajith Anand, Kevin McBride, Keith Lowe, Todd Jones, and Bill Gordon-Kamm. 2020. “Use of Non-Integrating Zm-Wus2 Vectors to Enhance Maize Transformation: Non-Integrating WUS2 Enhances Transformation.” *In Vitro Cellular & Developmental Biology - Plant* 17 (January): 43.
Holsman, K. K., A. C. Haynie, A. B. Hollowed, J. C. P. Reum, K. Aydin, A. J. Hermann, W. Cheng, et al. 2020. “Ecosystem-Based Fisheries Management Forestalls Climate-Driven Collapse.” *Nature Communications* 11 (1): 4579.
Hsiau, T., T. Maures, K. Waite, J. Yang, R. Kelso, and K. Holden. 2018. “Inference of CRISPR Edits from Sanger Trace Data.” *BioRxiv*. https://www.biorxiv.org/content/10.1101/251082v1.abstract.
Hu, Ye-Qin, Sheng Liu, Hong-Mei Yuan, Jing Li, DA-Wei Yan, Jian-Feng Zhang, and Ying-Tang Lu. 2010. “Functional Comparison of Catalase Genes in the Elimination of Photorespiratory H2O2 Using Promoter- and 3′-Untranslated Region Exchange Experiments in the Arabidopsis cat2 Photorespiratory Mutant.” *Plant, Cell & Environment*. https://doi.org/10.1111/j.1365-3040.2010.02171.x.
Hu, Yuncai, and Urs Schmidhalter. 2005. “Drought and Salinity: A Comparison of Their Effects on Mineral Nutrition of Plants.” *Journal of Plant Nutrition and Soil Science* 168 (4): 541–49.
Jacoby, Richard, Manuela Peukert, Antonella Succurro, Anna Koprivova, and Stanislav Kopriva. 2017. “The Role of Soil Microorganisms in Plant Mineral Nutrition—Current Knowledge and Future Directions.” *Frontiers in Plant Science* 8: 1617.
Jiang, Yina, Wanxiao Wang, Qiujin Xie, Na Liu, Lixia Liu, Dapeng Wang, Xiaowei Zhang, et al. 2017. “Plants Transfer Lipids to Sustain Colonization by Mutualistic Mycorrhizal and Parasitic Fungi.” *Science* 356 (6343): 1172–75.

Jinek, M., K. Chylinski, I. Fonfara, and M. Hauer. 2012. “A Programmable Dual-RNA–guided DNA Endonuclease in Adaptive Bacterial Immunity.” https://science.sciencemag.org/content/337/6096/816.abstract?casa_token=JzjuUK-ghFoAAAAA:_xcEHR0Y4Sr8Gct0JRIbrL3s45eXqhaaGk9T4Jimw1_NZ__njXvHZBvP83ApdE8VKHJvKhyqrL9p_w.

Johnson, Stephanie M., Ian Cummins, Fei Ling Lim, Antoni R. Slabas, and Marc R. Knight. 2015. “Transcriptomic Analysis Comparing Stay-Green and Senescent Sorghum Bicolor Lines Identifies a Role for Proline Biosynthesis in the Stay-Green Trait.” *Journal of Experimental Botany* 66 (22): 7061–73.

Johnstone, James A., and Todd E. Dawson. 2010. “Climatic Context and Ecological Implications of Summer Fog Decline in the Coast Redwood Region.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (10): 4533–38.

Jones, Todd, Keith Lowe, George Hoerster, Ajith Anand, Emily Wu, Ning Wang, Maren Arling, Brian Lenderts, and William Gordon-Kamm. 2019. “Maize Transformation Using the Morphogenic Genes Baby Boom and Wuschel2.” *Methods in Molecular Biology* 1864: 81–93.

Jusino, Michelle A., Mark T. Banik, Jonathan M. Palmer, Amy K. Wray, Lei Xiao, Emma Pelton, Jesse R. Barber, et al. 2019. “An Improved Method for Utilizing High-throughput Amplicon Sequencing to Determine the Diets of Insectivorous Animals.” *Molecular Ecology Resources* 19 (1): 176–90.

Kawasaki, Akitomo, Suzanne Donn, Peter R. Ryan, Ulrike Mathesius, Rosangela Devilla, Amanda Jones, and Michelle Watt. 2016. “Microbiome and Exudates of the Root and Rhizosphere of Brachypodium Distachyon, a Model for Wheat.” *PloS One* 11 (10): e0164533.

Khush, G. S. 1999. “Green Revolution: Preparing for the 21st Century.” *Genome / National Research Council Canada = Genome / Conseil National de Recherches Canada* 42 (4): 646–55.

Kiers, E. Toby, Marie Duhamel, Yugandhar Beesetty, Jerry A. Mensah, Oscar Franken, Erik Verbruggen, Carl R. Fellbaum, et al. 2011. “Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis.” *Science* 333 (6044): 880–82.

Kim, Daehwan, Ben Langmead, and Steven L. Salzberg. 2015. “HISAT: A Fast Spliced Aligner with Low Memory Requirements.” *Nature Methods* 12 (4): 357–60.

Kim, Young-Mo, Shane Nowack, Millie T. Olsen, Eric D. Becraft, Jason M. Wood, Vera Thiel, Isaac Klapper, et al. 2015. “Diel Metabolomics Analysis of a Hot Spring Chlorophototrophic Microbial Mat Leads to New Hypotheses of Community Member Metabolisms.” *Frontiers in Microbiology* 6 (April): 209.

Kind, Tobias, Gert Wohlgemuth, Do Yup Lee, Yun Lu, Mine Palazoglu, Sevini Shahbaz, and Oliver Fiehn. 2009. “FiehnLib: Mass Spectral and Retention Index Libraries for Metabolomics Based on Quadrupole and Time-of-Flight Gas Chromatography/mass Spectrometry.” *Analytical Chemistry* 81 (24): 10038–48.

Korenblum, Elisa, Yonghui Dong, Jedrzej Szymanski, Sayantan Panda, Adam Jozwiak, Hassan Massalha, Sagit Meir, Ilana Rogachev, and Asaph Aharoni. 2020. “Rhizosphere Microbiome Mediates Systemic Root Metabolite Exudation by Root-to-Root Signaling.” *Proceedings of the National Academy of Sciences of the*

United States of America 117 (7): 3874–83.
Kumlehn, Jochen, Janine Pietralla, Goetz Hensel, Michael Pacher, and Holger Puchta. 2018. “The CRISPR/Cas Revolution Continues: From Efficient Gene Editing for Crop Breeding to Plant Synthetic Biology.” *Journal of Integrative Plant Biology* 60 (12): 1127–53.
Labun, Kornel, Tessa G. Montague, Maximilian Krause, Yamila N. Torres Cleuren, Håkon Tjeldnes, and Eivind Valen. 2019. “CHOPCHOP v3: Expanding the CRISPR Web Toolbox beyond Genome Editing.” *Nucleic Acids Research* 47 (W1): W171–74.
Lanfranco, Luisa, Valentina Fiorilli, Francesco Venice, and Paola Bonfante. 2018. “Strigolactones Cross the Kingdoms: Plants, Fungi, and Bacteria in the Arbuscular Mycorrhizal Symbiosis.” *Journal of Experimental Botany* 69 (9): 2175–88.
Laurance, William F. 2008. “Theory Meets Reality: How Habitat Fragmentation Research Has Transcended Island Biogeographic Theory.” *Biological Conservation* 141 (7): 1731–44.
Laux, T., K. F. Mayer, J. Berger, and G. Jürgens. 1996. “The WUSCHEL Gene Is Required for Shoot and Floral Meristem Integrity in Arabidopsis.” *Development* 122 (1): 87–96.
Li, Aixia, Shangang Jia, Abou Yobi, Zhengxiang Ge, Shirley J. Sato, Chi Zhang, Ruthie Angelovici, Thomas E. Clemente, and David R. Holding. 2018. “Editing of an Alpha-Kafirin Gene Family Increases, Digestibility and Protein Quality in Sorghum.” *Plant Physiology* 177 (4): 1425–38.
Liao, Yang, Gordon K. Smyth, and Wei Shi. 2014. “featureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features.” *Bioinformatics* 30 (7): 923–30.
Lin, Yi-Hsien, Kui-You Pan, Ching-Hui Hung, Hsiang-En Huang, Ching-Lian Chen, Teng-Yung Feng, and Li-Fen Huang. 2013. “Overexpression of Ferredoxin, PETF, Enhances Tolerance to Heat Stress in Chlamydomonas Reinhardtii.” *International Journal of Molecular Sciences* 14 (10): 20913–29.
Li, Shao-Peng, Pandeng Wang, Yongjian Chen, Maxwell C. Wilson, Xian Yang, Chao Ma, Jianbo Lu, et al. 2020. “Island Biogeography of Soil Bacteria and Fungi: Similar Patterns, but Different Mechanisms.” *The ISME Journal* 14 (7): 1886–96.
Liu, Guoquan, Jieqing Li, and Ian D. Godwin. 2019. “Genome Editing by CRISPR/Cas9 in Sorghum Through Biolistic Bombardment.” *Methods in Molecular Biology* 1931: 169–83.
Liu, Hongwei, Laura E. Brettell, Zhiguang Qiu, and Brajesh K. Singh. 2020. “Microbiome-Mediated Stress Resistance in Plants.” *Trends in Plant Science* 25 (8): 733–43.
Lowe, Keith, Mauricio La Rota, George Hoerster, Craig Hastings, Ning Wang, Mark Chamberlin, Emily Wu, Todd Jones, and William Gordon-Kamm. 2018. “Rapid Genotype ‘Independent’ Zea Mays L. (maize) Transformation via Direct Somatic Embryogenesis.” *In Vitro Cellular & Developmental Biology. Plant: Journal of the Tissue Culture Association* 54 (3): 240–52.
Lowe, Keith, Emily Wu, Ning Wang, George Hoerster, Craig Hastings, Myeong-Je Cho, Chris Scelonge, et al. 2016. “Morphogenic Regulators Baby Boom and Wuschel Improve Monocot Transformation.” *The Plant Cell* 28 (9): 1998–2015.

Lundberg, Derek S., Scott Yourstone, Piotr Mieczkowski, Corbin D. Jones, and Jeffery L. Dangl. 2013. “Practical Innovations for High-Throughput Amplicon Sequencing.” *Nature Methods* 10 (10): 999–1002.
Mace, Emma S., Shuaishuai Tai, Edward K. Gilding, Yanhong Li, Peter J. Prentis, Lianle Bian, Bradley C. Campbell, et al. 2013. “Whole-Genome Sequencing Reveals Untapped Genetic Potential in Africa’s Indigenous Cereal Crop Sorghum.” *Nature Communications* 4: 2320.
Maciá-Vicente, Jose G., Bora Nam, and Marco Thines. 2020. “Root Filtering, rather than Host Identity or Age, Determines the Composition of Root-Associated Fungi and Oomycetes in Three Naturally Co-Occurring Brassicaceae.” *Soil Biology & Biochemistry* 146 (July): 107806.
Magoc, T., and S. L. Salzberg. 2011. “FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies. Bioinformatics 27, 2957e2963.”
Mai-Prochnow, Anne, Maryse Clauson, Jungmi Hong, and Anthony B. Murphy. 2016. “Gram Positive and Gram Negative Bacteria Differ in Their Sensitivity to Cold Plasma.” *Scientific Reports* 6 (December): 38610.
Malthus, Thomas Robert. 1809. *An Essay on the Principle of Population, as It Affects the Future Improvement of Society*.
Manion, Glenn, Matthew Lisk, Simon Ferrier, Diego Nieto-Lugilde, Matthew C. Fitzpatrick, Maintainer Matthew C. Fitzpatrick, Linkingto Rcpp, and Imports Rcpp. 2017. “Package ‘gdm.’” *A Toolkit with Functions to Fit, Plot, and Summarize Generalized Dissimilarity Models: CRAN Repository*. https://mran.microsoft.com/snapshot/2017-01-30/web/packages/gdm/gdm.pdf.
Mann, David G. J., Peter R. LaFayette, Laura L. Abercrombie, Zachary R. King, Mitra Mazarei, Mathew C. Halter, Charleson R. Poovaiah, et al. 2012. “Gateway-Compatible Vectors for High-Throughput Gene Functional Analysis in Switchgrass (Panicum Virgatum L.) and Other Monocot Species.” *Plant Biotechnology Journal* 10 (2): 226–36.
Mann, David G. J., Peter R. Lafayette, Laura L. Abercrombie, Zachary R. King, Mitra Mazarei, Mathew C. Halter, Charleson R. Poovaiah, et al. 2012. “Gateway-Compatible Vectors for High-Throughput Gene Functional Analysis in Switchgrass (Panicum Virgatum L.) and Other Monocot Species.” *Plant Biotechnology Journal* 10 (2): 226–36.
Maxwell, D. P., Y. Wang, and L. McIntosh. 1999. “The Alternative Oxidase Lowers Mitochondrial Reactive Oxygen Production in Plant Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 96 (14): 8271–76.
McMurdie, Paul J., and Susan Holmes. 2013. “Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data.” *PloS One* 8 (4): e61217.
Mejstrik, Vaclav, Arthur P. Kelley, and Others. 1979. “Mycorrhizae in Sequoia Gigantea Lindl. et Gard. and Sequoia Sempervirens Endl.” *Ceska Mykologie* 33 (1): 51–54.
Miller, F. R. 1984. “Registration of RTx430 Sorghum Parental Line.” *Crop Science* 24: 1224–1224.
Minkenberg, Bastian, Jianwei Zhang, Kabin Xie, and Yinong Yang. 2019. “CRISPR-PLANT v2: An Online Resource for Highly Specific Guide RNA Spacers

Based on Improved off-Target Analysis." *Plant Biotechnology Journal* 17 (1): 5–8.
Moe, Luke A. 2013. "Amino Acids in the Rhizosphere: From Plants to Microbes." *American Journal of Botany* 100 (9): 1692–1705.
Mony, Cendrine, Philomène Brunellière, Nathan Vannier, Anne-Kristel Bittebiere, and Philippe Vandenkoornhuyse. 2020. "Effect of Floristic Composition and Configuration on Plant Root Mycobiota: A Landscape Transposition at a Small Scale." *The New Phytologist* 225 (4): 1777–87.
Mony, Cendrine, Philippe Vandenkoornhuyse, Brendan J. M. Bohannan, Kabir Peay, and Mathew A. Leibold. 2020. "A Landscape of Opportunities for Microbial Ecology Research." *Frontiers in Microbiology* 11 (November): 561427.
Mookkan, Muruganantham, Kimberly Nelson-Vasilchik, Joel Hague, Zhanyuan J. Zhang, and Albert P. Kausch. 2017. "Selectable Marker Independent Transformation of Recalcitrant Maize Inbred B73 and Sorghum P898012 Mediated by Morphogenic Regulators BABY BOOM and WUSCHEL2." *Plant Cell Reports* 36 (9): 1477–91.
Mundia, Clara W., Silvia Secchi, Kofi Akamani, and Guangxing Wang. 2019. "A Regional Comparison of Factors Affecting Global Sorghum Production: The Case of North America, Asia and Africa's Sahel." *Sustainability: Science Practice and Policy* 11 (7): 2135.
Nair, N. V., A. Selvi, T. V. Sreenivasan, K. N. Pushpalatha, and Sheji Mary. 2005. "Molecular Diversity amongSaccharum, Erianthus, Sorghum, Zea and Their Hybrids." *Sugar Tech: An International Journal of Sugar Crops & Related Industries* 7 (1): 55–59.
Naylor, Dan, Stephanie DeGraaf, Elizabeth Purdom, and Devin Coleman-Derr. 2017. "Drought and Host Selection Influence Bacterial Community Dynamics in the Grass Root Microbiome." *The ISME Journal* 11 (12): 2691–2704.
Ngara, R., and B. K. Ndimba. 2014. "Model Plant Systems in Salinity and Drought Stress Proteomics Studies: A Perspective on Arabidopsis and Sorghum." *Plant Biology* 16 (6): 1029–32.
Ngumbi, Esther, and Joseph Kloepper. 2016. "Bacterial-Mediated Drought Tolerance: Current and Future Prospects." *Applied Soil Ecology: A Section of Agriculture, Ecosystems & Environment* 105 (September): 109–25.
Nguyen, Nhu H., Zewei Song, Scott T. Bates, Sara Branco, Leho Tedersoo, Jon Menke, Jonathan S. Schilling, and Peter G. Kennedy. 2016. "FUNGuild: An Open Annotation Tool for Parsing Fungal Community Datasets by Ecological Guild." *Fungal Ecology*. https://doi.org/10.1016/j.funeco.2015.06.006.
Noss, Reed F. 1999. "Assessing and Monitoring Forest Biodiversity: A Suggested Framework and Indicators." *Forest Ecology and Management* 115 (2): 135–46.
Nuccio, Erin E., James Anderson-Furgeson, Katerina Y. Estera, Jennifer Pett-Ridge, Perry De Valpine, Eoin L. Brodie, and Mary K. Firestone. 2016. "Climate and Edaphic Controllers Influence Rhizosphere Community Assembly for a Wild Annual Grass." *Ecology* 97 (5): 1307–18.
Nuccio, Erin E., Evan Starr, Ulas Karaoz, Eoin L. Brodie, Jizhong Zhou, Susannah G. Tringe, Rex R. Malmstrom, et al. 2020. "Niche Differentiation Is Spatially and Temporally Regulated in the Rhizosphere." *The ISME Journal* 14 (4): 999–1014.

Nunes, Cristina Isabel, Juan Leandro García Massini, Ignacio Hernán Escapa, Diego M. Guido, and Kathleen Campbell. 2020. “Conifer Root Nodules Colonized by Arbuscular Mycorrhizal Fungi in Jurassic Geothermal Settings from Patagonia, Argentina.” *International Journal of Plant Sciences* 181 (2): 196–209.

Nyholm, Lasse, Adam Koziol, Sofia Marcos, Amanda Bolt Botnen, Ostaizka Aizpurua, Shyam Gopalakrishnan, Morten T. Limborg, M. Thomas P. Gilbert, and Antton Alberdi. 2020. “Holo-Omics: Integrated Host-Microbiota Multi-Omics for Basic and Applied Biological Research.” *iScience* 23 (8): 101414.

Oksanen, J., R. Kindt, P. Legendre, and B. O’Hara. n.d. “The Vegan Package.” *Researchgate.net*. https://www.researchgate.net/profile/Gavin-Simpson-2/publication/228339454_The_vegan_Package/links/0912f50be86bc29a7f000000/The-vegan-Package.pdf.

Olanrewaju, Oluwaseyi Samuel, Bernard R. Glick, and Olubukola Oluranti Babalola. 2017. “Mechanisms of Action of Plant Growth Promoting Bacteria.” *World Journal of Microbiology & Biotechnology* 33 (11): 197.

Oldroyd, Giles E. D. 2013. “Speak, Friend, and Enter: Signalling Systems That Promote Beneficial Symbiotic Associations in Plants.” *Nature Reviews. Microbiology* 11 (4): 252–63.

Oldroyd, Giles E. D., and J. Allan Downie. 2008. “Coordinating Nodule Morphogenesis with Rhizobial Infection in Legumes.” *Annual Review of Plant Biology* 59: 519–46.

Orozco-Mosqueda, Ma del Carmen, Ma del Carmen Rocha-Granados, Bernard R. Glick, and Gustavo Santoyo. 2018. “Microbiome Engineering to Improve Biocontrol and Plant Growth-Promoting Mechanisms.” *Microbiological Research* 208 (March): 25–31.

Padamsee, Mahajabeen, Renee B. Johansen, S. Alexander Stuckey, Stephen E. Williams, John E. Hooker, Bruce R. Burns, and Stanley E. Bellgard. 2016. “The Arbuscular Mycorrhizal Fungi Colonising Roots and Root Nodules of New Zealand Kauri Agathis Australis.” *Fungal Biology* 120 (5): 807–17.

Panke-Buisse, Kevin, Stacey Lee, and Jenny Kao-Kniffin. 2017. “Cultivated Sub-Populations of Soil Microbiomes Retain Early Flowering Plant Trait.” *Microbial Ecology* 73 (2): 394–403.

Pascale, Alberto, Silvia Proietti, Iakovos S. Pantelides, and Ioannis A. Stringlis. 2019. “Modulation of the Root Microbiome by Plant Molecules: The Basis for Targeted Disease Suppression and Plant Growth Promotion.” *Frontiers in Plant Science* 10: 1741.

Paterson, Andrew H. 2008. “Genomics of Sorghum.” *International Journal of Plant Genomics* 2008: 362451.

Paterson, Andrew H., John E. Bowers, Rémy Bruggmann, Inna Dubchak, Jane Grimwood, Heidrun Gundlach, Georg Haberer, et al. 2009. “The Sorghum Bicolor Genome and the Diversification of Grasses.” *Nature* 457 (7229): 551–56.

Peay, Kabir G. 2016. “The Mutualistic Niche: Mycorrhizal Symbiosis and Community Dynamics,” November. https://doi.org/10.1146/annurev-ecolsys-121415-032100.

Peay, Kabir G. 2018. “Timing of Mutualist Arrival Has a Greater Effect on Pinus Muricata Seedling Growth than Interspecific Competition.” *The Journal of Ecology* 106 (2): 514–23.

Peay, Kabir G., Thomas D. Bruns, Peter G. Kennedy, Sarah E. Bergemann, and Matteo Garbelotto. 2007. “A Strong Species-Area Relationship for Eukaryotic Soil Microbes: Island Size Matters for Ectomycorrhizal Fungi.” *Ecology Letters* 10 (6): 470–80.

Peay, Kabir G., Peter G. Kennedy, and Jennifer M. Talbot. 2016. “Dimensions of Biodiversity in the Earth Mycobiome.” *Nature Reviews. Microbiology* 14 (7): 434–47.

Pebesma, Edzer, and Roger S. Bivand. 2005. “S Classes and Methods for Spatial Data: The Sp Package.” *R News* 5 (2): 9–13.

Qin, Ruijun, Yasuhiro Hirano, and Ivano Brunner. 2007. “Exudation of Organic Acid Anions from Poplar Roots after Exposure to Al, Cu and Zn.” *Tree Physiology* 27 (2): 313–20.

Raineri, D. M., P. Bottino, M. P. Gordon, and E. W. Nester. 1990. “Agrobacterium–Mediated Transformation of Rice (Oryza Sativa L.).” *Biotechnology* 8 (1): 33–38.

Ramírez-Puebla, Shamayim T., Luis E. Servín-Garcidueñas, Berenice Jiménez-Marín, Luis M. Bolaños, Mónica Rosenblueth, Julio Martínez, Marco Antonio Rogel, Ernesto Ormeño-Orrillo, and Esperanza Martínez-Romero. 2013. “Gut and Root Microbiota Commonalities.” *Applied and Environmental Microbiology* 79 (1): 2–9.

Ray, Deepak K., Paul C. West, Michael Clark, James S. Gerber, Alexander V. Prishchepov, and Snigdhansu Chatterjee. 2019. “Climate Change Has Likely Already Affected Global Food Production.” *PloS One* 14 (5): e0217148.

Redillas, Mark C. F. R., Jin S. Jeong, Youn S. Kim, Harin Jung, Seung W. Bang, Yang D. Choi, Sun-Hwa Ha, Christophe Reuzeau, and Ju-Kon Kim. 2012. “The Overexpression of OsNAC9 Alters the Root Architecture of Rice Plants Enhancing Drought Resistance and Grain Yield under Field Conditions.” *Plant Biotechnology Journal* 10 (7): 792–805.

Rehman, Naveed Ur, Mohammed Ali, Muhammad Zulfiqar Ahmad, Guo Liang, and Jian Zhao. 2018. “Strigolactones Promote Rhizobia Interaction and Increase Nodulation in Soybean (Glycine Max).” *Microbial Pathogenesis* 114 (January): 420–30.

Richter-Heitmann, Tim, Thilo Eickhorst, Stefan Knauth, Michael W. Friedrich, and Hannes Schmidt. 2016. “Evaluation of Strategies to Separate Root-Associated Microbial Communities: A Crucial Choice in Rhizobiome Research.” *Frontiers in Microbiology* 7 (May): 773.

Risso, Davide, Katja Schwartz, Gavin Sherlock, and Sandrine Dudoit. 2011. “GC-Content Normalization for RNA-Seq Data.” *BMC Bioinformatics* 12 (December): 480.

Roberts, David W., and Others. 2007. “Labdsv: Ordination and Multivariate Analysis for Ecology.” *R Package Version* 1 (1).

Rodriguez, Patricia A., Michael Rothballer, Soumitra Paul Chowdhury, Thomas Nussbaumer, Caroline Gutjahr, and Pascal Falter-Braun. 2019. “Systems Biology of Plant-Microbiome Interactions.” *Molecular Plant* 12 (6): 804–21.

Rosenzweig, Cynthia, and Martin L. Parry. 1994. “Potential Impact of Climate Change on World Food Supply.” *Nature* 367 (6459): 133–38.

Roth, Ronelle, and Uta Paszkowski. 2017. “Plant Carbon Nourishment of Arbuscular Mycorrhizal Fungi.” *Current Opinion in Plant Biology* 39 (October): 50–56.

Roy, B. A. 1993. “Floral Mimicry by a Plant Pathogen.” *Nature* 362 (6415): 56–58.
Rubin, Benjamin E., Spencer Diamond, Brady F. Cress, Alexander Crits-Christoph, Christine He, Michael Xu, Zeyi Zhou, et al. 2020. “Targeted Genome Editing of Bacteria Within Microbial Communities.” https://doi.org/10.1101/2020.07.17.209189.
Russell, Angela J., Martin I. Bidartondo, and Brian G. Butterfield. 2002. “The Root Nodules of the Podocarpaceae Harbour Arbuscular Mycorrhizal Fungi.” *The New Phytologist* 156 (2): 283–95.
Saikkonen, Kari, Riitta Nissinen, and Marjo Helander. 2020. “Toward Comprehensive Plant Microbiome Research.” *Frontiers in Ecology and Evolution* 8. https://jyx.jyu.fi/handle/123456789/68691.
Sakai, Masao, and Makoto Ikenaga. 2013. “Application of Peptide Nucleic Acid (PNA)-PCR Clamping Technique to Investigate the Community Structures of Rhizobacteria Associated with Plant Roots.” *Journal of Microbiological Methods* 92 (3): 281–88.
Salas-González, Isai, Guilhem Reyt, Paulina Flis, Valéria Custódio, David Gopaulchan, Niokhor Bakhoum, Tristan P. Dew, et al. 2020. “Coordination between Microbiota and Root Endodermis Supports Plant Mineral Nutrient Homeostasis.” *Science*, November. https://doi.org/10.1126/science.abd0695.
Saleem, Muhammad, Audrey D. Law, Mohammad Radhi Sahib, Zahida H. Pervaiz, and Qingming Zhang. 2018. “Impact of Root System Architecture on Rhizosphere and Root Microbiome.” *Rhizosphere*. https://doi.org/10.1016/j.rhisph.2018.02.003.
Santos-Medellín, Christian, Joseph Edwards, Zachary Liechty, Bao Nguyen, and Venkatesan Sundaresan. 2017. “Drought Stress Results in a Compartment-Specific Restructuring of the Rice Root-Associated Microbiomes.” *mBio* 8 (4). https://doi.org/10.1128/mBio.00764-17.
Santoyo, Gustavo, Gabriel Moreno-Hagelsieb, Ma del Carmen Orozco-Mosqueda, and Bernard R. Glick. 2016. “Plant Growth-Promoting Bacterial Endophytes.” *Microbiological Research* 183 (February): 92–99.
Sasse, Joelle, Enrico Martinoia, and Trent Northen. 2018. “Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?” *Trends in Plant Science* 23 (1): 25–41.
Schneider-Maunoury, Laure, Aurélie Deveau, Myriam Moreno, Flora Todesco, Simone Belmondo, Claude Murat, Pierre-Emmanuel Courty, Marcin Jąkalski, and Marc-André Selosse. 2020. “Two Ectomycorrhizal Truffles, Tuber Melanosporum and T. Aestivum, Endophytically Colonise Roots of Non-Ectomycorrhizal Plants in Natural Environments.” *The New Phytologist* 225 (6): 2542–56.
Schwendemann, Andrew B., Anne-Laure Decombeix, Thomas N. Taylor, Edith L. Taylor, and Michael Krings. 2011. “Morphological and Functional Stasis in Mycorrhizal Root Nodules as Exhibited by a Triassic Conifer.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (33): 13630–34.
Selosse, Marc-André, Marie-Pierre Dubois, and Nadir Alvarez. 2009. “Do Sebacinales Commonly Associate with Plant Roots as Endophytes?” *Mycological Research* 113 (Pt 10): 1062–69.
Selosse, Marc-André, Laure Schneider-Maunoury, and Florent Martos. 2018. “Time to Re-Think Fungal Ecology? Fungal Ecological Niches Are Often Prejudged.” *The New Phytologist*.

Shrawat, Ashok Kumar, and Horst Lörz. 2006. “Agrobacterium-Mediated Transformation of Cereals: A Promising Approach Crossing Barriers.” *Plant Biotechnology Journal* 4 (6): 575–603.
Simmons, Tuesday, Daniel F. Caddell, Siwen Deng, and Devin Coleman-Derr. 2018. “Exploring the Root Microbiome: Extracting Bacterial Community Data from the Soil, Rhizosphere, and Root Endosphere.” *Journal of Visualized Experiments: JoVE*, no. 135 (May). https://doi.org/10.3791/57561.
Simonin, Marie, Cindy Dasilva, Valeria Terzi, Eddy L. M. Ngonkeu, Diégane Diouf, Aboubacry Kane, Gilles Béna, and Lionel Moulin. 2020. “Influence of Plant Genotype and Soil on the Wheat Rhizosphere Microbiome: Evidences for a Core Microbiome across Eight African and European Soils.” *FEMS Microbiology Ecology* 96 (6). https://doi.org/10.1093/femsec/fiaa067.
Smil, Vaclav. 2000. “PHOSPHORUS IN THE ENVIRONMENT: Natural Flows and Human Interferences.” *Annual Review of Energy and the Environment* 25 (1): 53–88.
Smith, Gabriel R., Roger D. Finlay, Jan Stenlid, Rimvydas Vasaitis, and Audrius Menkis. 2017. “Growing Evidence for Facultative Biotrophy in Saprotrophic Fungi: Data from Microcosm Tests with 201 Species of Wood-Decay Basidiomycetes.” *The New Phytologist* 215 (2): 747–55.
Socolar, Jacob B., James J. Gilroy, William E. Kunin, and David P. Edwards. 2016. “Sparse Data Necessitate Explicit Treatment of Beta-Diversity: A Reply to Bush et Al.” *Trends in Ecology & Evolution*.
Sohngen, Brent, and Robert Mendelsohn. 2003. “An Optimal Control Model of Forest Carbon Sequestration.” *American Journal of Agricultural Economics* 85 (2): 448–57.
Songstad, D. D., C. L. Armstrong, W. L. Petersen, B. Hairston, and M. A. W. Hinchee. 1996. “Production of Transgenic Maize Plants and Progeny by Bombardment of Hi-II Immature Embryos.” *In Vitro Cellular & Developmental Biology - Plant* 32 (3): 179–83.
Spaepen, Stijn. 2015. “Plant Hormones Produced by Microbes.” In *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*, edited by Ben Lugtenberg, 247–56. Cham: Springer International Publishing.
Steen, Ingrid. 1998. “Phosphorus Availability in the 21st Century: Management of a Non-Renewable Resource.” *Phosphorus Potassium* 217: 25–31.
Steidinger, B. S., T. W. Crowther, J. Liang, M. E. Van Nuland, G. D. A. Werner, P. B. Reich, G. J. Nabuurs, et al. 2019. “Climatic Controls of Decomposition Drive the Global Biogeography of Forest-Tree Symbioses.” *Nature* 569 (7756): 404–8.
Stephens, Philip A., Steven W. Buskirk, Gregory D. Hayward, and Carlos Martinez del Rio. 2005. “Information Theory and Hypothesis Testing: A Call for Pluralism.” *The Journal of Applied Ecology* 42 (1): 4–12.
Stopnisek, Nejc, and Ashley Shade. 2021. “Persistent Microbiome Members in the Common Bean Rhizosphere: An Integrated Analysis of Space, Time, and Plant Genotype.” *The ISME Journal*, March. https://doi.org/10.1038/s41396-021-00955-5.
Subramanian, Aravind, Pablo Tamayo, Vamsi K. Mootha, Sayan Mukherjee, Benjamin L. Ebert, Michael A. Gillette, Amanda Paulovich, et al. 2005. “Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression

Profiles.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (43): 15545–50.
Sudhakar Reddy, Palakolanu, Dumbala Srinivas Reddy, Kaliamoorthy Sivasakthi, Pooja Bhatnagar-Mathur, Vincent Vadez, and Kiran K. Sharma. 2016. “Evaluation of Sorghum [Sorghum Bicolor (L.)] Reference Genes in Various Tissues and under Abiotic Stress Conditions for Quantitative Real-Time PCR Data Normalization.” *Frontiers in Plant Science* 7: 529.
Suzuki, Nobuhiro, Shai Koussevitzky, Ron Mittler, and Gad Miller. 2012. “ROS and Redox Signalling in the Response of Plants to Abiotic Stress.” *Plant, Cell & Environment* 35 (2): 259–70.
Takahashi, Shunsuke, Junko Tomita, Kaori Nishioka, Takayoshi Hisada, and Miyuki Nishijima. 2014. “Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using next-Generation Sequencing.” *PloS One* 9 (8): e105592.
Taylor, D. Lee, William A. Walters, Niall J. Lennon, James Bochicchio, Andrew Krohn, J. Gregory Caporaso, and Taina Pennanen. 2016. “Accurate Estimation of Fungal Diversity and Abundance through Improved Lineage-Specific Primers Optimized for Illumina Amplicon Sequencing.” *Applied and Environmental Microbiology* 82 (24): 7217–26.
Terrat, Sébastien, Walid Horrigue, Samuel Dequiedt, Nicolas P. A. Saby, Mélanie Lelièvre, Virginie Nowak, Julie Tripied, et al. 2017. “Mapping and Predictive Variations of Soil Bacterial Richness across France.” *PloS One* 12 (10): e0186766.
Traoré, O., V. Groleau-Renaud, S. Plantureux, A. Tubeileh, and V. Boeuf-Tremblay. 2000. “Effect of Root Mucilage and Modelled Root Exudates on Soil Structure.” *European Journal of Soil Science* 51 (4): 575–81.
Tremblay, Julien, Kanwar Singh, Alison Fern, Edward S. Kirton, Shaomei He, Tanja Woyke, Janey Lee, Feng Chen, Jeffery L. Dangl, and Susannah G. Tringe. 2015. “Primer and Platform Effects on 16S rRNA Tag Sequencing.” *Frontiers in Microbiology* 6 (August): 771.
Tuomi, M., T. Thum, H. Järvinen, S. Fronzek, B. Berg, M. Harmon, J. A. Trofymow, S. Sevanto, and J. Liski. 2009. “Leaf Litter decomposition—Estimates of Global Variability Based on Yasso07 Model.” *Ecological Modelling* 220 (23): 3362–71.
United States Department of Agriculture, Agricultural Research Service. n.d. “GRIN.” Germplasm Resources Information Network. Beltsville (MD). Accessed 2007-2017. http://www.ars-grin.gov/.
Vannette, Rachel L., Devin R. Leopold, and Tadashi Fukami. 2016. “Forest Area and Connectivity Influence Root-Associated Fungal Communities in a Fragmented Landscape.” *Ecology* 97 (9): 2374–83.
Van Pelt, Robert, Stephen C. Sillett, William A. Kruse, James A. Freund, and Russell D. Kramer. 2016. “Emergent Crowns and Light-Use Complementarity Lead to Global Maximum Biomass and Leaf Area in Sequoia Sempervirens Forests.” *Forest Ecology and Management* 375 (September): 279–308.
Varoquaux, Nelle, Benjamin Cole, Cheng Gao, Grady Pierroz, Christopher R. Baker, Dhruv Patel, Mary Madera, et al. 2019. “Transcriptomic Analysis of Field-Droughted Sorghum from Seedling to Maturity Reveals Biotic and Metabolic Responses.”

Proceedings of the National Academy of Sciences of the United States of America, December. https://doi.org/10.1073/pnas.1907500116.

Walhout, Albertha J. M., Gary F. Temple, Michael A. Brasch, James L. Hartley, Monique A. Lorson, Sander van den Heuvel, and Marc Vidal. 2000. "[34] GATEWAY Recombinational Cloning: Application to the Cloning of Large Numbers of Open Reading Frames or ORFeomes." In *Methods in Enzymology*, edited by Jeremy Thorner, Scott D. Emr, and John N. Abelson, 328:575–IN7. Academic Press.

Wall, Christopher B., Cameron P. Egan, Sean I. O. Swift, and Nicole A. Hynson. 2020. "Three Decades Post-Reforestation Has Not Led to the Reassembly of Arbuscular Mycorrhizal Fungal Communities Associated with Remnant Primary Forests." *Molecular Ecology* 29 (21): 4234–47.

Wang, Qi, Jinge Liu, and Hongyan Zhu. 2018. "Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions." *Frontiers in Plant Science* 9 (March): 313.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. "Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy." *Applied and Environmental Microbiology* 73 (16): 5261–67.

Willing, Claire E., Grady Pierroz, Devin Coleman-Derr, and Todd E. Dawson. 2020. "The Generalizability of Water-Deficit on Bacterial Community Composition; Site-Specific Water-Availability Predicts the Bacterial Community Associated with Coast Redwood Roots." *Molecular Ecology* 29 (23): 4721–34.

Worm, Boris, Edward B. Barbier, Nicola Beaumont, J. Emmett Duffy, Carl Folke, Benjamin S. Halpern, Jeremy B. C. Jackson, et al. 2006. "Impacts of Biodiversity Loss on Ocean Ecosystem Services." *Science* 314 (5800): 787–90.

Wu, Emily, Brian Lenderts, Kimberly Glassman, Maya Berezowska-Kaniewska, Heather Christensen, Tracy Asmus, Shifu Zhen, Uyen Chu, Myeong-Je Cho, and Zuo-Yu Zhao. 2014. "Optimized Agrobacterium-Mediated Sorghum Transformation Protocol and Molecular Data of Transgenic Sorghum Plants." *In Vitro Cellular & Developmental Biology.* 50 (1): 9–18.

Xu, Ling, Zhaobin Dong, Dawn Chiniquy, Grady Pierroz, Siwen Deng, Cheng Gao, Spencer Diamond, et al. 2021. "Genome-Resolved Metagenomics Reveals Role of Iron Metabolism in Drought-Induced Rhizosphere Microbiome Dynamics." *Nature Communications* 12 (1): 1–17.

Xu, Ling, Dan Naylor, Zhaobin Dong, Tuesday Simmons, Grady Pierroz, Kim K. Hixson, Young-Mo Kim, et al. 2018. "Drought Delays Development of the Sorghum Root Microbiome and Enriches for Monoderm Bacteria." *Proceedings of the National Academy of Sciences of the United States of America* 115 (18): E4284–93.

Xu, Ling, Grady Pierroz, Heidi M-L Wipf, Cheng Gao, John W. Taylor, Peggy G. Lemaux, and Devin Coleman-Derr. 2021. "Holo-Omics for Deciphering Plant-Microbiome Interactions." *Microbiome* 9 (1): 69.

Yadava, Pranjal, Alok Abhishek, Reeva Singh, Ishwar Singh, Tanushri Kaul, Arunava Pattanayak, and Pawan K. Agrawal. 2016. "Advances in Maize Transformation Technologies and Development of Transgenic Maize." *Frontiers in Plant Science* 7: 1949.