

Perennial grass ecotype ecological and genetic response to local and non-local microbial
inoculum is ecotype-specific

by

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Abstract

Andropogon gerardii Vitman (big bluestem) is a dominant grass of the Great Plains that accounts for roughly 70% of the biomass of tallgrass prairies. Its broad distribution across a steep rainfall gradient in the Great Plains has given rise to locally adapted wet and dry ecotypes of this grassland species. Abiotic factors, such as rainfall, in the formation of ecotypes and local adaptation have been well-studied. However, much less is known about the role of biotic factors, such as soil microbiomes, in local adaptation. Soil microbiomes play a fundamental role in plant drought resistance and nutrient uptake. We used a reciprocal inoculation experiment in greenhouse to determine the role of local and non-local microbial communities on *A. gerardii* performance and local adaptation. We collected seed and rhizosphere soil samples from six *A. gerardii* populations, three from western Kansas (580 mm rainfall per year) representing the dry ecotype and three from southern Illinois (1167 mm rainfall per year) representing the wet ecotype. We isolated microbes from roots and native soil, cultured them in Reasoners 2A agar broth, and reciprocally inoculated wet and dry microbes (plus no-microbe control) weekly into common garden soil for 12 weeks under greenhouse conditions (6 replicates per treatment combination). Plant form and function were measured weekly for a variety of ecological responses. At the end of the experiment, leaf samples were collected for mRNA extraction to investigate differential gene expression. We found that ecotype determined most of our results. In addition to ecotype effects, inoculation increased total biomass, particularly of the dry ecotype by ~30% compared to control. Furthermore, ecotypes had higher chlorophyll absorbance when grown with local soil microbes, thus providing evidence of biotic local adaptation. Gene expression differed between the two ecotypes regardless of inoculum with over seven thousand genes differentially expressed between ecotypes. For the dry ecotype, selected upregulated

genes involved those in photosynthesis, disease resistance, and zinc ion binding. Conversely, for the wet ecotype, notable selected genes were involved in response to auxin, detoxification, and defense. Inoculum was not a major driver of gene expression. However, of the few differentially expressed genes, plants grown with dry inoculum showed upregulation of genes relating to nitrate reductase and lipid binding, while plants grown with the wet inoculum showed upregulation of genes involved with abscisic acid biosynthesis, detoxification, and heat response. Furthermore, when ecotypes were inoculated with their local soil microbes, with whom they share an evolutionary history, we found major effects of inoculum origin on plant growth and development. In the dry ecotype, there was a strong positive correlation with increased chlorophyll absorbance and co-expression of differentially expressed genes related to electron capture, light response, and plant growth. For the wet ecotype, the co-expression of genes relating to biosynthesis, transcriptional regulation, and auxin signaling in the wet ecotype strongly correlated with its leaf area and biomass. Thus, our results showed diverse effects of inoculum origin on many unanticipated aspects of plant growth and development depending on ecotype. Our study highlights transcriptional pathways in *A. gerardii* ecotypes that are modulated by microbial communities thus impacting host form, function, and process. These results provide insight into how plants interact with native microbes and how researchers might harness microbiomes to increase plant productivity. As grasslands worldwide face climate pressures and anthropogenic impacts, the ability to harness beneficial microbial communities will be essential to preserve these ecosystems now and into the future. By matching plant hosts to specific beneficial microbes, we can potentially increase plant success and productivity.

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Chapter 1 - Introduction and Overview

Abstract

Andropogon gerardii Vitman is a dominant grass of the Great Plains that accounts for roughly 70% of the biomass of tallgrass prairies. Its broad distribution across a steep rainfall gradient in the Great Plains has given rise to locally adapted wet and dry ecotypes. Abiotic factors, such as rainfall, in the formation of ecotypes and local adaptation have been well-studied. However, much less is known about the role of biotic factors, such as soil microbiomes, in local adaptation. Soil microbiomes play a fundamental role in plant drought resistance and nutrient uptake. Similar to plant ecotypes, soil microbiomes also vary across the Great Plains. In Chapter 2, we first investigated using a greenhouse experiment how local soil microbes affect *A. gerardii* growth and whether specific plant ecotypes are matched to local soil microbes in terms of morphology, physiology, and biomass. We predicted that each ecotype would respond positively to their local microbial inoculum as assessed by an increase in performance, thus indicating local adaptation. In Chapter 3, we performed a transcriptional analysis on the same experimental plants to investigate differential gene expression in ecotypes inoculated with local or non-local soil microbes. We predicted that while ecotypic differences would drive differential gene expression, ecotypes would show greater expression of genes involved in metabolism (*gtl*, *tb1*, and *MAX3*) and growth (*Aux/IAA*, *GH3*, and *SAUR* gene families) when inoculated with their respective local soil microbes. For materials and methods, we collected seed from six *A. gerardii* populations, three from western Kansas (580 mm rainfall per year) representing the dry ecotype and three from southern Illinois (1167 mm rainfall per year) representing the wet ecotype and grew them in the greenhouse in common garden soil. We isolated microbes from

roots and native soil, cultured them in R2A broth, and reciprocally inoculated wet and dry microbes (plus no-microbe control) weekly into separate pots with wet and dry ecotypes for 12 weeks under greenhouse conditions (6 replicates per treatment combination). Plant form and function were measured weekly for a variety of responses. At the end of the experiment, leaf samples were collected for extraction of mRNA to investigate differential gene expression. We investigated gene expression in several different ways: 1) across ecotypes while combining inocula allowing us to identify sets of genes differentially expressed in ecotypes regardless of inocula, 2) across inocula while combining ecotypes allowing us to identify genes expressed regardless of ecotype, 3) next, we compared the effect of ecotype (wet and dry) within each inoculum and 4) finally, we evaluated gene expression of each ecotype grown with their “homesite” inoculum, i.e., dry ecotype matched with dry microbial inoculum and wet ecotype matched with wet inoculum. Thus, we examined how shared evolutionary histories of ecotype with microbial communities impact ecotype gene expression and local adaptation. Here, we provide evidence of a context specific response to local soil microbes in a grassland species in terms of enhanced physiology, increased productivity, and microbial-modulated gene expression with implications for grassland systems worldwide. Interactions between plants and their local soil microbes and the genetic underpinnings of these interactions will continue to be an important and productive line of inquiry in plant science research. As grasslands worldwide face climate pressures and anthropogenic impacts, the potential ability to harness beneficial microbial communities is urgent and will be essential to preserving these ecosystems now and into the future.

Introduction

Plants are colonized by a vast number of microbial symbionts (Berg et al., 2016). In most of these symbioses, plant cells are largely outnumbered by the microbial cells that inhabit them and with which they interact (Berg et al., 2016). The myriad of symbionts housed by plants have profound effects on plant performance and success (Weyens et al., 2009; Berendsen et al., 2012; Mendes et al., 2013; Zuppinger-Dingley et al., 2014). Further, symbioses with soil microbial communities play fundamental roles in performance (Ulrich et al., 2019, Fig. 1.1), especially during times of stress (Rolli et al., 2015). Figure 1.1 summarizes our conceptual basis for our understanding of microbiome and plant host interactions where microbes can benefit plants directly by conveying drought tolerance or enhancing nutrient uptake. Or, microbes can indirectly benefit plant response through pathogen exclusion and increased disease resistance (Fig. 1.1). Many studies document the role of soil plant growth-promoting bacteria (PGPB) in mitigating plant response to initial and subsequent drought (Ulrich et al., 2019; Panke-Buisse et al., 2020), strengthening plant disease resistance (Andrews, 1992; Berendsen et al., 2012; Zamioudis & Pieterse, 2012; Mendes et al., 2013), and supplementing nutrient availability for plant hosts (Berendsen et al., 2012; Zhao et al., 2016). The nature and strength of these plant-microbe symbioses are often host species and symbiont-specific (Fitzpatrick et al., 2018; Gdanetz et al., 2021) leading to a wide diversity of interactions. However, most of this research has been done on crop plants in agricultural settings (Agrahari et al., 2020; Harris et al., 2020; Iqbal et al., 2022), not those grown in the wild, which is a gap this research on a dominant prairie species seeks to fill.

Plant local adaptation has been a major area of interest for centuries (Wangenheim, 1787; Langlet, 1971; Wadgyr et al., 2022), and such studies often focused on population divergence along environmental gradients (Wadgyr et al., 2022). Numerous researchers have

documented plant population responses along temperature gradients (Wilczek et al., 2008; Fournier-Level et al., 2011; Hancock et al., 2011, Ågren & Schemske, 2012; Laskey et al., 2012, DeMarche et al., 2018). Others have investigated the role of precipitation gradients (Eckhart et al., 2004; Lenssen et al., 2004; Hall & Willis, 2006; Exposito-Alonso et al., 2018), latitudinal gradients (McMillan 1959, 1965; Chapin et al. 1981; McGraw et al. 2015), and edaphic conditions (Kruckeberg, 1951; Rajakaruna & Bohm, 1999; Guggisberg et al. 2018) in plant local adaptation. However, far fewer studies investigate the role of biotic factors, namely plant-microbe interactions, in the process of local adaptation (Brachi et al., 2022). The few studies that address plant ecotype interaction with microbiomes have particularly expanded our understanding of local adaptation, especially across environments. For example, microbial communities mitigate warmer-drier conditions for their plant host and increase plant fitness when microbial communities of the plant environment are kept intact (Remke et al., 2022). Additional studies indicate that 'home' and 'away' microbial communities differentially impact the growth and development of *Panicum hallii* ecotypes (Khasanova et al., 2022). Local genotypes of *Panicum virgatum* can preferentially recruit local microbes, resulting in an affinity between plant genotypes and local microbiota (Edwards et al., 2023). Thus, the scarcity of studies on biotic influences in local adaptation is a major motivation to explore the intersection of local adaptation and microbial biotic interactions in this study.

Modern genetic techniques provide new opportunities to understand the underlying and mechanistic nature of plant-soil microbe interactions. By utilizing tools to investigate mRNA expression in plants in response to soil microbes, we can obtain insights into the mechanisms underlying these interactions (Agarwal et al., 2014). Recent studies investigating plant-microbe interactions using transcriptomic tools have documented the role of microbial symbionts in

altering plant gene expression (Li et al., 2019; Daval et al., 2020; Wang et al., 2022). These studies have shown an upregulation of genes involved in defense signaling in response to pathogen infection (Kim and Hwang, 2014) as well as genes involved in metabolic pathways and defense (Van der Ent et al., 2009). Another recent study examined gene expression in pear seedlings inoculated with local and commercial AMF (arbuscular mycorrhizal fungi) inocula (Shao et al., 2022). Plants inoculated with either AMF treatment showed increases in growth and nutrient acquisition, but also differences in gene expression in response to local and commercial AMF inocula (Shao et al., 2022). Thus, investigating plant host transcriptional response to microbial origin promises to expand our knowledge of mechanisms of host-microbial interactions and the role of the microbiome in local adaptation.

Background

To place our work in a broader context, we surveyed primary literature published within the last 15 years (2009 – May 2023) using the search terms (rhizobiome OR microbiome AND plant genotype AND host) and found 291 relevant articles. A main finding was that 59% of surveyed studies relating to plant-microbe interactions lacked plant measurements (Fig. 1.2a) and focused instead on microbial community composition. This striking result indicates a major gap in our knowledge and one which our research helps fill by focusing on the plant host partner. Additionally, 40% of these studies lasted less than one month or were field observational surveys of microbial diversity (Fig. 1.2b). Most of the studies (66%) investigated crop species while wild plants represented only 34% of studies (Fig. 1.2c), illustrating the need for these studies to extend beyond agriculture to explore wild plants. Most studies (76%) were at least partially interested in the rhizosphere microbes, with less than half of all studies investigating

microbiomes in different plant organs (Fig. 1.2d). Less than a third of the studies utilized some inoculation treatment to elucidate the relationship and interactions between plants and microbes. Thus, most studies were descriptive and not experimental in nature and contrast with our experimental microbial inoculation studies.

A few notable studies in our survey included plant measurements, lasted longer than a month, and investigated wild grasses. Panke-Buisse and colleagues (2020) used drought simulations on *Festuca arundinacea* to investigate plant and microbial tolerance to and recovery from drought. Their findings suggest that microbiomes modify plant traits such as flowering time. Remke et al. (2022) transplanted *Bouteloua gracilis* ecotypes with sympatric or allopatric soil communities across an elevation gradient in a three-year field experiment and showed that inoculum origin predicted plant performance and success when plants were transplanted to warmer and drier sites. Khasanova et al. (2022) showed that 'home' and 'away' microbial communities differentially impacted the growth and development of *Panicum hallii* ecotypes and showed that functional traits such as root and shoot biomass were altered in the presence of native microbiomes.

An additional literature survey focused on plant-microbial interactions with a focus on transcriptomics to grasp the current understanding of plant gene expression in relation to microbes. We surveyed primary literature published from 2004 until 2023 using the search terms (rhizobiome OR microbiome AND plant genotype AND host AND transcriptome) and found 112 publications of which 28 were relevant. Though relatively few, these studies provide useful insights into the current state of the literature. First, each of these relevant studies investigated plant response at the transcriptional scale. Similar to our first review, 36% of studies were a month or less in duration. A large percentage of studies also investigated crop

species at the transcriptional scale with only 7% of studies investigating wild plants. The majority of the studies (96%) were at least partly interested in the impact of the rhizosphere community on plant transcriptional regulation and response.

Few studies in our transcriptomics literature survey investigated wild plant species. Notably, Liao et al. (2019) showed that in *Populus trichocarpa* the expression of genes for lipid and gibberellin signaling and nutrient uptake were upregulated in plants inoculated with the fungi *Mortierella elongata*, but not *Ilyonectria europaea*. Sharma et al. (2022) found that flowering gene expression in *Brachypodium distachyon* differed between plants inoculated with diverse strains of *Bacillus velezensis*. In summary, in this review of the literature, we observe that many studies are short in duration, investigate crop species, and mostly focus on the rhizosphere microbial communities rather than the plant host partner. It also highlights that in the era of genomics to probe plant host-microbe interactions, still, many studies are focusing on the microbes rather than the plant response.

Of the studies that address plant host gene expression response, authors have described major roles of microbiomes in modulating these processes (Kim and Hwang, 2014; Syed-Ab-Rahman et al., 2022). These and others (Li et al., 2019; Daval et al., 2020; Wang et al., 2022) have shown that distinct soil microbe communities elicit a unique plant transcriptomic response. That is, plants respond differently at the gene expression level to varying soil microbes (Kim and Hwang, 2014; Syed-Ab-Rahman et al., 2022). At the intraspecific level, Sun et al., (2019) observed that in ecotypes of *Cistanche deserticola*, the expression of the pathway for phenylethanoid glycosides production was distinct between ecotypes inhabiting different soil communities. Furthermore, these differences in glycoside production can be directly linked to the composition of the ecotype-specific soil microbe communities. Thus, capturing the various

effects of soil microbe communities on ecotype gene expression is tractable and can be particularly informative to reveal mechanism (Sun et al., 2019).

Study species *Andropogon gerardii*

Andropogon gerardii is a warm-season C4 perennial bunchgrass with a wide distribution across North America. This plant dominates tall-grass prairie communities and accounts for roughly 70% of the biomass of these systems (Knapp et al., 1998; Blair et al., 2014). Within the Great Plains, *A. gerardii* and its corresponding local soil microbes occur along a sharp mean annual precipitation (MAP) gradient from western Kansas (580 mm rainfall per year) to Illinois (1,167 mm rainfall per year), a climate gradient that has been in place for ~10,000 years (Axelrod 1985) (Fig. 1.3), allowing ample time for natural selection to work on both *A. gerardii* and associated soil microbes. This sharp precipitation gradient of the Midwest has given rise to dry and wet ecotypes of *A. gerardii* which are locally adapted to rainfall regimes of the Midwest (Gray et al., 2014; Johnson et al., 2015; Gallart et al., 2019; 2020). The soil microbes vary along the rainfall gradient (Zhao et al., 2016; Sarkar et al., 2022a), in a pattern similar to variation in ecotypes. Genetically distinct plants also select for different soil microbial communities (Hein et al., 2008; Kazarina et al., 2023), and this has been supported by results in *A. gerardii* rhizosphere communities along this Midwest precipitation gradient (Sarkar et al., 2022a).

Overarching goals and approach

Our research focuses on the role of soil microbes in the morphology, physiology, biomass production, and gene expression of genetically distinct *A. gerardii* regional climate ecotypes.

Because of the vital role these microbes play in plants in general (Sun et al., 2020; Cordovez et al., 2021; Latz et al., 2021), understanding how they interact with *A. gerardii* is key to understanding its biology and has major applications in restoration and grassland productivity. To study local adaptation in *A. gerardii* ecotypes to local soil microbes, we took advantage of the historic Midwest precipitation gradient that has strongly selected on *A. gerardii* populations (Johnson et al., 2015; Galliart et al., 2019; 2020) and their corresponding soil microbial communities (Sarkar et al., 2022a).

We investigate the local adaptation of wet and dry *A. gerardii* ecotypes to local soil microbes under controlled greenhouse conditions by examining the plant morphological and physiological response, and the plant host genes differentially expressed from these interactions. Growing these plants in common garden soils in the greenhouse allows for experimental manipulations and isolation of the interactions between ecotypes and their soil microbes under optimal conditions and quantifying differences in morphology, physiology, and gene expression in response to soil microbe origin.

We used a reciprocal-inoculation greenhouse study to address our overarching goal to determine if *A. gerardii* ecotypes are matched to their respective local soil microbes at morphological, physiological, and transcriptomic scales. Reciprocal inoculation involves growing plants of the wet and dry *A. gerardii* ecotypes in the greenhouse and inoculating them weekly with either their local or non-local soil microbes (or no-microbe controls). This approach allows us to answer our overarching questions: 1) Are *A. gerardii* ecotypes locally adapted to their soil microbes morphologically and physiologically under control conditions in greenhouse? 2) Are genes differentially expressed in ecotypes exposed to local and non-local soil microbes?

Our research takes a three-pronged approach to understand the interplay of plant data associated with microbiomes (Fig. 1.4A), genetic data (Fig. 1.4B), and phenotype (Fig. 1.4C) in our study to gain a better understanding of plant-microbe relations in *A. gerardii*. Many studies focus their investigations on one of these prongs (Wilczek et al., 2008; McGraw et al. 2015; DeMarche et al., 2018) or have investigated the interaction between any two of these points of our conceptual model (Fitzpatrick et al., 2018; Li et al., 2019; Ulrich et al., 2019; Daval et al., 2020) (Fig. 1.4A-C). However, by broadly investigating the interplay of the three, we can gain a broad and realistic insight into the nature of the interactions. Combining the results of these three interconnected facets will further contribute to ultimately allow us to model how *A. gerardii* ecotypes and their associated soil microbiomes will respond to future climatic conditions. By addressing each of these three areas together, we will be able to address our overarching goal to determine the role of microbes in local adaptation, by integrating experimental manipulation with differential gene expression analyses.

Significance of the research

Andropogon gerardii is the major component of tallgrass prairie biomass and the preferred major forage for cattle in the Midwest. Cattle ranching is a vital industry throughout the Midwest; in Kansas, it annually contributes ~\$8.7 billion to the state economy (Kansas Department of Agriculture, 2018). So, identifying if ecotypes of *A. gerardii* are locally adapted to their soil microbe can allow for more successful establishment and productivity for this valuable forage grass. This grass is widely used in conservation and restoration. The USDA Conservation Reserve Program plants *A. gerardii* for conservation and consists of 2.4 million acres in Kansas alone and 4.3 million acres in a five-state Midwest region

(<http://www.fsa.usda.gov>), with the purpose of restoring grasslands from marginal agricultural lands. Thus, understanding these plant-microbe interactions and genes differentially expressed in these interactions can maximize success in these prairie restoration efforts. More broadly, we investigate the response to soil microbes in a grassland species with implications for grassland systems worldwide. The observed interactions will help elucidate the role of soil microbes on plant productivity, and interactions such as these must continue to be a major interest in grassland plant communities. As global grasslands face climatic pressures and anthropogenic impacts (IPCC, 2021), harnessing the utility of beneficial microbial communities is urgent and, arguably, essential to preserving these ecosystems now and into the future.

Objectives and hypotheses of the chapters

Chapter 2: Ecotypes Modulate Response to Local and Non-Local Rhizobiome Inoculum in a Perennial Grass

This chapter aims to determine if *A. gerardii* ecotypes and local soil microbes are matched in terms of plant morphology and physiology under controlled greenhouse conditions. This chapter is being prepared for publication in the Journal of Ecology. Here we address the following questions: 1) What is the interplay between plant hosts and their microbes? 2) How do local microbial communities influence the process of local adaptation in their plant hosts? 3) Specifically, how do local soil microbial communities disproportionately influence local adaptation of plant hosts in different populations across multiple environments?

To answer these questions, we used a reciprocal-inoculation experiment in a greenhouse setting to determine if *A. gerardii* ecotypes are locally adapted to their respective local soil microbes. We grew plants of the wet and dry ecotypes in the greenhouse and inoculated them

weekly with either their local or non-local soil microbes (or no-microbe control). We hypothesized that:

(i) Ecotypes will differ in their growth and performance i.e., an ecotype effect (Fig. 1.5a)

We have prior studies to indicate that the ecotype effect is strong, with clearly differentiated phenotypes of wet and dry ecotypes (Johnson et al., 2015; Gallart et al., 2019; 2020).

(ii) Ecotypes inoculated with microbes will show an increase in performance in comparison to no-microbe control plants i.e., an inoculum effect (Fig. 1.5b). Many studies suggest the beneficial role of microbes (Weyens et al., 2009; Berendsen et al., 2012; Mendes et al., 2013).

(iii) Ecotypes will show an increase in performance when grown with their local soil microbes compared to non-local microbes thereby showing evidence of local adaptation of each ecotype to its local soil microbiome i.e., ecotype x inoculum interaction (Fig. 1.5c).

Studying plant-microbe interactions under controlled conditions allows us to clearly identify interactions, both positive and negative. Mimicking ideal conditions in greenhouse gives the best insight into how these plants and soil microbes interact while unfortunately minimizing ecological realism. Chapter 2 characterizes plant host response morphologically and physiologically and complements Chapter 3 where, using the same plant material, we study differences in gene expression to local vs. non-local inoculum.

Chapter 3: Host Gene Expression in Perennial Grass Ecotypes Grown with Local and Non-Local Soil Microbial Inocula is Dependent on Ecotype

This chapter aims to quantify genes differentially expressed in ecotypes exposed to local and non-local microbes in a controlled greenhouse setting. This chapter is being prepared for

submission in New Phytologist. Here we address the following questions: 1) Can we detect evidence of local adaptation to soil microbes at the transcriptional scale? 2) Do ecotypes show an increase in expression in genes relating to fitness and metabolism such as *gt1*, *tb1*, and *MAX3* (Kandel et al., 2018) when inoculated with local soil microbes and an increase in defense and immunity genes like *VpRPW8s*, *HvSERK2*, and *EuCHIT2* (Diaz, 2018) when inoculated with their non-local soil microbes?

We used samples collected from the same reciprocal-inoculation greenhouse experiment in Chapter 2 to determine if *A. gerardii* ecotypes show evidence of local adaptation to soil microbes at the transcriptomic scale. We collected leaf samples for mRNA isolation and gene expression analysis after 12 weeks of experimental treatment from ecotypes inoculated with their local or non-local microbial inoculum (plus no-microbe control). We extracted, sequenced, and analyzed transcripts from leaf samples to investigate differential gene expression in ecotypes in response to local vs. non-local microbial inoculum. We hypothesized that plants would show:

(i) strong ecotypic differences in the expression of genes involved in their stark difference in morphology and function (Galliart et al., 2019).

(ii) differences in gene expression in response to each microbial inocula. Many studies document the role of microbial communities in eliciting and modulating broad gene expression responses in plant hosts (Ortiz-Castro et al., 2009; Subramanian et al., 2015; Shi et al., 2018).

(iii) greater differences in gene expression within a given ecotype due to the specificity of these interactions. That is, the effect of inocula will be unique to each ecotype in terms of gene expression.

(iv) plants inoculated and matched with their local soil microbes will reflect local adaptation at the transcriptional scale. Fitness-related genes will be upregulated in ecotypes only

when they are inoculated with their local soil microbes, thus reflecting the important role these communities play in plant host performance and local adaptation. Ecotypes will show the greatest upregulation in metabolism-related genes such as *gt1*, *tb1*, and *MAX3* (Kandel et al., 2018) and growth-related genes (*Aux/IAA*, *GH3*, and *SAUR* gene families) (Overvoorde et al., 2005) due to local adaptation with these communities. In contrast, we expect upregulation of defense and immunity genes (*VpRPW8s*, *HvSERK2*, and *EuCHIT2*, (Diaz, 2018)) in plants inoculated with their non-local soil microbes.

By examining gene expression in *A. gerardii* ecotypes in response to local or non-local microbial communities, we can gain a better understanding of the nature of these symbioses (Agarwal et al., 2014) and can draw deeper insights into the mechanistic role of soil microbes on *A. gerardii* ecotype performance and growth and local adaptation. Identifying upregulated genes in each ecotype x inoculum treatment group will provide context and help explain the differences in morphology, physiology, and biomass production. Thus, by combining our findings, we can provide a more complete understanding of these plant-microbial interactions and local adaptation between *A. gerardii* and its local soil microbes across multiple scales.

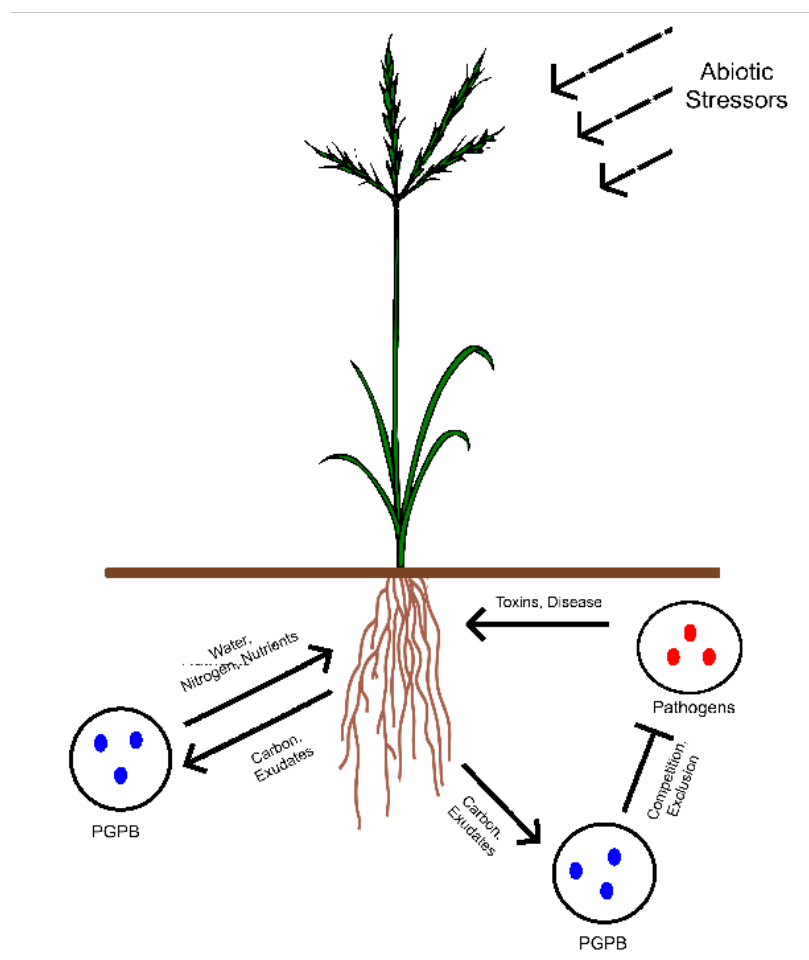


Figure 1.1. Role of plant growth-promoting bacteria (PGPB, blue) in soil and roots alters plant performance, fitness, and response to environmental conditions as well as the exclusion of pathogens (red).

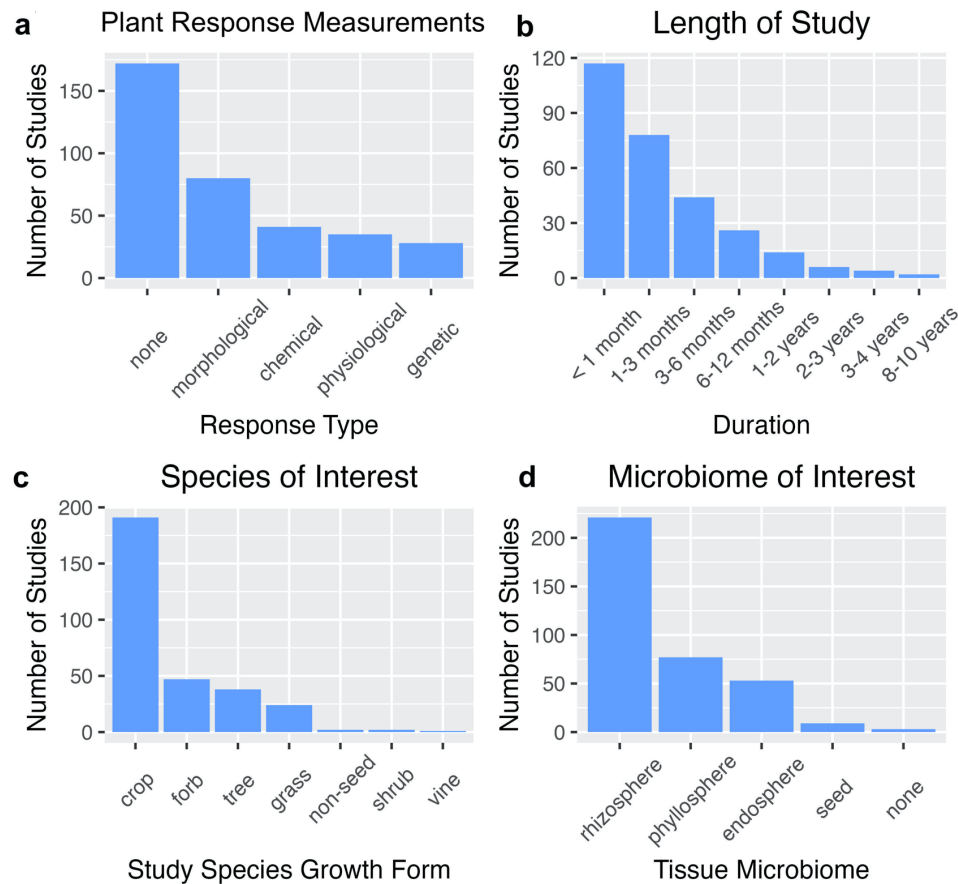


Figure 1.2. Results of literature survey. We assessed 427 articles of which 291 were relevant.

(a) Types of measurements collected on study plants (grouped by broad discipline classifications), (b) Study duration, (c) Species of interest classified first by crop or wild plant, and within wild plants by growth form, (d) Microbiome compartment investigated. Note, study designs that applied to more than one classification (e.g., studies that investigated both rhizosphere and phyllosphere compartments) were counted in both, and thus counts often do not add to 291.

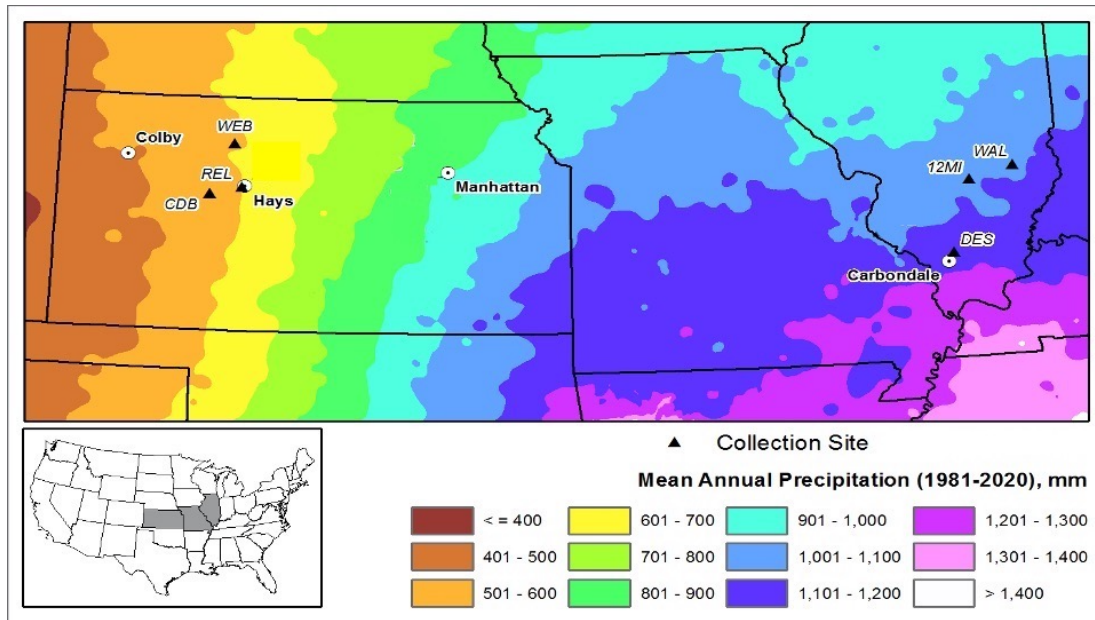


Figure 1.3. Mean annual precipitation map of the US Midwest with seed and soil source population sites (triangles).

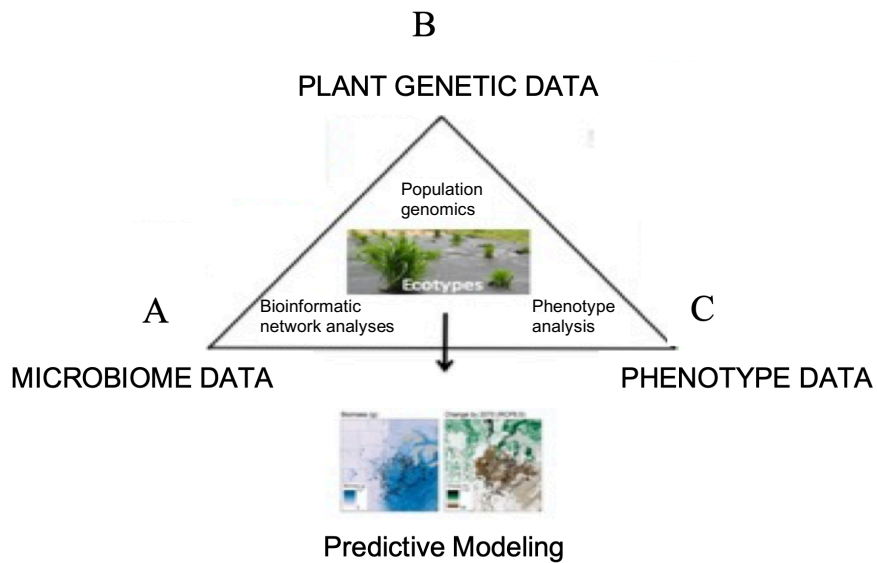


Figure 1.4. The research takes a three-pronged approach composed of a) microbiome data, b) plant genetic data, and c) phenotype data to ultimately help create predictive models to understand current and future *A. gerardii* distributions.

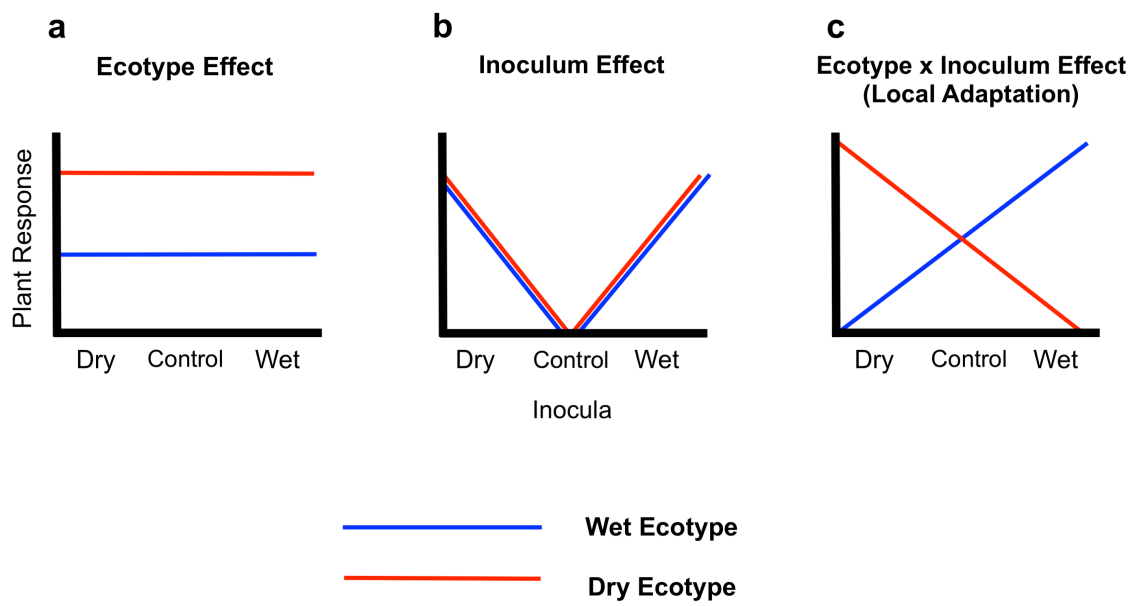


Figure 1.5. Predictions of the effect of ecotype and microbes on plant performance (Not all possibilities shown).

Chapter 2 - Ecotype Modulates Response to Local and Non-Local Rhizobiome Inoculum in a Perennial Grass

Abstract

Andropogon gerardii is a dominant grass of the Great Plains that accounts for roughly 70% of the biomass of tallgrass prairies. Its broad distribution across a steep rainfall gradient in the Great Plains has given rise to locally adapted wet and dry ecotypes. Abiotic factors, such as rainfall, in the formation of ecotypes has been well-studied. However, much less is known about the role of biotic factors, such as soil microbiomes, in such local adaptation. Here, we investigated how soil microbes affect growth of *A. gerardii*, and whether ecotypes are matched to microbes. We collected seed and rhizosphere soil samples from six *A. gerardii* populations from western Kansas (500 mm rainfall per year) and Illinois (1200 mm rainfall per year). We isolated microbes from roots and native soil, cultured them in R2A agar broth, and reciprocally inoculated wet and dry microbes (plus no-microbe control) weekly into garden soil in greenhouse where plants were grown for 12 weeks with 6 replicates per treatment. Plant form and function were measured weekly for a variety of responses. We hypothesized that ecotypes (i) will differ in growth, (ii) when inoculated with microbes, will perform better and (iii) when matched with local soil microbes, will perform best, and show evidence of local adaptation to soil microbes. All response variables showed ecotype differences, and that ecotypes differed in biomass, leaf area and width, and height. Wet ecotypes produced more biomass, greater leaf area, and were taller than the dry ecotype. For inoculation effects, physiological traits, such as chlorophyll absorbance, a proxy for photosynthesis, were enhanced in ecotypes growing with their local microbes, and thus showed evidence of local adaptation to soil microbes. Even more notable, the dry ecotype produced ~30% more biomass when it was matched with its local

microbiome. These results suggest ecotype specific and microbe-mediated effects on nutrient availability. Additionally, results show how plants interact with microbes and how microbes could potentially benefit plant growth. As grasslands worldwide face climatic pressures, harnessing the utility of beneficial microbes is urgent and essential to preserving these ecosystems into the future.

Introduction

Populations of widespread plant species experience different abiotic and biotic conditions. Distinct environmental conditions can result in selection on local populations that, when coupled with partial geographic isolation, can lead to genetic and phenotypic divergence, and, ultimately, local adaptation of these populations (Gregor, 1944; Turrill, 1946; Linhart & Grant, 1996; Geburek et al., 2008). The study of local adaptation has been a major area of interest for centuries (Wangenheim, 1787; Langlet, 1971; Wadgymer et al., 2022), and such studies have often focused on population divergence along environmental gradients (Wadgymer et al., 2022). In early seminal studies, Clausen and colleagues (1940; 1941; 1949) used *Potentilla glandulosa* and *Achillea millefolium* for studying local adaptation along an elevational gradient. Since then, numerous researchers have documented plant population responses along temperature gradients (Wilczek et al., 2008; Fournier-Level et al., 2011; Hancock et al., 2011, Ågren & Schemske, 2012; Laskey et al., 2012; DeMarche et al., 2018). Others have investigated the role of precipitation gradients (Eckhart et al., 2004; Lenssen et al., 2004; Hall & Willis, 2006; Exposito-Alonso et al., 2018), latitudinal gradients (McMillan 1959; 1965; Chapin et al. 1981; McGraw et al., 2015), and edaphic conditions (Kruckeberg, 1951; Rajakaruna & Bohm, 1999; Guggisberg et al., 2018) in plant local adaptation. Thus, ample evidence exists for local

adaptation in response to various abiotic conditions. However, it remains unclear to what extent biotic factors can also drive local adaptation.

Biotic factors, specifically plant-microbe interactions, have a long history of research (Kamienski, 1881; Frank, 1885; Leake et al., 2004). The importance of symbiotic microbes in plant function and productivity have been well documented (Zuppinger-Dingley et al., 2014; Pérez-Izquierdo et al., 2019; Sun et al., 2020; Cordovez et al., 2021; Latz et al., 2021). Plant-microbe interactions can differentially influence plant response to initial and subsequent drought (Ulrich et al., 2019; Panke-Buisse et al., 2020), and strengthen plant disease resistance (Andrews, 1992; Berendsen et al., 2012; Zamioudis & Pieterse, 2012;). Much work documents the microbial role in the availability and uptake of nutrients for plant hosts (Heath & Tiffin, 2009; Berendsen et al., 2012; Boivin & Lepetit, 2020), and highlights the microbial role in nutrient translocation (Gianinazzi et al., 2010; Adeleke et al., 2012; Johnson & Graham, 2013), and availability (Rainey, 1999; Adnan et al., 2022; Khan et al., 2023). However, a complete understanding of plant response to these microbial communities, especially in the process of local adaptation, is still largely unknown (Brachi et al., 2022). Thus, an effort to understand the potential benefit plants receive from their microbial symbionts remains a significant but urgent challenge (Brachi et al., 2022).

From these studies on plant local adaptation and plant-microbe interactions, several major outstanding questions remain: are different populations (ecotypes) of widely distributed plant species locally adapted to their native soil microbes? Do ecotypes perform better when grown with their local soil microbes? More broadly, how do microbial communities influence the process of local adaptation in their plant hosts? Numerous papers investigated plant microbiomes, but few focused on the ecotype-specific plant response to the microbiome and its

role in local adaptation. This indicates a crucial gap in our knowledge and acts as motivation for this study.

The few studies that address plant ecotype interaction with microbiomes have particularly expanded our understanding of these relationships, especially across environments. Microbial communities mitigate overall warmer-drier conditions for their plant host and increase host fitness when microbial communities of a plant's environment are kept intact (Remke et al., 2022). Additional studies indicate that 'home' and 'away' microbial communities differentially impact growth and development of *Panicum hallii* ecotypes (Khasanova et al., 2022), and that local genotypes of *P. virgatum* can preferentially recruit local microbes (Edwards et al., 2023). We focus on plant-microbiome interactions in US Great Plains grasslands which cover 30% of the US (Bailey, 1988). Specifically, we study the relationship between ecotypes of a dominant prairie grass *Andropogon gerardii* Vitman and their local soil microbes. *Andropogon gerardii*, a warm-season perennial bunchgrass, is the focal species because its wide distribution across the rainfall gradient of the Great Plains (Weaver & Fitzpatrick, 1932) and the diverse soils it occupies have led to regionally adapted ecotypes. Soil microbes vary across the diverse soils of the Great Plains (Zhao et al., 2016; Sarkar et al., 2022a), and likely play an essential role in the local adaptation of this species.

Andropogon gerardii dominates tallgrass prairies where it contributes roughly 70% of the biomass to these plant communities (Knapp et al., 1998; Blair et al., 2014) and, as a foundation species (Weaver & Fitzpatrick, 1932; Inouye & Tilman, 1988; Gibson et al., 2016), shapes the structure and function of the US Great Plains grasslands (McCain et al., 2010; Dietrich & Smith, 2016). *Andropogon gerardii* is widely used in restoration and conservation plantings of

(Conservation Reserve Program Statistics, 2022) and for forage in rangelands (Gould and Shaw, 1983; Springer, 1989) make the study of this species particularly urgent.

Ecological and genetic differences in ecotypes of *A. gerardii* have been shaped by the sharp rainfall gradient of the US Great Plains (Caudle et al., 2014; Johnson et al., 2015; Sytsma et al., in review; Gray et al., 2014; Galliart et al., 2019; 2022) that has been in place for roughly 10,000 years (Axelrod, 1985) (Fig. 2.1-2.2). Regions of low rainfall in the western portion of the range of *A. gerardii*, have selected for dwarfed drought-tolerant *A. gerardii* ecotypes. The eastern range, where precipitation is high and plant growth is light-limited, has favored large and robust wet *A. gerardii* ecotypes (Caudle et al., 2014; Johnson et al., 2015; Kramer et al., 2018; Galliart et al., 2020). What remains unclear is the extent to which these regional ecotypes are also adapted to their native soil microbiomes in addition to rainfall.

The strength and nature of the role of the microbiome in ecotype adaptation is still unknown in grasslands. Composition of soil rhizosphere bacterial communities differed among *A. gerardii* ecotypes growing in the field (Sarkar et al., 2022a). Host ecotype genetic variation led to differential recruitment of rhizosphere communities where ecotypes are more successful in recruiting unique microbial specialists at their homesites (Kazarina et al., 2023). Furthermore, the rhizosphere of *A. gerardii* in the dry part of its range has nitrogen-fixing stress tolerant *Pseudomonas* bacteria with potential to influence nutrient uptake and productivity of the host in dry locations (Sarkar et al., 2022b). These studies provide motivation to experimentally investigate local adaptation in *A. gerardii* in relation to its rhizosphere bacterial communities by manipulating microbiomes and ecotypes to test for local vs non-local matching and local adaptation of ecotypes to their local soil microbes. The relative importance of microbiomes in

shaping local adaptation is poorly understood in most plant species (Brachi et al., 2022) and is a gap this paper seeks to fill.

We used a reciprocal-inoculation experiment in a greenhouse setting to address our overarching goal to determine if *A. gerardii* ecotypes are locally adapted to their respective local soil microbes. We grew plants of wet and dry ecotypes in greenhouse with common garden soil and inoculated them weekly with local or non-local soil microbes (or no-microbe control). We hypothesized that (i) ecotypes will differ in their growth and performance, i.e., an ecotype effect (Fig. 2.3a). We have prior studies to indicate that the ecotype effect is strong, with clearly differentiated phenotypes of wet and dry ecotypes (Johnson et al., 2015; Galliart et al., 2020) (ii) ecotypes inoculated with microbes will perform better compared to no-microbe control plants, i.e., an inoculum effect (Fig. 2.3b). Many studies suggest the beneficial role of microbes (Weyens et al., 2009; Berendsen et al., 2012; Mendes et al., 2013) and (iii) ecotypes will perform better when grown with their local soil microbes compared to non-local microbes thereby showing evidence of local adaptation of each ecotype to its native soil microbiome, i.e., an ecotype x inoculum interaction (Fig. 2.3c).

This study should provide insight into how *A. gerardii* interacts with its local soil microbes and ultimately, how land managers may utilize these soil communities to optimize plant production in prairie restoration and rangeland sustainability. Beyond this, our results may increase our understanding of plant-microbe interactions in grasslands globally and how these interactions affect plant performance and potentially can be harnessed in these widespread grassland ecosystems. In this study, we focused on the plant responses to the inoculation while companion studies focus on the microbiome response to the ecotypes (Kazarina et al., 2023).

Materials and methods

Collection sites: Six populations of *Andropogon gerardii* Vitman were chosen for seed and soil collection (Table 2.1): three in western Kansas (580 mm rainfall) representing the dry ecotype (Relict Prairie (REL), Webster Reservoir (WEB), and Cedar Bluffs Reservoir (CDB), and three in southern Illinois (1,167 mm rainfall) representing the wet ecotype (Desoto Railroad Prairie (DES), 12 Mile Railroad Prairie (12M), and Walters Prairie (WAL) (Fig. 2.1, Table 2.1). These populations have already been intensively studied in terms of divergence of wet and dry ecotypes (Caudle et al., 2014; Johnson et al., 2015; Galliard et al., 2019, 2020). Collecting from pristine prairies provide a window into how *A. gerardii* has historically interacted with its soil microbes in native soil without agricultural disturbance.

Seed and Soil Collection: Seed of *A. gerardii* was collected from multiple plants during fall 2020 from the six populations described above. Seeds were stored at 4°C until the start of the experiment. Soil from these populations was collected in June 2021. Three soil cores 1.25 cm in diameter to a depth of 15 cm were taken from each population at the base of an *A. gerardii* plant. Soil collections corresponding to each ecotype were pooled into one representing the ecotypes for microbial isolation: Soils from Western Kansas for the dry ecotype inocula and soils from Southern IL for the wet ecotype inocula.

Greenhouse experimental design: The seeds were scarified by light rubbing and planted in shallow germination trays with Berger BM6 all-purpose garden soil (Berger; Saint-Modeste, QC) in February 2021 under natural light conditions of the greenhouse. In May 2021, individual seedlings were transplanted into single plastic pots (12.5 cm x 11 cm x 8 cm Square Injection Molded Pots: Greenhouse Megastore, Danville, IL) with the same common garden soil. Individual plants were placed in a randomized block design with six replicate blocks. Each

population was represented with 12 plants per block and 432 total plants across the six replicate blocks, overall (6 x 12 x 6). Within each block, plants were randomly assigned to one of three inoculation treatments: dry soil inocula, wet soil inocula, or no-microbe control inocula. Plants were grown under natural light conditions in the greenhouse from Julian days 132 to 209 where temperature ranged from 20 to 30 °C. (Note that there were no block effects on response variables and hereafter are not considered.)

Culturing of microbes and inoculation: We cultured bacterial communities from soil samples of dry and wet ecotypes on R2A (Reasoner's 2A medium) broth (de Raad et al. 2021). The dry and wet ecotype soils (50 mg) were added to 5 ml of broth and incubated overnight at 30°C under 150 rpm shaking conditions in culture tubes. Cells were then diluted using phosphate-buffered saline (PBS) (pH 7) and ultrapure water (R2A broth culture: PBS: water 1:1:8). For the no-microbe control, we used PBS and water only (PBS: water 2:8). Ten ml of the diluted dry or wet inocula or no-microbe control inocula were then reciprocally injected into the soil using a 20 ml syringe weekly for six weeks starting on Julian day 174. Note that this method with R2A cultured only bacteria, not fungi.

Plant responses and data collection: We measured several plant performance-related traits: height, leaf width, leaf surface area, chlorophyll absorbance, and biomass at the end of the experiment before plants had begun to flower.

Height: Maximum height in centimeters was recorded on each plant weekly and collected five times during this experiment on Julian days 169, 181, 190, 195, and 201. Measurements were taken from the base of each plant to the end of the tallest outstretched leaf. Full data set will be available in archive upon acceptance.

Leaf surface area: Leaf surface area was recorded using images collected on a NIKON D5500 DSLR camera mounted on a two-meter tripod. Plants were placed on a large white canvas and exposed soil was covered with white paper. The tripod was placed over the plant and canvas; pictures were taken directly from above. A reference ruler was included in each photo for digitized leaf area analysis. Images were taken at between 10:00AM and 2:00PM and processed and analyzed within FIJI-ImageJ (Schindelin et al., 2012) (<https://imagej.net/Fiji>). Surface area was measured twice; first from Julian days 179 to 181, and two weeks later Julian days 193 to 195. Full data set will be available in archive.

Leaf width: Leaf width in millimeters was recorded four times on Julian days 169, 182, 196, and 201. The widest leaf on each plant during a given sampling period was chosen and measured at its widest part. Data are presented from Julian day 169. Full data set will be available in archive.

Chlorophyll absorbance: Chlorophyll absorbance was measured six times on Julian days 172, 175, 190, 195, and 200. We measured chlorophyll absorbance using a SPAD-502 Plus Chlorophyll Meter (Konica-Minolta, Japan) as a proxy for leaf nitrogen content and photosynthetic rate. We selected the youngest fully expanded vegetative leaf for each measurement. Chlorophyll absorbance was recorded between 10:00AM and 2:00PM. Data are presented from Julian days 172 and 200. Full data set will be available in archive.

End-of-season biomass harvest: Plants were harvested by block after 11 weeks of growth in the greenhouse. Aboveground biomass was collected by cutting plants at the soil surface and placing material in paper bags. For belowground biomass, which composed of the summed root and rhizome weights, roots and the rhizome were separated, and excess soil was removed from the roots by shaking. Roots and rhizomes were washed with deionized water on a 2 mm sieve.

Samples were dried at 55°C for 48 hours before weighing using an Ohaus TS400D Precision Standard Electronic Balance. Each plant had three biomass measurements recorded: aboveground biomass, root biomass, and rhizome biomass, which were combined for total biomass. Plants were harvested before flowering.

Plant Genotyping: We characterized genetic differences between ecotypes as we expected genetic background would strongly affect the relationship with the microbes. For the plant DNA samples, we used tissue from the same populations (not the same plants to avoid destructive harvest) to characterize genetic differences between ecotypes. DNA from the same population sources (Galliart et al. 2019) was extracted using a modified CTAB protocol (Doyle & Doyle, 1987). Extracted DNA was assessed both visually on an 0.8% agarose gel and quantified for concentration with PicoGreen (ThermoFisher.com) and normalized for library preparation. Samples were prepared for genotyping-by-sequencing (GBS) (Poland & Rife, 2012; Elshire et al., 2011). A pair of two restriction enzymes (PstI and MspI) was used for digestion. Sequencing was performed on Illumina HiSeq 200 using 200 base paired-end sequencing. Final SNP (single nucleotide polymorphism) calling was performed using the UNEAK pipeline in the TASSEL 3.0 Standalone Pipeline (Lu et al., 2013). For full sequencing and SNP calling parameters and procedure see Galliart et al. (2019). For Principal Coordinate Analyses (PCoA), we included 8,110 SNP markers from 67 individuals from the dry ecotype (23 CDB, 20 REL, 24 WEB) and from 73 individuals from the wet ecotype (23 DES, 24 12M, 26 WAL). PCoA of genetic distance was calculated from the SNP markers using the R package Adgenet (Jomart, 2008).

Metabarcoding Sequencing of Native Soils and Inocula: We characterized the differences in the microbial communities associated with both six field soils and microbial inocula used in

the inoculation experiment. The environmental DNA from the field soils and the microbial DNA from the inocula were extracted using the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Environmental/total DNA was extracted from aliquot of 0.25g of manually mixed soil. To prepare the inocula for the DNA extraction, 20-25mL of the inocula broth was aliquoted in 2mL centrifuge tubes and centrifuged for 10 min at 8000rpf at 4°C. The supernatant was carefully decanted from each tube. A total 1000µL of PBS buffer was added to one of the 2mL centrifuge tube to resuspend the pellet; the resuspension was transferred to the next tube to acquire one pooled 2ml centrifuge tube per inocula. This 2mL tube was centrifuged again for 10 min at 8000rpf at 4°C to pellet the cells and supernatant discarded prior to the DNA extraction as per manufacturer's protocol.

Illumina MiSeq platform was used to sequence 16S rRNA V4 amplicons using 515F and 806R [515F-GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGGTWTCTAAT] primers with the barcodes (Illumina, San Diego, CA, USA). QIIME 2 v2021.4 (Bolyen et al., 2019) was used for raw sequence processing. The QIIME 2 plugin cutadapt (Martin 2011) was used to remove the primer sequences. For initial quality control, any sequences with ambiguous bases, no primer, with greater than 0.1 error rate mismatch with primer or any mismatches to the sample-specific 12 bp molecular identifiers (MIDs) were discarded. We used the DADA2 pipeline to extract Amplicon Sequence Variants (ASVs) (Callahan et al., 2016). Pre-trained SILVA database (v.138) was used for the taxonomic assignment of the bacteria (Quast et al., 2013). We rarefied the data to 10,000 reads per sample. We estimated pairwise Bray-Curtis distances to compare the bacterial communities among the different locations and visualized these data using Non-Metric Multidimensional Scaling (NMDS) ordinations. We sequenced each weekly inocula to ensure microbial composition stability over time.

Statistical analysis of plant responses: To test for differences among plant ecotypes and to inoculation treatments, plant responses were analyzed for ecotype and inoculum main effects as well as for their interaction using generalized linear models and ANOVA in SAS ® software (Proc Reg, SAS 9.4, SAS Institute Cary NC). The least-squares means procedure was used to examine differences in the means of main effects. Significance was assigned at $\alpha = 0.05$.

Contrasts between means in biomass and chlorophyll absorbance results were found using post hoc Tukey Honestly Significant Difference (HSD) tests in SAS software (Proc Reg, SAS 9.4, SAS Institute Cary NC).

Results

3.1 Genetic Differences among Ecotypes, Prairie Soils and Microbial Inocula

Ecotypes: We identified 8,110 SNPS to characterize differences between ecotypes. The PCoA of the SNP data separated the wet and dry ecotypes into two main clusters corresponding to the two ecotypes (Fig. 2.4). The first PCoA axis accounted for the most variation (16.38%) with the first and second axes together accounting for 20.19% of the variation.

Field Soils: PERMANOVA of the 16S rRNA amplicon metabarcode data indicated there were marginally significant differences between the original field soils sampled from the dry and wet ecotype populations ($p=0.1$), a result likely attributable to low replication due to pooling of soil samples within a site and the resultant limited statistical power. However, soil communities did visually cluster separately in NMDS ordinations (Fig. 2.5a). Compositionally, the relative frequencies of the dominant families differed between the communities associated with the two *A. gerardii* ecotypes (Fig. 2.5b). These analyses suggest baseline differences among the soil bacterial communities used for the inocula in our experiment.

Microbial Inocula: Wet and dry soil inocula clearly differed compositionally in the analyses of the 16S rRNA amplicon metabarcode data (Fig. 2.6). In the dry inocula, *Paenibacillus*, *Lysinibacillus*, and *Brevibacillus* were abundant, accounting for $43.7\% \pm 4.2$ of the microbial community (Fig. 2.6). While common in the dry inocula, these three genera were almost entirely absent in the wet inocula. Furthermore, *Clostridium*, *Aneurinibacillus*, and *Acinetobacter* contributed $38.6\% \pm 6.4$ of the relative abundance of the microbial community in the wet inocula while almost entirely absent from the dry inocula (Fig. 2.6). Genus *Bacillus* was the most common taxon in both inocula and accounted for $45.7\% \pm 5.6$ of each microbial community, although it was typically more abundant in the wet inocula (Fig. 2.6). Notably, the above-described differences between the two inocula were consistent throughout the six inoculations of the experiment.

3.2 Height, Leaf Width, and Leaf Area Differ Between Ecotypes

The observed differences in plant height, leaf area, and leaf width were explained only by ecotype supporting our first hypothesis that ecotypes would differ in their performance (Fig. 2.3a, $p < 0.0001$), thus demonstrating a strong genetic constraint on host plant morphology. Wet ecotypes were significantly taller than dry ecotypes across all five measurement periods of the growing season. The wet ecotype was 13-19 cm taller than the dry ecotype ($p < 0.0001$, Fig. 2.7a), ranging from $58.4 \text{ cm} \pm 0.6$ early in the experiment to $95.2 \text{ cm} \pm 1.3$ five weeks later. The dry ecotype height ranged from $43.5 \text{ cm} \pm 0.6$ to $82.3 \text{ cm} \pm 1.3$ during the experiment. The wet ecotype also had significantly greater leaf area in both sampling periods (Julian days 181 and 195). In the first sampling, just after the first inoculation, the wet ecotype had an average leaf area of $258.7 \text{ cm}^2 \pm 5.30$, more than double the leaf area for the dry ecotype (118.9

$\text{cm}^2 \pm 5.30$, $p < 0.0001$). Similarly, in the second measurement period on Julian days 193 to 195, the wet ecotype had an average leaf area of $442.8 \text{ cm}^2 \pm 7.8$, and again nearly double the leaf surface area of the dry ecotype ($228.1 \text{ cm}^2 \pm 7.8$, $p < 0.0001$, Fig. 2.7b).

Leaf width also differed between the two ecotypes with the wet ecotype having significantly wider leaves. In our first sampling (Julian day 169) (Fig. 2.7c), the wet ecotype had a maximum leaf width of $10.1 \text{ mm} \pm 0.1$, compared to the dry ecotype ($8.4 \text{ mm} \pm 0.1$, $p < 0.0001$). Results for all three sampling periods were similar and corroborated the difference observed in the first sampling ($p < 0.0001$). In summary, leaf area, leaf width, and height showed only an ecotype effect, with no evidence of inoculum main effect or ecotype x inoculum interaction.

3.3 Chlorophyll Absorbance Shows Evidence of Local Adaptation

Chlorophyll absorbance also showed a main ecotype effect in every sampling period, again supporting our first hypothesis that ecotypes would differ in their performance (Fig. 2.3a). The dry ecotype had significantly higher chlorophyll absorbance than the wet ecotype for all sampling periods. In our first sampling period on Julian day 172, the dry ecotype chlorophyll absorbance was 46.7 ± 0.32 compared to the wet ecotype 42.8 ± 0.32 ($p < 0.0001$, Fig. 2.8a). This main ecotype effect was consistent across all sampling periods (Fig. 2.8a-2.8c). Chlorophyll absorbance decreased for both ecotypes as the growing season progressed, but the dry ecotype was always significantly greater than the wet ecotype ($p < 0.0001$).

In addition to the strong ecotype effect driving differences in chlorophyll absorbance for early sampling periods, an interaction effect (ecotype x inoculum) emerged during our fifth ($p = 0.014$) and sixth ($p < 0.0001$) sampling periods (Julian days 195 and 200) which supported our third hypothesis that ecotypes will show an increase in performance and evidence of local

adaptation when grown with their local soil microbes (Fig. 2.3c). This interaction explained 2% of the ANOVA model variation in the fifth sampling period and 6% in the sixth sampling period compared to 12% and 17% explained by the ecotype in the same sampling periods, respectively. Chlorophyll absorbance of the ecotypes responded positively when grown with their local microbial inoculum and negatively when grown with their non-local inoculum in comparison to the no-microbe control treatment ($p < 0.0001$, Fig. 2.8b-2.8c) based on post-hoc tests. For the dry ecotype, from our sixth sampling round, pairwise comparisons showed that chlorophyll absorbance of plants inoculated with their local dry soil microbe inoculum averaged 41.7 ± 0.6 and was significantly higher than the no-microbe control (38.5 ± 0.6 , $p = 0.0065$, Fig. 8c) and non-local wet soil inoculum (37.6 ± 0.6 , $p = 0.0002$, Fig. 2.8c). Chlorophyll absorbance did not differ between dry ecotypes with no-microbe control and wet inoculum. For the wet ecotype, chlorophyll absorbance of plants inoculated with their local soil microbe inoculum (35.7 ± 0.6) differed from the dry soil inoculum (32.3 ± 0.6 , $p = 0.0032$) but not from the no-microbe control (34.1 ± 0.6 , $p = 0.32$). These results are consistent with those from our fifth sampling period of chlorophyll absorbance on Julian day 195 ($p = 0.014$, Fig. 2.8b).

3.4 Host Plant Biomass Differed Between Ecotypes and Inoculation Treatments

Total, aboveground, and belowground biomasses differed between the ecotypes and explained most of the variation (Fig. 2.9a-2.9c) again supporting our first hypothesis (Fig. 2.3a). Total biomass strongly differed between the two ecotypes with the dry ecotype weighing $6.3 \text{ g} \pm 0.3$ compared to nearly double that for the wet ecotype ($11.3 \text{ g} \pm 0.3$, $p < 0.0001$, Fig. 2.9c) and explained 46% of the ANOVA model variation. Belowground biomass mirrored this trend with the biomass of the dry ecotype ($2.7 \text{ g} \pm 0.1$) about half that of the wet ecotype ($4.8 \text{ g} \pm 0.1$,

$p < 0.0001$, Fig. 2.9b). The aboveground biomass of the dry ecotype ($3.6 \text{ g} \pm 0.16$) was again roughly half of the wet ecotype ($6.4 \text{ g} \pm 0.16$, $p < 0.0001$, Fig. 9a). No significant ecotype x inoculum interaction effects were detected for the biomass.

In addition to an ecotype effect, total biomass responded to inoculation treatment ($p = 0.02$) supporting our second hypothesis that plants inoculated with microbes perform better compared to no-microbe control plants (Fig. 2.3b). Inoculation explained little (2%) of the ANOVA model variation compared to ecotype (46%). Combining inocula across ecotypes, pairwise post hoc comparisons showed that total biomass responded to inoculation. Plants inoculated with the dry inoculum ($9.5 \text{ g} \pm 0.3$) were significantly larger than those inoculated with the wet inoculum ($8.6 \text{ g} \pm 0.3$, $p = 0.046$) or the no-microbe control ($8.3 \text{ g} \pm 0.3$, $p = 0.007$). Wet inoculum and no-microbe control did not differ from each other ($p = 0.47$). Examining further, we found that these differences in total biomass were especially evident in the dry ecotype when inoculated with the dry inoculum. Specifically, total biomass of the dry ecotype grown with its local dry inoculum ($7.4 \text{ g} \pm 0.4$) differed significantly from the no-microbe control ($5.6 \text{ g} \pm 0.4$, $p = 0.004$); a roughly 30% increase (Fig. 2.9c).

Interestingly, while there was an inoculum effect on total biomass, however, there was no effect of inoculum on belowground biomass (Fig. 2.9b, $p = 0.21$). In contrast, aboveground biomass responded to inoculum further supporting our second hypothesis (Fig. 2.3b) that plants would perform better with ecotype adapted microbes. In addition to an ecotype effect, aboveground biomass increased in response to the dry inoculum ($p = 0.011$, Fig. 2.9a). Inoculum explained 3% of the ANOVA model variation compared to ecotype (40%). Pairwise comparisons showed that aboveground biomass of plants that received the dry soil inoculum ($5.4 \text{ g} \pm 0.2$) were significantly greater than the no-microbe control ($4.6 \text{ g} \pm 0.2$, $p = 0.0029$, Fig. 2.9a).

Plants inoculated with the wet microbial inoculum did not differ from the dry inoculum ($p=0.09$) or no-microbe control in our post hoc comparisons ($p=0.19$).

Discussion

Here we provide insights into the relationship between *A. gerardii* ecotypes and their local soil microbes. Plant ecotype differences and their distinct soil microbiomes in native soils (Sarkar et al., 2022a; Kazarina et al., 2023) provide the foundation to investigate host local adaptation to biotic drivers. The two locally adapted ecotypes varied in their responses. This was especially evident in morphological differences in height, leaf area, and leaf width (Fig. 2.3a). However, some responses, e.g., biomass and chlorophyll absorbance, were modulated by microbial inoculation (Fig. 2.3b) or the interaction between inoculum and ecotype (Fig. 2.3c), respectively. The interaction between ecotype and inoculum where ecotypes showed an increase in performance when grown with their local soil microbes compared to non-local microbes and thereby shows evidence of physiological local adaptation to soil microbes in *A. gerardii*. Below we place our work into the context of plant-microbial interactions, consider the role of ecotype genetics in constraining response, provide evidence of microbial matching and local adaptation, and discuss the microbial impact on plant productivity.

4.1 Ecotype Genetic Background Drives Most Plant Responses

Andropogon gerardii ecotypes show strong morphological differences in response to selection driven by the Great Plains precipitation gradient. Previous studies have revealed the genetic underpinnings of these differences (Gray et al., 2014, Gallart et al., 2019). Here, all response variables differed between ecotypes: the wet ecotype produced greater biomass, had

greater leaf width and canopy area, and was taller than the dry ecotype across all treatments. However, the dry ecotype had higher chlorophyll absorbance than the wet ecotype across all treatments. These ecotypic differences corroborate previous studies on *A. gerardii* ecotypes (Olsen et al., 2013; Gibson et al., 2013; Johnson et al., 2015; Galliard et al., 2019; 2020). This also corroborates studies by Gao et al. (2020) and Wagner (2021) which describe the deterministic influence of host genetics on plant responses and that the microbiome is strongly governed by host genotype and phenotype.

Our results parallel those of other studies focused on the role of precipitation and aridity gradients in shaping genetic variation (Hall & Willis, 2006; Siepielski et al., 2017; Exposito-Alonso et al., 2018; Bowsher et al., 2020; Christie et al., 2022). In wheat, Dahan-Meir et al. (2022) found that genotypes associate with distinct microhabitats. Genotypes showed sharp transitions occurring along different slope positions, and local adaptation of these genotypes to these ecological microhabitats was strongly stable over time (Dahan-Meir et al., 2022). In these and our study, we detect a strong response to selection from environmental heterogeneity along large climate and small-scale topographic gradients.

4.2 Ecotype x Inoculum Interactions Influence Physiology, Suggesting Local Adaptation

Physiological measurements of chlorophyll absorbance showed an interactive effect of ecotype and inoculum in addition to the main ecotype effect, and thus present evidence of local adaptation of ecotypes to microbial inoculum. Both ecotypes showed an increase in chlorophyll absorbance when inoculated with microbes from their native locations and a relative decrease when inoculated with a non-local inoculum. Other studies similarly show evidence of local adaptation to local microbial communities. In *Pinus albicaulis*, pathogen resistance was

correlated with the combined effects of both host plant genetic background and endophyte community composition (Bullington et al., 2018). Further, in *Populus trichocarpa*, interactive effects of host genotype and microbial species inoculation affected disease susceptibility (Leopold & Busby, 2020). Thus, the combined effect of host genetics and microbial community composition act as a major driver of plant performance and success.

The onset of physiological local adaptation detected in chlorophyll absorbance was delayed in our study, and the interactive effect was not observable until three weeks after our first inoculation. These delays likely reflect the time necessary for these host-microbial interactions to develop and be detected. Short study durations were common in our literature survey, and if delayed interactions are common in other systems, many studies would have failed to observe these protracted effects. Our results warrant longer studies to better understand these interactions.

4.3 Microbial Inocula Modulate Plant Biomass Response

Soil microbial communities are subject to strong abiotic pressures and vary along ecological gradients (Zhao et al., 2016). This is illustrated in the relative frequency of the most prevalent members of each ecotype's soil microbial inocula shaped by different rainfall regimes. The soil microbial community composition can be largely explained by changes in soil water and nutrient availability across ecological gradients (Xiong et al., 2012; Zhang et al., 2018). Thus, rainfall clines exert selective pressure on and shape the resident soil microbial community in our system (Sarkar et al., 2022b).

The composition of the soil microbial inocula in our study likely reflects evolutionary selection imposed by the Great Plains' rainfall gradient. Stark differences in the relative

frequency of major microbial community members between inocula likely reflect differential selection and may explain some of our results. The genera *Paenibacillus*, *Brevibacillus*, and *Lysinibacillus* were abundant in the dry ecotype inoculum and are notable for their role as putative nitrogen fixers (Ma et al., 2007; Nehra et al., 2016; Jyolsna et al., 2021). This link to N-fixing bacteria may provide a nutrient-based explanation for the substantial increase in biomass production for both ecotypes and for higher chlorophyll absorbance in the dry ecotype when inoculated with the dry inoculum as mentioned above.

Recent field soil culture metagenome studies (Sarkar et al., 2022b) also support our results and further our interest in nutrient availability as a mechanism to explain the increased plant productivity for plants treated with the dry soil inoculum. In the arid western portion of the range of *A. gerardii*, Sarkar et al. (2022b) found in the soils bacterial metabolic pathways involved in nitrogen transformation and stress response in metagenome assembled genome (MAG)-*Pseudomonas*. Such findings highlight the potential of microbes to benefit plant growth during stressful conditions and to increase nitrogen availability. Additional work confirmed that these bacterial metabolic pathways can help fulfill a host plant's nitrogen requirements (Fox et al., 2016). Thus, the presence of nitrogen fixing bacteria in the dry ecotype inoculum here as well as in arid sites in the western range of *A. gerardii* (Sarkar et al., 2022b) further supports the hypothesis that the dry inoculum increases nitrogen availability in the dry ecotype. However, it is important to recognize with these results that 1) standard techniques cannot culture many microorganisms (Riesenfeld et al., 2004), 2) an endemic seed microbiome, not controlled for here, may impact our results (Hone et al., 2021), and 3) we did not investigate fungi in our study. Our results may also be an indirect consequence of complex microbe-microbe interactions (Denison et al., 2003; Schlaeppi et al., 2013; Bakker et al., 2014; Cardinale et al., 2015). Studies

have found *Pseudomonas* bacteria that colonize roots and produce antifungal compounds leading to disease-suppressive soils (Cook et al., 1995; Weller et al., 2002). It remains a possibility that the increased productivity of ecotypes lies in non-pathogenic symbionts excluding pathogens. Further work will be necessary to understand the nature of these interactions and the benefits of the dry inoculum to the dry ecotype more fully. However, the short time frame casts doubt on whether there was enough time to permit pathogen accumulation.

4.4 Implications for Grassland Productivity, Conservation, and Restoration

Most studies on intraspecific variation focus on abiotic factors in driving selective pressures and ecotype formation (Gregor, 1944; Turrill, 1946; Geburek et al., 2008). This study and others provide evidence that biotic factors also shape plant fitness and success (Ulrich et al., 2019; Bergelson et al., 2021; Brachi et al., 2022). Our study demonstrates microbial modulation of plant responses and local adaptation to soil microbes in *A. gerardii*. These results have major implications and potential for use in prairie restoration and management. The USDA Conservation Reserve Program widely uses *A. gerardii* for restoration efforts across 4.3 million acres in a five-state Midwest region (<http://www.fsa.usda.gov>), with the purpose to restore grasslands from marginal agricultural lands. By coordinating seeding efforts with endemic soil microbial communities, we can maximize the success and production of *A. gerardii* and prairie communities in general.

More broadly, we provide evidence of a strong positive response to soil microbes in a grassland species in terms of physiology and productivity with implications for grassland systems worldwide. The observed interactions imply large-scale impacts due to soil microbes, and interactions such as these will remain a major interest in grassland communities. As global

grasslands face climatic pressures and anthropogenic impacts (IPCC, 2021), harnessing the utility of beneficial microbial communities is urgent and will be essential to preserving these ecosystems into the future.

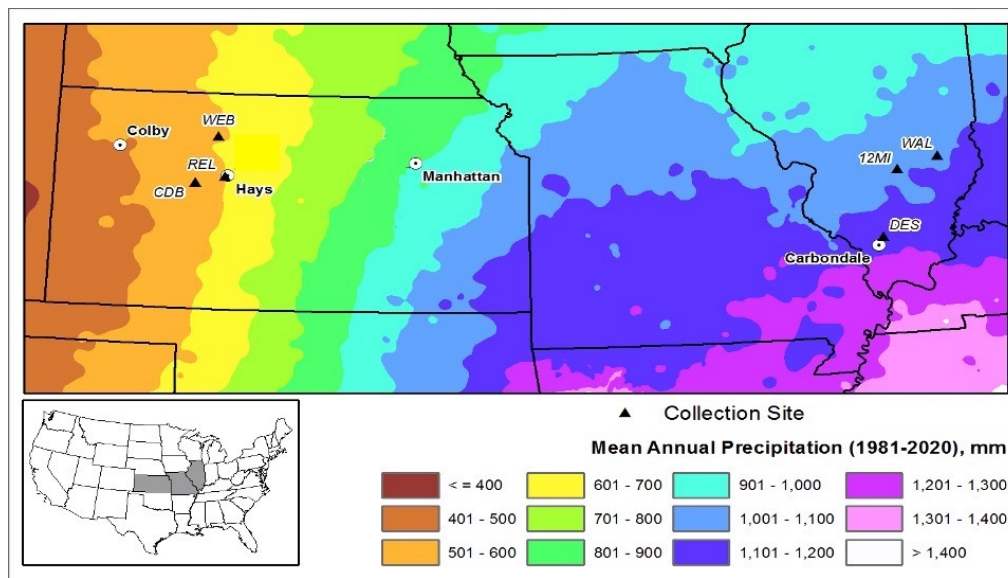


Figure 2.1. Mean annual precipitation map of the US Midwest with seed and soil source population sites (triangles).

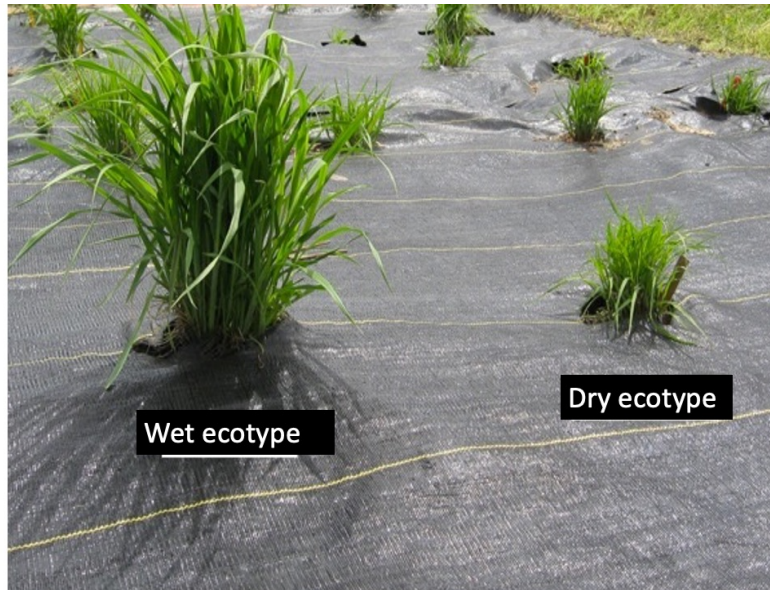


Figure 2.2. Strong morphological differences between the *Andropogon gerardii* ecotypes. Left: Wet ecotype sourced from southern Illinois, Right: Dry ecotype sourced from central Kansas.

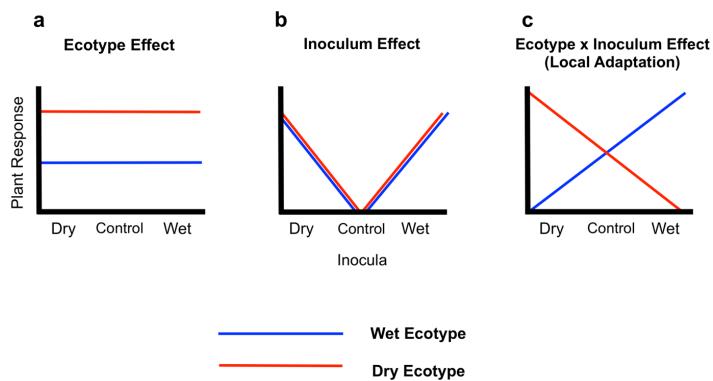


Figure 2.3. Predictions of the effect of ecotype and inoculum on *A. gerardii* plant performance.

(a) a main effect of ecotype with no inoculum effect, (b) a main effect of inoculum with no ecotype effect, (c) an interaction effect of ecotype and inoculum (local adaptation). (Not all possibilities shown)

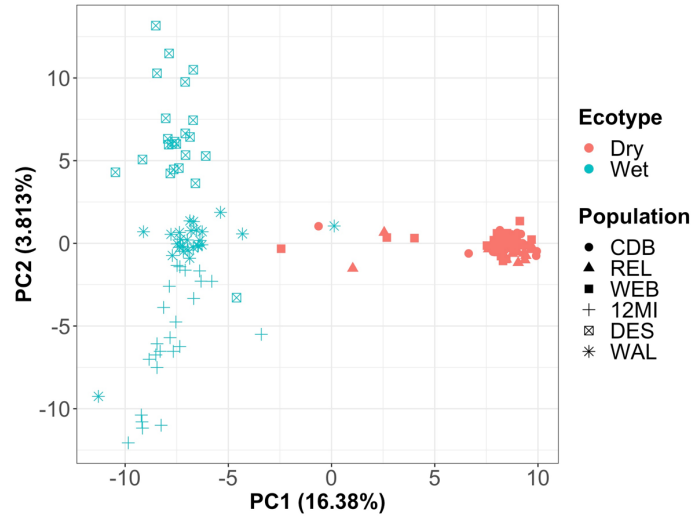


Figure 2.4. Scatter plot of the first two principal coordinates scores for allelic frequency of 8,011 *A. gerardii* single nucleotide polymorphism (SNP) marker loci. Colors correspond to regional ecotypes (Blue = wet ecotype; Red = dry ecotype), and symbols represent regional populations sampled. Principal Coordinate 1 explains 16.38% of variation and Principal Coordinate 2 explains 3.813% of variation.

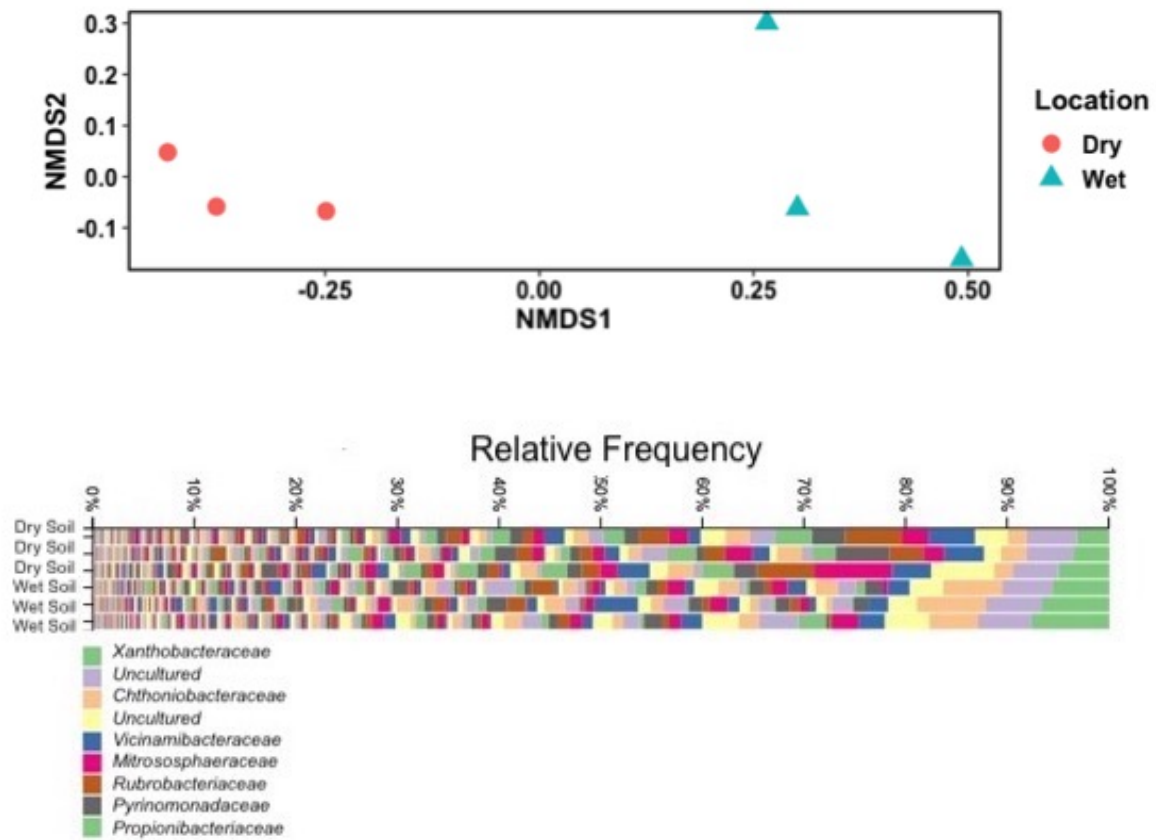


Figure 2.5. a) NMDS based on base soil bacterial communities isolated from dry and wet soils. Permanova: $F_{1,5} = 3.2$, $P = 0.1$. Circles represent dry ecotype population soils. Triangles represent wet ecotype population soils. b) Stacked bar plots of taxonomic profile to family level for base soil from dry (above) and wet (below) ecotype soils based on 16S v4 sequences.

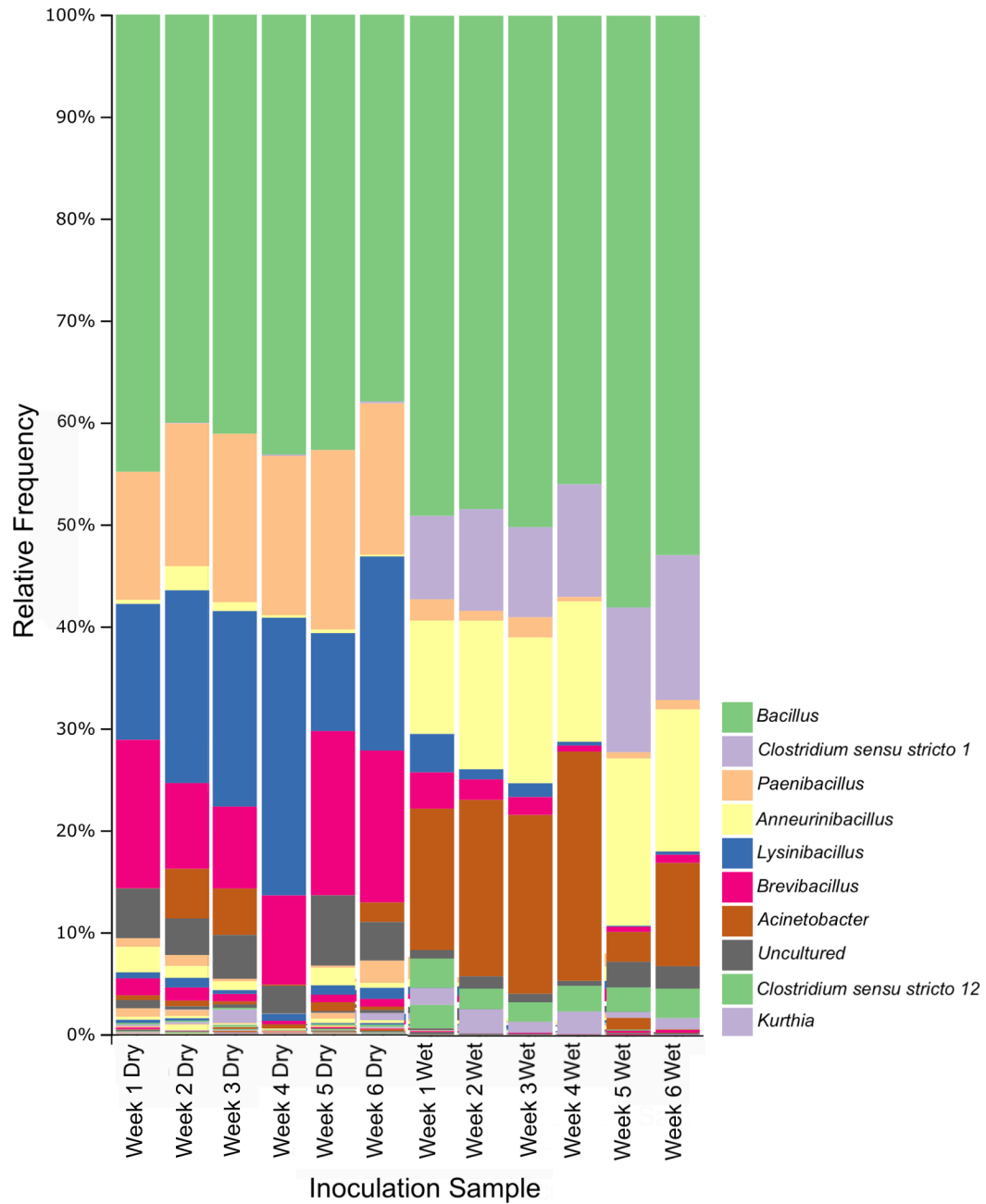


Figure 2.6. Stacked bar plots of taxonomic profile to genus level for dry (left) and wet (right) inocula based on 16S v4 sequences over time. Each bar represents the composition of one of the six weekly inoculation treatment ordered from left to right.

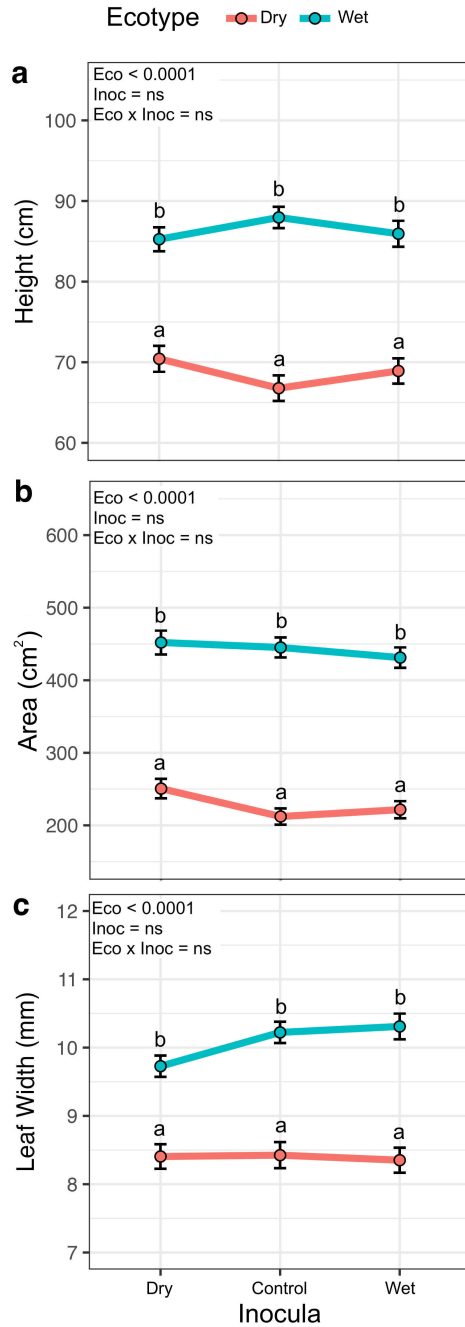


Figure 2.7. Effect of plant Ecotype (Eco), inoculum treatment (Inoc) and their interaction (Eco x Inoc) on *A. gerardii* plant morphological traits. Traits: (a) Height; (b) Leaf area (cm²); (c) leaf width (mm). Data are means $\pm$ one s.e. and p-values are presented from the ANOVA tests for each factor.

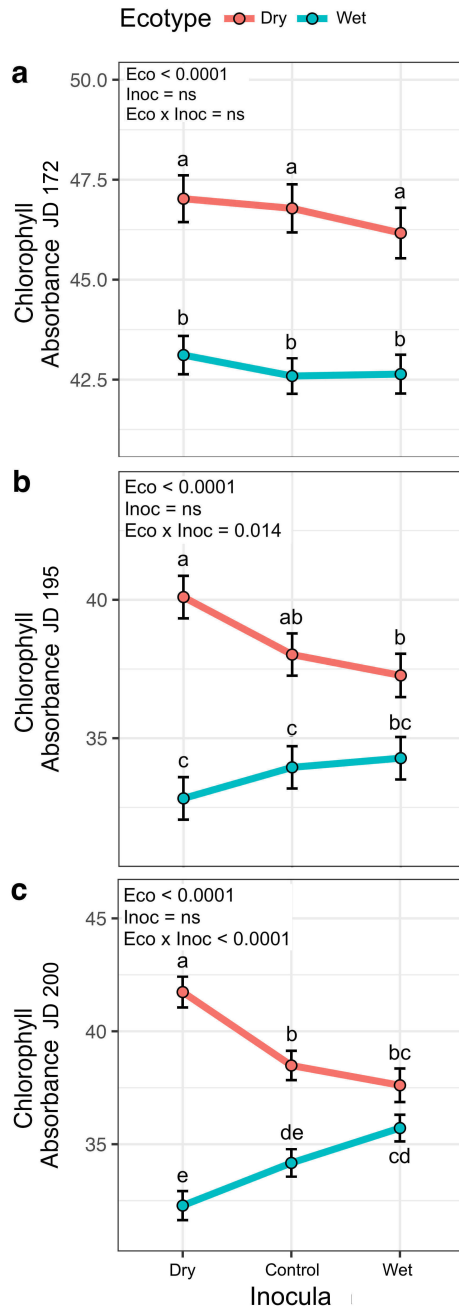


Figure 2.8. Effect of plant Ecotype (Eco), inoculum treatment (Inoc) and their interaction (Eco x Inoc) on *A. gerardii* chlorophyll absorbance rates from three sampling rounds: (a) Julian day (JD) 172; (b) Julian day 195; (c) Julian day 200. Data are means $\pm$ one s.e. and p-values are presented from the ANOVA tests for each factor.

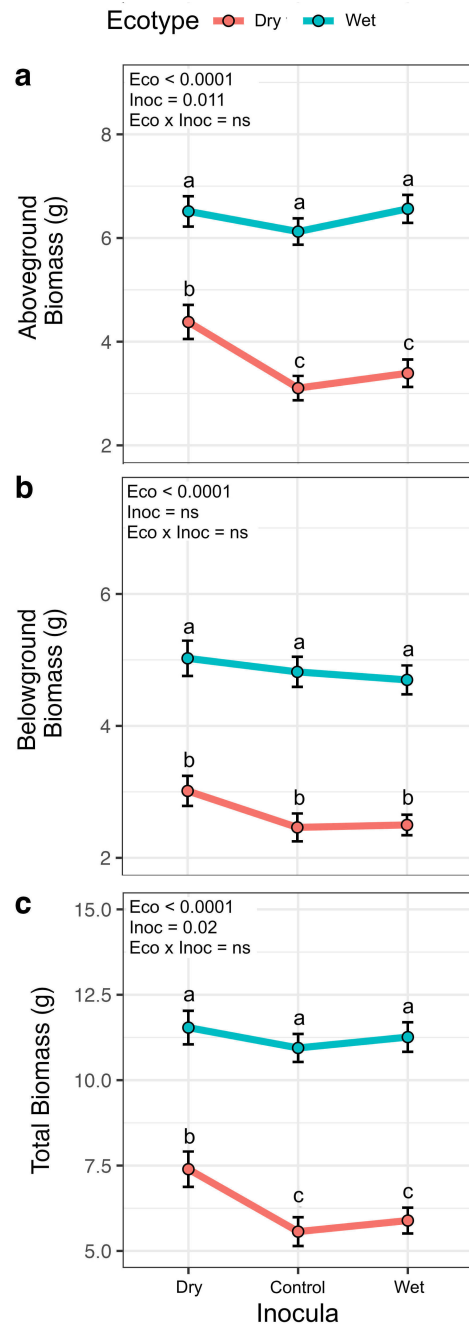


Figure 2.9. Effect of plant Ecotype (Eco), inoculum treatment (Inoc) and their interaction (Eco x Inoc) on *A. gerardii* plant biomasses (g): (a) Aboveground; (b) Belowground; (c) Total. Data are means $\pm$ one s.e. and p-values are presented from the ANOVA tests for each factor.

Ecotype	Collection Site	Latitude (N)	Longitude (W)	Elevation (m)	Size (ha)	Mean Annual Rainfall (mm)
Western Kansas	Relic Prairie	38°51'	99°22'	659	14	580
	Webster Res.	39°24'	99°32'	606	356	587
	Cedar Bluffs Res.	38°45'	99°46'	668	445	533
Southern Illinois	Twelve Mile Railroad Prairie	38°46'	88°50'	160	28	1076
	Desoto Railroad Prairie	37°51'	89°14'	119	0.4	1159
	Walters Prairie	38°59'	88°09'	150	5	1040

Table 2.1. Locality information for *A. gerardii* seed and soil collection sites.-

Chapter 3 - Host Gene Expression in Perennial Grass Ecotypes Grown with Local and Non-Local Soil Microbial Inoculum is Dependent on Ecotype

Abstract

Numerous studies investigate the diversity of soil microbial communities and the effect of host plant identity on rhizosphere composition. However, few studies investigate how microbe origin influences plant response, local adaptation, and gene expression in widespread species. Here, we investigate how rainfall-adapted ecotypes of the widespread North American prairie grass *Andropogon gerardii* respond at the transcriptional scale to local and non-local microbial inocula. We predicted that plants would show i) strong differences in gene expression due to ecotype, ii) differences in expression due to each inocula, iii) greater differences in gene expression within each ecotype, and iv), evidence of local adaptation at the transcriptional scale when inoculated with their local soil microbes. We collected seed and rhizosphere soil samples from six *A. gerardii* populations, from western Kansas (580 mm rainfall per year) representing the dry ecotype and southern Illinois (1167 mm rainfall per year) representing the wet ecotype. We isolated microbes from roots and native soil, cultured them in Reasoners 2A agar broth, and reciprocally inoculated wet and dry microbes (plus no-microbe control) weekly into common garden soil for 12 weeks under greenhouse conditions. After 12 weeks, leaf samples were collected for mRNA extraction to investigate differential gene expression in ecotypes in response to these local and non-local inocula. We found that gene expression strongly differed between the two ecotypes regardless of inoculum with 7097 genes differentially expressed between ecotypes. Overall, inoculum composition was a secondary driver of gene expression, however,

within each ecotype, several genes were differentially expressed due to inoculation treatment. Within the dry ecotype, genes relating to nitrate reduction (*NIA1*), chlorophyll light capture (chlorophyll a-b binding protein), and disease resistance (*RGA4*) were upregulated when grown with its local soil inoculum. Within the wet ecotype, genes related to lipid metabolism (*PAD4*), auxin transport (*WAT1*), and phosphorylation (serine/threonine-protein kinase B120) were upregulated when grown with its local inoculum. When inoculated with their local soil microbes, we found major and diverse effects of inoculum origin on many diverse aspects of plant growth and development, beyond the defense and immunity genes initially expected. For example, ecotype-specific inoculum resulted in striking differences in genes involved in growth (*PYL9*), differentiation (*ROTUNDIFOLIA*), metabolism (*gag-pol*, *PAD4*) and transcription factors (*BEL1*, *grassy tillers1*). Our results document strong controls on gene expression exerted by intrinsic host genetics. We also show the context dependency of plant microbial interactions at the transcript scale.

Introduction

Plants interact with a plethora of microorganisms that inhabit their entirety (Berg et al., 2016). Numerous studies document the role these symbionts play in host growth and development (Finkel et al., 2020), productivity (Poveda et al., 2019), and overall fitness (Weston et al., 2012a, b). Thus, examining these relationships has been and continues to be a major area of interest and application in plant science. In and surrounding their roots, plant cells are vastly outnumbered by rhizosphere microbial communities (Berg et al., 2015) that modulate major processes such as nutrient uptake (Freschet et al., 2021; Klein et al., 2021), disease resistance (Andrews, 1992; Weller et al., 2002), and drought tolerance (Ulrich et al., 2019). Studies on the

Arabidopsis thaliana rhizosphere found that these microbial communities are strongly influenced by soil type but also vary between plant development stage and genotype (Lundberg et al., 2012). Moreover, soil type defines root-bacterial community composition while host genotype determines these profiles to a limited extent (Bulgarelli et al., 2012). These and other model plant-microbe interaction studies provide the foundation for numerous further investigations (Pascale et al., 2020).

More recent studies of interactions between *Panicum hallii* genotypes and ‘home’ and ‘away’ microbial communities document their differential impacts on ecotype growth and development depending on soil microbiome origin (Khasanova et al., 2022). Additionally, the affinity between plant genotypes and local microbiota is reflected in genotype ability to preferentially recruit local microbes (Edwards et al., 2023). Perhaps the most well-studied model in our understanding of these host-microbial interactions is that of the legume-*rhizobia* mutualism (Young & Johnston, 1989; Oldroyd et al., 2011) where the legume plant provides carbon to bacteria located in root nodules in exchange for nitrogen (Prell & Poole, 2006). These studies have had major implications in scientific endeavors and applications for land management wherein matching plant hosts to beneficial microbes can benefit plant productivity. Manipulating the rhizobiome and harnessing these benefits for plant growth has major environmental (Dessaux et al., 2016; Bibi et al., 2018), agricultural (Johnson & Gibson, 2021), and biotechnological (Hakim et al., 2021) applications. Thus, the impact and value of understanding these relationships cannot be understated (Ramakrishna et al., 2019).

Plant local adaptation has been a major area of interest for centuries (Wangenheim, 1787; Langlet, 1971; Wadgyamar et al., 2022), and such studies often focused on population divergence along environmental gradients (Wadgyamar et al., 2022). Numerous researchers have

documented plant population responses along climatic gradients (Wilczek et al., 2008; Fournier-Level et al., 2011; Hancock et al., 2011, Ågren & Schemske, 2012; Laskey et al., 2012, DeMarche et al., 2018). However, far fewer studies investigate the role of biotic factors, namely plant-microbe interactions, in the process of local adaptation (Brachi et al., 2022). Thus, the lack of studies on biotic influences in local adaptation is a major gap in our understanding and a motivation to explore the intersection of local adaptation and microbial biotic interactions.

Today, modern genetic techniques provide new opportunities to investigate and understand the underlying mechanistic nature of plant-soil-microbe interactions (Shelake et al., 2019) and plant local adaptation (Gamalero et al., 2022). Utilizing genetics in ecology provides a finer scale understanding of natural processes (Feder & Mitchell-Olds, 2003; Johnson et al., 2007; Ungerer et al., 2008; Hoffman & Smith, 2017), and is advancing many areas within the field of ecology and plant-microbial symbioses (Des Marais et al., 2017; Groen and Purugganan, 2016; Hamann et al., 2021). With the advent of new genetic tools, we have been able to investigate these symbionts in manners not previously possible (Chaudhary et al., 2021; Gamalero et al., 2022). Thus, progress in these technologies and their utilization has played a crucial role in expanding our understanding of these complex relationships (Afridi et al., 2022).

Transcriptomic tools are powerful means to investigate plant responses (Baldoni, 2022), and additionally provide avenues into studying complex non-model organisms at the genetic level (Guo et al., 2021). By utilizing current techniques to investigate mRNA expression in plants in response to soil microbes, we can obtain insight into the nature and mechanism of these interactions (Agarwal et al., 2014). Many studies investigating plant-microbe interactions using transcriptomic profiling have characterized the role of microbial symbionts in altering plant gene expression (Labbe et al., 2014; Li et al., 2019; Daval et al., 2020; Wang et al., 2022). So,

exploring gene expression in *A. gerardii* ecotypes in response to local and non-local inocula poses a promising endeavor.

Here, we investigate differential gene expression in wet and dry ecotypes of *A. gerardii* Vitman (Poaceae), a warm season perennial prairie grass, in response to inoculation with local and non-local soil bacterial communities and how these interactions influence host local adaptation. We chose *A. gerardii* as our study species due to its distribution across much of North America (Weaver & Fitzpatrick, 1932) leading to interactions with diverse soil communities (Sarkar et al., 2022a), and the formation of locally adapted regional climate ecotypes. Ecological and genetic differences in ecotypes of *A. gerardii* have been shaped by the sharp rainfall gradient of the US Great Plains (Caudle et al., 2014; Johnson et al., 2015; Sytsma et al., in review; Gray et al., 2014; Galliard et al., 2019; 2022) that has been in place for roughly 10,000 years (Axelrod, 1985). Regions of low rainfall in the western portion of the range of *A. gerardii*, have selected for dwarfed drought-tolerant *A. gerardii* ecotypes. The eastern range, where precipitation is high and plant growth is light-limited, has favored large and robust wet *A. gerardii* ecotypes (Caudle et al., 2014; Johnson et al., 2015; Kramer et al., 2018; Galliard et al. 2020). By examining gene expression in *A. gerardii* wet and dry ecotypes in response to local or non-local microbial communities derived from wet and dry prairie sites, we can gain a better understanding of the nature of these interactions (Agarwal et al., 2014) and can draw stronger insights into the mechanistic role soil microbes have on *A. gerardii* phenotypes, productivity, and local adaptation.

Previous studies on *A. gerardii* and its corresponding soil microbial communities motivate our current research. Galliard et al., (in prep) found strong differences in gene expression between wet and dry *A. gerardii* ecotypes within sites in a large field study. Sharp

differences in gene expression between ecotypes, and transcriptomic signatures of each ecotype predicted ecological differences and local adaptation to rainfall (Galliart et al., in prep).

However, we do not know the extent to which microbes are affecting this response.

Other research documents the selective pressure that rainfall clines exert on the resident soil microbial across the Midwest precipitation gradient (Sarkar et al., 2022a). Further studies in these same field sites found that the composition of soil bacterial communities differed among *A. gerardii* ecotypes growing within a site (Sarkar et al., 2022b). Moreover, genetic variation in ecotypes led to differential recruitment of rhizosphere communities where ecotypes were more successful in recruiting unique microbial specialists at their homesites (Kazarina et al., 2023). Additionally, these studies on the *A. gerardii* rhizosphere in the western portion of its distribution have identified nitrogen-fixing stress tolerant *Pseudomonas* and its potential capacity to influence nutrient uptake and productivity in its *A. gerardii* hosts in dry locations (Sarkar et al., 2022b). In summary, these studies provide a foundation of fundamental differences in gene expression between ecotypes (Galliart et al., in prep), broad differences in soil communities across the range of *A. gerardii* (Sarkar et al., 2022a), differences in rhizosphere community recruitment (Sarkar et al., 2022b; Kazarina et al., 2023), and the capacity of rhizosphere communities to benefit plant productivity (Sarkar et al., 2022b). Major remaining questions regarding these symbioses between *A. gerardii* and its soil microbes are how do these ecotypes respond to these discrete rhizosphere communities, and how are existing differences in ecotype gene expression modulated by soil microbe origin? By investigating gene expression in wet and dry ecotypes reciprocally inoculated with these soil microbial communities, we aim to fill this knowledge gap.

This study stands as a complement of our previous work (Hartung et al., in review) where we used a reciprocal-inoculation experiment in a greenhouse setting to characterize plant response in ecotypes morphologically and physiologically due to origin of the soil microbial inoculum. We collected seed and rhizosphere soil samples from six *A. gerardii* populations from western Kansas (580 mm rainfall per year) and Illinois (1167 mm rainfall per year) (Table 3.1). We grew plants of the wet and dry *A. gerardii* ecotypes in greenhouse and reciprocally inoculated them weekly with either their local or non-local soil microbes (or no-microbe control) to examine the influence of soil microbe inocula on plant performance.

In this study, all our response variables in our greenhouse experiment (Chapter 2, Hartung et al., in prep) showed strong ecotypic differences and thus reflect the strong genetic control over plant ecotype performance and productivity. However, we detected additional effects due to microbial inocula origin and the interaction between ecotype and its local microbial inocula (Fig. 3.1). The inoculum, especially the dry inoculum, increased total biomass particularly for the dry ecotype by ~30% compared to the no-microbe control (Hartung et al., in review, Chapter 2). Chlorophyll absorbance showed evidence of physiological local adaptation to microbial inocula. In addition to the ecotypic drivers of chlorophyll absorbance, both ecotypes showed a relative increase in chlorophyll absorbance when matched with their respective local microbe inocula and a relative decrease in chlorophyll absorbance when mismatched to the non-local microbial inocula. Thus, we detected a positive response when matching ecotypes with local microbes and a negative response when ecotypes were mismatched to non-local microbes (Hartung et al., in prep) suggesting local adaptation of host ecotype and inoculum.

We predicted that ecotypes would show strong differences in gene expression when inoculated with the different inocula treatments with the overarching expectation that plants should perform best when matched with local microbes. Specifically, we hypothesized that plants would show (i) strong ecotypic differences in expression of genes involved in their stark difference in morphology (Galliat et al., 2019). (ii) differences in gene expression in response to each microbial inocula. (iii) greater differences in gene expression within a given ecotype due to the context specificity of these interactions. That is, the effect of inocula will be unique to each ecotype in terms of gene expression. (iv) Plants inoculated and matched with their local soil microbes will reflect local adaptation at the transcriptional scale. Major fitness related genes will be upregulated in ecotypes only when they are inoculated with their local soil microbes, thus reflecting the important role these communities play on plant host performance. Ecotypes will show the greatest upregulation in metabolism-related genes such as *gt1*, *tb1*, and *MAX3* (Kandel et al., 2018) and growth-related genes (*Aux/IAA*, *GH3*, and *SAUR* gene families) (Overvoorde et al., 2005) due to local adaptation with these communities. In contrast, we expect upregulation of defense and immunity genes (*VpRPW8s*, *HvSERK2*, and *EuCHIT2*, (Diaz 2018)) in plants inoculated with their non-local soil microbes.

This study will provide insight into how local and non-local microbial inocula influence the strength of local adaptation in gene expression in *A. gerardii* ecotypes. By examining gene expression in *A. gerardii* ecotypes in response to local or non-local microbial communities, we can gain a better understanding of the nature of these symbioses (Agarwal et al., 2014), and can gain stronger insights into the mechanistic role soil microbes may have on *A. gerardii* ecotype performance and local adaptation. Genetic approaches are instrumental to understand ecotypic differentiation and adaptation of symbiotic interactions and genes of interest that may be

potential targets of microbially-mediated plant success. Additionally, identifying upregulated genes in each ecotype x inoculum will provide context and a more complete understanding of these plant-microbial interactions between *A. gerardii* and its corresponding matched soil microbes at the genetic scale.

Materials and methods

Plant and Soil Sample Collection

Collection sites: A total of six populations of *Andropogon gerardii* Vitman were chosen for seed and soil collection: three in western Kansas (580 mm rainfall) representing the dry ecotype (Relict Prairie (REL), Webster Reservoir (WEB), and Cedar Bluffs Reservoir (CDB) (Fig. S3.1), and three in southern Illinois (1,167 mm rainfall) representing the wet ecotype (Desoto Railroad Prairie (DES), 12 Mile Railroad Prairie (12M), and Walters Prairie (WAL). Collecting from these never restored prairies provide a window into how *A. gerardii* has historically interacted with its soil microbes in native soil without disturbance from agriculture.

Seed and Soil Collection: Seed of *A. gerardii* was collected from multiple plants from the six populations during fall 2020. Seeds were stored at 4°C until the start of the experiment. Soil from these populations was collected in June 2021. Three soil cores 1.25 cm in diameter to a depth of 15 cm were taken from each of the six populations at the base of a mature *A. gerardii* tillers. Soil collections corresponding to each ecotype were pooled resulting in two composite samples for microbial isolation: Western Kansas for the dry ecotype inoculum and Southern Illinois for the wet ecotype inoculum.

Microbial Inoculation Preparation

Culturing of microbes, inoculation, and sequencing: We cultured bacterial communities from soil samples of dry and wet ecotypes on R2A broth (de Raad et al., 2021). The dry and wet ecotype soils (50 mg) were added to 5 ml of broth and incubated overnight at 30°C under 150 rpm shaking conditions. The cells were then diluted using phosphate-buffered saline (PBS) (pH 7) and ultrapure water (R2A broth culture: PBS: water 1:1:8). For the no-microbe control, we used uninoculated R2A broth, PBS, and water (R2A broth: PBS: water 1:1:8). Ten ml of the diluted dry and wet inoculum and mock control inoculum were then reciprocally injected into the soil using a 20 mL syringe weekly for six weeks.

We characterized the differences in the microbial communities associated with both six field soils and microbial inocula used in the inoculation experiment. The environmental DNA from the field soils and the microbial DNA from the inocula were extracted using the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Environmental/total DNA was extracted from aliquot of 0.25g of manually mixed soil. To prepare the inocula for the DNA extraction, 20-25mL of the inocula broth was aliquoted into 2mL centrifuge tubes and centrifuged for 10 min at 8000rpf at 4°C. The supernatant was carefully decanted from each tube. A total 1000µL of PBS buffer was added to one of the 2mL centrifuge tube to resuspend the pellet; the resuspension was transferred to the next tube to acquire one pooled 2ml centrifuge tube per inocula. This 2mL tube was centrifuged again for 10 min at 8000rpf at 4°C to pellet the cells and supernatant discarded prior to the DNA extraction as per manufacturer's protocol (Hartung et al., in prep).

Greenhouse experimental design

The *A. gerardii* seeds were scarified by gentle rubbing and planted in shallow germination trays with Berger BM6 all-purpose garden soil (Berger; Saint-Modeste, QC) in February 2021 under natural light conditions of the greenhouse. In May 2021, individual seedlings were transplanted to single plastic pots (12.5 cm x 11 cm x 8 cm Square Injection Molded Pots: Greenhouse Megastore, Danville, IL) with the same potting media. Each population was represented with 12 plants per block and 432 total plants across the six replicate blocks, overall (6 x 12 x 6). Within each block, plants from each population were randomly assigned to one of three inoculation treatments: dry soil inoculum, wet soil inoculum, or mock control inoculum. Individual plants were placed in a randomized block design with six replicate blocks. Plants were grown under natural light conditions in the greenhouse where temperature ranged from 20 to 30 °C for twelve weeks. Upon harvest, after twelve weeks of growth, leaf samples were collected from each individual for differential gene expression analyses. Leaves were frozen on dry ice and transferred to -80°C freezer at the laboratory until extraction.

RNA Extraction

Total RNA was extracted from four replicates of each population x inoculation treatment combination for a total of 72 leaf samples (4 x 6 x 3). For each sample, 100mg of frozen leaf tissue was ground into a fine powder using a mortar and pestle. Samples were submerged in liquid nitrogen throughout the grinding process to prevent thawing. RNA was extracted from samples using RNeasy plant mini kits (#74904, Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. Extracted samples were subjected to an additional in solution DNase treatment to eliminate genomic DNA contamination using RNase-Free DNase Sets (#79254, Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. The Nanodrop 2000

spectrophotometer (Thermo Scientific, Wilmington, DE) was used to determine RNA concentration as well as reagent and protein contamination (indicated by the A260/A280 nm and A260/A230 nm ratios) in the sample. The A260/A280 nm ratios for all samples ranged between 1.8 and 2.2 (majority of samples had ~2.0).

cDNA library construction and sequencing

RNA quality was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) for 28S/18S rRNA band intensity (2:1) and RNA integrity number (RIN). Construction of cDNA libraries from messenger RNA was performed at the Kansas State Integrated Genomics Facility. Messenger RNA was selected from 1 µg of high-quality total RNA and converted to indexed RNA-seq paired-end libraries using TruSeq Stranded mRNA Library prep kits (Illumina, San Diego, CA). RNA-seq libraries were barcoded with TruSeq RNA CD dual 10 bp indexes (Illumina Inc., USA).

Sequencing on the Illumina HiSeq2000 was performed at the University of Kansas Genome Sequencing Facility. The pooled libraries were loaded on six lanes of NovaSeq 6000 at a concentration of 8nM and sequenced for 300 cycles from each 150bp paired end fragments according to the manufacturer's instructions (Illumina Inc., San Diego, CA). In total, 137,208,750 paired-end reads were obtained. Raw files were converted to fastq files with Casava 1.8.2 (Illumina, San Diego, CA). Average quality scores were > 30 across sequencing cycles. Reads were 150bp in length with an average cDNA fragment length of 335bp.

Bioinformatic Assembly, Annotation and Analyses

Our pipeline for bioinformatic analyses is illustrated in the generalized workflow (Fig. S3.2). Initial read quality assessment was performed using Fastqc (Andrews, 2010). De novo transcriptome assembly were conducted using Trinity v2.13.03. Using Trimmomatic (Bolger et al., 2014), any remaining Illumina adapters and low phred quality score reads were removed. Contigs from the de novo assembly were clustered using default similarity thresholds CD HIT EST v4.8.1 (Li & Godzik, 2006). Following clustering, TransDecoder v5.5.0 (<https://github.com/TransDecoder>) was used to identify and predict coding regions from the clustered sequences. Finally, we used BUSCO v4.0.2 (Simão et al., 2015) to identify orthologs in the assembly and assess the quality of our assembled transcriptome. We used STAR v2.7.5 (Dobin et al., 2013) index our clustered transcriptome and to align and map transcripts from each of our 72 samples to the indexed reference transcriptome. Gene counts were obtained using the rsem-calculate-expression procedure in STAR v2.7.5. To identify differentially expressed genes in these gene counts, we used DESeq2 v3.112 (Love et al., 2014). Differentially expressed genes ($p=0.05$) were then identified using BlastX and BLAST2GO (Götz et al. 2008) was then used to determine gene annotations and GO terms and functional enrichment of genes based on KEGG pathways. GO terms were analyzed in REVIGO (Supek et al., 2011) for functional analysis. Finally, we used the “WGCNA” R package (Langefelder & Horvath, 2008) to identify significantly co-regulated gene cluster modules. These modules are clusters of genes with similar expression patterns. Following the module detection steps, we explored the correlation between module expression, ecotype identity, inocula origin, and plant phenotype data.

Results

Metrics on assembly:

The metrics and statistics for our assembly reflected that the transcriptome was successfully assembled (Table 3.2). Our transcriptome contained a total of 1,478,933 contigs after our initial Trinity (Grabherr et al., 2011) transcriptome assembly with an average contig length of 537.59 bp. Subsequent clustering with CDHIT (Fu et al. 2012) reduced our total contig number to 801,653. Contig number was further reduced to 672,856 using TransDecoder (Haas et al., 2013) to identify translatable transcripts within the transcriptome. These 672,856 contigs act as our final assembly which we compared to near-universal single-copy orthologs using BUSCO (Manni et al., 2021). Of the 425 total plant orthologs in our transcriptome, 91.1% were complete BUSCO matches while 7.2% and 1.3% were fragmented and missing matches, respectively. Thus, BUSCO results indicate the assembly of a relatively complete transcriptome.

Design of analyses:

In our experimental study design, we compared effects of ecotype and inocula origin on differential gene expression. To do this, we investigated gene expression in several different ways: 1) across ecotypes while combining inocula allowing us to identify sets of genes differentially expressed in ecotypes regardless of inocula, 2) across inocula while combining ecotypes allowing us to identify genes expressed regardless of ecotype, 3) across each inoculum within each ecotype to compare the unique effect of inoculum for each ecotype. Similarly, we compared the effect of ecotype (wet and dry) within each inoculum. However, these results are covered in supplements due to space limitations, and 4) across each ecotype grown with their “homesite” inoculum, i.e., dry ecotype matched with dry microbial inoculum and wet ecotype matched with wet inoculum. Thus, we examined how shared evolutionary histories of ecotype with microbial communities impact ecotype gene expression. A list of selected differentially

expressed genes that highlights candidate genes discussed throughout the results and discussion can be found in supplemental (Table S3.1).

Comparison 1-Ecotype effect produced the largest number of differentially expressed genes.

Between wet and dry *A. gerardii* ecotypes, 7097 genes were differentially expressed based on Deseq2 (Table S3.1), thus reflecting the importance of genetic background of ecotypes in gene expression. As visualized in the heatmap (Fig. 3.2a), most genes grouped into clusters that sharply differed in expression between the two ecotypes. The volcano plot (Fig. 3.2b) and table of differentially expressed genes (Table S3.2) indicate 3249 genes were upregulated in the dry ecotype. In the dry ecotype, functional gene enrichment plots (based on KEGG GO enrichment terms, Fig. 3.2c) showed that dry ecotype gene expression was significantly enriched in terms relating to protein kinase (*liguleless narrow1*, *RLK1*, *MAP3K*), chloroplast localization (*OEE2*, ascorbate peroxidase 2), and catalytic activity (*Aly3*, *UVH6*, *SFH13*) compared to the wet ecotype (Fig. 3.2c, Fig. S3.3). For the dry ecotype, selected upregulated genes beyond the GO enrichment terms were involved in photosynthesis (*HCF243*), disease resistance (*RPM1*), and zinc ion binding (*At3g08650*) (Table S3.2). The REVIGO functional gene enrichment analyses TreeMap showed enriched GO terms relating to chloroplast organization (*Tsip1*), nitrogen metabolism: (*MTA/SAH*), salt and water stress (*DEAD-box*, *WRKY*, *BOBBER 1*) and zinc ion binding (*COL13*, *NFXL1*) in the dry ecotype (Fig. S3.4a).

Based on Weighted Gene Co-expression Network Analysis (WGCNA) in our ecotype comparison, ten distinct gene module clusters emerged within the dry ecotype. Notably, five module clusters exhibited a high correlation with ecotype and phenotype data (Chapter 2, Hartung et al. in prep): Clusters 1, 5, 8, 9, and 10 (Fig. 3.3d). Notably, Cluster 5 showed a strong

positive correlation with the dry ecotype and its greater chlorophyll absorbance (Fig. 3.2d). In Cluster 5, co-expression of differentially expressed genes were related to disease resistance (*RF45*, *RPM10*), response to heat (*HSFP30*) and salt stress (*MSRA4*), and water transport (*TIP1-2*, *NIP1-2*).

In the wet ecotype, 3484 genes were upregulated in the wet ecotype as visualized in the volcano plot (Fig. 3.2b, Table S3.1). The wet ecotype showed significantly more KEGG enrichment of terms relating to carbohydrate binding (*GOS9*) and hydrolase activity (*DEAH13*) compared to the dry ecotype (Fig. S3.3). Notable selected genes beyond the KEGG GO enrichment terms upregulated in the wet ecotype were involved in response to auxin (auxin response factor 6,8), detoxification (cytochrome p450), and defense (L-type lectin receptor kinase) (Table S3.2). The REVIGO functional gene enrichment analyses TreeMap showed GO terms in the wet ecotype related to hormone response (auxin response factor 8), defense (*NDRI/HINI*-like protein 10), and transmembrane transport (*NRT1*) (Fig. S3.4b). Additionally, Cluster 8 in our ecotype WGCNA analysis showed a strong positive correlation with the wet ecotype and high biomass (Fig. 3.2d). Co-expression of genes was related to several transcription factors (*MYB*, *TUB*, *AP2*, *HBPI-α*), auxin transport (*AUX1*), and lignin biosynthesis (caffeoyl-CoA 3-O-methyltransferase 1).

Comparison 2- Inocula treatment led to few differentially expressed genes

Here we focus mainly on comparing differential expression in wet and dry inocula (see Supplemental Information for more details on results comparing inocula to the no-microbe control). Between wet and dry inocula, we detected only 38 differentially expressed genes as visualized in the heatmap (Fig. 3.3a, Table S3.1) and volcano plot (Fig. 3.3b). Thus, it was not

possible to detect a strong main effect of inocula on ecotype gene expression when ecotypes were combined. Therefore, KEGG, REVIGO and WGCNA were not informative and not included for this comparison. Of the few genes that were differentially expressed (Table S3.2), plants grown with dry inoculum showed upregulation of genes relating to nitrate reductase (*NIA1*) and lipid binding (lipid binding protein) in comparison to plants grown with wet inoculum (Table S3.2). Conversely, plants grown with the wet inoculum showed upregulation of selected genes involved with abscisic acid biosynthesis (9-cis-epoxycarotenoid dioxygenase 1) detoxification (cytochrome P450), and heat response (*OPI10*) (Table S3.2).

Comparison 3- Inoculum effect on host gene expression depends on ecotype

Within Dry Ecotype:

We next investigated the effect of inocula within each ecotype because we did not detect strong main effects of inocula combined across ecotypes due to the overwhelming effect of ecotype genetic background on expression. Within the dry ecotype, a total of 338 genes were differentially expressed when inoculated with wet and dry inocula (Table S3.1). Based on the heatmap, a strong diametric pattern in expression is detected between inocula (Fig. 3.4a). In the dry ecotype, 162 genes were upregulated by the dry inoculum (Fig. 3.4b). The KEGG enrichment plots showed upregulation in genes related to nitrogen metabolism (*NIA1*, *Atg8*), cell division (*KIN-12D*), and metal ion binding (BTB/POZ and TAZ domain-containing protein 1) compared to the wet inoculum (Fig. 3.4c, Fig. S3.7) (Table S3.2). Upregulation of selected genes were involved in nitrate reduction (*NIA1*), chlorophyll light capture (chlorophyll a-b binding protein), and disease resistance (*RGA4*). The REVIGO functional gene enrichment analyses TreeMap showed enriched GO terms related to nitrogen metabolism (*NIA1*), cell

division (*KIN-12D*), and metal ion binding (BTB/POZ and TAZ domain-containing protein) (Fig. S3.8a).

In the dry ecotype, the dry inoculum resulted in upregulation of 176 genes were upregulated as visualized in the volcano plot (Fig. 3.4b, Table S3.1). The KEGG analysis showed enrichment of genes involved broadly in biosynthesis (*IRX9-L*) and macromolecule metabolic pathways (*gag-pol*) (Fig. S3.7). Upregulation of selected genes were involved in ethylene responsive transcription factor production (*AP2-EREBP*) disease resistance (*RPP13*), and lignin biosynthesis (cinnamoyl-CoA reductase 2) (Table S3.2). The REVIGO functional gene enrichment analyses TreeMap showed enriched GO terms relating to metabolism (*gag-pol*) and catalytic activity (TUB) (Fig. S3.8b). Due to few genes within ecotype, WGCNA were not informative and not included.

Within Wet Ecotype:

Within the wet ecotype, 156 were differentially expressed genes between wet and dry inocula and they showed a strong diametric pattern in expression between inoculation as visualized in the heatmap (Fig. 3.5a, Table S3.1). Visualized in the volcano plot, 71 genes were upregulated with the dry inoculum (Fig. 5b). In the wet ecotype with dry inoculum, the KEGG enrichment plot (Fig. 3.5c) showed significant upregulation of genes related to nitrogen metabolism (*NIA1*), nucleic acid binding (*BG2*, *GSTU6*), and ion binding (*CML21*). The dry inoculum showed upregulation of selected genes involved in shoot development (*ROTUNDIFOLIA*), photosynthesis (Ferredoxin--NADP reductase), and fatty acid metabolism (4-coumarate—CoA) (Table S3.2). REVIGO functional gene enrichment analyses TreeMap

showed GO terms relating to catalytic activity (*TUB*), and metabolism (Protein kinase domain protein) (Fig. S3.12a).

In the wet ecotype with the wet inoculum, 85 genes were upregulated with the wet inoculum compared to the dry as shown in the volcano plot (Fig. 3.5b, Table S3.1). The KEGG plots showed significant enrichment of genes related to transmembrane activity (peptide transporter, *WAT1*) and transporter activity (potassium transporter 3, *CAX3*) as visualized in the functional gene enrichment plot (Fig. S3.9). Specific notable upregulated genes were involved in lipid metabolism (*PAD4*), auxin transport (*WAT1*), and phosphorylation (serine/threonine-protein kinase B120) (Table S3.2). The REVIGO TreeMap showed GO terms relating auxin transport (*WAT1*) and transmembrane transport (peptide transporter) (Fig. S3.12b). Due to few genes within ecotype, WGCNA were not informative and not included.

Comparison 4- “Homesite” comparison provides insight into natural interactions between ecotypes and inocula due to their shared evolutionary history

Finally, we performed a “homesite” comparison of gene expression in the dry ecotype matched with dry soil inoculum and the wet ecotype inoculated matched with wet soil inoculum, thus reflecting the shared evolutionary interactions that occur and have evolved in nature. Overall, 2639 genes were differentially expressed between the dry ecotype x dry inoculation homesite compared to the wet ecotype x wet inoculation homesite (Table S3.1). Here, a strong diametric pattern of gene expression is detected between the homesite groups shown in the heatmap (Fig. 3.6a). While the expression of many genes may be driven by a combination of ecotype and inoculum effects, this comparison provides insight how these interactions in the wild impact plant gene expression.

In dry ecotype x dry inoculum, 1307 genes were upregulated as shown in the volcano plot (Fig. 3.6b). The KEGG enrichment plot showed genes related to chloroplast localization (*OEE2*), nucleic acid binding (*Aly3*, *IRX9-L*), and zinc ion binding (*TSO2*) (Fig. 3.6c). Specifically, selected upregulated genes were involved in nitrate reduction (*NI1*), photosynthesis (Chlorophyll a-b binding protein), light capture (*FAR1*), growth and development (*PYL9*), and asymmetric cell division (*SCARECROW* protein 9, 34) (Table S3.2). REVIGO functional gene enrichment analyses TreeMap showed GO terms relating to nitrogen metabolic processes (nucleoredoxin 1), salt stress (*GsSRK*), and DNA binding (*VIPI*) (Fig. S3.20a). The analyses of WGCNA for our “dry homesite” showed eight module clusters exhibited a high correlation with ecotype identity and our phenotype data: Clusters 2, 3, 4, 5, 6, 8, 9, and 10 (Fig. 3.6d). Specifically, Cluster 9 showed a strong positive correlation with increased chlorophyll absorbance (Fig. 3.6d) and co-expression of differentially expressed genes related to electron capture (electron carrier protein), light response (*FAR1*), and plant growth (*GIDIL2*).

In the wet ecotype x wet inoculum, 1332 genes were upregulated as visualized in the volcano plot (Fig. 3.6b). In our KEGG enrichment analyses, genes relating to protein kinase activity (*LOC4325243*, *CRK-10*), response to stress (annexin D7-like), and response to external stimuli (*TNT*, *NBS-LRR*) were enriched as visualized in the functional gene enrichment plot (Fig. S3.19). Specifically, selected upregulated genes were involved in transcription factor production (*MYB*, *TUB*, *NF-E2*), growth and development (*GIDIL2*), pathogen resistance (*RPP13*), and sugar transport (sugar phosphate translocator) (Table S3.2). REVIGO functional gene enrichment analyses TreeMap showed GO terms relating to transcription regulation (*BEL1*, *grassy tillers1*), response to auxin (*ARF*), and primary metabolism (*EMS1*) (Fig. S3.20b).

In our “homesite” WGCNA analysis, Cluster 8 showed a strong positive correlation with the wet ecotype inoculated with the wet inoculum with increased leaf area, and biomass (Fig. 3.6d). In this cluster, there was significant co-expression of genes relating to growth (O-fucosyltransferase protein) and cellulose biogenesis (cellulose synthase protein).

Discussion

Here we provide insights into the relationship between *A. gerardii* ecotypes and their local soil microbial communities at the transcriptional scale. By examining differential gene expression in host ecotypes in response to local and non-local microbial inocula, we highlight potential mechanisms underpinning the shared evolutionary history of these interactions as well as insights into how these interactions influence local adaptation in *A. gerardii*. In our study, the two locally adapted ecotypes strongly differed in their gene expression as illustrated by the 7097 differentially expressed genes. We detected few differentially expressed genes when we compared inocula across the two ecotypes. However, within each ecotype we found a strong context-specific pattern. That is, gene expression strongly differed depending on which ecotype was being inoculated and with which inoculum. Surprisingly, we found diverse effects of inoculum origin on many unanticipated aspects of plant growth and development, far beyond defense and immunity. We further support this context specificity when we compare these effects in each ecotype treated with its “homesite” inoculum. Our “homesite” analysis reflects plant gene expression when plants were grown with microbial communities with whom they have a shared evolutionary history in an effort provide insights into these interactions in the wild. We consider the effect of host genetics on constraining gene expression, the specific and

contextual nature of plant-microbial symbioses, and implications of these interactions in natural settings.

4.1 Ecotype genetic background drives differential gene expression and constrains response to inoculum

The genetic background differences between *A. gerardii* ecotypes strongly determined differential gene expression which supported our first hypothesis that plants would strongly differ because of their ecotypic differences. The differentially expressed genes among the ecotypes represent distinct functions. For example, the dry ecotype showed upregulation of genes involved in photosynthesis (*HCF243*), nitrogen metabolism (*NIA1*) and leaf development (*At3g08640*) which correlate with the observed increased chlorophyll absorbance (Fig. 3.7a). Our results supported similar findings from others working with *A. gerardii*. Caudle et al. (2014) and Johnson et al. (2015) showed similar patterns in chlorophyll absorbance where the dry ecotype in their study also increased chlorophyll absorbance. Further, Maricle et al. (2017) found this same pattern where dry ecotypes higher leaf nitrogen contents supporting higher photosynthetic rates than wet ecotypes across reciprocal garden sites. Thus, we can link our gene expression of nitrogen related genes to field-based validation of these nitrogen dependent processes.

Conversely, the wet ecotype showed upregulation of genes related to lignin biosynthesis (caffeol-CoA 3-O-methyltransferase) and hormone transport (*AUX1*) which potentially drive the greater size, height, and robustness of the wet ecotype (Fig. 3.7a). Galliart et al. (2020) further provides mechanism for the greater size in the wet ecotype. Authors detailed that increased frequency of the “tall” allele for the *GAI* gene in the wet ecotype in their study supporting its increased height. From our WGCNA analyses, the co-expression of genes relating to

biosynthesis, transcriptional regulation, and auxin signaling in the wet ecotype strongly correlate with increases in height, leaf area, and biomass (Chapter 2, Hartung et al. in review). We find additional support from Gallart et al. (in prep) where authors found strong differences in ecotype gene expression in a field experiment and showed strong upregulation in the ethylene production pathway in the wet ecotype. Thus, greater expression of these genes relating to hormone processes in the wet ecotype likely plays a role in the greater height, leaf area, and biomass in this ecotype.

Our work corroborates previous studies on the genetic patterning of genotypes and ecotypes in other species. Sun et al. (2019) showed similar patterns of ecotypic differences in gene expression in *Cistanche deserticola* where ecotype expression is likely a product of the different environments these ecotypes grow. Further, MacQueen et al. (2021) were able to identify phenological trait related loci in distinct genotypes of *Panicum virgatum* across populations. Authors found environmental similarity across populations coincides with genetic convergence with implications for genomic x environmental interactions (MacQueen et al., 2021). In *Brassica rapa*, populations experiencing different precipitation regimes differed in genes relating to flowering with drier populations showing an upregulation of these genes and earlier flowering times (Hamann et al., 2020). Thus, intrinsic host genetics provide a fundamental baseline of expression that can be modulated the local microbiome.

4.2 Inocula, combined across ecotypes, leads to only slight changes in gene expression

Across the two ecotypes, we did not find major patterns in differential gene expression due to inocula. We did, however, find a few genes that were differentially expressed due to inoculum which supported our second hypothesis that inocula composition would modulate host

gene expression (Fig. 3.7b). These findings likely reflect differences in ecotypes and the specificity of these interactions in nature (Mendes et al., 2013). In these settings, host genes are differentially expressed depending on plant and bacterial genotype where authors control for both (Boivin & Lepetit, 2020). Whether or not a microbial community elicits a similar response across plant hosts is thus likely determined by the nature of these interactions (Li et al., 2019; Liao et al., 2019) as well as the evolutionary history of the plant host and its associated microbiomes (Spoerke & Parker, 1996). For example, Li et al. (2019) found that monocropping *Arachis hypogaea* leads to not only a reduction in soil microbial diversity, but also to a distinct host plant response to the less diverse microbiome.

Field studies of ecotypes of *A. gerardii* growing in common gardens support these results. In common gardens, where bulk soil microbial community is the same, ecotypes of *A. gerardii* selectively recruit different microbial communities from the common microbial pool (Kazarina et al., 2023). Similar results detail selective recruitment in local sites in *Panicum virgatum* (Edwards et al., 2023) where local genotypes show strong affinities to local soil community members. Similarly, Bowsher et al. (2020) found a similar pattern in *Mimulus guttatus* where each ecotype associated with a set of exclusive taxa even though ecotypes recruit similar microbial communities across environments. So, in our study, even if inoculation is the same across the two ecotypes, preferential recruitment likely acts as a filter on rhizosphere community composition. Thus, ecotype regulation over gene expression is strong and unique enough that detecting broad response patterns due to inocula alone is unlikely.

4.3 Inoculum responses depend on ecotype context

Inocula origin was not a strong determinant of differential gene expression across ecotypes as described above. However, we did detect greater effects on gene expression within each ecotype, thus reflecting the context specificity of ecotype-microbe interactions in our system. This supports the framework of shared evolutionary histories for matched ecotype x inoculum (Thrall et al., 2007). Specifically, repeated contact between hosts and symbionts over time leads to the coevolution of mechanisms that increase fitness for both partners (Younginger & Friesen, 2019). The specificity of these selective interactions and their genetic underpinning is further illustrated by Kawaharada et al. (2015). In that work authors document the role of exopolysaccharide production by compatible *Rhizobia* strains in eliciting Epr3 transcription factor expression in legume hosts ultimately leading to nodule formation (Kawaharada et al., 2015). In our study, the dry ecotype when inoculated with the dry inoculum, several upregulated genes relating to photosynthesis and nitrate reduction were detected supporting our third hypothesis that within each ecotype we would find an increased number of differentially expressed genes (Fig. 3.7c). The expression of these genes likely support the observed increased chlorophyll absorbance we found (Chapter 2, Hartung et al. in review) and corroborate increased photosynthetic rates in the dry ecotype found in other studies (Caudle et al., 2014; Johnson et al., 2015; Galliard et al., 2019, 2020).

We can additionally connect these findings to the 16S metabarcode inferred of our inocula. With a high relative abundance of putative nitrogen fixing genera *Paenibacillus*, *Brevibacillus*, and *Lysinibacillus* in the dry inocula (Fig. 2). Ours and other studies (Ma et al., 2007; Nehra et al., 2016; Masood et al., 2020; Jyolsna et al., 2021) confirm the potential of soil nitrogen fixers to upregulate host nitrogen metabolism genes. In contrast, we do not see this pattern in the wet inoculum growing with the dry ecotype. Instead, genes involved in disease

resistance and general metabolism were upregulated. Thus, only when the dry ecotype is inoculated with the dry inoculum are nutrient pathway genes upregulated resulting in major responses in plant growth and physiology (Chapter 2, Hartung et al., in review).

The wet ecotype, when inoculated with the wet soil inoculum, showed an upregulation of genes relating to transcription regulation (*RAP2-4*), hormone response (*AUX1*), and biosynthesis (galacturonosyltransferase) (Fig. 3.7c). These findings corroborate Galliart et al. (in prep) who mirrored this pattern of strong upregulation of genes relating to hormone processes in the wet ecotype in its homesite. Khasanova et al. (2022) found that ‘home’ and ‘away’ microbial communities differentially impact growth and development of *Panicum hallii* ecotypes. Similarly, in maize, Wang et al. (2022) showed that treatment of different strains of *Rhizophagus irregularis* led to differences in phosphorus uptake in plant hosts. More beneficial strains elicited greater expression of phosphorus related genes and increased phosphorus uptake in their plant hosts (Wang et al., 2022). So, beneficial microbes in the wet soil inocula putatively play a role in supporting the robust stature and high biomass of *A. gerardii*.

The within ecotype results corroborate other studies that document that transcriptome responses depend on host context (Berendsen et al., 2012). Elhady et al. (2021) showed this specificity in cultivated and wild genotypes of barley and beneficial soil microbiomes were better at facilitating cultivars to resist nematode infection through eliciting upregulation of salicylic acid related genes (Elhady et al., 2021). Additionally, our results are supported by morphological findings such as increased biomass in *Brassica rapa* due to selection for beneficial microbes in nutrient poor conditions (Garcia et al., 2022), and by physiological findings where specific beneficial microbes increase plant access to nutrients and suppress pathogens (Mendes et al., 2013). Daval et al. (2020) showed evidence of this context

dependency in *Brassica napus*. Gene expression in plant hosts was linked to soil microbial diversity and genes involved in molecular transport were upregulated when plants were grown with more diverse soil communities (Daval et al., 2020). Additionally, in cucumber, different commercial genotypes showed differential gene expression in response to *Fusarium* inoculation showing that plant response to fungal pathogens are also host background context specific (Gao et al., 2020). Furthermore, the more resistant genotype showed increased expression of genes relating to isoquinoline alkaloid biosynthesis (Gao et al., 2020). Thus, plant gene expression responds in a specific context to microbial community diversity (Daval et al., 2020).

4.4 “Homesite” comparison provides insight into gene expression in ecotypes grown with co-evolved microbial communities

Our “homesite” comparison provides insights into the nature of how ecotypes and soil microbial communities may interact in nature with implications for microbiomes influencing local adaptation in ecotypes. Plants are often locally adapted to regional climate (Wangenheim, 1787; Langlet, 1971; Wadgyman et al., 2022). However, in general the role of biotic interactions in affecting local adaptation is poorly known (Brachi et al., 2021; Johnson et al., 2022). In our study, we found major effects of inoculum origin on many diverse aspects of plant growth and development, beyond the defense and immunity genes initially expected. For example, ecotype-specific inoculum effect resulted in striking differences in genes involved in growth (*PYL9*), differentiation (*ROTUNDIFOLIA*), metabolism (*gag-pol*, *PAD4*) and transcription factors (*BEL1*, *grassy tillers1*) and may underlie local adaptation to microbes (O’Brien et al., 2021; Wagner 2021) (Fig. 3.7d). Thus, the differentially expressed genes we identified due to each inoculum likely play major roles in performance, fitness, and ultimately the local adaptation of

each ecotype. While our inocula reflect a subset of this diverse soil community, we expect that our observed effects of microbiome on hosts are either an underestimate (Bonk et al., 2018) or overestimate (Magnoli and Bever, 2023) of what may happen in nature.

When dry ecotypes are matched with their dry soil inocula, reflecting their shared environmental context, we found upregulation of genes involved in light capture (*FAR1*), photosynthesis (*HCF243*), nitrogen metabolism (*NIA1*) and overall plant fitness (*PLY4*). In contrast, when wet ecotypes are matched with their wet soil inocula, genes involved in auxin hormone response (*ARF*), growth (*GIDIL2*), and metabolism (*EMS1*) were upregulated (Fig. 3.7d). In both, these findings support our fourth hypothesis that plants inoculated with their local soil microbes would show upregulation of genes related to growth and development. We can also connect these expression patterns to our previous results supporting local adaptation to soil microbes in the two ecotypes (Chapter 2, Hartung et al., in prep). High expression of photosynthesis related genes (*HCF243*, *NIA1*, *FAR1*) in the dry ecotype corroborates increased chlorophyll absorbance as well as the increased contribution of nitrogen-fixers to the dry inocula. In contrast, increased expression of growth and development related genes (*GIDIL2*, *BEL1*, *grassy tillers1*) in the wet ecotype inoculated with the wet inoculum support our findings of greater biomass and increased height, leaf area, leaf width in this ecotype. Taken together, in both ecotypes we detect a suite of performance related genes that are expressed in response to local microbial community that reflect the shared evolutionary history between the symbionts and support our findings biotic local adaptation at the transcriptional scale.

Our results corroborate others that document host and site dependencies for the relationship between local plant hosts and resident microbes (Kazarina et al., 2023). In a study on tomato genotypes, authors showed that native soils activate a first level defense against *Fusarium*

infection and act as general suppressors of disease (Chialva et al., 2018). Edwards et al. (2023) found recruitment affinities between local genotypes of *P. virgatum* and local soil microbial community members. Shao et al. (2022) showed similar differences in gene expression in pear seedlings inoculated with local and commercial AMF (arbuscular mycorrhizal fungi) inocula. Plants inoculated with either AMF treatment showed increases in growth and nutrient acquisition, but plants showed upregulation in gene expression relating to fatty acid and flavonoid biosynthesis in response to local AMF inocula (Shao et al., 2022). Thus, our results support a growing number of studies documenting the positive and locally adaptive potential of local soil communities on plant host performance (Petipas et al., 2021).

Conclusions

Few studies investigate biotic interactions and particularly the interplay of plant-microbial interactions and plant genetics in plant local adaptation (Brachi et al., 2022). Here, we addressed *A. gerardii* ecotype-microbe interactions at the transcriptional scale and provide mechanisms for how these interactions may operate in natural environments. Further, we provide evidence of local adaptation to soil microbial communities at the transcriptional scale. Unique sets of genes were differentially expressed in each ecotype when matched to their local soil community supporting morphological and physiological findings of other studies in this field (Caudle et al., 2014; Johnson et al., 2015; Exposito-Alonso et al., 2018; Bowsher et al., 2020; Khasanova et al., 2022). Our study highlights pathways in *A. gerardii* ecotypes that are modulated by microbial communities thereby impacting host form, function, and process. Genetic approaches such as ours are instrumental to understanding ecotypic differentiation and local adaptation coinciding with symbiotic interactions. Additionally, we identified genes of

interest that may be potential targets of microbially mediated plant success with implications for land management. By matching plant hosts to specific beneficial microbes, we can potentially increase plant success and productivity. So, by identifying the genetic pathways that microbial communities modulate in their plant hosts, we can match these beneficial microbes with wild plants for more successful restoration and management efforts.

Our study also demonstrates strong context specific effects of inoculum communities with implications for the field as a whole. Ecotype response to inocula was unique for the two ecotypes, and broad benefits on plant performance were not detected for either inocula. However, the effects of inoculum was ecotype specific, with inocula modulating the expression of genes in major pathways relating to nutrient uptake, plant productivity, and transcription factor cascades. Thus, we found major and diverse effects of inoculum origin on many diverse aspects of plant growth and development. Our results highlight the need for continued investigation of plant-microbe interactions, especially in wild plants growing in the field. As we discuss the cost or benefit of microbial communities on plant host performance, the specificity of these interactions needs to be considered.

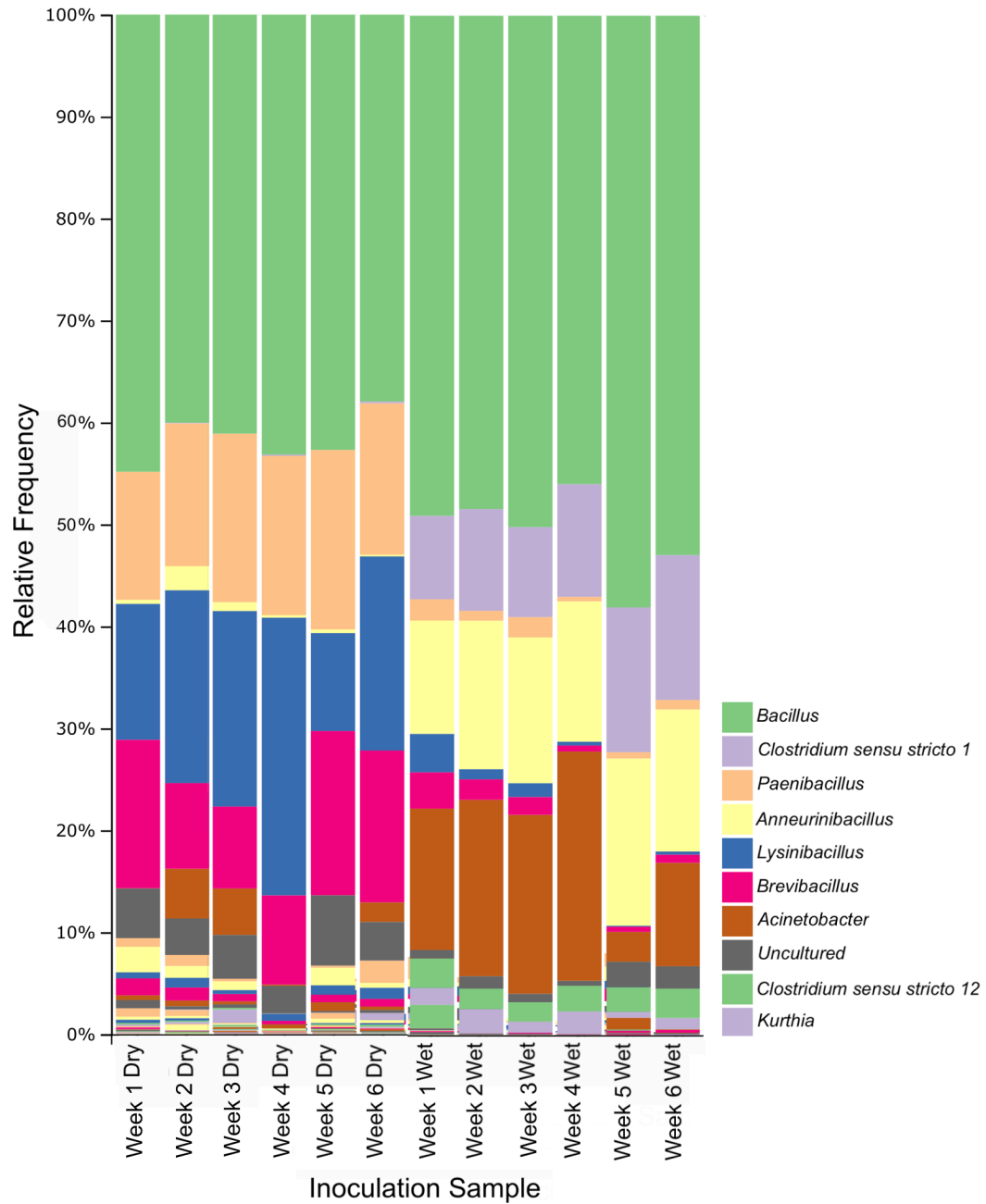


Figure 3.1. Stacked bar plots of taxonomic profile to genus level for dry (left) and wet (right) inocula based on 16S v4 sequences over time. Each bar represents the composition of one of the six weekly inoculation treatment ordered from left to right.

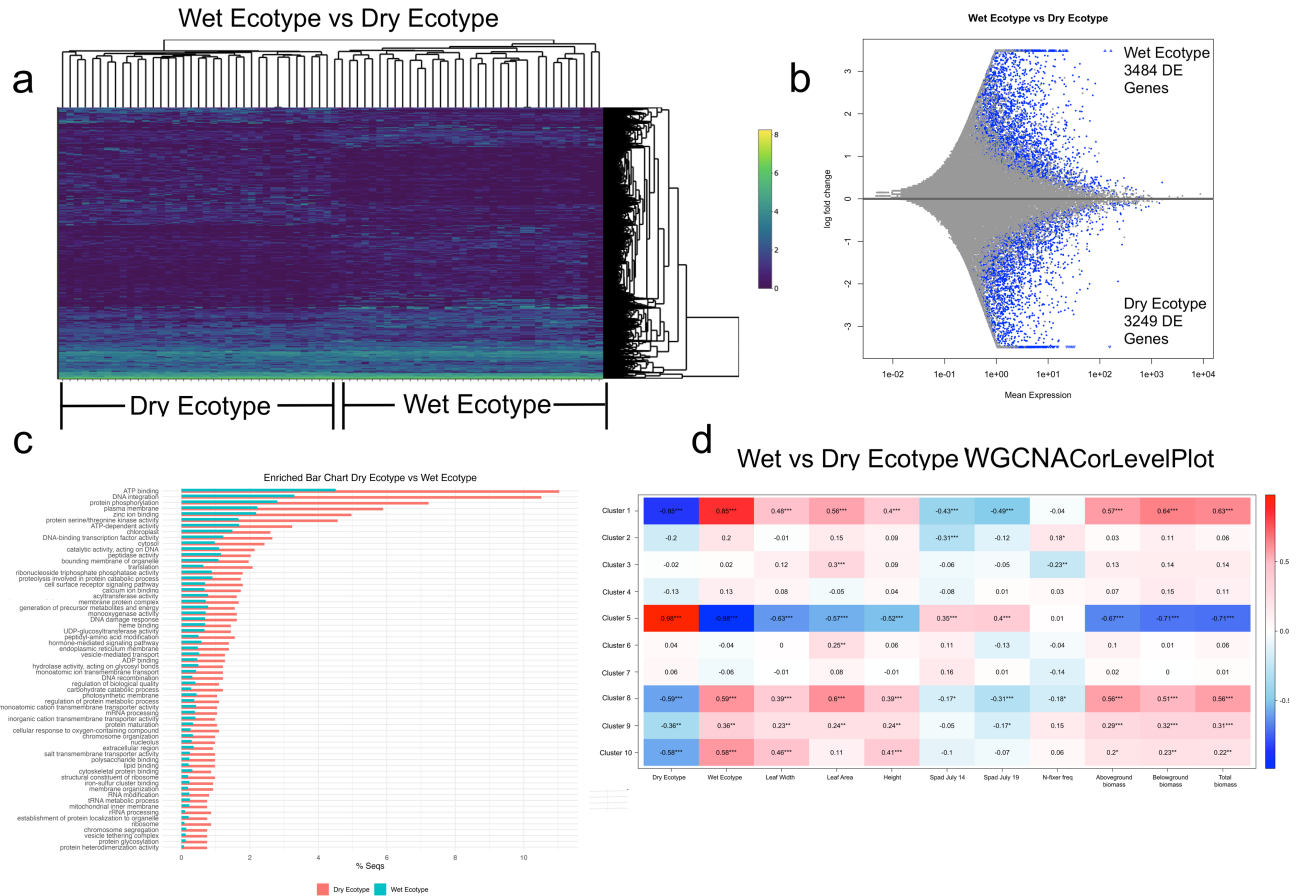


Figure 3.2. a) Heat map showing all differentially expressed genes between the two *A. gerardii* ecotypes. Yellow indicates higher expression and purple indicates lower expression. b) Volcano plots from DeSeq2 differential gene expression analysis (above 0 = upregulated in the wet ecotype, below 0 = upregulated in the dry ecotype). Blue dots indicate significant differentially expressed genes based on an Alpha of 0.05. c) Differential distribution of Gene Ontology (GO) terms. Bar charts show the GO terms that were significantly (alpha < 0.05) enriched in the dry ecotype in comparison to the wet ecotype reference using Fisher's exact test in BLAST2GO. d) WGCNA CorLevelPlot results of calculating the feature correlation coefficients between gene modules and plant response data. Red cells indicate positive correlation between the module gene expression and the response variable. Blue cells indicate a negative correlation and white cells indicate no correlation.

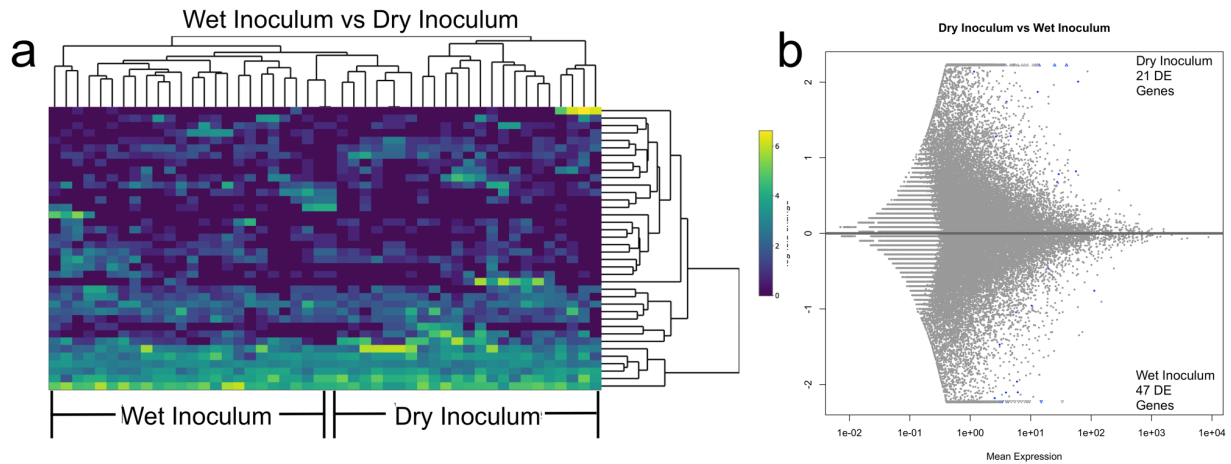


Figure 3.3. a) Heat map showing all differentially expressed genes between plants treated with the wet and dry inoculation treatments. Yellow indicates higher expression and purple indicates lower expression. b) Volcano plots from DeSeq2 differential gene expression analysis (above 0 = upregulated in plants inoculated with the dry inoculum, below 0 = upregulated in plants inoculated with the wet inoculum). Blue dots indicate significant differentially expressed genes based on an Alpha of 0.05.

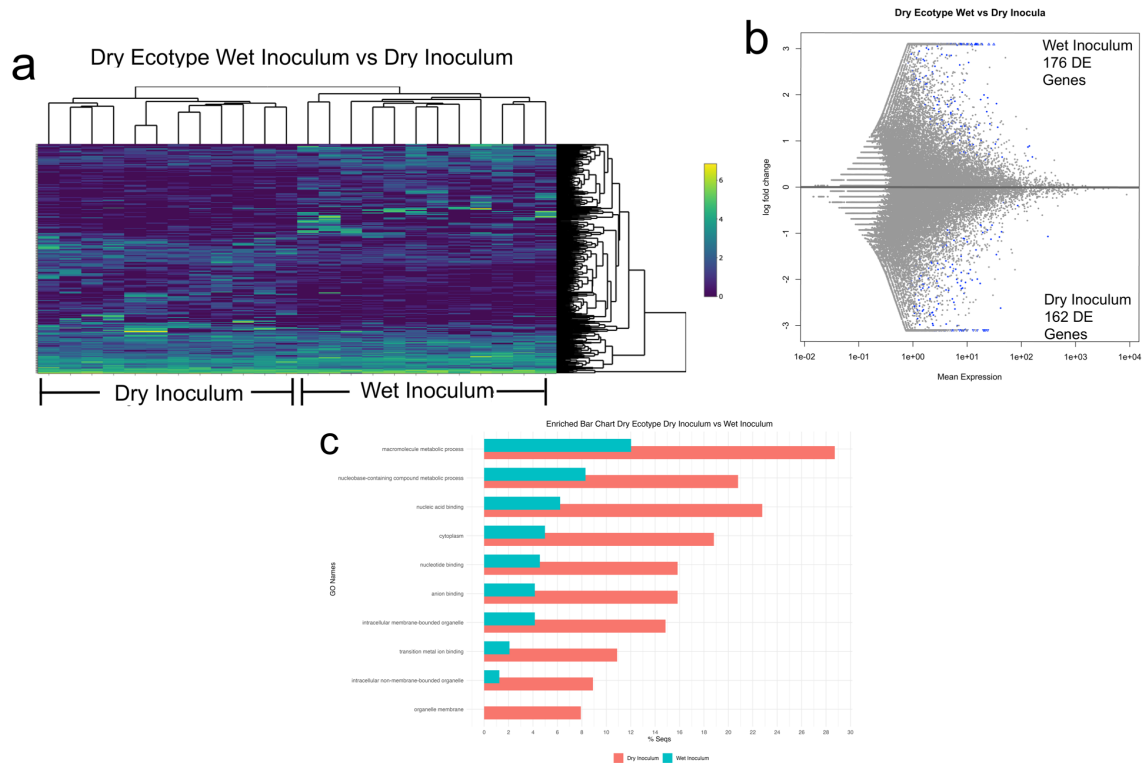


Figure 3.4. a) Heat map showing all differentially expressed genes within the dry *A. gerardii* ecotype between the wet and dry inoculation. Yellow indicates higher expression and purple indicates lower expression. b) Volcano plots from DeSeq2 differential gene expression analysis (above 0 = upregulated in plants inoculated with the wet inoculum, below 0 = upregulated in plants inoculated with the dry inoculum). Blue dots indicate significant differentially expressed genes based on an Alpha of 0.05. c) Differential distribution of Gene Ontology (GO) terms. Bar charts show the GO terms that were significantly (alpha < 0.05) enriched in the dry ecotype inoculated with the dry inoculum in comparison to the dry ecotype inoculated with the wet inoculum reference using Fisher's exact test in BLAST2GO.

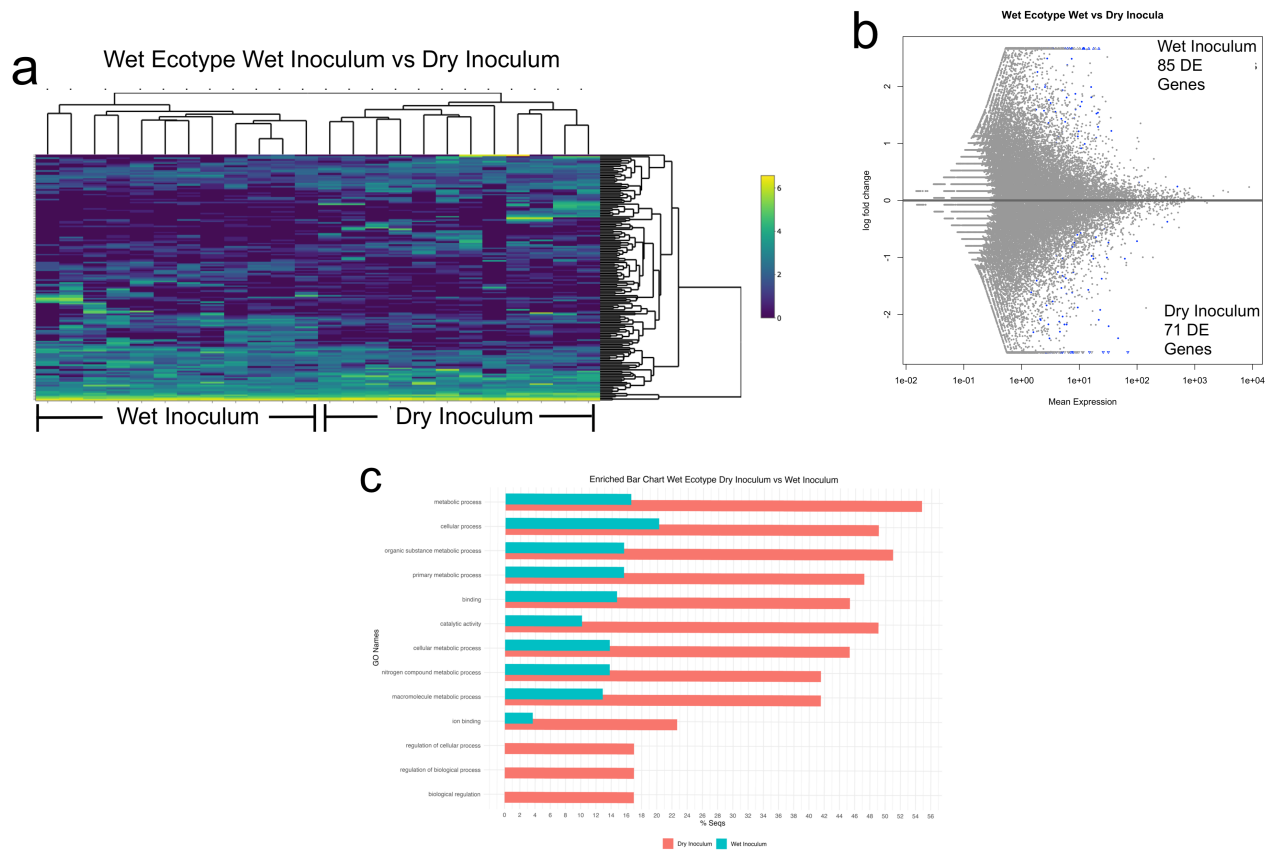


Figure 3.5. a) Heat map showing all differentially expressed genes within the wet *A. gerardii* ecotype between the wet and dry inoculation. Yellow indicates higher expression and purple indicates lower expression. b) Volcano plots from DeSeq2 differential gene expression analysis (above 0 = upregulated in plants inoculated with the wet inoculum, below 0 = upregulated in plants inoculated with the dry inoculum). Blue dots indicate significant differentially expressed genes based on an Alpha of 0.05. c) Differential distribution of Gene Ontology (GO) terms. Bar charts show the GO terms that were significantly (alpha < 0.05) enriched in the wet ecotype inoculated with the dry inoculum in comparison to the wet ecotype inoculated with the wet inoculum reference using Fisher's exact test in BLAST2GO.

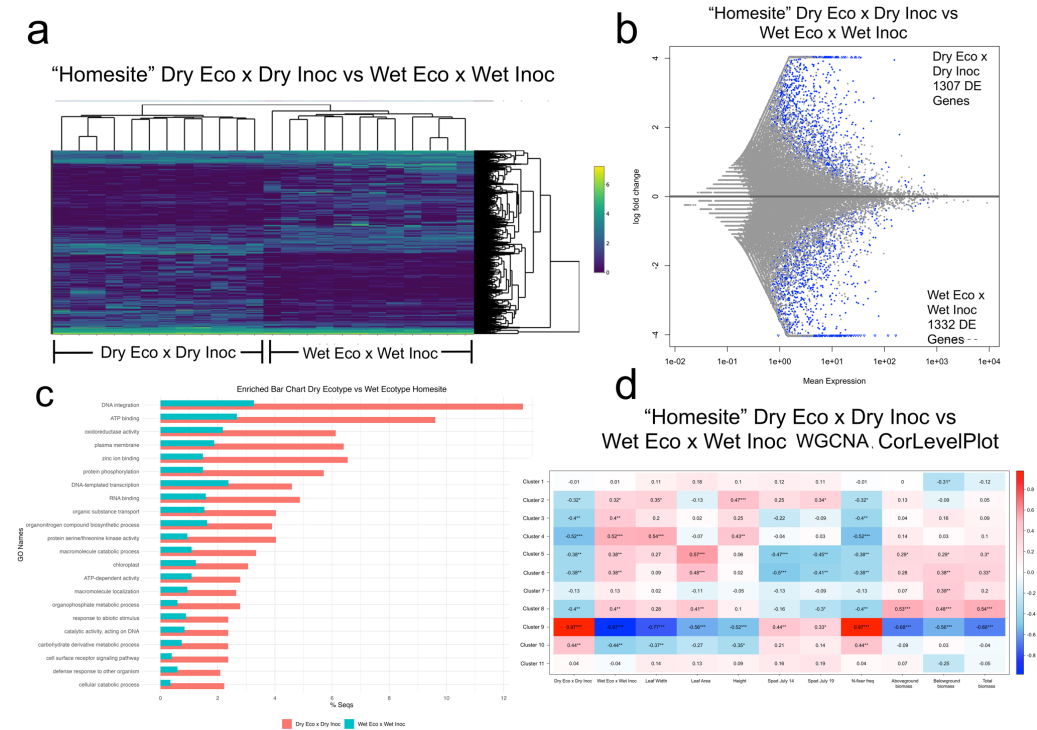


Figure 3.6 a) Heat map showing all differentially expressed genes between the two *A. gerardii* ecotypes inoculated with their “homesite” inocula. Yellow indicates higher expression and purple indicates lower expression. b) Volcano plots from DeSeq2 differential gene expression analysis (above 0 = upregulated in the dry ecotype inoculated with the dry inoculum, below 0 = upregulated in the wet ecotype inoculated with the wet inoculum). Blue dots indicate significant differentially expressed genes based on an Alpha of 0.05. c) Differential distribution of Gene Ontology (GO) terms. Bar charts show the GO terms that were significantly (alpha < 0.05) enriched in the wet ecotype inoculated with the wet inoculum in comparison to the dry ecotype inoculated with the dry inoculum reference using Fisher's exact test in BLAST2GO. d) WGCNA CorLevelPlot results of calculating the feature correlation coefficients between gene modules and plant response data. Red cells indicate positive correlation between the module gene expression and the response variable. Blue cells indicate a negative correlation and white cells indicate no correlation.

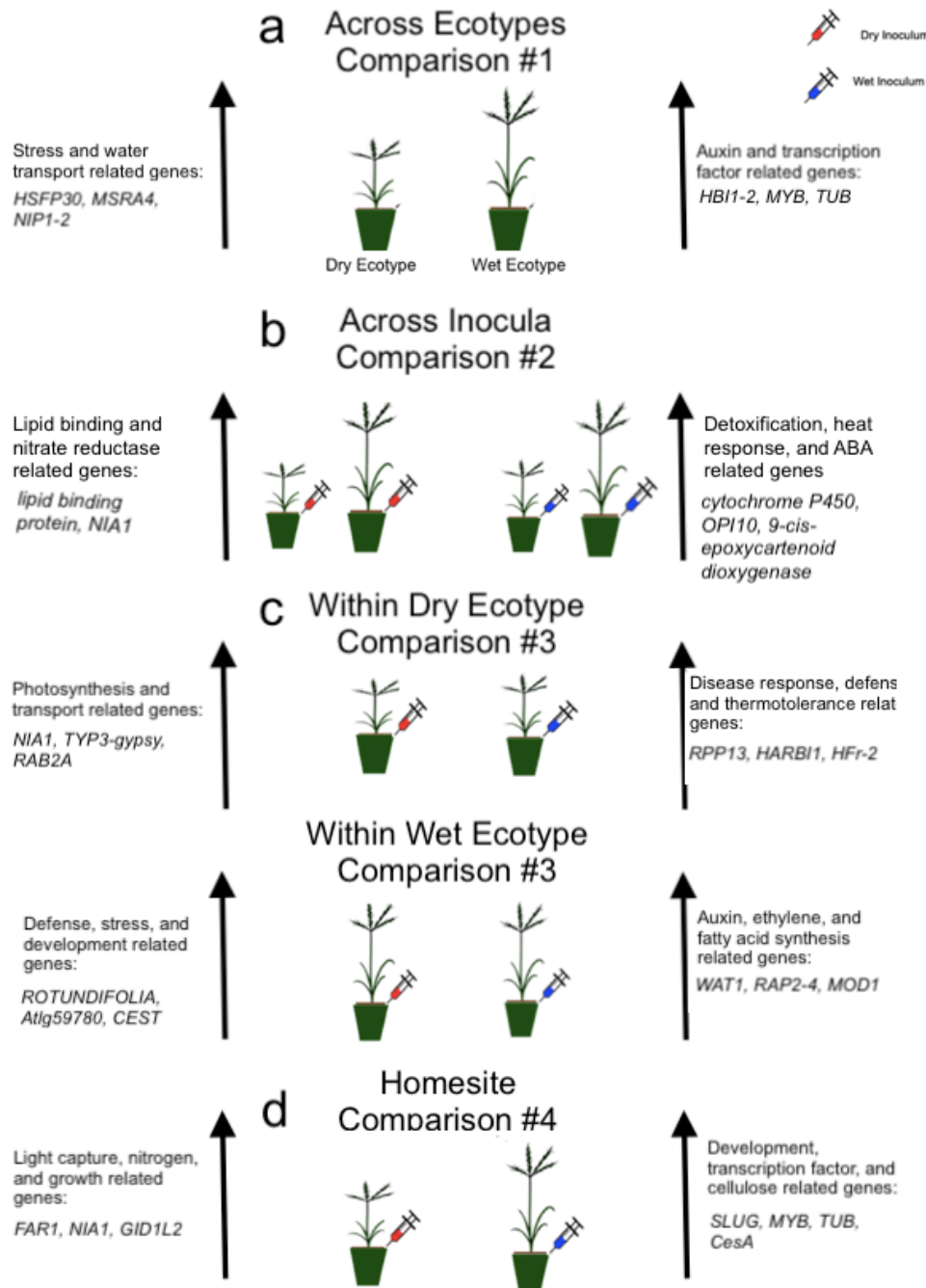


Figure 3.7. Conceptual diagram summarizing differential gene expression comparisons and selected upregulated genes and their function. a) comparing across ecotypes (comparison #1), b) comparing across inocula (comparison #2), c) comparing inocula within each ecotype (comparison #3), and d) “homesite” comparison (comparison #4).

Ecotype	Collection Site	Latitude (N)	Longitude (W)	Elevation (m)	Size (ha)	Mean Annual Rainfall (mm)
Western Kansas	Relic Prairie	38°51'	99°22'	659	14	580
	Webster Res.	39°24'	99°32'	606	356	587
	Cedar Bluffs Res.	38°45'	99°46'	668	445	533
Southern Illinois	Twelve Mile Railroad Prairie	38°46'	88°50'	160	28	1076
	Desoto Railroad Prairie	37°51'	89°14'	119	0.4	1159
	Walters Prairie	38°59'	88°09'	150	5	1040

Table 3.1. Locality information for *A. gerardii* seed and soil collection sites

Step in assembly.	# of Seqs	BUSCO	Complete BUSCOs (C)	Complete and single-copy BUSCOs (S)	Complete and duplicate BUSCOs (D)	Frag. BUSCOs (F)	Miss. BUSCOs (M)
Trinity CDHit Trans-Decoder	1,478,933 801,653 672,856	C:91.0% [S:26.8%, D:64.2%], F:7.3%, M1.7%, n:425	387	114	273	31	7

Table 3.2. Transcriptome assembly statistics after Trinity assembly, CDHit Clustering, and

TransDecoder". BUSCO scores are based on the assembly post TransDecoder. See Figure S1 for RNA bioinformatic workflow.

Chapter 4 - Conclusions and Future Directions

Abstract

By utilizing a reciprocal inoculation experiment in common garden soil in the greenhouse, we have been able to experimentally investigate the relationship between wet- and dry-adapted *A. gerardii* ecotypes and their microbial communities. *Andropogon gerardii* has been the focus of many studies aiming to expand our understanding of this ecologically dominant prairie grass (Knapp et al., 1998; Blair et al., 2014) and economically invaluable forage species (Gould and Shaw, 1983; Springer, 1989; Donatelli, 2013). Here, our studies build upon the existing knowledge surrounding local adaptation in this species (Olsen et al., 2013; Gibson et al., 2013; Johnson et al., 2015; Galliard et al., 2019; 2020) as well as the genetic underpinnings of local adaptation to regional rainfall regimes (Gray et al. 2014, Galliard et al., 2019). Much is known about local adaptation of species across environmental gradients, yet few studies address the role of biotic interactions, and specifically, the soil microbiome, as a driver of local adaptation. This thesis helps to fill this gap in our knowledge and has provided insights into the morphological, physiological, and genetic responses to soil microbes in the locally adapted ecotypes of *A. gerardii* and their interactions. This final chapter will conclude by summarizing key findings, connecting these to our overarching questions, and discussing the contributions of this work to a broader understanding of plant-microbe interactions. Lastly, we also review the limitations of our studies and present opportunities for future work.

Key findings

Chapter 2 of this thesis broadly addressed our first overarching question: Are *A. gerardii* ecotypes locally adapted to their soil microbes morphologically and physiologically under controlled conditions in greenhouse? Our findings from Chapter 2 document strong ecotypic constraints over plant performance regardless of inoculum. All response variables in our greenhouse experiment varied significantly due to ecotypic differences and reflect the strong genetic control over plant ecotype performance and productivity. Biomass production was strongly different between the two ecotypes with the wet ecotype weighing almost double the total biomass of the dry ecotype. Chlorophyll absorbance, a proxy indicator for photosynthetic rate, of the dry ecotype was consistently significantly greater than that of the wet ecotype,

In addition to these strong ecotypic drivers of plant performance, we detected additional effects due to microbial inoculum and the interaction between ecotype and microbial inoculum origin. Inoculum, especially the dry inoculum, increased total biomass for both ecotypes and particularly increased total biomass of the dry ecotype by ~30% compared to the no-microbe control.

Chlorophyll absorbance showed evidence of physiological local adaptation to microbial inoculum confirming our overarching question for this chapter: Are *A. gerardii* ecotypes locally adapted to their soil microbes morphologically and physiologically under control conditions in greenhouse? In addition to the ecotypic drivers of chlorophyll absorbance, both ecotypes showed a relative increase in chlorophyll absorbance when matched with their respective local inoculum and a relative decrease in chlorophyll absorbance when mismatched to the non-local microbial inoculum compared to no-microbe control. Thus, we detected a positive response when matching ecotypes with local microbes and a negative response when ecotypes were mismatched with non-local microbes. So, our chlorophyll absorbance results show evidence that

ecotypes are locally adapted to their soil microbes under control conditions in greenhouse in addition to the roles of the genetic background of each ecotype and microbial inoculum on plant performance.

Chapter 3 investigated differential gene expression in *A. gerardii* ecotypes in response to local and non-local microbial inoculum. This chapter addressed the question: are genes differentially expressed in ecotypes exposed to local and non-local soil microbes? As expected, gene expression was strongly different between ecotypes regardless of inoculum with over seven thousand differentially expressed genes between the two ecotypes. Because phenotype and gene expression were taken on the same plants, we can relate these gene expression patterns to phenotype results in Chapter 2. For the dry ecotype, selected upregulated genes were related to photosynthesis (*HCF243*), disease resistance (*RPM1*), and zinc ion binding (*At3g08650*). Dry ecotypes had consistently higher chlorophyll absorbances than the wet ecotype and these photosynthesis and fitness-related genes support those results. Conversely, for the wet ecotype, notable selected genes were involved in response to auxin (auxin response factor 6,8), detoxification (cytochrome p450), and defense (L-type lectin receptor kinase). These growth-related genes likely support the greater stature of wet ecotypes in the wild (Johnson et al., 2015; Galliard et al., 2020), and support our findings of greater height, leaf area, leaf width, and biomass in the wet ecotype as described in Chapter 2.

Inoculum was not a major driver of gene expression. However, of the few differentially expressed genes, plants grown with dry inoculum showed upregulation of genes relating to nitrate reductase (*NIA1*) and lipid binding (lipid-binding protein) while plants grown with the wet inoculum showed upregulation of selected genes involved with abscisic acid biosynthesis (9-cis-epoxycarotenoid dioxygenase 1) detoxification (cytochrome P450), and heat response

(*OPI10*). Thus, though few, inoculum composition still had a significant effect on fitness-related genes in both ecotypes.

The effects of inoculum on gene expression were much greater within the context of each ecotype and differences were even greater in the “homesite” comparison. When inoculated with their local soil microbes in the “homesite” comparison, major effects of inoculum were detected on genes relating to plant growth, development, and fitness. In the dry ecotype matched with the dry inoculum, there was a strong positive correlation with increased chlorophyll absorbance and the co-expression of differentially expressed genes related to photosynthesis (Chlorophyll a-b binding protein), light capture (*FAR1*), and growth and development (*PYL9*). So, by applying our gene expression results in Chapter 3 to our physiological results in Chapter 2, we provide a potential mechanism for the increased chlorophyll absorbance in dry ecotypes inoculated with the dry inoculum. Further, these genes are likely involved in this interactive ecotype x inoculum effect we detected and play a role in the physiological local adaptation of *A. gerardii* to local soil microbes. For the wet ecotype inoculated with the wet inoculum, upregulated genes were related to transcription factor production (*MYB*, *TUB*, *NF-E2*), growth and development (*GID1L2*), and pathogen resistance (*RPP13*) that we can relate to increased leaf area and biomass in the wet ecotype. These genes also support the consistent findings of larger wet ecotypes in terms of plant cover and biomass in the field (Johnson et al., 2015; Galliart et al., 2019; 2020; Sytsma et al., in prep). Thus, in both ecotypes we found diverse effects of inoculum origin on the genetic underpinnings of many unanticipated aspects of plant growth and development. Further, we highlight genetic mechanisms of local adaptation to soil microbes in wet and dry ecotypes and expand upon previous findings of local adaptation in this species.

Limitations and challenges

Considering results from Chapters 2 and 3, several limitations impair the extent to which we can interpret and generalize our results. Firstly, standard laboratory techniques fail to culture many microorganisms (Riesenfeld et al., 2004). This issue raises questions regarding if the inocula treatments reflect a true soil microbial community that *A. gerardii* ecotypes interact with in natural settings (Daniel, 2004; Aslam et al., 2010). So, whether our culturing methodologies artificially selected for novel microbial communities remains an open question and may affect the interpretation of our results. Additionally, our culturing techniques and inoculation preparation protocols selected for and cultured only bacterial communities, leaving the fungal component of these rhizosphere communities uninvestigated in our work. Other studies have documented the important role fungal symbionts play in the success of *A. gerardii* (Schultz et al., 2001) and grasses in general (Garcia-Parisi & Omacini, 2017; Hume et al., 2020; Fernando et al., 2021). The impact of these fungal symbionts on the strength and nature of these results remains unknown and requires investigation under more realistic conditions with complex interaction networks including fungi.

An endemic seed microbiome, not controlled for in our studies, may also have impacted our results (Hone et al., 2021). The strength and influence of these vertically inherited microbial communities have been well documented in other species (Johnston-Monje et al., 2016; Rybakova et al., 2017; Shade et al., 2017), but remain unaccounted for here. Whether or not the intrinsic seed microbiome plays a major role in *A. gerardii* ecotype performance also remains unanswered.

Lastly, and likely the most problematic, findings from greenhouse studies may not be generalizable into realistic field conditions (Gibson et al., 1999; Lekberg et al., 2018).

Greenhouses are an invaluable tool experimentally as they allow for strong control and manipulation over environmental factors, however, excluding these confounding variables often over-simplifies the processes observed (Cortois & De Deyn, 2012), and patterns detected in greenhouse may result from these unique controlled conditions (Van der Putten et al., 2016). While invaluable for experimentally manipulating and investigating plant-microbial symbioses, future studies must be conducted under a true, field setting (Lekberg et al., 2018; Van der Putten et al., 2016).

Significance

This study and others provide evidence that biotic factors also shape plant fitness and success (Ulrich et al., 2019; Bergelson et al., 2021; Brachi et al., 2022). Our study in Chapter 2 demonstrates microbial modulation of plant morphological and physiological responses and local adaptation to soil microbes in *A. gerardii*. These results have major implications and potential for use in prairie restoration and management where utilizing beneficial microbial communities can lead to increases in plant performance and productivity. The USDA Conservation Reserve Program widely uses *A. gerardii* for restoration efforts across 4.3 million acres in a five-state Midwest region (<http://www.fsa.usda.gov>), with the purpose of restoring grasslands from marginal agricultural lands. By coordinating seeding efforts with endemic soil microbial communities, we can maximize the success and production of *A. gerardii* and prairie communities in general across these widespread restoration efforts.

More broadly in Chapter 2, we provide evidence of how plants interact with soil microbes in a grassland species in terms of physiology and productivity with implications for grassland systems worldwide by utilizing soil microbes to benefit plant growth. The observed

interactions imply large-scale impacts due to soil microbes, and interactions such as these will remain a major interest in grassland communities. As global grasslands face climatic pressures and anthropogenic impacts (IPCC, 2021), harnessing the utility of beneficial microbial communities is urgent and will be essential to preserving these ecosystems now and into the future.

In Chapter 3, we show that local and non-local soil microbial communities have strong effects on *A. gerardii* ecotype gene expression. Thus, broadly, we provide evidence of major modulations of genetic activity in plant hosts in response to microbial community composition. Beyond this, our study highlights genetic pathways in *A. gerardii* ecotypes that are influenced by microbial communities and thus impact host form, function, and local adaptation. Additionally, we identified genes of interest that may be potential targets of microbe-mediated plant success with implications for land management. By matching plant hosts to specific beneficial microbes, we can potentially increase plant success and productivity through microbe-mediated upregulation of fitness-related genes.

Our Chapter 3 results also demonstrate strong context-specific effects of inoculum with implications for the field of plant-microbe interactions overall. For most of our differentially expressed genes, the effect of inoculum was ecotype specific. Inoculum origin modulated the expression of genes in major pathways relating to nutrient uptake, plant productivity, and transcription factor cascades, but only in the context of each ecotype's genetic background. Thus, the diverse effects of inoculum on plant growth and development are context-specific and bound within a window of intrinsic host genetics unique to each ecotype. Our results highlight the need for continued investigation of plant-microbe interactions, especially in wild plants growing in the field with emphasis on the specificity of these interactions. As we discuss the cost

or benefit of microbial communities on plant host performance, the genetic background of the host plant, the microbial community composition, and the specificity of these interactions need to be considered.

Future directions and opportunities

Limitations in our studies provide suggestions for future studies and investigations. Issues with the culturability of many microorganisms and including fungal symbionts can be overcome by repeating this study in greenhouse with field soils instead of weekly inoculation treatments using a subset of cultured microbes. By basing this follow-up study using more complete microbial communities and reducing unintentional selection during the culturing process, we could obtain even stronger confidence in results from inoculation experiments. Thus, simply repeating this study with field soils and intact microbial communities will provide stronger insights into these symbioses and further our understanding of local adaptation to microbial communities in *A. gerardii* ecotypes.

Additional projects will be necessary to answer questions surrounding the endemic seed microbiome in the *A. gerardii* ecotypes. Addressing the endemic seed microbiome in *A. gerardii* ecotypes, while challenging, poses an important avenue to understand these relationships and further investigate the role of microbial symbionts on *A. gerardii* ecotype performance and productivity and there are numerous avenues to explore in this relatively understudied domain (Kaminsky et al., 2019; Wassermann et al., 2020). First, seed endophyte communities are most often species-specific (Wassermann et al., 2020). Investigating this community should start with broad capture, culturing, and characterization of this microbiome (Kaminsky et al., 2019). After these first major steps, opportunities in manipulating this microbiome and identifying function in

planta become available. Capturing and manipulating these communities allows for insightful inoculation and synthetic community experiments that can be executed solely or in tandem with our inoculation study (Kaminsky et al., 2019). Thus, by identifying and manipulating the seed microbiome in *A. gerardii* ecotypes, the relationship between this microbiome and its corresponding ecotype as well as the relationship with other major microbiomes can be revealed.

Second, extending studies like this into a field setting and examining these relationships in the wild is likely the most insightful and meaningful direction to continue this project. Mirroring our greenhouse study under realistic field conditions is the optimal route to understanding the complexity and true nature of these plant-microbe interactions as well as validating current results (Gibson et al., 1999; Lekberg et al., 2018). Our greenhouse study provides a strong direction for a follow-up field study with recommendations to examine and confirm the observed patterns. Implementing parallel studies in the field will not only provide insights into the realistic ecotype-inocula interactions, but also lend themselves to investigating how these relationships respond to variable conditions, competitive interactions, and long-term patterns governing these interactions.

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