Title
Fast-growing high-yield forage crops via a novel biotechnology platform

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Fast-growing high-yield forage crops via a novel biotechnology platform

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SUMMARY

Alfalfa and sorghum are two major forage crops in the U.S. each offering advantageous traits for the agricultural value chain. Alfalfa, a perennial dicot, generally offers high protein content. Sorghum, an annual monocot, offers more cellulose and other nutritious carbohydrates. As forage crops, traits such as rapid growth rates, increased harvest yields, and high quality (digestibility) are desirable. 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 support by DOE SBIR fast-track program FY14, LBNL and AFINGEN previously demonstrated engineered healthy switchgrass plants which grew with 22% to 54% more fermentable sugar release, 25% less lignin, faster growth, and more biomass yields than control plants. In this CRADA project, AFINGEN collaborated with JBEI/LBNL again and have transferred the simple tissue-targeting technology from the demonstrated switchgrass to two forage crops, alfalfa and sorghum. In addition to characterizing biomass quality and quantity in transgenic plants, RNAseq detected significant increase of a series of secondary metabolite enzyme gene expression specific to engineered lines, suggesting that enhanced secondary metabolite production contributes to fast-growth and high yielding trait.

OBJECTIVE

• This CRADA project was aimed at generating significantly improved forage alfalfa and sorghum with a combination of three beneficial traits - accelerated growth, increased biomass per acre, and improved digestibility (high quality). Enabled by Afingen's biotechnology platform, the high quality animal feeds could also 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 objective for early project duration was to generate a total of 8 constructs combining targeted promoters and transcription factors for alfalfa and sorghum. A total of 6 constructs including one control was transformed to alfalfa, and a total of 4 constructs including one control was transformed to sorghum for production of transgenic lines expressing the genes of interest. The first generation of transgenic lines was characterized by Afingen and JBEI/LBNL for viability, T-DNA integration and transgene expression, and for selected lines, detailed morphological and quality analyses performed. We identified the two best strategies in each crop - alfalfa and sorghum - for validation and additional beneficial trait development during future two years CRADA project from USDA SBIR Phase II.

ACCOMPLISHMNETS/ DELIVERTABLES

• Products during this project are a series of DNA Plasmids - AFINGEN's binary backbone vectors (pAFINGEN) with kanamycin resistance selection marker for alfalfa and basta resistance selection marker for sorghum. Also the vector derivatives with a series of combination of promoters and transcription factors genes were 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.

LBNL/JBEI and AFINGEN Inc. under the CRADA No.FP00004847. JBEI/LBNL PI in this CRADA project is Dr. Chris Petzold, who was also PI with our switchgrass project for metabolite analysis. 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 alfalfa. High quality alfalfa and sorghum production in this project was enabled by a simple combinatory gene assembly, a strategy that has already been successfully applied in the energy model crop switchgrass.

Table 1. The CRADA project milestones and their completion time

	Work Done by:		Time-frame with each Milestone											
			2017									2018		
Task/Milestone	JBEI/ LBNL	AFINGEN	4	5	6	7	8	9	10	11	12	1	2	3
1. Generation of 8 constructs		AO	x	x										
2. Transform alfalfa and sorghum plants		AO	x	x	x	x	x	x	x	x				
3. Detailed morphological, biomass quality, metabolite analyses	CP	AO								x	x	x	x	x
4. Selection of the best two lines in alfalfa and sorghum	CP	AO											x	x

1. Generation of 8 constructs combining targeted promoters and transcription factors (Afingen): We generated one binary vector backbone with a simple transcription factor cassette driven by the tissue targeting promoters. The cassettes was assembled by a PCR based Gibson cloning, which is one of the highest efficiency assembly techniques, for modular recombination of a total of 5 TFs with 4 promoters. This 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 alfalfa transformation, and three combinations and one vector were integrated into monocot binary vector for sorghum transformation. The generation of 8 different strategies allowed us to better identify the best combination of traits for detailed characterization. Promoters and codon optimized transcription factors were synthesized by Genscript. Afingen synthesized dicot promoters and transcription factors genes for alfalfa and monocot genes for sorghum constructs.

2. Transform alfalfa and sorghum plants with these constructs and re-generate transgenic plantlets in tissue culture expressing the genes of interest (Afingen): All 10 constructs was sent to two third parties, the University of Missouri Plant Transformation Core Facility and the Ralph M. Parsons Foundation Plant Transformation Facility at University of California Davis (fee for service facilities). We generated 5 independent lines per transformation, which was kept in tissue culture until delivery to Afingen. At the late middle of this CRADA project, we had all 10 transformations at the re-generation stage. Plant viable lines was completed using our three-screen procedure (viability, T- DNA integration and transgene expression). USDA-APHIS permit was successfully approved and obtained for transportation of generated plants from Missouri to California.

3. Detailed morphological and biomass quality analyses of selected lines (Afingen and JBEI/LBNL): We had a total of 8 different strategies combining the promoter and transcription factor, and one empty vector control. During this CRADA project, 12 months was devoted not only to generate mature plants from these lines, but also to initial characterization of morphology, biomass properties and forage quality analysis. Whole stem tissue samples from transgenic plants lines were taken from equivalent side lateral stems at a stage immediately after formation of the first florets (approximately 3 months from soil transfer). At this stage, sufficient fiber formation occurred in the lateral tissues. These samples were used for biochemical characterization. For biochemical characterization of biomass, lateral stem and root samples was ground and assayed for polysaccharide and lignin analyses using standard protocols. Total polysaccharide content and composition was determined on cell wall preparations 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 (TFA) to assay for monosaccharide composition of the matrix polysaccharide fraction of the cell wall (pectins and hemicelluloses) were carried out using HPLC and GC/MS. Cellulose content was 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.

4. Selection of the best two lines in alfalfa and sorghum for CRADA renewal project (Afingen and JBEI/LBNL): Based on results from the plant phenotyping, morphology studies, and biomass quality analyses, we selected the best performing strategies using the following criteria: (i) Increased biomass accompanied by fast growth and (ii). Reduced lignin content and enhanced biomass deposition. 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 allow us to better quantify the differences between strategies and the vector control lines. . In addition to characterizing biomass quality and quantity in transgenic plants, RNAseq detected significant increase of a series of secondary metabolite enzyme gene expression specific to engineered lines, suggesting that an increase of secondary metabolites contributes to fast-growth and high yielding trait.