Fast growing high-yield wheat and canola for efficient nutrient recycling systems

Lawrence Berkeley National Laboratory
CRADA No. FP00005224

Sponsored by
NASA STTR FY17 Phase I Program
Project Duration:
10/01/2017-09/30/2019

Lawrence Berkeley National Laboratory (LBNL)
Joint Bioenergy Institute (JBEI)
Project Team: Christopher Petzold Ph.D.
5885 Hollis ST. ESE 4th Floor, Emeryville CA 94608

AFINGEN Inc
Project Team: Ai Oikawa, Ph.D. (PI). Yang Tian
6550 Vallejo ST., Emeryville CA 94608

SUMMARY

Wheat and canola are two major rotation crops in the U.S. each offering advantageous traits for the agricultural value chain. Canola, a dicot rotation crop, generally offers high seed oil content. Wheat, a monocot rotation crop, offers more cellulose and other nutritious carbohydrates. As rotation crops, traits such as rapid growth rates, increased harvest yields, and high quality (digestibility, deconstructability) are desirable for food, biorefinery, and also nutrient recycling for crop rotation. Improvement of such traits has been the goal of numerous biotechnology efforts. However, alternative biotechnology techniques are typically constitutive and when applied to commercial crops have resulted in net negative consequences. For example, efforts to improve quality by lowering lignin biosynthesis have resulted in plants with poor structural integrity and diminished mass at maturity. With supports by SBIR programs from DOE and USDA, JBEI/LBNL and AFINGEN previously demonstrated engineered healthy switchgrass and alfalfa which grew with 20-30% more fermentable sugar release, 25% less lignin, faster growth, and more biomass yields than control plants. In this CRADA project supported by NASA STTR program, AFINGEN again collaborated with JBEI/LBNL and transferred the simple tissue-targeting technology from the demonstrated switchgrass and alfalfa to the two major rotation crops wheat and canola for efficient nutrient recycling systems.

BACKGROUD

JBEI's mission is to do the fundamental research that is needed to enable the economically and environmentally sound conversion of biomass to advanced biofuels. The goal is to provide the nation with clean, renewable transportation fuels identical to gasoline, diesel and jet fuel. JBEI is focused on fundamental research and break-through inventions, while the ultimate commercialization of research should be done by companies. AFINGEN is a start-up company from JBEI, with a novel technology for engineering advanced feedstocks in ways that substantially increase yield. Among a suite of synthetic biology methods, Afingen's APFL technology offers a robust path to produce high-value biochemicals from inedible biomass-derived substrates with minimal cis-genetic manipulation and improved genetic stability compared to conventional bio-engineering. By amplifying and/or reducing target compounds with unprecedented specificity and improved tolerance, engineered food-, feed-, and biofuel crops (e.g. switchgrass, wheat, canola, corn, soybeans, alfalfa, tomato, potato) may offer higher yields of biomass and enhance degradation in the inedible biomass to facilitate nutrient recycling.

The technology was developed at JBEI and has been further developed by Afingen in collaboration with JBEI. The projects fit well into the mission of JBEI since the objective is to employ a novel, and possibly disruptive, synthetic biology technology that will deliver economically viable advanced biofuels and chemicals. As potential NASA commercial applications, the engineered crops will be able to provide food and be converted to advanced degradable feedstocks that provide nutrient recycling as next generation organic fertilizers. The proposed biotech platform would also allow variety of different crops to 1) grow better even at limited spaces and resources, 2) accelerate rooting systems compatible to microgravity, and 3) increase their photosynthetic organs (green vegetative tissues: leaves and stems) to convert carbon dioxide to oxygen for more sustainable and efficient cultivation systems on Mars. As potential non-NASA commercial applications enabled the combination of three beneficial traits, accelerated rooting growth, increased biomass, and enhanced (bio)-degradability of inedible biomass will contribute to U.S. agricultural production, self-sustainability, economy, food security, and bioenergy.

Project Key words: Biomass Growth, Crop Production, Essential Life Resources (Oxygen, Water, Nutrients), Food (Preservation, Packaging, Preparation), Remediation/Purification, Sources (Renewable, Nonrenewable), Waste Storage/Treatment

OBJECTIVE

- This CRADA project supported by NASA STTR program was aimed at generating significantly improved rotation crops wheat and canola with a combination of three beneficial traits: 1) accelerated rooting growth, 2) increased grain yield and vegetative biomass, and 3) enhanced degradability of inedible biomass, for efficient nutrient recycling. We intended to increase biomass and grain yield by at least 30%. An additional trait, 20% less lignin, was also anticipated to provide better degradability for nutrient recycling. Enabled by Afingen's biotechnology platform, the

rotation crop crops could be converted to advanced lignocellulosic biofuel feedstocks that are easier to process. The proposed fast-growth trait would also allow crops to reduce exposure to potential abiotic and biotic stresses.

- For the goal, main technical objective for early project duration was to generate to generate a total of eight genetic contracts incorporating designed traits for wheat and canola. A total of six constructs including one control is to be transformed to canola, and a total of four constructs including one control is to be transformed to wheat for production of transgenic lines expressing the genes of interest. The first generation of transgenic lines was characterized for viability, T-DNA integration and transgene expression, and for selected lines, detailed morphological and biomass quality/degradability analyses were performed. We aimed at identifying the two best strategies in each crop - canola and wheat - for validation of efficient nutrient recycling and cultivation system development during CRADA renewal project. In this project, we intended to increase yields of both the biomass and grain by at least 30% as well as reducing lignin by 20% for better nutrient recycling and cultivation systems.

ACCOMPLISHMENTS/DELIVERTABLES

- Products during this project are a series of DNA Plasmids - AFINGEN's binary backbone vectors (pAFINGEN) with kanamycin resistance selection marker for canola and hygromycin resistance selection marker for wheat. The vector derivatives with a series of combination of promoters and transcription factors genes were also produced through Gibson DNA assembly method.

- To record the assembly history and features, internal database of the DNA constructs was established. The Internal database also includes series of results from generated transgenic plants with growth, morphological and quality analyses.

- As an invention from this CRADA project, the results were also described in "*COMPOSITIONS AND METHODS FOR INCREASING PLANT GROWTH AND IMPROVING MULTIPLE YIELD-RELATED TRAITS*" U.S. Application No. 62/623,279; International Application No.: PCT/US2019/015688

- JBEI/LBNL and AFINGEN under the CRADA No. FP00005224: JBEI/LBNL's team lead in this CRADA project was Dr. Chris Petzold, who was also the team lead with our switchgrass and alfalfa projects. JBEI provided most of the laboratory space and state-of-the-art facilities and equipment needed for this project and plant growth chamber space for growing transgenic canola. High quality canola production in this project was enabled by a simple combinatory gene assembly, a strategy that has already been successfully applied in switchgrass and alfalfa.

Table 1. The CRADA project milestones and their completion time

Phase I	Year 1				Year 2			
Tasks/Milestones	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
1. - Generation of eight constructs combining targeted promoters and transcription factors	x	x						
2. - Transform wheat and canola plants with these constructs and regenerate transgenic plantlets in tissue culture expressing the genes of interest	x	x	x	x	x	x	x	x
3. - Characterization of the first generation of transgenic lines					x	x	x	x
4. - Detailed morphological and biomass quality analyses of selected lines						x	x	x
5. - Selection of the best two lines in canola for CRADA Renewal							x	x

1. - Generation of eight constructs combining targeted promoters and transcription factors (Afingen): We generated two binary vector backbone with a simple transcription factor cassette driven by the tissue targeting promoters. The promoters and codon optimized transcription factors were synthesized by GenScript. The cassettes were assembled by a PCR-based Gibson cloning, which is one of the most efficient assembly techniques, for modular recombination of a total of five TFs with four promoters. The assembling generated a total of 10 independent transformation plasmids for Agrobacterium-mediated transformation of plants. Five combinations and one vector control was integrated into dicot binary vector for canola transformation, and three combinations and one vector were integrated into monocot binary vector for wheat transformation. The generation of eight different strategies allowed us to identify the best combination of traits for detailed characterization. The master destination vector contained a spectinomycin resistance marker for selection in E. coli, and hygromycin/kanamycin resistance genes for selection of transformed plants. Positive clones were confirmed by sequencing the insert regions before being sent to the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (UCD) for introduction into Agrobacterium tumefaciens strain EHA105 by electroporation. Transformed A. tumefaciens cells was used for co-culture with phytoembyogenic calli for plant transformation, as described below. All cloning reactions, plasmid preparations, as well as selection of positives clones in E. coli and A. tumefaciens was carried out at JBEI by Afingen employees.

2. and 3. - Transform canola and wheat plants with these constructs and regenerate transgenic plantlets in tissue culture expressing the genes of interest. Characterization of the first generation of transgenic lines (Afingen): All 10 constructs will be sent to a third party, the Ralph M. Parsons Foundation Plant Transformation Facility at UCD. At the late middle

of this CRADA project, we had all 10 transformations at the regeneration stage. Since the transformation was carried out at the transformation facility within California, where Afingen is located, the Interstate Transit Permit from USDA Animal and Plant Health Inspection Services was unnecessary for this project. For the 10 transformations, we observed whether or not plants are able to regenerate in tissue culture. During this tissue culture period, regeneration speeds and regeneration growths were measured by comparing with vector controls as preliminary phenotyping analysis. For the regenerated lines, we genotyped each plant by PCR using genomic DNA and primer pairs that are specific for the hygromycin/kanamycin and engineering cassettes. This confirmed whether hygromycin/kanamycin-resistant plants in tissue culture are transgenic and contain the appropriate cassettes from Afingen's constructs. We extracted RNA from PCR positive lines to see expression of the transgenes in older leaves using Q-PCR, since selected promoters should be expressed in both leaf vasculature and stems. The viability screens, genotyping and Q-PCR experiments were all carried out at JBEI by Afingen employees. As lines selected from tissue culture, they were transferred to soil and kept in plant growth chambers and/or the growth room at JBEI and greenhouse facility at UC Berkeley (UCB). The transformants and the empty vector controls were phenotyped on the basis of plant height, stem and root length, and the number of lateral tissues will be measured in order to get an initial evaluation of plant biomass yield. The work was also performed by Afingen employees who are also responsible for watering, fertilization, pest control and overall plant care at JBEI and UCB.

4. - Detailed morphological and inedible biomass quality analyses of selected lines (Afingen and JBEI/LBNL): During this CRADA project, 14-20 months were devoted not only to generating mature plants from these lines, but also to initial characterization of biomass properties including degradability. Whole stem tissue samples from transgenic plant lines were then taken from equivalent side lateral stems at a stage immediately after formation of the first flower (approximately three months from soil transfer). At this stage, sufficient fiber formation occurred in the lateral tissues. These samples was used for biochemical characterization analyses. Lateral stem and root samples will be ground and assayed for polysaccharide, lignin and other metabolite analyses using standard protocols. Total polysaccharide content and composition were determined on cell wall preparations (also known as alcohol insoluble residue, or AIR) after sulfuric acid treatment as previously described for total sugar hydrolysis prior to monosaccharide analysis. For detailed carbohydrate analysis, cell wall preparations digested with trifluoroacetic acid to assay for monosaccharide composition of the matrix polysaccharide fraction of the cell wall (pectins and hemicelluloses) were carried out using high-performance anion exchange chromatography. Cellulose content will be determined by the Updegraff method. Detailed lignin analysis was performed by measuring both content (using the Klason lignin method) and S/G ratio using a standard procedure as described previously. During this project, the PI and Afingen technician carried out plant maintenance and tissue collection, cell wall analysis, mechanical strength testing, biomass substrate release experiments and all other lab work at UCB and JBEI. Throughout the life of the project, the PI coordinated all research activities and was responsible for project management, milestone tracking and writing of reports for NASA. The PI was responsible for coordination of the use of facilities and equipment by interfacing with the UCB, JBEI, and the Ralph M. Parsons Foundation Plant Transformation Facility at UCD.

5. - Selection of the best two lines in canola for CRADA renewal project (Afingen and JBEI/LBNL): Based on results from the plant phenotyping, morphology studies, and inedible biomass quality analyses, we selected the best-performing strategies using the following criteria: 1) Increased biomass accompanied by fast growth and 2) Reduced lignin content and enhanced biomass degradability. The best two strategies out of the eight were selected for validation of the traits in the second generation during new two years CRADA project. We generated biological replicates of the best lines within each strategy by events and clonal productions, which allowed us to better quantify the differences between strategies and the vector control lines.