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Participants

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Brief Summary of Minutes of Annual Meeting

The 2023 NC-1203 meeting was held as a hybrid in person and Zoom meeting on August 14, 2023, at the University of Nebraska, Lincoln. The meeting was opened by the host Tom Clemente. A time of introductions was followed by presentations by the participants. Multistate Project Administrative Advisor Carolyn Lawrence-Dill joined later to provide a summary of information corresponding to multistate projects and opportunities for additional funding support. Accomplishments for each of the objectives are summarized below. The presentations were followed by a discussion of future plans for each of the aims and the timing of the 2024 annual meeting co-hosted by Joe Louis (President) and Rebecca Roston (Secretary) at the University of Nebraska Lincoln one day ahead of the ISPL meeting in July 2024. Future meeting sites: In 2025 we plan to hold the annual meeting in Kansas City (alternatively, at Kansas State University) (hosted by Kathrin Schrick, who will serve as Vice President in 2024), and in 2026 the meeting will be held at the University of Nevada, Reno (hosted by Dylan Kosma, who will serve as Vice President in 2025). As part of the itinerary of the next year's 2024 annual meeting, a new Secretary will be elected for 2025.

NC-1203 2022-2023 Accomplishments

Activities and accomplishments related to each of the project's three objectives are described below. The report also summarizes the collaborative research efforts of LIPIDS of Crops

members including the establishment of new collaborations as well as the outcomes of existing collaborations in the form of publications and/or grant activities.

Objective 1: Improve and extend methods for lipid characterization and measurement

Welti, Markham, and Lee groups, in collaboration with Durrett, are working toward developing a semi-quantitative and semi-targeted catalog of the occurrence and amounts of lipids from Arabidopsis and crop plants. They continued to work on optimizing analysis conditions and standardizing a protocol for comprehensive fatty acid-based negative precursor and positive neutral loss scanning and on incorporation of data from Arabidopsis leaves of varied accessions and camelina seeds into a visual lipidome map. They collected a dataset on wild type camelina and transgenic camelina expressing an acetyltransferase (and making acetyl-triacylglycerol) during seed development, germination, and seed aging. The data are being analyzed in conjunction with Trupti Joshi's group at the University of Missouri-Columbia.

The Lee group has optimized MALDI-MS imaging conditions to visualize camelina seed lipids. Additionally, an in vivo isotope labeling method is currently being developed for MS imaging.

The Bates, Durrett and Welti groups developed and optimized three different approaches for analysis of molecular species of diacylglycerol, a key metabolic intermediate in lipid biosynthesis, catabolism, and signaling (Parchuri et al., 2023).

Objective 2: Identify lipid-related mechanisms to increase agricultural resilience

Welti, Durrett, and Schrick collaborated on characterization of plant genes identified in a lipid profiling screen of Arabidopsis mutants in which the lipid profiles are significantly different from wild-type plants. Various mutants identified in the lipid profiling screen affect genes with functions in fatty acid desaturation, cutin metabolism, and transacylase activities. Welti and Durrett collaborated with colleagues in India to characterize acyltransferases of the Himalayan plant, *Buglossoides arvensis*, incorporating polyunsaturated fatty acids into seed triacylglycerols (Parchuri et al., 2022). Welti and Durrett are also characterizing the lipid and gene expression changes in camelina seeds during development and germination.

The Schrick lab demonstrated that a lipid-sensing domain from plant-specific homeodomain leucine-zipper (HD-Zip IV) transcription factors is critical for both protein stability and homodimerization (Mukherjee et al., 2022). The group also showed that although fluorescent protein tags are useful for live imaging and affinity purification, they may lead to enhanced protein stability and changes in agronomically important traits (Subedi and Schrick, 2022). Ongoing work highlights the role of lysophosphatidylcholines in binding to the lipid sensor from a family member that regulates cell-type differentiation of the epidermis. Studies highlighting HD-Zip IV transcription factors are helping us to understand how gene expression is modulated by lipid metabolites (Schrick et al., 2023).

With funding from the USDA-NIFA, Narayanan and Welti are characterizing the heat tolerance of members of a soybean recombinant inbred line population (derived from a genetic cross between a heat-tolerant and a susceptible genotype) and elite soybean genotypes by measuring physiological responses and identifying molecular markers associated with heat-induced lipid metabolic changes. Narayanan lab also investigated lipid alterations under heat stress conditions that might affect seed oil quality. Their recent work found that heat stress during the early flowering stage does not affect seed fatty acid contents in conventional oleic peanut varieties (Kakati et al., 2022). On the other end of the temperature stress spectrum, the Welti and Wang groups collaborated to identify specific lipid changes associated with plant freezing tolerance (Vu et al., 2022).

The Hoffmann-Benning lab has been studying phloem lipids and lipid-binding proteins with a role in long distance signaling. They studied gene expression profiles in response to various abiotic stresses, as well as localization, and lipid-binding properties. Gene expression profiles were compared to that of lipases that might generate the lipid ligands. The lab established an optogenetics method that allows researchers to induce gene expression in a single leaf and monitor movement of these lipid-binding proteins throughout the plant. Next, a FRET approach will be taken to monitor protein-lipid interaction during movement. Molecular dynamic simulations provided clues about the physical interaction between our protein of interest (PLAFP, Phloem Lipid-Associated Family Protein) and its ligand phosphatidic acid (PA). These simulations showed that PA inserts itself into the protein and that, upon PA binding, PA is released from the membrane (Kulke et al., 2023). A combination of mutagenesis, lipid-binding assays and optogenetics/movement studies is currently being used to study the importance of specific amino acids on protein-lipid interaction and movement. Additionally, the lab has started a collaboration with Dr. Phil Lewis at the USDA (Otis Lab) to identify phloem compounds conveying resistance against spotted lanternfly.

The Thelen, Bates, and Koo labs together demonstrated that expression of the pea *alpha-CT* gene in *Camelina sativa* increases seed oil accumulation (Wang et al., 2022). Lipid flux analysis in these lines demonstrated increased fatty acid biosynthesis that favored triacylglycerol accumulation, but still utilized the membrane lipid phosphatidylcholine as a key intermediate of oil biosynthesis. These data emphasize the overlapping nature of essential membrane lipid biosynthesis with storage oil synthesis. Camelina lines and transgenic Arabidopsis lines overexpressing the same *alpha-CT* construct, as well as *badc1;badc3* double knockout lines, were subject to RNA-seq and TRAP-seq analysis. The TRAP-seq technique complements the proteomics approach to analyze the translational efficiency of ribosome-associated RNAs that are actively engaged in protein translation. The Koo lab in collaboration with Dong Xu at University of Missouri analyzed the data and found several lipid-related genes with significant changes in translational efficiency.

The Louis lab in collaboration with Welti lab is working towards identifying the involvement of host lipids in sorghum defense against sugarcane aphids (SCA), a relatively new and devastating pest of sorghum in the US. Previously, the Louis lab identified varied levels of resistance to SCA in founder lines of the sorghum NAM population compared to RTx430, the common parent used for these NAM lines (Grover et al., 2022). SCA reproduction was low on the inbred line SC265, intermediate on RTx430, and high on SC1345 lines. Electrospray ionization mass spectrometry (ESI-MS) was used by the Welti lab to compare the lipids of SCA resistant, susceptible, and RTx430 sorghum plants in response to SCA feeding. A total of 227 lipids were identified in ESI-MS from SCA infested or uninfested leaves sampled at early and late timepoints in three sorghum genotypes. This work aims to identify the specific role of sorghum lipid(s) in resistance to aphids to uncover novel aspects of plant lipid metabolism after aphid infestation. In addition, the lab recently demonstrated that sorghum cuticular waxes impact host plant selection by aphids, plant age is a determinant for SCA feeding, and subtle changes in triterpenoids and available sugars influence SCA establishment on sorghum plants (Cardona et al., 2023a, b).

The Tamborindeguy lab started working towards identifying the involvement of host lipids in solanaceous defenses against the potato psyllid, a phloem-feeding hemipteran, and liberibacter, the bacterial pathogen it transmits. LC-MS/MS analysis was used to quantify different oxylipins in tobacco plants following infection and infestation. Two oxylipins were found to be decreased in infected plants (Levy et al., 2023).

The Kosma and Cahoon labs are working towards identifying the enzymes required for the synthesis of falcarins, a unique class of lipids with known antimicrobial and anticancer activities. Falcarins are a subgroup of polyacetylenic lipids that contain multiple triple bonds and are found in specific, diverse taxa including plants from the Apiaceae (e.g., carrot, celery, parsley), Asteraceae (e.g., sunflowers), and Araliaceae (e.g., ginseng). In 2021, Kosma and Cahoon received funding from NIFA to study the falcarin biosynthetic pathway in carrot and other species and published a review paper on falcarin biosynthesis (Santos et al., 2022). More recently the Cahoon lab developed T0, T1, and T2 transgenic carrot lines overexpressing known and candidate genes from the falcarin biosynthetic pathway. The Kosma lab verified that several of these lines produce higher levels of falcarins with significant differences in falcarin composition. The Kosma lab has developed an extensive tissue and developmental atlas of falcarin content and composition in carrots. The Cahoon lab has generated multigene knockout lines, with edits in at least three different FAD2 or FAD2-variant genes. Chemical types of these lines await confirmation. Kosma lab has recreated the initial desaturation steps of the falcarin biosynthetic pathway in a heterologous, transient expression system (*Nicotiana benthamiana*) and is currently testing candidate genes for downstream steps of the pathway. Kosma has been working with collaborator Won Yim (UNR) on a revised annotation of the carrot genome that has led to the identification of numerous additional candidate falcarin genes. The initial estimation of FAD2 and FAD2-variant genes has increased from 24 to 42 with the new annotation. Furthermore, a tissue-specific RNA-seq data set profiling the transcriptomes of carrot

taproot periderm (skin), xylem, and phloem, as well as aerial tissues, was generated and employed to develop a new co-expression matrix that includes several hundred datasets from the Sequence Read Archive (SRA) at NCBI.

The Cahoon lab, working in collaboration with investigators at Lund University and Swedish University of Agricultural Sciences, developed camelina lines that produce oils enriched in (Z)-11-hexadecenoic acid, a sex pheromone precursor in several moth species. These oils provided a feedstock from which the precursor was isolated, purified and transformed into the final pheromone. Trap lures containing this pheromone were assessed for their capacity to manage moth pests in the field. Plant-derived pheromone lures proved equally effective as synthetic pheromone lures in monitoring the diamondback moth, *Plutella xylostella*, in cabbage and disrupting mating of cotton bollworm, *Helicoverpa armigera*, in common bean fields. This study demonstrated the biological efficacy and economic feasibility of pheromone production in plant factories by metabolic engineering of an oilseed crop (Wang H-L et al., 2022).

The Roston and Schrick labs collaborated to identify that cold tolerance does depend on sterols, and this beginning effort will be followed up. The Roston and Schachtman labs identified that sorghum root exudates include variable amounts of sorgoleone, which changes the ability of sorghum to recruit a strong root microbiome (Wang et al., 2022). The Roston lab is working toward identifying the evolutionary similarities in membrane changes in response to severe low temperature, publishing one paper describing evolutionary similarities in TGDG accumulation in response to low temperature (Barnes et al., 2023). A second is submitted on evolutionary similarities in low temperature response in the panicoid grass family that includes maize, sorghum, and millet crops.

The Cahoon lab contributed to a study that explored the cryo-EM structure of the plant serine palmitoyltransferase (SPT) complex, the first and rate-limiting enzyme for sphingolipid long-chain base synthesis. Regulation of sphingolipid homeostasis in plants is critical for maintaining sufficient glycosphingolipid amounts for growth while limiting accumulation of biosynthetic intermediates that trigger programmed cell death, until needed for microbial pathogen defense. Central to sphingolipid homeostatic regulation is the ORM protein, which functions as a negative regulator of SPT. Gene-edited Arabidopsis mutants lacking ORMs have non-viable seeds that hyperaccumulate ceramides from the loss of SPT regulation. ORMs reversibly control SPT biosynthetic flux in response to intracellular sphingolipid concentrations: SPT-repression in response to excess intracellular sphingolipids and SPT de-repression when sphingolipids are required for growth. Plants have the additional need to accumulate the apoptotic-inducers ceramides and long-chain bases to rapidly induce programmed cell death for the hypersensitive response for bacterial and fungal pathogen defense. The mechanism for reversible ORM regulation of SPT has been unclear. Recent evidence from the cryo-EM structure of the Arabidopsis SPT complex revealed that ceramides bind ORMs to repress SPT activity. Most effective for SPT repression are ceramides with trihydroxy long-chain bases that are typically

paired with very-long chain fatty acids, derived from LOH1 and LOH3 ceramide synthase activity.

The Bates and Allen labs have been studying leaves engineered to produce high levels of lipids. Their work describes the capacity of some plants, such as tobacco, to store starch in leaves non-transiently over the course of development. The sink capacity of these leaves can be converted to store lipid at significant levels which appears to be less successful in plants without the non-transient starch capacity. The work resulted in a publication (Chu et al., 2022) and led to questions about the ability of these plants to subvert abiotic stresses. Given that membrane lipids must maintain a semi-fluid state, the labs are currently investigating the consequences of high lipid production in leaves on temperature stress tolerance.

The Yandeau-Nelson lab is focused on dissecting the genetic networks underlying cuticle formation and untangling cuticle structure-function relationships relative to biotic and abiotic stress responses, using maize silks as a model system. In collaboration with Gabriella Consonni (University of Milan), the team assessed the roles of the transcription factor *Fused Leaves 1* (*FDL1*) and *Glossy2,* a gene encoding a protein that supports chain-length elongation in the Fatty Acid Elongation (FAE) pathway, on cuticle composition on maize silks and how changes in cuticular wax composition impact the silks' response to biotic stress, specifically infection by the fungal pathogen, *Fusarium verticillioides.* This work resulted in a publication asserting that the cuticle likely plays an active role in the response to *F. verticillioides* infection (Castorina et al., 2023). The Yandeau-Nelson team, in collaboration with previous NC-1203 member Basil Nikolau and others, have started a new effort that incorporates synthetic biology approaches in plants and microbes to engineer *de novo* the cuticular wax biosynthetic pathways in systems naturally devoid of a cuticle (plant roots and yeast) to dissect the regulatory pathways and kinetics involved in cuticle synthesis. Specifically, the team has engineered yeast strains for which different iterations of the maize FAE pathway have replaced the endogenous yeast pathway, providing a system to study the extensive genetic redundancy that exists in the maize pathway. Already, they have demonstrated that different maize Ketoacyl-CoA Synthetase (KCS) enzymes in FAE produce very different VLCFA profiles. To study the transcriptional regulation of cuticle biosynthesis, transcription factors that have been identified via multi-omics approaches to be associated with cuticle dynamics are now being expressed in root protoplasts to assess impacts on expression of different genes involved in FAE and downstream cuticle synthesis pathways.

Additionally, the Yandeau-Nelson team has probed weather parameters at different stages of plant development that impact cuticular wax composition on mature maize silks. Using joint statistical analysis of weather data across the growing season and cuticular wax profiles from mature silks for large genetic diversity panels grown in three unique environments, the team identified associations between solar radiation patterns at early stages of silk development and precipitation patterns just prior to silk emergence into the external environment that influence

cuticular wax compositions of mature silks. This work enhances our understanding of abiotic influence on cuticle deposition, namely the impacts of weather as it modulates cuticular wax composition in genotype-specific manners.

The Wang lab in collaboration with Daniel Schachtman and Charles Shapiro (University of Nebraska-Lincoln) addressed how camelina seed oil production and composition respond to low input environments, such as phosphorus (P), in field conditions (Li. et al., 2023). Lipidomic profiling revealed that P deficiency in field settings triggered extensive leaf lipid remodeling that decreased the ratio of phospholipids to non-P-containing galactolipids under P sufficient to deficient conditions. P deficiency increased seed oil content per seed weight and altered seed fatty acid composition, with increases in monounsaturated 18:1 and 20:1 and decreases in polyunsaturated 18:3, but total seed production decreased greatly under P deficiency. The results from field and greenhouse conditions indicate that P deficiency increases seed oil content, alters fatty acid composition, and decreases seed production, suggesting that achieving a high yield and quality of camelina seed oil is positively linked to P status of soil.

In addition, the Wang lab investigated how lipids are involved in plant response to heat (Kim et al., 2022). They previously reported that in response to heat stress, cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) accumulated in the nucleus to modulate transcription and thermotolerance. Heat-induced GAPC nuclear accumulation and plant heat tolerance were reduced in Arabidopsis phospholipase D (PLD) knockout mutants of *pldδ* and *pldα1;pldδ*, but not of *pldα1*. Heat stress elevated the levels of the PLD product phosphatidic acid (PA) in the nucleus in wild type, but not in *pldδ* plants. The heat-induced nuclear co-localization of PA and GAPC. Our data suggest that PLDδ and PA are critical for the heat-induced nuclear translocation of GAPC.

The Huang Lab joined the project in September 2023 to study the lipid droplets-mediated regulation of Cersosporin, a perylenequinone toxin produced by *Cercpspora* fungus, that damages plant host cells during infection. The *Cercospora* fungal pathogens are the causal agents of several economically important crop diseases, including soybean and sugar beet. The active cercosporin was found to be sequestrated in lipid droplets of *Cercpspora* fungus which can be important for its self-resistance to Cersosporin. The Huang Lab is going to study the role of the lipid droplets of plant host and *Cercpspora* fungus on toxin production and release for developing effective disease management.

Objective 3: Develop crops with improved yield and/or functionality

The Bates lab characterized lipid metabolic genes that act in a novel triacylglycerol remodeling pathway in *Physaria fendleri*. Their study showed that PfeSDP1 expression during seed development reduces both total seed oil and levels of the industrially valuable hydroxylated fatty acid, lesquerolic acid (Azeez et al., 2022). Additionally, the Bates lab evaluated the fatty acid

selectivity of various acyltransferases to accumulate valuable and unusual fatty acids (Shockey at al, 2023).

The Durrett and Allen labs collaborated to alter the partitioning of carbon between oil, oligosaccharides, and protein in soybeans. Specifically, by suppressing the GmSDP1 lipases expressed late in seed development they were able to increase seed oil content and reduce accumulation of unwanted oligosaccharides (Aznar-Moreno et al., 2022). The lines resulted in larger seeds, which is under further investigation.

Additional studies by the Allen lab in collaboration with the Clemente lab focused on soybeans expressing malic enzyme alleles to increase malic enzyme activity, resulting in lines with increased lipid levels (Morley et al., 2023). Malic enzyme activity was enhanced specifically in the mitochondria or in the chloroplast. The former resulted in expected changes in amino acid profiles, favoring those that are pyruvate-derived at the expense of aspartate-derived amino acids. This is consistent with the role of malic enzyme to move carbon to pyruvate and therefore provide a 'push' to those amino acids. The enhanced activity in the plastids resulted in increased lipid levels in seeds by providing more pyruvate there for fatty acid biosynthesis and concomitantly a supply of reducing equivalents because malic enzyme and pyruvate dehydrogenase both produce reduced cofactor products. Changes in the fatty acid profile were observed depending on the subcellular location of the enzyme activity and suggested that the utilization of malate in each location resulted in changing cofactor balances and likely affected malate shuttles that normally distribute cofactors between locations. Measurements of cofactors further supported this hypothesis. The studies support prior metabolic flux analyses published by the Allen lab in soybean, confirmed the importance of subcellular location, and emphasize that a single gene alteration can positively impact final biomass composition. Such studies that build on flux analyses provide a strategy for rational metabolic engineering.

Acetyl-TAGs are unusual TAG molecules that possess reduced viscosity due to the presence of an sn-3 acetate group. Acetyl-TAGs can be synthesized in transgenic oilseeds by expressing DAG acetyltransferases isolated from species that naturally produce acetyl-TAG. By suppressing or mutating endogenous enzymes that compete for the supply of DAG and acetyl-CoA substrates, the Durrett lab has successfully generated transgenic pennycress capable of accumulating up to 98 mol% acetyl-TAG in seed oil. These levels of acetyl-TAG are stable through multiple generations. Transgenic seeds germinate slower than wild-type seeds, but no other effects on seed properties or plant growth are evident. In collaboration with the Lee lab, the residual 2% endogenous TAG was found to localize to the embryonic axis of developing seeds.

Research led by the Cahoon lab discovered that seed oils of many Thunbergia species contain up to 92% of the unusual monounsaturated petroselinic acid (18:1Δ6), one of the highest reported levels for a single fatty acid in plants. To elucidate the biosynthetic origin of petroselinic acid, they identified a Δ6-stearoyl-acyl carrier protein (18:0-ACP) desaturase from *Thunbergia*

laurifolia, closely related to a previously identified Δ6-palmitoyl-ACP desaturase that produces sapienic acid (16:1Δ6)-rich oils in *Thunbergia alata* seeds. Guided by a *T. laurifolia* desaturase crystal structure obtained in this study, enzyme mutagenesis identified key amino acids for functional divergence of Δ6 desaturases from the archetypal Δ9-18:0-ACP desaturase and mutations that result in nonnative enzyme regiospecificity. Furthermore, the team demonstrated the utility of the *T. laurifolia* desaturase for the production of unusual monounsaturated fatty acids in engineered plant and bacterial hosts. Through stepwise metabolic engineering, the team provided evidence that divergent evolution of high-level petroselinic acid and sapienic acid production arises from biosynthetic and metabolic functional specialization and enhanced expression of specific enzymes to accommodate metabolism of atypical substrates (Gan et al., 2022).

The Clemente lab, working in collaboration with the Ana Alonso lab (University of North Texas) and the Leah McHale lab (Ohio State University), evaluated the metabolic and transcriptional responses to AtWRI1 and AtDGAT1 expression in soybean seeds. AtWRI1 is a master regulator of fatty acid (FA) biosynthesis, and AtDGAT1 encodes an enzyme catalyzing the final and rate-limiting step of triacylglycerol biosynthesis. Expressing these genes in the embryo did not show an increase in total FA content, but did result in changes in the oil and carbohydrate composition. Transcriptomic studies revealed a down-regulation of genes putatively encoding for oil body packaging proteins, and a strong induction of genes annotated as lipases and FA biosynthesis inhibitors. Novel putative AtWRI1 targets, presenting an AW-box in the upstream region of the genes, were identified by comparison with an event that harbored only AtWRI1. Lastly, targeted metabolomics analysis showed that carbon from sugar phosphates was used in FA competing pathways, such as starch and cell wall polysaccharides, contributing to the restriction in oil accumulation. These results allowed the identification of key cellular processes that need to be considered to break the embryo's natural restriction on uncontrolled seed lipid increase (Arias et al., 2022).

The Cahoon and Clemente labs developed camelina and soybeans for aquaculture feed that produce seed oils enriched in the high-value carotenoid, astaxanthin [(3S,3′S)-3,3′-Dihydroxy-β,β-carotene-4,4′-dione)]. Astaxanthin is a red lipophilic pigment derived from β-carotene and is distinguished by keto groups on each ionone ring ("ketocarotenoid"). Research focused on the discovery of sustainable sources and cost-effective production of natural astaxanthin for use in aquaculture feed and as a natural pigment. Flower petals of Adonis (*Adonis aestivalis*) are one of the few plant sources of this high-value ketocarotenoid. The team focused on the transfer of the Adonis astaxanthin biosynthetic pathway to camelina and soybean seeds for cost-effective, oilseed-based production. Three genes, encoding phytoene synthase (PSY), Adonis β-carotene ketolase (CBFD2) and Adonis β-carotene hydroxylase (HBFD1), were introduced into camelina under the control of seed-specific promoters. The production of ketocarotenoids (~136 μg/g DW) containing astaxanthin (~47 μ/g DW) was obtained in "Asta-camelina" seeds. In addition to the production of astaxanthin, Asta-camelina seeds also produced a novel ketocarotenoid containing

one ketone and three hydroxy groups, which is considered an intermediate or derivative in astaxanthin biosynthetic pathways. The astaxanthin-containing oil extracted from Asta-camelina seeds had higher oxidative stability during storage duration than normal camelina seed oil. Astaxanthin biosynthetic genes, CBFD2 and HBFD1, also functioned when transiently expressed in *Nicotiana benthamiana* leaves. Furthermore, it was observed that *N. benthamiana* leaves expressing a combination set of Adonis and bacterial (*crtW* and *crtZ*) astaxanthin-biosynthetic genes more effectively converted biosynthetic intermediates to astaxanthin than leaves expressing bacterial genes alone. Overall, results indicated that Adonis genes are a valuable genetic source for astaxanthin metabolic engineering in oilseed crops and for generating vegetable oils with enhanced antioxidant capacity and high-value aquaculture traits. The Cahoon lab identified candidate genes for astaxanthin from a transcriptome generated from astaxanthin-rich petals of Adonis. From this transcriptome, new biosynthetic genes and astaxanthin ester synthase genes were identified. The function of these genes was established in camelina. Using up to six transgene combinations, the Cahoon lab was able to engineer camelina seeds that accumulate >200 μg/g DW of ketocarotenoids, which were present as >95% pure astaxanthin. These seeds, in contrast to the Asta Prototype 1, also showed little or no impairment in germination and seed oil content.

Through CRISPR-Cas9-based gene editing, the Kosma lab has developed potato lines with enhanced storage life via reduced sprouting (enhanced dormancy) and reduced shrinkage through targeting specific transcription factors. A provisional patent was filed for one of these lines (Vulavala et al., 2023). The Kosma lab continues to investigate transcriptional regulation of suberin through CRISPR-Cas9-targeted editing of transcription factors believed to regulate suberin. In total, 51 independent events targeting 6 different transcription factors have been generated and two transcription factors were validated to function as positive regulators of wound suberin in the past year.

Thelen and Van Doren labs are studying the structure-function relationships between catalytic and regulatory subunits of ACCase. They are investigating the biochemical nature and structural basis of the dynamic interactions of catalytic and regulatory subunits. This features biotin carboxylase (BC) interactions with biotin carboxyl carrier proteins (BCCPs), BADCs, and PII subunits, as well as carboxyltransferase (CT) interactions with carboxyltransferase interactors (CTIs). They have been probing the interactions with microscale thermophoresis, NMR spectroscopy, mass spectrometry, and computed structural predictions by Alpha Fold Multimer. These reductionist and pairwise studies of recombinant proteins are complemented by quantitative proteomics of plant samples (Arabidopsis and pennycress) using the newly expanded ACCase AQUA-MRM assay that includes PII and CTI isoforms. These measurements of interactions and abundance *in vivo* will provide a basis for predictions about ACCase form and function *in situ.*

The Dhankher lab previously developed *Camelina sativa* lines co-expressing *AtDGAT1* and yeast *GPD1* with a more than 60% increase in overall seed and oil yields. They utilized metabolomic and transcriptomic profiling approaches and identified metabolic bottlenecks that control oil production and accumulation in seeds of *AtDGAT1+ScGPD1* camelina lines. Accordingly, they selected several candidate genes/enzymes for metabolic engineering of camelina. They targeted the overexpression of the camelina *PDCT* gene, a homolog of the Arabidopsis *Reduced Oleate Desaturation 1* (*ROD1*) gene, which encodes phosphatidylcholine:diacylglycerol cholinephosphotransferase 1 enzyme. PDCT is proposed to act as a gatekeeper responsible for the interconversions of diacylglycerol (DAG) and phosphatidylcholine (PC) pools. To test the hypothesis that increased PDCT activity in developing camelina seeds would enhance carbon flux toward increased levels of TAG and alter oil composition, the Dhankher group overexpressed the *CsPDCT* gene under the control of the seed-specific phaseolin promoter. Transgenic camelina plants exhibited significant increases in seed mass and seed oil content, higher seed and oil yields per plant and altered polyunsaturated fatty acid (PUFA) content compared to their parental wild-type plants. Results from [14C] acetate labeling, in collaboration with Yair Shachar-Hill (MSU), of developing camelina embryos in culture indicated increased rates of fatty acid incorporation into glycerolipids. This resulted in higher total radiolabeled lipid content in the *PDCT* transgenic lines, particularly in TAG and DAG lipid classes, relative to wild-type embryos. Therefore, overexpression of *PDCT* appears to cause a synergistic effect on flux through the TAG synthesis pathway, thereby increasing oil yields in camelina (Abdullah et al., 2023, under review).

The Koo lab has developed a transgenic plant system referred to as "416," designed for the rapid and significant induction of TAG (triacylglycerol) in vegetative tissues. Within this system, a plastid-localized phospholipase A1 protein, known for its involvement in jasmonic acid biosynthesis (Mulaudzi et al., 2023), is expressed under an inducible system. The induction of this gene resulted in a substantial increase in TAG levels in leaves. However, mutant lines expressing a modified version of *DAD1* at its active site failed to accumulate oil. When leaves were stained for neutral lipids, they revealed significant increases in oil bodies. In a direct comparison with seven previously published high-oil lines, 416 demonstrated higher TAG levels. Lipidomic analysis in collaboration with the Welti lab revealed that the increase in TAG comes at the expense of chloroplast galactolipids. FAME analysis in collaboration with the Allen lab indicated that the FA profiles in TAG from 416 resemble the leaf FA profile, which is distinct from the seed neutral lipid FA profile. Transient expression experiments in *N. benthamiana* and stable transformation of soybean lines both exhibited similar increases in biomass TAGs, underscoring the biotechnological potential of this system.

The Cahoon lab explored the feasibility of maximizing tocochromanol production in the oilseed crop camelina by combining seed-specific homogentisate geranylgeranyl transferase (HGGT) expression with increased biosynthesis and/or reduced homogentisate catabolism. Plastid-targeted *Escherichia coli* TyrA-encoded chorismate mutase/prephenate dehydrogenase and

Arabidopsis hydroxyphenylpyruvate dioxygenase (HPPD) cDNA were co-expressed in seeds to bypass feedback-regulated steps and increase flux into homogentisate biosynthesis. Homogentisate catabolism was also suppressed by seed-specific RNAi of the gene for homogentisate oxygenase (HGO), which initiates homogentisate degradation. In the absence of HGGT expression, tocochromanols were increased by ~2.5-fold with HPPD/TyrA co-expression, and ~1.4-fold with HGO suppression compared to levels in non-transformed seeds. No further increase in tocochromanols was observed in HPPD/TyrA lines with the addition of *HGO* RNAi. HGGT expression alone increased tocochromanol concentrations in seeds by ~four-fold to ≤1400 μg/g seed weight. When combined with HPPD/TyrA co-expression, an additional 3-fold increase in tocochromanol concentrations was obtained, indicating that homogentisate concentrations limit HGGT's capacity for maximal tocochromanol production. The addition of *HGO* RNAi further increased tocochromanol concentrations to 5000 μg/g seed weight, an unprecedented tocochromanol concentration in an engineered oilseed (Konda et al., 2023).

The Wang lab in collaboration with others has identified a lipid-metabolizing gene for non-specific phospholipase C4 (NPC4), which improves plant response to phosphate deficiency and enhances growth and seed production in oil seed crops, such as rapeseed (*Brassica napus*). NPC4 hydrolyzes phosphosphingolipids and phosphoglycerolipids in roots, with a greater change in glycerolipids than sphingolipids in leaves, under phosphate deficiency conditions. Increased *NPC4* expression led to the upregulation of genes involved in lipid metabolism, phosphate release, and phosphate transport and an increase in free inorganic phosphate in leaves in rapeseed (Yang et al., 2023).

Impact Statements

The LIPIDS of Crops multi-state research project has an overarching goal to increase the value of crop oilseeds by increasing seed oil content, making unusual and economically important fatty acids, finding new markets for existing or future vegetable oils and oilseed crops (e.g., camelina), and also adding value to the defatted meal particularly for niche crops like camelina. Each of these goals has the potential to impact the economy and move towards renewable energy independence. Additionally, LIPIDS of Crops is working to improve crop resilience to environmental stresses, including those associated with climate change. The NC-1203 group has interacted collaboratively to achieve project milestones during the year as indicated by milestones and 52 publications, 28 grant proposals funded, and 3 patents listed below, as well as standards and protocols that have been shared among participants. Future work will focus on completing the remaining and future milestones: See next page.

Milestones:

2023

● Development of on-tissue chemical derivatization for mass spectrometry imaging. The Lee group has optimized MALDI-MS imaging conditions to visualize camelina seed lipids. Additionally, an in vivo isotope labeling method is currently being developed for MS imaging.

● Identification of genes with altered expression in transgenic plants with modified lipid metabolism. Combined transcriptomic and lipidomic analyses of developing seeds identified differentially expressed genes and altered lipid molecular species correlated with the accumulation of high levels of acetyl-TAG in transgenic camelina lines.

● Transcriptomic studies of soybean lines engineered for enhanced fatty acid and TAG flux revealed a down-regulation of genes putatively encoding for oil body packaging proteins, and a strong induction of genes annotated as lipases and FA biosynthesis inhibitors.

●Work on surface lipids addressed the following 2023 milestones: Identification of gene transcripts and/or lipids with altered levels due to changing environmental factors in wildtype, mutant, and transgenic plants; characterization of genes involved in synthesizing surface lipids protective against environmental stresses; and engineered potato and camelina generated for enhanced suberin and other wound healing targets.

These milestones were largely met through quantitative analysis of the role of surface lipids in crop resilience against biotic and/or abiotic stress resulting in the following outputs, 1) targeted knockout of a transcription factor implicated to regulate wound suberin deposition led to unexpected phenotypes of delayed tuber sprouting and reduced shrinkage in cold storage (with a provisional patent); 2) specific weather parameters at specific stages in maize development were identified that impact cuticular wax composition on maize silks and will ultimately provide metabolite biomarkers for protective capacity against solar radiation and drought stresses; 3) a relationship between the cuticle and biotic stress response was established via observations that cuticle-related gene expression was altered upon *Fusarium* spp. infection of maize silks, and infection parameters were impacted by changes in cuticle composition in cuticle-related mutants.

● Quantitative analysis of the stress response of phloem lipid-binding proteins. A heatmap displaying the gene expression of 10 phloem lipid binding proteins as well as several phospholipases in response to multiple abiotic environmental factors has been generated. The gene expression studies together with localization and lipid-binding studies are currently being compiled into a manuscript. A second manuscript detailing the molecular dynamics simulations of the PLAFP-PA interaction is currently under review at *Plant Science*. Missense mutations that are predicted to affect PLAFP-membrane interactions were shown to be dispensable for PA

binding specificity. A computational approach will be taken to identify the amino acid requirements for interaction with PA.

● Characterization of the mechanism underlying subcellular localization of transcription factors in response to lipid changes. A predicted nuclear localization signal (NLS) was found to be necessary and sufficient for nuclear localization of two HD-Zip IV transcription factors. While the NLS overlaps with the DNA binding domain, mutant analysis shows that the two functions can be separated. Protein-protein interaction studies and mutant analysis indicate that an alpha importin is required for nuclear import.

● Lipid content has been quantified in camelina seeds engineered for reduced lipase activity and overexpression of acyltransferase genes. Putative Cas9-generated mutations in target lipases have also been identified in the T2 generation and will be confirmed in the T3 generation.

● Validation of ≥3 candidate genes for enhanced astaxanthin production in model systems. Camelina and soybean lines were generated that accumulate >100 μg/g astaxanthin per seed weight.

Milestone was met. Four candidate genes for the biosynthesis of astaxanthin and astaxanthin esters were identified in the transcriptome of astaxanthin-rich Adonis petals. Introduction of these genes into camelina under seed-specific promoters. These seeds accumulated astaxanthin in high purity to >200 μg/g seed weight.

● Phenotypic and genotypic data for sorghum lines engineered with first iteration of a vegetative oil construct completed. Sorghum lines developed that accumulate up to 7% DW TAG in leaves and 4% DW TAG in stems.

Milestone was met. Target levels in TAG in leaves and stems were met in field grown lines of engineered sorghum.

● Camelina with >5% higher seed oil content due to overexpression of the least abundant catalytic subunit to ACCase. Targeted quantitation of the catalytic subunits of the plant heteromeric ACCase showed that the alpha-carboxyltransferase subunit was up to 10-fold lower in absolute abundance than its partner subunit beta-carboxyltransferase during Arabidopsis seed development. It was hypothesized that overexpression of the alpha subunit could enhance ACCase activity, resulting in a greater “push” of carbon into de novo fatty acid synthesis and ultimately into storage lipids. Using the pea ortholog and expressing both the full length and catalytic region alone, ACCase specific activity and seed oil content in Arabidopsis were enhanced. The same constructs were expressed in camelina and an increase in seed oil content was observed, supporting the hypothesis that the alpha-carboxyltransferase subunit is an important and perhaps limiting subunit for ACCase activity.

● Evaluation of enzymes responsible for the novel TAG remodeling pathway in *Physaria fenderli:* Their use for controlling seed oil composition in engineered plants will be ascertained.

● Gaining insight on the genetic underpinnings that govern soybean seed protein and oil reserve content. Created a set of edited lineages in major seed protein storage gene models, which manifest a proteome rebalancing, these lines have been re-transformed with TRAP-seq reagents to allow for the monitoring of transcript level/ translational competence determination. Second set of edited soybean lines carrying INDELs in the gene model that underlies the major protein QTL on chromosome 20, and its paralog on chromosome 10. These lines are being sown across multiple field environments, and a series of complementation studies are ongoing.

● Identification of gene transcripts and/or lipids with altered levels due to changing environmental factors in wild type, mutant, and transgenic plants.

● Identification of a lipid, trigalactolipid (TGDG) that accumulates to different extents in response to survivable low temperatures in tolerant and non-tolerant species. Results in test species indicate non-linear evolution of the response, suggesting that the accumulation is not always adaptive (Barnes et al. 2023). Follow-up work investigates the response of TGDG in non-responding species, cotton.

● Investigation of the importance of diacylglycerol acyltransferases (DGATs) and phospholipid:diacyglycerol acyltransferases (PDATs) in response to low temperatures, finding that all these genes appear to be involved in TAG accumulation to measurable extents.

Publications

1. Abdullah HM, Pang N, Chilcoat B, Shachar-Hill Y, Schnell DJ, Dhankher OP. (2023) Overexpression of the phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT) gene increases carbon flux toward triacylglycerol (TAG) synthesis in *Camelina sativa* seeds. Plant Physiology and Biochemistry (resubmitted after 2nd major revision).

2. Arias CL, Quach T, Tu H., Nguyen H, Moretti A, Shi Y., Guo M, Rasoul A, Kyujung V, McHale, L, Clemente TE, Alonso AP, Zhang C. (2022) Expression of AtWRI1 and AtDGAT1 during soybean embryo development influences oil and carbohydrate metabolism, Plant Biotech J 20:1327-1345.

3. Azeez A, Parchuri P, Bates PD. (2022) Suppression of *Physaria fendleri* SDP1 increased seed oil and hydroxy fatty acid content while maintaining oil biosynthesis through triacylglycerol remodeling. Front Plant Sci 13:931310.

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9. Busta L, Dweikat I, Sato SJ, Qu H, Xue Y, Zhou B, Gan L, Yu B, Clemente TE, Cahoon EB, Zhang C. (2022) Chemical and genetic variation in feral Cannabis sativa populations across the Nebraska climate gradient. Phytochemistry 200:113206 DOI: 10.1016/j.phytochem.2022.113206

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Grants awarded

1. PI: Laura Bartley; Co-PI(s): Gary Stacey, Jay Thelen, Dong Xu; Agency: NSF; Program: PGR; Dates: 09/01/2020-08/01/2024; Title: RESEARCH PGR: Genome-enabled characterization of orphan receptor like kinases in plants; Total cost: $2,000,000.

2. PI: Lee, Young-Jin; Agency: NSF; Program: ISO-PGR; Dates: 02/01/2022-01/31/2025; Title: Mass Spectrometry Imaging of in vivo Isotope Labeling; Total cost: $635,498 (direct $466,167).

3. PI: Jiujiu Li; Co-PI(s): Rebecca Roston; Agency: NIH; Program: National Institute of Diabetes & Digestive & Kidney Diseases Dates: 04/01/2022-03/01/2025; Title: Role of chive-derived exosome-like nanoparticles in suppressing inflammation in obesity; Total cost: $1,734,810.

4. PI: Kathrin Schrick. Agency: NSF-MCB; Dates: 9/15/2016 - 8/30/2024; Title: START Lipid/Sterol Binding Domains in Homeodomain Transcription Factors from Plants; Total cost: $385,150.

5. PI: Abraham J Koo; Agency: National Science Foundation; Program: IOS; Dates: 01/01/2022-12/01/2024; Title: Regulation of the early steps of wound-activated jasmonate biosynthesis; Total cost: $846,276.

6. PI: Abraham J Koo; Agency: CAFNR Joy of Discovery (University of Missouri); Dates: 02/01/2023-1/31/2025; Title: Novel Ways to Increase Biomass Oil Production; Total cost: $17,412.

7. PI: Phil Lewis; Collaborator: Susanne Hoffmann-Benning; Agency: USDA; Program: APHIS; Dates: 09/30/2022-09/29/2024; Title: Identifying Attractive Components of Phloem Sap for Spotted Lanternfly Control; Total cost: $75,625.

8. PI: Sruthi Narayanan; Co-PI(s): Rustgi S (Clemson University); Collaborator(s): Fallen B (USDA-ARS Raleigh, NC), Smith J (USDA-ARS Stoneville, MS), and Welti R (Kansas State University); Agency: USDA NIFA; Program: AFRI Foundational Program ; Dates: 01/01/2022-12/31/2026; Title: Improving soybean’s efficiency for heat tolerance with an integrated metabolic and genetic approach; Total cost: $649,895.

9. PI: Marna Yandeau-Nelson; Co-PI(s): Basil J. Nikolau; Collaborator(s): Erin E. Sparks (University of Delaware); Rajib Saha (University of Nebraska); Agency: National Science Foundation; Program: MCB BIO; Dates: 08/01/2022-07/31/2025; Title: Collaborative Research: PlantSynBio: Deciphering the roles of genetic and biochemical redundancy and pathway regulation via refactoring the protective plant cuticle; Total cost: $2,647,000.

10. PI: Candice Hirsch; Co-PI(s): Marna Yandeau-Nelson; Agency: NSF; Program: PGRP; Dates: 10/01/2016-09/01/2023; Title: ECA-PGR: Dissecting Natural Mechanisms for Genome Content Variation and the Impact on Phenotypic Variation; Total cost: $2,198,800.

11. PI: Jay Thelen; Co-PI(s): Phil Bates, Abe Koo, Doug Allen, Dong Xu; Agency: NSF; Program: PGR; Dates: 11/01/2018-10/01/2022; Title: RESEARCH-PGR: Discovering new metabolic constraints and regulatory nodes in oilseeds engineered for enhanced fatty acid synthesis and seed oil; Total cost: $3,437,639.

12. PI: Rustgi S. (Clemson University); Co-PI(s): Narayanan S (Clemson University), Kulis M.D., Kim E. (both from The University of North Carolina), Dunne J.C., and Andres R. (both from North Carolina State University); Agency: Foundation for Food and Agriculture Research; Program: Seed the solutions; Dates: 01/01/2022-12/31/2026; Title: Reduced-immunogenicity high-oleic peanuts through applied innovation (genome-editing and breeding); Total cost: $609,816.

13. PI: Ed Cahoon; Co-PI(s): D Allen, P Bates, T Durrett, J Fox, M Gehan, T Joshi, C Lu, M Smanski, J Thelen, R Welti, D Xu; Agency: DOE; Program: Bioenergy Research; Dates: 10/01/2022-09/01/2027; Title: B5: Bigger Better Brassicaceae Biofuels and Bioproducts ; Total cost: $12,800,000.

14. PI: Fallen B. (USDA-ARS, Raleigh, NC); Co-PI(s): Narayanan S (Clemson University), Mian R (USDA-ARS Raleigh, NC), Walker D (USDA-ARS Raleigh, NC), Lorenz A (University of Minnesota); Agency: United Soybean Board; Program: ; Dates: 10/01/2021-09/30/2022; Title: Developing high protein, low oil content soybeans that provide enhanced nutritional and economic value; Total cost: $184,702.

15. PI: T. Durrett, co-PIs: YJ. Lee, U. Yucel. Agency: USDA-NIFA; Dates: 2020-2023; Title: Optimizing the temporal and spatial synthesis of functional lipids In developing seeds; Total cost: $500,000.

16. PI: E. Cahoon, co-PIs: A. Ramer-Tait, P. Verlander. Agency: USDA-NIFA Research and Extension Experiences for Undergraduates; Dates: 1/1/2022-12/31/2026. Title: Expanding Opportunities in Agricultural Sciences: Crop-to-Food Innovation; Total cost: $742,000.

17. PI: S. Sanjaya, co-PI: E. Cahoon. Agency: USDA-NIFA; Dates: 3/1/2022-2/28/2025. Title: Storage Compound Biosynthetic Mechanisms and Photosynthetic Processes for Enhancing Crops Yield; Total cost: $150,000.

18. PI: A, Leakey, Investigators: T. Clemente, E. Cahoon. Agency: DOE Bioenergy Research Center; Dates; 11/1/2017-10/30/2022. Title: Center for Advanced Biofuels and Bioproducts Innovation (CABBI); Total cost: $3,800,000 to UNL.

19. PI: Om P. Dhankher, coPIs: B Xing, J. White, V Dhandapani. Agency: NIH RO1 award; Dates: 03/09/2021 - 12/31/2025. Title: A novel strategy for phytoremediation of arsenic. (We are engineering non-food oil seed crop *Crambe abyssinica* for arsenic accumulation and detoxification). Total cost: $1,177,305.

20. PI: K. Glowacka, coPIs: R. Roston, N. Buan, J. Stone. Agency: Nebraska Agricultural Experiment Station (NEAES) ; Dates: 10/1/2022- 9/30/2027. Title: Regulation of Photosynthetic Processes (NC-1200); Total cost: $250,000.

21. PI: T. Durrett, co-PI: D. Allen. Agency: United Soybean Board; Dates 10/1/2021-9/30/2022. An Innovative 'Push-Pull-Protect' Approach to Improving Protein Quality. Total cost: $189,306

22. PI: P. Bates, co-PI: A. Azeez. Agency: USDA-NIFA 2/1/2023 - 1/31/2026. *Physaria fendleri* Crop Improvement by Gene Editing and Bioengineering for a Domestic High Yielding Source of Industrially Valuable Fatty Acids. Total Cost: $650,000.

23. PI: P. Bates, co-PIs: A. Smertenko, J. Shockey, D. Allen, J. Thelen. Agency: National Science Foundation. Dates 3/1/2023 - 2/28/2026. Beyond static metabolic maps - Understanding the cellular organization and dynamics of lipid flux for enhanced seed oil production. Total cost: $1,278,068.

24. PI: X. Wang, co-PI: B. Yang **(**MU/DDPSC). NSF NATL SCI FNDTN (8/1/2022 -7/30/2025) Collaborative Research: Mechanisms of pollen-specific phospholipases in maize haploid production. Total cost: $1,197,471

25. PI: R. Roston. USDA-AFRI; 2023 Plant Lipids: Structure, Metabolism and Function Gordon Research Conference & Seminar (10/1/2022 - 9/30/2023). Total cost: $10,000

26. PI: R. Roston. DOE BES/BER; 2023 Plant Lipids: Structure, Metabolism and Function Gordon Research Conference & Seminar (10/1/2022 - 9/30/2023). Total cost: $20,000

27. PI: R. Roston. NSF-MCB; 2023 Plant Lipids: Structure, Metabolism and Function Gordon Research Conference & Seminar (10/1/2022 - 9/30/2023). Total cost: $15,000

28. PI: R. Roston, coPIs M. Naldrett, B Altartouri. DOE-BES; Photosynthetic membrane lipid transport through chloroplast membrane contact site homologs. Total cost: $632,874

Software

None to report.

Patents

1. Koo AJ, Kimberlin A (2022) Genetic means to increase neutral oil in vegetative tissues by conditional induction of membrane lipid hydrolysis. US Patent #P13628US01

2. Neumann N, Nazarenus TJ, Aznar Moreno JA, Durrett TP, Cahoon EB (2023) Improved camelina plants and plant oil, and uses thereof. US Patent App. No. 17770466

3. Vulavala VKR, Santos P, Kosma DK (2023) Potato variety named 'UNR-01'. US Provisional Application #63/509898.