ENGINEERING THE OPTIMIZED BIOFUEL CROP

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PROJECT TITLE: Engineering Enzymes in Energy Crops
PROGRAM: OPEN 2009
AWARD: $3,914,527
PROJECT TEAM: Agrivida (Lead), University of Illinois at Urbana-Champaign
PROJECT TERM: January 2010 – March 2015
PRINCIPAL INVESTIGATOR (PI): Michael Raab

TECHNICAL CHALLENGE

Biofuels offer alternative approaches to transportation fuels that support U.S. energy security, reduce dependence on energy imports, and can reduce net greenhouse gas (GHG) emissions dramatically. Domestically produced biofuels must address challenges in the sustainable production of bio-energy crops, as well as be cost-effective. Sustainability requires crops with high yields per land area, the ability to use land that is less useful for food crops, and reduced agronomic inputs (e.g. water, fertilizer, etc.). One approach to achieving this goal is to use cellulosic biomass, the stems and leaves of plants, as a feedstock for biofuels. However, cellulose is much more difficult to chemically process into biofuel compared to corn grain, and this challenge needs to be addressed to decrease the capital and operating costs of cellulosic biorefineries.

TECHNICAL OPPORTUNITY

Advances in computational biology and structural biology have allowed researchers to rationally design enzymes with specific properties to optimize them for applications such as biomass deconstruction and fermentation. These technologies are allowing researchers to incorporate engineered enzymes into plants to produce enhanced bioenergy crops. Switchgrass, *Panicum virgatum*, is grass native to the United States (Figure 1) and was identified by the Department of Energy as one of the high priority crops for development as a dedicated bioenergy crop. Switchgrass is capable of high biomass yields with low levels of agronomic input, and because it is a perennial crop with a deep root system, it can effectively sequester carbon below ground. Lastly, the efficiency of genetic transformation for switchgrass has improved significantly, allowing researchers to routinely engineer desired traits into the plant.

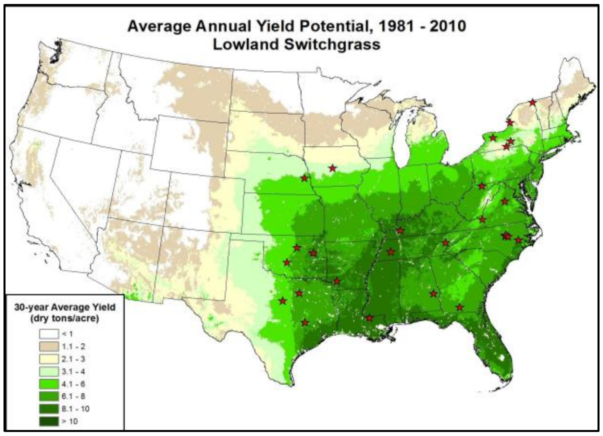

Figure 1. Potential yield mapping of switchgrass, as determined by the PRISM Climate Group (*energy.gov/sites/prod/files/2014/11/f19/daly_biomass_2014.pdf)*

INNOVATION DEMONSTRATION

Agrivida's goal was to develop plant varieties that would be better feedstocks for cellulosic biofuels. Prior to the ARPA-E award, enzymes had been isolated from microbes that naturally degrade the cell walls in plant biomass into fermentable sugars, but these enzymes are costly to produce. Agrivida proposed to engineer plants so they directly produce these cell wall degrading (CWD) enzymes *in planta,* significantly decreasing the cost of enzyme production and improving their efficiency by placing the CWD enzymes next to the plant cell wall. The goal was to deliver switchgrass varieties expressing CWD enzymes and demonstrate how this biomass can be hydrolyzed with less severe pretreatment conditions. To achieve the project goals, Agrivida first had to optimize the enzymes for performance *in vitro*, and then demonstrate the transgenic switchgrass lines had improved processing.

Agrivida optimized the CWD enzymes through protein engineering so that they would be inactive during the life of the plant, protecting the plant from being damaged by the CWD enzymes while it was growing. This was done by inserting a blocker (intein) into the enzyme. The intein would be deactivated by a high temperature (> 60°C) trigger after harvest, and yield a functional CWD enzyme. Agrivida tested 157 intein variants. The best blocker candidate reduced enzyme activity 10-fold when present in the enzyme, but yielded almost 80% of the activity of the native protein after heat treatment (Figure 2). The most promising inteins were engineered into a variety of CWD enzymes and transformed into switchgrass and corn.

After promising results in greenhouse studies, Agrivida obtained an environmental release permit from the United Sates Department of Agriculture (USDA) Animal and Plant Health Inspection Service to perform a field trial with two varieties of intein-CWD containing switchgrass. The transgenic switchgrass with the CWD enzyme grew as well as the native Alamo control variety. Pilot scale processing at both Agrivida's laboratory and a collaborator at the University of Illinois demonstrated that with only 20% of the normal full enzyme cocktail, the two transgenic switchgrass lines achieved the theoretical glucose yields of 68-71%. This compared with 52% for the negative control Alamo switchgrass line, represented a 40% improvement in glucose yield over the control Alamo biomass (Figure 3).

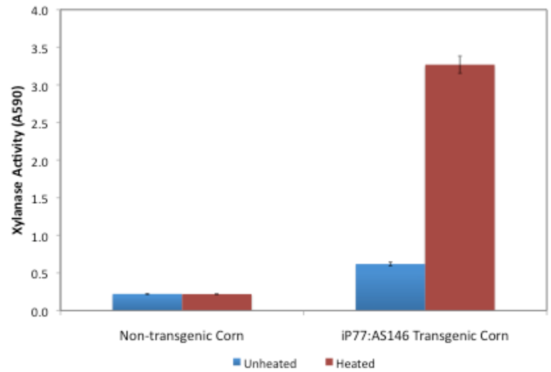

Figure 2. Level of enzymatic activity of control corn compared with transgenic intein-CWD corn. Samples were heated to 60°C to excise the intein and activate the enzyme.

The project demonstrated both Agrivida's intein technology to control activity of enzymes *in planta*, and the effectiveness of embedding these intein-CWD enzymes in the plant to reduce recalcitrance of plant biomass. By modeling the state-of-the-art cellulosic biorefining process in 2015, Agrivida estimated that ethanol produced from its intein-CWD switchgrass would cost approximately $0.80 less per gallon from a conventional switchgrass feedstock, a reduction of 30% of the total cost.

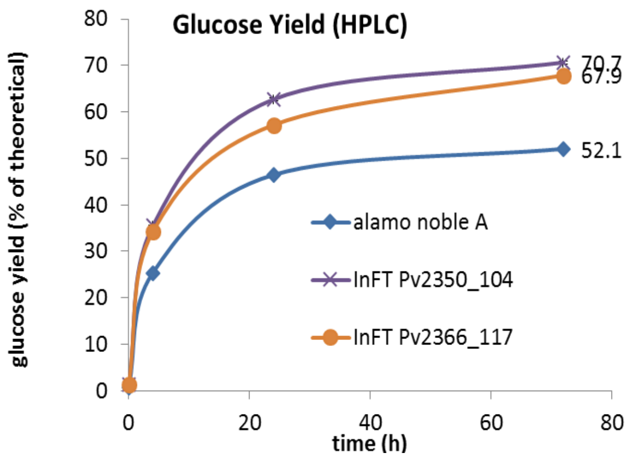

Figure 3. Level of glucose released using reduced enzyme loading from the biomass of two intein-CWD switchgrass compared to wild type Alamo. Percent glucose yield is calculated relative to the theoretical yield from the total cellulose in the plant.

PATHWAY TO ECONOMIC IMPACT

In 2012, Agrivida established a joint development agreement with POET Research, Inc., one of the world's largest ethanol producers. POET worked with Agrivida to utilize Agrivida's technology platforms with the goal of significantly reducing the capital and operating costs of commercial cellulosic ethanol production facilities. However, with low oil prices, the economics of cellulosic ethanol production have been too challenging for POET to deploy Agrivida's enhanced crop feedstocks at scale in its biorefineries.

Agrivida is therefore developing an alternative path to first market entry, by providing enzymes in grain that are routinely added to animal feed products to improve the digestibility or increase the nutritional content, and consequently reduce the energy impacts of food production. When applied externally to animal feed, cell wall hydrolyzing enzymes improve the conversion efficiency of the feed by animals, reducing the quantity of feed needed, along with the energy used to produce the crops for feed. Agrivida has performed animal feeding studies following the completion of its ARPA-E award. These studies indicated that Agrivida's *in planta* enzyme technologies provide value to feed, and Agrivida is targeting the commercial release of feed products in the next two years, including those incorporating intein variants based upon work under its ARPA-E award.

Since Agrivida received its ARPA-E award, it has closed four rounds of private funding, totaling $61M, to further develop and commercialize its technologies for the fuel and feed markets.

LONG-TERM IMPACTS

Agrivida's intein-CWD enzyme enhanced feedstocks have demonstrated their efficacy in easing the processing of cellulose post-harvesting, while still allowing healthy plant growth. Success in the first-market strategy outlined above is expected to provide a foundation for future commercialization efforts in advanced biofuels and bio-based products. Agrivida's enhanced crops have been field tested, and its biomass demonstrated at the pilot scale. When the economics of cellulosic biofuel production become more favorable, Agrivida's feedstocks will be ready for scale-up and commercial deployment. Furthermore, Agrivida's enhanced crops could become a significant feedstock for animal feed and their improved efficiency as feed could both reduce the energy inputs in meat production and the acreage needed to grow the feed.

INTELLECTUAL PROPERTY

As of December 2016, the Agrivida team has generated four invention disclosures, three patent applications with the U.S. Patent and Trademark Office (PTO) and two patents:

AGRPT010.2WO, "*Intein-modified enzymes, their production and industrial applications*". U.S. Patent No. 9,464,333.

AGRPT015.1WO, "*Consolidated Pretreatment and Hydrolysis of Plant Biomass Expressing Cell Wall Degrading Enzymes*". U.S. Patent No. 9,249,474.