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STI PRODUCT TITLE

IDENTIFYING GENES CONTROLLING FERULATE CROSS-LINKING FORMATION IN GRASS CELL WALLS

STI PRODUCT TYPE

Final report

STI PRODUCT REPORTING PERIOD

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DOE Plant Feedstock Genomics

SUBJECT CATEGORIES

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DESCRIPTION/ABSTRACT

This proposal focuses on cell wall feruloylation and our long term goal is to identify and isolate novel genes controlling feruloylation and to characterize the phenotype of mutants in this pathway, with a spotlight on cell wall properties.

We have shown previously that the expression of an *Aspergillus niger* ferulic acid esterase (FAEA) in different grass species resulted in a substantial reduction in cell-wall-esterified ferulates and diferulates with significant impact cell wall hydrolysis, resulting in increased yield of reducing sugars by cellulase treatment as well as increased digestibility, reinforcing the importance of feruloylation and cross linking for cell wall degradability.

Currently, the genes underlying AX feruloylation have not been identified and the isolation of such genes could be of great importance in manipulating ferulates accretion to the wall. Mutation of the feruloyl transferase gene(s) should lead to less ferulates secreted to the cell wall and reduced ferulate cross-linking. Our current research is based on the hypothesis that controlling the level of total feruloylation will have a direct impact on the level of cross-linking and in turn impact biomass utility for forage and biofuel production. To identify these genes we are taking a forward genetic approach combined with a spectroscopic screen followed by detailed genetic and phenotype analyses and have chosen *Brachypodium distachyon* as our model grass system. We are also taking a Bioinformatics approach to try to identify and test putative candidates for AX feruloyl transferases (FTs) in *Brachypodium*.

Our results/accomplishments for this project so far include:

1. ***Mutagenised Brachypodium population***. We have developed EMS mutagenized populations of model grass species *Brachypodium distachyon*. EMS populations have been developed from over 28,000 mutagenized seeds generating 5,184 M2 families. A total of 20,793 plants have been screened and 1,233 were originally selected.
2. **Selected Brachypodium mutants**: Potential mutants on their *levels of cell wall ferulates and cell wall AX* – have been selected from 708 M2 families. A total of 303 back-crosses

to no-mutagenized parental stock have been done, followed by selfing selected genotypes in order to confirm heritability of traits and to remove extraneous mutations generated by EMS mutagenesis. We are currently growing 12 F5 and F6 populations in order to assess CW composition. If low level of ferulates are confirmed in the candidate lines selected the mutation could be altered in different in one or several kinds of genes such as genes encoding an AX feruloyl transferase; genes encoding the arabinosyl transferase; genes encoding the synthesis of the xylan backbone; genes encoding enzymes of the monolignol pathway affecting FA formation or genes encoding transcription factors that control feruloylation. So it will require further investigations to confirm if we have a mutation on the ferulloyltransferase gene(s). We have also identified severe phenotypes which showed a significant change in the level of cell wall ferulates and sugars and have not survived. As this genotype did not reach flowering stage there was no seed production and so further analysis could not be done.

3. **Candidate Gene Approach**: Because of the likely long time expected to generate and identify candidate with mutation(s) on the feruloyltransferase gene, from our screening, we have in addition taken a bioinformatics approach in order to try to identify candidates gene(s) involved in feruloylation. Homologues of the rice feruloyl transferase genes belonging to Pfam PF02458 family were identified in *Brachypodium distachyon* by blasting EST sequences of putative rice arabinoxylan feruloyl transferase genes against *Brachypodium* and homologous sequences identified were tested for their expression level in *Brachypodium*. Sequences of the two *Brachypodium* genes, which showed highest expression and similarity to rice sequences, were used to design primers for construction of RNAi and over-expression vectors. These were transformed into *Brachypodium* using *Agrobacterium* transformation and plants generated have been analyzed for levels of cell wall ferulates and diferulates over generations T0 to T2 or T3. Our data shows a significant reduction if ferulates monomers and dimers from plants generated from RNAi::BdAT2 over 2-3 generations indicating that this gene might be a positive candidate for feruloylation in *Brachypodium*. However when BdAT2 was up regulated there was not much increase in the level of ferulates as would be expected. This lack of effect on the level of cell wall ferulates could be due to the *CaMV::35S* promoter used to drive the expression of the putative BdAT2 gene. We have shown

previously that *Aspergillus* FAEA expression in tall fescue under *CaMV::35S* resulted in 1.9 fold decrease in activity compared to activity when FAEA was driven by the rice actin promoter (Buanafina et al., 2008) and indicates that the CaMV*::35S* promoter might not be ideal to drive gene expression in grasses. Our results also shows that the level of cell wall esterified ferulates and diferulates did not change when BdAT8 was down regulated indicating that wall ferulates indicating this is not a feruloyl transferase candidate. We are currently preparing plant material from these selected transgenic plants to assess putative feruloyltransferase transcripts levels among different transgenic lines produced.

4. **Cell Wall Characterization of *Brachypodium accessions***. We have also been assessing how the level of cell wall esterified ferulates and diferulates, arabinoxylan, lignification and cellulose mediated sugar release varies among different *Brachypodium* diploid and tetraploid lines at different stages of development. Considerable variation has been found for the different cell wall components studied. We have also found significant variation for cellulase-mediated release of sugars from leaves of different *Brachypodium* accession lines. This will give us a good ground to assess the mutants and will be useful for producing a mapping population. The analyses are still ongoing as plant material needs to collected from different genotypes at the flowering stage for cell wall characterization. We aim to have this study completed and published.

5. Based on our previous findings where FAE expression *in planta* resulted in increased cell wall degradability and the positive synergism between FAEA and xylanase, we have tested if **co-expression of FAEA and XYN2 *in planta*** could improve the digestion of polysaccharides and increase cell wall degradability and post harvest cell wall deconstruction in grasses more effectively than expression of xylanase (Buanafina et al., 2012) or FAE (Buanafina et al. 2008, 2009, 2010) alone. As such we have studied the effects of constitutive co-expression of FAEA in the vacuole or apoplast combined with senescence inducible expression of XYN2 in the apoplast, on plant growth, levels of cell wall hydroxycinnamic acids (HCAs) and cell wall sugar composition, lignification and cell wall degradability of tall fescue (as we had this material available). FAE+XYN expression showed to be more efficient than FAE or XYN expression alone in changing

HCA and AX levels, increasing cell wall *in vitro* dry matter digestibility, and increasing release of reduced sugars when plants were treated with cellulase compared with sugar release by the action of cellulase alone. These results reinforce the role of cell wall ferulates in hindering cell wall degradability and the relevance of such approach for the bioethanol industry. An additional interesting aspect of the work is that it showed that FAEA+ XYN2 expression *in planta* does not alter total Klason lignin levels but significantly increases the level of acetyl bromide soluble lignin from about 56% in controls for up to 86 % in some plants co-expressing FAEA +XYN with a significant negative correlation between ferulates and acetyl lignin. **THESE RESULTS ARE BEING WRITTEN FOR PUBLICATION.**

6. **New protein identified**: In the course of our work we have also identified a new protein (enzyme). We have characterised the novel enzyme in maize and this **WORK IS CURRENTLY BEING WRITTEN FOR PUBLICATION.** We have also attempted to identify the putative gene sequence in maize and respective UF*Mu* seed stocks with *Mu* inserts in each of the putative sequences and one Mu illumina seed stock with inserts common to all four putative genes identified. These seeds have been ordered from MaizeGDB.org and are to be tested. It will be important to clone the gene in order to study the its role in plant processes. As a longer term goal it might also be possible to over express the cloned gene in the cell wall of maize or other biofuel crops, aiming to improve their degradability as it might be more effective than the fungal enzymes at degrading maize lignocellulose if overexpressed in the cell wall.

Deliverables

- ***Publications supported by this grant:***

Buanafina, M.M. de O. (2009). Feruloylation in Grasses: Current and Future Perspectives. **Molecular Plant,** 2:861-872.

Buanafina, M. M. de O., Langdon, T., Hauck, B., Dalton, S., Timms-Taravella, E. and Morris, P. (2010). Targeting expression of a fungal ferulic acid esterase to the apoplast, endoplasmic

reticulum or golgi can disrupt feruloylation of the growing cell wall and increase the biodegradability of tall fescue (*Festuca arundinacea*): **Plant Biot. J**. 8:316-33.

Michael L. Robbins, Ansuman Roy, Po-Hao Wang, Iffa Gaffoor, Rajandeep S. Sekhon, **Marcia M. de O. Buanafina**, Jai S. Rohila, Surinder Chopra (2013). Comparative Proteomics Analysis by DIGE and iTRAQ Provides Insight into the Regulation of Phenylpropanoids in Maize. Journal of Proteomics (In Press http://dx.doi.org/10.1016/j.jprot.2013.06.018))

- **Publications in Preparation**

Marcia M. de O. Buanafina, Sue Dalton, Tim Langdon, Emma-Timms-Tavarella, Erica A. Shearer, and Phillip Morris. Improved post harvest cell wall deconstruction of tall fescue by co-expression of a fungal ferulic acid esterase with a β1-4-endoxylanase.

Marcia M. de O. Buanafina, Mandeep Sharma, Howard W. Fescemyer and Erica A. Shearer. Functional testing of two PF02458 homologues of putative rice arabinoxylan feruloyl transferase genes in *Brachypodium distachyon.*

Marcia M. de O. Buanafina and Erica A. Shearer. Novel protein identified in maize and other grasses.

- **Presentations/ Posters presented at major scientific meetings**:

'Identifying genes controlling feruloylation in grass cell walls'.
Genomics: GTL Annual Contractor-Grantee Workshop held jointly with the USDA-DOE Plant Feedstock Genomics for Bioenergy awardees meeting. February 8-11/**2009**, Bethesda, Maryland.

'Identifying genes controlling feruloylation in grass cell walls'. The American Society of Plant Biologists (ASPB). July 18-22, **2009** Honolulu, Hawaii at the 'Bioenergy Crops and Biofuels' section

"Identifying genes controlling feruloylation in grass cell walls". Plant & Animal Genome Conference XVIII **and Plant Feedstock Genomics for Bioenergy Workshop** January 9-13, **2010.** Town & Country Hotel - San Diego, CA. Poster

"Identifying genes controlling feruloylation in grass cell walls". Genomic Sciences Contractor-grantee Meeting IX USDA-DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting April 10-13, **2011**.Cryatal City, Virginia.

"Brachypodium distachyon-pathogen interactions: exploring the cell wall barrier and defense-related compounds". ASPB July 20-24, **2012**, Austin TX. **Presentation and Poster**

"Expression of putative arabinoxylan feruloyl transferase genes in *Brachypodium distachyon*" Genomic Sciences Contractor-Grantee Meeting XI USDA-DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting 2013 February 24-27, **2013** Bethesda, MD. Poster

"Differential responses *of Brachypodium distachyon"* genotypes to fungal pathogens: exploring the cell wall barrier and defense-related compounds. First International Brachypodium Conference. June 19-21, **2013**. Modena, Italy. **Presentation**

"Ferulates in Cell Walls of Forage Grasses: Their Significance for Wall Degradability and Resistance to Insects and Pathogens". Plant Pathology and Environmental Microbiology Fall Seminar Series 13 October **2013**, The Pennsylvania State University. **Invited Presentation**.

- **Training**

Technical personnel:

Sharon Hoover: *Brachypodium* mutagenesis, growing mutagenized lines; plant harvesting sample preparation and spectroscopy-based leaf assay screening, cell wall preparation and ferulate composition analysis; *in vitro* cellulase assays.

Ruth Haldeman (replaced Sharon Hoover who resigned for illness): growing mutagenized *Brachypodium* populations, spectroscopy-based leaf assay screening, cell wall preparation and ferulates composition analysis; cell wall sugar analysis; *in vitro* cellulase assays; microscopy.

Erica Sheere (replaced Ruth Haldeman whose performance was well bellow expectation): growing, harvesting and preparing cell wall material from *Brachypodium* mutagenisded populations, spectroscopy-based leaf assay screening*;* growing *Festuca* transgenic lines co-expressing FAE and XYN, harvesting and cell wall material preparation for sugar and ferulates

analysis, *in vitro* assays such as cellulase digestion; Growing and harvesting RNAi and overexpressing putative feruloyl transferase *Brachypodium* lines for sugar and ferulates analysis, in vitro assays such as cellulase digestion, lignin quantification, RNA extraction, cDNA synthesis and RTPCR setup; Growing maize, pollinating; protein extraction, purification, quantification (Bradford, SDS, ammonium sulfate precipitation).

Students:

Andrea Hendershot: growing mutagenized *Brachypodium* population, cell wall preparation screening for changes in cell wall ferulates and degradability.

Nil Dhanani:, Growing *Brachypodium*, harvesting and cell wall preparation for cell wall ferulates composition analysis, spectroscopy-based leaf assay screening and cellulase digestion assay

Anthony Rosario: Growing mutagenized *Brachypodium* populations, cell wall preparation for screening for changes in cell wall ferulates; microscopy/ lignin staining.

Kathleen Tanner: growing *Brachypodium*, embryo culture and *Agrobacterium* transformation

David Apont: Growing *Brachypodium*, harvesting plant material and cell wall preparation and *in vitro* assays such as cellulase digestion and measurement of reduced sugars released.

Brandon Armsted: Growing *Festuca* plants, cell wall preparation for sugar analysis, separation and sugars quantification.

Maria Frrnanda Buanafina Maia: protein extraction, quantification, growing *Brachypodium*, embryo culture and *Agrobacterium* transformation.

Vishakha Mahajan: growing maize, pollination, DNA extraction and PCR.

Postdoctoral Fellow:

Mandeep Sharma: mutagenesis, growing mutagenized *Brachypodium* populations, spectroscopy-based leaf assay screening methods, cell wall preparation, sugar analysis and

quantification; crossing Brachypodium; RNAi and overexpression vector construction; lignin staining/microscopy and extraction; protein extraction; *in vitro* digestibility assays; embryo culture and *Agrobacterium* transformation.