COVER PAGE

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Project Objectives

Of the myriad specialized metabolites that plants deploy to adapt to environmental challenges, terpenoids form the most diverse class. In many major crops, unique terpene blends serve as key biotic and abiotic stress defenses that directly impact plant fitness and biomass yield. In addition, terpenoids, such as bisabolene and pinene, are used for producing renewable biofuels. Essential to advancing a broader use of terpenoids for biofuel crop optimization is a system-wide knowledge of the diverse biosynthetic machinery and stress-defensive potential of often species-specific terpenoid blends. This project integrated genome-wide enzyme discovery with comparative –omics, protein structural and plant biochemical and genetic studies to elucidate the diversity, biosynthesis and stress-defensive functions of the switchgrass (*Panicum virgatum*) terpenoid network. These insights can be applied toward designing plants with desirable terpene blends for higher productivity and biofuel production on marginal lands. Specific project objectives included:

(Aim 1) Define the metabolic capacity of the switchgrass terpenoid network. Generate a genome-wide atlas of switchgrass terpenoid metabolism and identify the underlying molecular mechanisms by pairing rapid pathway discovery with enzyme structure-function studies.

(Aim 2) Identify the role of terpenoid metabolism in switchgrass abiotic stress tolerance. Combine systems-level -omics and transient genetics studies of key pathway nodes with the investigation of root-microbiome interactions to elucidate the impact of terpenoids on switchgrass abiotic stress adaptation.

(Aim 3) Develop switchgrass lines with enhanced stress resilience and terpene composition. Optimize terpenoid blends for enhanced stress tolerance and biofuel production by combining knock-out and over-expression of key terpene pathways using CRISPR/Cas9 genome engineering.

Major Project Accomplishments

Aim 1:

Prior to the start of the project our group identified a family of 75 terpene synthases (TPSs) in the allotetraploid switchgrass (*Panicum virgatum* AP13) genome, including 31 predicted diterpene synthases (diTPSs) (Pelot et al. 2018 *Plant Physiol*. 178:54-71; PMC6130043). This gene family is more than twice as large as any other monocot crop studied to date, supporting the hypothesis that switchgrass produces a diverse array of bioactive terpenoid metabolites. To test this hypothesis, Aim 1 of the project focused on identifying the underlying biosynthetic genes, enzymes and pathways. During the project duration, we employed multi-gene co-expression assays in *E. coli* and *Nicotiana benthamiana* to biochemically characterize 15 diTPS, 32 mono-/sesqui-TPS and 12 cytochrome P450 monooxygenase (P450) enzymes, that together form the core of the switchgrass terpenoid network (Publications # 3-5,7-8). We could demonstrate that many of these enzymes show substrate/product promiscuity and form a modular pathway network, where class II diTPS, class I diTPS and P450 enzymes with distinct and often promiscuous functions can interact in different combinations to generate a large group of diterpenoids. This includes metabolites that commonly occur in monocot crops and the broader plant kingdom, as well as terpenoids that, to current knowledge, are uniquely produced in switchgrass. In particular, we discovered a group of furanoditerpenoids, designated as panicoloids, that accumulate in switchgrass root tissue in response to stress (see Aim 2). Panicoloids are formed through the activity of a small group of 5 P450s (CYP71Z25-29) that convert three different class II diTPS products into furanoditerpenoids of different stereochemistry. Notably, panicoloid biosynthesis revealed a previously unrecognized diterpenoid pathway organization, where CYP71Z25-29 convert class II diTPS products, thus differing from the common pairwise reaction of class II and class I diTPS prior to P450-catalyzed functional modifications observed in most diterpenoid pathways (Publication # 3). Additional structure-function analysis of select diTPS and P450 enzymes identified key active site residues that control substrate and product specificity. Near complete functional

interconversion of select enzymes with only a few residue mutations further highlighted possible evolutionary routes toward the substantial divergence of the switchgrass diterpenoid network (Publications # 3, 9).

Collaborative work led by the laboratory of Prof. Dorothea Tholl (Virginia Polytechnic Institute, VA) with support through a prior joint DOE Joint Genome Institute DNA synthesis award (grant #2568, Lead PI: P. Zerbe) further identified the function of 32 mono- and sesqui-TPS enzymes, producing a range of mono- and sesqui-terpenoids (Publication #8). This work showed that switchgrass leaves and roots emit blends of mono- and sesqui-terpenoids upon insect stress and in response to the treatment of roots with defense hormones.

Research during the last year of the project period further identified two CYP701 enzymes in switchgrass, functioning as an *ent*-kaurene oxidase with a probable role in gibberellin metabolism and in the formation of a previously unrecognized *syn*-pimara-9(11),15-diene-2-ol compound (a diterpenoid so far unique to switchgrass), respectively. These findings indicate the presence of a more extensive pathway of *syn*-CPP-derived metabolites in switchgrass, possibly similar to those involved in stress defenses and allelopathic functions in rice. Furthermore, combinatorial functional characterization of ten CYP99 enzymes has revealed several novel enzyme products that are currently being purified for NMR-based structural identification. Manuscripts describing these findings are presently in preparation and anticipated to be submitted in the summer of 2024.

In summary, this work identified a complex, modular diterpenoid network in switchgrass that provides the foundational knowledge needed to elucidate the physiological roles of common and switchgrass-specific diterpenoids in crop growth and stress resilience. The identified enzymes and generated variants with alternate substrate and/or product specificity provide a rich resource for natural product engineering. All gene and enzyme sequences have been deposited to major repositories for broad community use. Constructs of native enzymes and engineered protein variants are available upon request. In addition, we have optimized a combinatorial pathway engineering platform for producing and purifying a broad range of diterpenoids (Publication #6).

Aim 2:

To investigate if enzyme products identified in Aim 1 are present *in planta* and to elucidate the physiological relevance of terpenoids in response to abiotic stress, drought-treatment studies of two switchgrass varieties (lowland ‘Alamo AP13’ and upland ‘Cave-in-Rock’) with contrasting drought tolerance were performed. In alignment with the phenotypic assessment of the plants, the lowland ecotype displayed a less severe response to the drought treatment on a transcriptomic level than the upland ecotype (Publication # 1). The relevant co-expressed genes included TPSs (especially diTPSs and triterpene synthases), P450s and glucosyltransferases with known or predicted functions in terpenoid metabolism. Gene co-expression of select *TPS*, *CYP701* and *CYP99* genes further supports the presence of a co-regulated pathway in switchgrass roots. In addition, extensive metabolomics analyses of drought-stressed tissue samples via targeted GC-MS and untargeted LC-MS analyses (collaboration with Dr. Robert Last, MSU) identified over 4,000 mass fragment features and yielded insights into metabolic processes possibly involved in the contrasting drought tolerance of different switchgrass ecotypes. Consistent with the gene expression data outlined above, multiple diterpenoids, including panicoloids, *syn*-pimara-9(11),15- diene-2-ol, and yet unidentified CYP99 products (unpublished data), ranked among the metabolites showing the most significant changes in response to drought stress, thus supporting a biological role of these metabolites in abiotic stress responses (Publications # 1, 3).

In addition, metabolite analysis of root exudates of switchgrass plants grown in soil, as well as in EcoFABs fabricated ecosystems (collaboration with Dr. Trent Northern, DOE JGI, LBNL),

demonstrated the presence of diterpenoids and other metabolites, thus providing evidence that select diterpenoids are exuded from root tissue (unpublished data). These findings are consistent with the excretion of mono- and sesqui-terpenoid blends from switchgrass roots exposed to pest attack or stress hormone treatment (Publication # 8). Planned experiments in collaboration with Dr. Trent Northen to investigate the impact of terpenoid exudates on synthetic switchgrass microbiome communities could not be successfully pursued within the project period, due to substantial research and hiring delays caused by the Covid-19 pandemic.

In summary, the project revealed the presence of panicoloids and other identified diTPS/P450 enzyme products predominantly in switchgrass root tissue and root exudates. Accumulation of these compounds in response to biotic and abiotic stressors supports the hypothesis that these compounds play important roles in mediating switchgrass stress responses. Recent identification of at least 12 additional diterpenoids in switchgrass roots that are yet to be structurally identified suggest an even broader diversity of speciazlied diterpenoids in switchgrass, consistent with the large size of the switchgrass TPS and P450 gene families. Although the physiological roles of switchgrass diterpenoids in plant-environment interactions require further study, these findings provide fundamental insights to facilitate such downstream analyses and gene targets for optimizing switchgrass stress resilience.

Aim 3:

Activities with focus on Aim 3 were, regrettably significantly delayed, due to research and hiring delays caused by the Covid-19 pandemic. Efforts to establish a more efficient transformation protocol for \switchgrass did not yield the desired results and ultimately was abandoned. However, identification of several TPS enzymes producing farnesene and bisabolene as relevant terpenes for biofuel production can provide key gene targets to apply genetic engineering to develop switchgrass plants for enhanced biofuel capacity. In addition, as a alternative to stable genetic modification of switchgrass, we have optimized a protocol for Virus-Induced Gene Silencing (VIGS) for switchgrass using foxtail mosaic virus (FoMV). This work demonstrated that leaf rub-inoculation is a suitable method for systemic gene silencing in switchgrass. Using three visual marker genes phenotypic changes were observed in leaves, albeit at different intensities. Gene silencing efficiency was verified by RT-PCR for all tested genes. Notably, systemic gene silencing was also observed in roots, although silencing efficiency was stronger in leaves as compared to roots. Plants at a later developmental stage were moderately less amenable to VIGS than younger plants, but also less perturbed by the viral infection (Publication # 2). At present, this protocol is applied to down-regulate core diTPS and P450 genes for further investigation of the physiological relevance of the underlying biosynthetic pathways and end products.

Project Products

Journal Articles

1. Tiedge K, Li X, Merrill AT, Davisson D, Chen Y, Yu P, Tantillo DJ, Last RL, Zerbe P (2022) Comparative transcriptomics and metabolomics reveal specialized metabolite drought stress responses in switchgrass (*Panicum virgatum*). *New Phytol*. 236:1393-1408. [PMID: 36028985]
2. Tiedge K, Destremps J, Solano-Sanchez J, Arce-Rodriguez ML, Zerbe P (2022) Foxtail mosaic virus-induced gene silencing (VIGS) in switchgrass (*Panicum virgatum* L.). *Plant Methods* 18:71. [PMID: 35644680]
3. Muchlinski A, Jia M, Tiedge K, Fell JS, Pelot KA, Chew L, Davisson D, Chen Y, Siegel J, Lovell JT, Zerbe P (2021) Cytochrome P450-catalyzed biosynthesis of furanoditerpenoids in the bioenergy crop switchgrass (*Panicum virgatum* L.). *Plant J*. 108:1053-68. [PMID: 34514645]
4. Tiedge K, Muchlinski A, Zerbe P (2020) Genomics-enabled analysis of specialized metabolism in bioenergy crops: Current progress and challenges. *Syn Biol*. 5: ysaa005. [PMID: 32995549]
5. Murphy KM, Zerbe P (2020) Specialized diterpenoid metabolism in monocot crops: Biosynthesis

and chemical diversity. *Phytochemistry* 172:112289. [PMID: 32036187]

6. Murphy KM, Chung S, Fogla S, Minsky HB, Zhu KY, Zerbe P. (2019) A Customizable approach for the enzymatic production and purification of diterpenoid natural products. *J Vis Exp*. doi: 10.3791/59992. [PMID: 31633697]
7. Karunanithi PS, Zerbe P (2019) Terpene synthases as metabolic gatekeepers in the evolution of plant terpenoid chemical diversity. *Front Plant Sci*. 10:1166. [PMID: 31632418]
8. Muchlinski A, Chen X, Lovell JT, Köllner TG, Pelot KA, Zerbe P, Ruggiero M, Callaway L 3rd, Laliberte S, Chen F, Tholl D (2019) Biosynthesis and Emission of Stress-Induced Volatile Terpenes in Roots and Leaves of Switchgrass (*Panicum virgatum* L.). *Front Plant Sci*. 10:1144. [PMID: 31608090]
9. Pelot KA, Hagelthorn DM, Hong YJ, Tantillo DJ, Zerbe P (2019) Diterpene Synthase-Catalyzed Biosynthesis of Distinct Clerodane Stereoisomers. *Chembiochem* 20:111-7. [PMID: 30393911]

Thesis/Dissertation

Kyle Adam Pelot, "Exploration of Diterpenoid Metabolism in Medicinal and Bioenergy Crop Plants", *University of California at Davis*.

Presentations

Results from this project have been disseminated primarily through contributed and invited presentations by at professional conferences and guest lectures, including:

12/2023	aBIOTECH Intl. Seminar series (Webinar)
08/2023	TERPENT Intl. Meeting, Davis, CA USA
04/2023	DOE Genomic Sciences Program PI meeting, Washington DC USA
01/2023	Michigan State University, East Lansing, USA
08/2022	IKIAM Regional Amazon University, Tena, Ecuador
07/2022	American Society of Plant Biologists (ASPB) annual meeting, Portland, OR USA
03/2022	Plant Science Symposium University of Manitoba (Webinar)
06/2021	DOE JGI Molecular Structural Workshop (Webinar)
02/2022	DOE Genomic Sciences Program PI meeting (Webinar)
01/2021	Academia Sinica, Taipei, Taiwan (Webinar)
01/2021	Osaka University, Osaka, Japan (Webinar)
02/2021	DOE Genomic Sciences Program PI meeting (Webinar)
07/2020	DOE JGI Engagement Webinar (Webinar)
03/2020	Iowa State University, Ames, IA, USA
02/2020	DOE Genomic Sciences Program PI meeting, Washington, DC, USA
12/2019	Max Planck Institute for Chemical Biology, Invited Speaker, Jena, Germany
12/2019	UCD West Coast Metabolomics Center Seminar Series, Davis, CA, USA
11/2019	Agricultural Genomics Institute Shenzhen (AGIS), Shenzhen, China
11/2019	International Center for Biotechnology, Osaka University, Osaka, Japan
10/2019	University of Nebraska-Lincoln Plant Science Symposium, Lincoln, NB, USA
08/2019	TERPNET International meeting, Halle, Germany
07/2019	PSNA annual meeting, Johnson City, TN, USA
06/2019	Donald Danforth Plant Science Center, St. Louis, IL USA
04/2019	Switchgrass Microbiome Symposium, Berkeley, CA, USA
04/2019	San Francisco Exploratorium After Dark Seminar Series, San Francisco, CA, USA
03/2019	Virginia Polytechnic Institute, Blacksburg, VA USA
02/2019	DOE Genomic Sciences Program PI meeting, Tyson's Corner, VA USA

Training and Professional Development Provided by the Project

During the full period 2018-23, the project provided training for four postdoctoral scholars (Drs. Andrew Muchlinski, Kira Tiedge, Lisette Arce Rodriguez, Meirong Jia), two junior specialists (Janessa Destremps, Janet Solano-Sanchez), two graduate students (Kyle Pelot, Gabrielle Wyatt), and research internships for 14 undergraduate students, many of whom successfully moved on to positions in industry or academia, graduate school or medical school. Notably, Dr. Andrew Muchlinski secured a research scientist position at Firmenich Inc., Dr. Kira Tiedge secured a faculty position at the University of Groningen (Netherlands), and Dr. Meirong Jia secured a faculty position Chinese Academy of Medical Sciences (China). Junior specialists Janessa Destremps and Janet Solano-Sanchez entered graduate programs at UC Davis and the University of Washington (Seattle), respectively. Graduate student Dr. Kyle Pelot secured a position in the biotechnology industry and graduate student Gabby Wyatt is presently working toward completion of her degree with support fo a NSF Graduate Student Research Fellowship.