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"Trojan Horse" Strategy for Deconstruction of Biomass for Biofuels Production

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Abstract

Production of renewable biofuels to displace fossil fuels currently consumed in the transportation sector is a pressing multi-agency national priority (DOE/USDA/EERE). Currently, nearly all fuel ethanol is produced from corn-derived starch. Dedicated “energy crops” and agricultural waste are preferred long-term solutions for renewable, cheap, and globally available biofuels as they avoid some of the market pressures and secondary greenhouse gas emission challenges currently facing corn ethanol. These sources of lignocellulosic biomass are converted to fermentable sugars using a variety of chemical and thermochemical pretreatments, which disrupt cellulose and lignin cross-links, allowing exogenously added recombinant microbial enzymes to more efficiently hydrolyze the cellulose for “deconstruction” into glucose. This process is plagued with inefficiencies, primarily due to the recalcitrance of cellulosic biomass, mass transfer issues during deconstruction, and low activity of recombinant deconstruction enzymes. Costs are also high due to the requirement for enzymes and reagents, and energy-intensive cumbersome pretreatment steps.

One potential solution to these problems is found in synthetic biology -engineered plants that self-produce a suite of cellulase enzymes. Deconstruction can then be integrated into a one-step process, thereby increasing efficiency (cellulose-cellulase mass-transfer rates) and reducing costs. The unique aspects of our approach are the rationally engineered enzymes which become Trojan horses during pretreatment conditions. During this study we rationally engineered Cazy enzymes and then integrated them into plant cells by multiple transformation techniques. The regenerated plants were assayed for first expression of these messages and then for the resulting proteins. The plants were then subjected to consolidated bioprocessing and characterized in detail. Our results and possible

implications of this work on developing dedicated energy crops and their advantage in a consolidated bioprocessing system.

ACKNOWLEDGMENTS

We would like to thanks Dr Philippe Vain at the John Innes Center -an institute of the Biotechnology and Biological Sciences Research Council United Kingdom for helpful advice, guidance and suggestions during the course of this project. We also thank Dr Bernd Reiss from the Max Planck Institute (Cologne, Germany) for his generous help with reagents, guidance and suggestions throughout the course of this project.

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1. Introduction:

Reducing the dependence of the United States on foreign energy supplies, specifically on imported petroleum is an important national security priority (DOE/USDA/EERE). The production of transportation fuels derived from biomass to displace fossil fuels is an important element of our national energy policy and the Department of Energy has set a goal to replace 30% of liquid petroleum transportation fuels with biofuels by 2025. Currently, almost all of domestic ethanol production is from corn-derived starch, which in itself is a high value energy food and feed commodity. An alternative is lignocellulosic biomass (e.g. grasses, wood, agriculture waste etc.), which is renewable, cheap and readily available to the tune of 50 billion tons per year. Rice straw is over half of the worlds cultivated biomass and is burned to waste causing environmental problems (Kim and Dale 2004).

Dedicated energy crops (e.g. herbaceous materials and short rotation woody crops) and agricultural waste are the preferred long-term feedstock solutions for renewable, cheap and globally available biofuels. The cellulosic and hemicellulosic components within these types of biomass are carbohydrate polymers that make up the walls of plant cells. Conversion of these polymers to fermentable sugars typically occurs with a two-step process:
(1) Chemical pretreatments that disrupt cellulose, hemicellulose, and lignin cross-linking.
(2) Exogenously added recombinant microbial enzymes to hydrolyze cellulose and hemicellulose into 6- and 5-carbon sugars, respectively. The current efficiency of this process is low due to the inherent recalcitrance of cellulosic biomass to these process steps, mass transfer issues during the deconstruction unit operations and low activity/high cost of recombinant deconstruction enzymes. Costs are also high due to low net efficiency, the requirement for enzyme reagents and energy intensive and cumbersome pretreatment steps. The major economic shortcomings of biomass refineries include the pretreatment processing and the cost of microbial cellulases (which are added exogenously) to convert the cellulose into fermentable sugars.

One solution is to insert an actuator (or set of actuators) that enables the hydrolysis of targeted plant cell wall polysaccharides using synthetic biology. This project proposed to introduce multiple synthetic genetic circuits to drive a biological process – one of the least explored areas in synthetic biology. We developed metabolic networks that express these actuators (enzymes) within targeted regions of model plant systems using a genomics-based approach. Engineering plants that self-produce a suite of cell wall degradation enzymes compartmentalized to a specific location in the plant cell. These enzymes were able to cleave the linkages that are

at the core of biomass recalcitrance. If this process is initiated during normal plant development, the structural integrity of the cell wall will be compromised negatively impacting plant viability. The genes encoding the degradation enzymes (the actuators) we proposed to use were obtained from extremophilic-organisms that grow at high temperatures and extreme pH. These enzymes remained inactive during the life cycle of the plant but become active during pretreatment that occurs at elevated temperatures (typically 90-150°C). These genetically tailored actuators thereby provide a unique thermal trigger within the biomass. With the successful integration, expression and accumulation of these enzymes, biomass deconstruction can theoretically be consolidated into a one-step process - thereby increasing the overall system efficiency (cellulose-cellulase mass transfer rates) and reducing costs by eliminating the need for adding exogenously produced enzymes at a much higher cost than those that are potentially expressed *in planta*.

2. Material and Methods:

Source of New parts

Industrial applications of microorganisms and enzymes for sustainable production of fuels from renewable resources, chemicals, biopolymers and materials are part of a growing global effort in green chemistry. Although a majority of the current industrial enzymes are from bacteria and fungi, isolates from archaea (aka extremophiles) are increasingly finding applications in industrial process due to the fact that most of the archaeal species (Fig 2) characterized to date are from extreme environment (conditions that exhibit industrial process – high temperature, pressure and extreme pH). Our initial parts for the actuators are two extremophilic enzymes – CelA from *Alicyclbacillus acidocaldarius* (a family 9, 1,4-glucanase with pH optimum of 4-5.5 and exhibits no detectable activity below 55 ^{o}C) and sso1949 from *Sulfolobus solfotaricus* (a family 12 glycosyl hydrolase with a pH optimum of 1.8 and exhibit no detectable activity below 60 ^{o}C).

Bioinformatics Analysis:

We performed extensive bioinformatics analysis of the open reading frames (ORFs); identifying and removing targeting signals, cryptic splice sites, etc., we then systematically optimized the codon preferences for efficient transcription and translation in Arabidopsis and Brachypodium. The optimized ORFs were then synthesized using a commercial vendor assembled at Sandia and cloned into several plant expression vectors. See below.

Construction of Actuator:

In the original proposal we had proposed to insert the actuator in Arabidopsis (model system) and rice (energy crop). We then included *Brachypodium sylvaticum* and *Saccharomyces cerevisiae* systems in the scope of this study. All ORFs were amplified by polymerase chain reaction and cloned into vectors obtained from collaborators at USDA or from Cambia (Australia). Standard molecular biology techniques were used according to Sambrook et al., (1986). All reagents were obtained from New England Biolabs (MA) and all chemicals were from Sigma chemicals (St Louis, MI). Integrity of the DNA ORFS were confirmed by sequencing using a commercial sequencing service and all sequence assembly was performed using Vector NTI. *pMM23AS* vector was constructed by modifying pMM23 (Qin et al. 1994) by replacement of the *Xba*I site with an *Asc*I site between *35S* and *cre*, and a *Spe*I site was added to the *Sph*I site between *cre* and *nos3'*. The vector was as follows HindIII-35S promoter-AscI-Cre ORF-SpeI-nos3' terminator-SacI-pUC18 backbone. *pC35-Cre* vector from pCambia 2300 (http://www.cambia.org/daisy/cambia), is a binary vector with the kanamycin resistance gene (*nptII*) for plant selection and the kanamycin

resistance gene (*aphIII*) for bacterial selection was cleaved with *Hin*dIII and *Sac*I for insertion of the *Hin*dIII-*35S-Cre-nos3'- Sac*I fragment from pMM23AS. *pC35-SSO* contains the plant codon optimized *sso1949* coding sequence from (Huang et al. 2005) was digested with AscI and SpeI and cloned into the pC35-Cre binary vector at the AscI and SpeI sites

Testing of Targeting Signals:

Actuator containing Agrobacterium were used to perform floral dip transformation of Arabidopsis according to published procedures at the USDA plant transformation facility. Transformants were selected and after 4-6 week plants were analyzed visually using a hand held VU lamp. Positives were allowed to grow for another 1-2 weeks and harvested and imaged using a hyperspectral imager.

Transient Testing of the Actuator for Expression in Brachypodium and Allium cells:

The actuators were introduced into *Allium sativa* epidermis cells (a.k.a. onion cells) and *Brachypodium sylvaticum* leaf tissue using a gold nano-particle based biolistics delivery device and expression of co-segregating beta-D-glucuronidase (GUS) was assayed in order to determine actuator compatibility as well as expression levels and effects of surrogate gene on the host system.

Introduction of Actuator into Arabidopsis for Stable Expression:

Arabidopsis thaliana Col-0 ecotype was used throughout this study. Seeds were stratified at 4°C for 2 days to synchronize germination and then grown in Sunshine mix #1 (SunGrow Horticulture Distribution, Bellevue WA) under 16 hour photoperiod at 22°C in a greenhouse or on medium in a growth chamber with a 16 hour photoperiod, at constant temperature of 22°C and a light intensity of 50 $mE/m^2/s$. Plants were transformed using the floral dip method with *Agrobacterium tumefaciens* strain GV3101 as previously described (Clough and Bent, 1998).

Introduction of Actuator into Brachypodium for Stable Expression:

Immature Brachypodium embryos were excised and used to introduce the actuators into Brachypodium cells. Agrobacterium was used for this transformation (typically a 6-8 month cycle). Stably transformed putative plantlets encoding recombinant proteins were selected and acclimated to the greenhouse. During this process we developed several QA/QC steps in order to reduce the number of false positives/escapes during this transformation.

PCR analysis of Putative Stable Transgenics:

Genomic DNA was extracted by grinding a single leaf in 400 ml of buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After centrifugation, the isopropanol precipitated pellet was washed with 70% ethanol and resuspended in 50 ml of H_2O. Two μl of genomic DNA in 25 μl volume was used per PCR reaction. Primers used were:
SSO F60 5'- CGTCAAGACCAGTAGTATTACCGTTACAACG-3' and
SSO R60 5'- CACGTAGCCGTTCGTAACCTTCC-3'.

RT-PCR Analysis:

Total RNA was extracted from stably transformed Arabidopsis plant tissue using Qiagen RNA-easy plant mini extraction kit (Valencia, CA) according to manufacturers recommendations. Random hexamers were used to synthesize cDNA using a complete cDNA synthesis kit (Clontech, CA) according to manufacturers recommendations and samples were normalized to a final concentration of 500ng/ul. Transcript analysis was performed using Roche DNA taq polymerase, FP: gagtttgcgaacctctcctg, RP: aacggcaacctcattggtag reaction was run according to the following conditions: 2 min at 94C; then 30 amplification cycles with 30 sec at 94 C, 1 min at 55 C, 3 min at 72 C and a final extension time for 5 min at 70 C. The primers used for Cel 9A were as follows: The product size was 576 bp. Additional end primers were used to confirm that the whole transcript was present.
The primers for SSO 1949 were as follows:
Forward primer: cgttacaacgaacgagacca
Reverse primer: cacgtagccgttcgtaacct
The product size was 713 bp. Additional end primers were used to confirm that the whole transcript was present
The products were separated on a 1.2% agarose gel.
For SSO PCR: annealing temperature: 55 C for 30 cycles
1. 1949 F1 + 1949 R1 (713 bp)
2. 1949 F2 + 1949 R1 (393 bp)
3. 1949 F3 + 1949 R1 (176 bp)

Southern blot analysis:

Total DNA was isolated using a rapid protocol (Dellaporta et al., 1983) from designated pC35-SSO plant lines. The DNA samples were digested with restriction endonuclease HindIII, blotted to nylon membrane, and probed with a ^{32}P-labeled *celA* sequence amplified with the SSO F60 and SSO R60 primers described above. Probe hybridization was accomplished following the Rapid Hyb (Amersham) protocol. The membranes were washed at high stringency in 0.5XSSC and0.1% SDS at 65°C for 15 minutes before exposing to x-ray film.

Total Soluble Proteins Extraction:

Total soluble proteins (TSP) extraction from leaf tissues as described by Ziegelhoffer et al. (2001). Briefly,100 mg fresh leaf tissue was ground on ice (or liquid nitrogen) in sodium acetate grinding buffer (50 mM sodium acetate pH 5.5, 100 mM NaCl, 10% v/v glycerol, 0.5 mM ethylenediaminetetra- acetic acid (disodium salt), 1 mM phenylmethyl- sulfonyl fluoride, 1 mg/l aprotinin, 1 mg/l leupeptin, 1 mg/l pepstatin) at a ratio of 5 ul per mg of sample. Soluble extract was recovered from insoluble debris by centrifugation for 5 min in a microfuge at full speed. A solution of saturated ammonium sulfate was added to the extracts to achieve a final concentration of 2.7 M ammonium sulfate, followed by incubation on ice for 30 min, the resulting precipitate was recovered by centrifugation for 5 min. The ammonium sulfate pellet was resuspended in 5 ul of grinding buffer for each 2 ul of starting crude extract. We quantifed extracts using the Bradford method using a standard curve generated from bovine serum albumin (BSA) (Ziegelhoffer et al. 2001).

Protein Extraction and Heat Fractionation:

Transformed plant sample were ground in liquid nitrogen to a fine powder using mortar and pestle. The powder was resuspended in extraction buffer (100 mM Tris pH7.5, 1 M NaCl and 5% glycerol) and the slurry transferred to a 50 ml falcon tube and vortexed for 2 minutes. The suspension was centrifuged at maximum speed in a table top centrifuge and the supernatant was transferred to a new tube and incubated at 70 ^{o}C for one hour and centrifuged again for maximum speed for 10 minutes. The supernatant was concentrated using a centricon XX K (Fisher Scientific) and the proteins quantified using a micro BCA assay (Fisher Scientific) according to manufacturers guidelines. Samples were normalized and three concentrations were used to setup saccrification assays in 100 mM K Phosphate pH 1.8 and 1.25% CMC for 20 hrs at 80 ^{0}C. At the end of the saccrification reaction 1ul of 10N NaOH to each well to change the pH of the sample.
Half of the saccrification reaction (50ul) was transferred to a new tube and mixed with to equal volume of DNS reagent and incubate at 70 ^{0}C for 30 minutes.

DNS Reducing Sugar Assay:

DNS assay reagent was prepared as the followings for each 100mL solution: mixed 1g of DNS (Sigma # D0550) in 50mL of H2O, stirred to dissolve at 40C. 30g of KNa Tartrate (Sigma # S2377) and 20mL of 2N NaOH (J.T. Baker # 3727-01) were subsequently added. Solution was brought to final volume with H2O and stored in brown ample bottle at RT. Saccharification samples in 96-well PCR plate were cooled to RT, if the samples were in acidic condition (pH < 5.5), small amount of 10N NaOH was added to each sample to bring its pH into alkaline environment. 50uL of samples were transferred to new PCR plate, where it contained 50uL of DNS reagent. Mixed well and quick spun down to

prevent bubbles. For standard curve, 10mM D-glucose, 1:2 serial dilutions, were used. Plate was sealed and incubate at 30min at 70C. After incubation, assay plate was spun down at 2000RMP for 15min. 95uL of each reaction was transferred to Costa 96-well, black, clear bottom for reading absorbance at 540nm.

EnzyChrom Glucose Assay:

EnzyChrom Glucose Assay kit (EBGL-100) was from BioAssay Systems. Colometric procedure was utilized. Briefly, all components were equilibrated to RT. Thawed enzyme was kept on ice. 20uL of standards or saccharification samples were put into Costar 96-well, black, clear bottom. In each well, 80uL of working reagent (85uL of Assay Buffer, 1uL of Enzyme Mix and 1uL of Dye Reagent) was added and mixed well. Standards were prepared with provided Glucose, starting from 300uM, 1:2 serial dilutions. Quick spun down to eliminate bubbles. Reaction was incubated for 30min at RT and the absorbance Plate read at 570 nm.

Scanning Electron Microscopy:

Actively growing stem tissues was harvested from the greenhouse and first embedded into 8 % agarose in water for stability and then sliced into 100 µm thick sections using a Leica VT1000S vibratome (Leica Microsystems, Wetzlar, Germany). The sections wit were picked up from the water and directly transferred onto brass sample stubs. The pretreatment was done on these sample stubs with drops of either 1.2% (w/w) sulfuric acid (for SSO samples), a buffer or Cel-A samples or water (as a control for both). Samples were incubated in an oven for 1 and 3 h at 70 °C, keeping them under a moist atmosphere using wet filter paper to avoid evaporation of the drops.

After pretreatment the sample stubs with the pretreated plant sections were washed twice with PBS, fixed for 2 h in 2.5% Glutaraldehyde (RT), washed again twice with PBS and dehydrated with subsequent Ethanol steps (30%, 50%, 70%, 80%, 90%, 3x 100%), followed by critical point drying (using an Autosamdri-815, Tousimis, Rockville, MD, USA). The samples were then sputter coated with approximately 30 Angstrom of Au/Pd. Subsequently images were acquired with a 10 kV accelerating voltage using a Hitachi S-5000 microscope (Hitachi America, Terrytown, NY, USA) at up to 25,000x magnification.

Expression of Actuator in Yeast cells:

We cloned and expressed the sso1949 ORF in yeast expression system. This was attempted in order to have an adequate supply of protein which could be used to develop an assay for the future transgenic plants as biomass from transgenic plants is quite limiting and extremely valuable and using it to develop an assay is not only economically prohibitive but can be a significant time and labor constraint.

3. Results and Conclusions:

Bioinformatics Analysis:

After extensive literature and bioinformatics analysis (using web based tools) targeting and localization signals were identified for sequestering these enzymes within specific plant cell compartments where they will not interfere with normal growth and can be activated during processing. These genes were then synthesized Blue Herron (Seattle, WA)

Optimized sequence for the CELA PLANT AVG CODON SEQ

GGCGCGCCGCCACC ATG GCA CCG TCC CGT GTT CCT AAG TCT ATC TTT
TAT AAC CAG GTG GGG TAC CTC ATT AGC GGT GAC AAG AGG TTC TGG
ATT CAG GCG CAC GAA CCG CAA CCG TTC GCT CTC CGT ACT CCG GAG
GGG CAG GCC GTT TTC GCC GGC ATG ACT AAA CCC GTC GGA GGT AAC
TGG TAT GTC GGA GAC TTT ACC GCC CTT CGC GTG CCT GGG ACT TAT
ACC CTG ACG GTG GGT ACC CTC GAG GCG CGT GTT GTC ATC CAC CGG
AGA GCT TAT CGC GAT GTC TTG GAG GCC ATG CTT CGT TTC TTC GAC
TAC CAG TTG TGC GGG GTC GTG CTT CCG GAG GAC GAG GCA GGC CCG
TGG GCA CAC GGT GCA TGC CAT ACT TCC GAC GCT AAA GTG TTT GGT
ACG GAG AGG GCA TTG GCT TGC CCC GGC GGA TGG CAT GAT GCG GGA
GAT TAC GGC AAG TAT ACA GTG CCT GCT GCG AAG GCT GTG GCT GAT
CTC CTC CTT GCT CAT GAG TAC TTC CCG GCC GCG TTG GCT CAC GTG
CGC CCT ATG CGG TCC GTG CAC AGG GCG CCG CAC CTG CCT CCC GCT
CTG GAG GTC GCG AGG GAG GAA ATT GCT TGG CTT TTG ACG ATG CAG
GAT CCC GCC ACT GGG GGA GTT TAC CAC AAG GTG ACC ACG CCG TCA
TTC CCG CCT CTG GAC ACT CGC CCA GAG GAT GAC GAT GCT CCG CTG
GTC CTA AGC CCG ATC TCC TAT GCA GCA ACG GCT ACA TTC TGT GCT
GCC ATG GCG CAT GCT GCG CTC GTT TAT CGT CCC TTC GAT CCG GCC
CTG TCC TCA TGT TGC GCG GAT GCG GCC AGG AGG GCA TAC GCT TGG
CTC GGT GCG CAC GAG ATG CAG CCA TTT CAC AAC CCC GAC GGG ATT
CTG ACG GGT GAG TAT GGC GAT GCC GAA CTT CGC GAT GAA CTG CTA
TGG GCT AGC TGT GCG TTG CTC AGG ATG ACG GGC GAT TCT GCG TGG
GCT AGA GTT TGC GAA CCT CTC CTG GAT CTT GAT TTG CCC TGG GAA
CTC GGG TGG GCA GAT GTT GCT CTG TAC GGT GTT ATG GAT TAT CTG
CGC ACC CCC AGG GCT GCC GTT AGT GAC GAT GTT AGG AAC AAG GTC
AAG TCC CGC CTT CTG AGA GAA CTT GAT GCC TTG GCC GCC ATG GCA
GAA TCC CAT CCA TTT GGT ATA CCT ATG CGG GAC GAT GAC TTC ATC
TGG GGC TCT AAT ATG GTC CTT TTG AAC CGG GCC ATG GCT TTC CTC
CTG GCA GAG GGC GTT GGC GTG TTG CAC CCT GCT GCC CAT ACT GTC
GCC CAG AGA GCT GCC GAC TAT CTG TTC GGC GCA AAC CCC CTG GGT
CAA TGC TAC GTG ACA GGC TTC GGG CAG AGG CCG GTT AGG CAC CCG
CAT CAT CGG CCG AGC GTC GCA GAT GAC GTG GAC CAT CCG GTG CCT
GGG ATG GTG GTG GGG GGC CCC AAC AGG CAT CTT CAA GAC GAA ATT
GCG CGC GCA CAG CTA GCT GGC CGC CCA GCC ATG GAA GCC TAC ATC
GAT CAT CAG GAT AGC TAC TCT ACC AAT GAG GTT GCC GTT TAT TGG
AAC TCG CCC GCG GTC TTC GTG ATA GCG GCA CTT TTG GAG GCC CGC
GGT CGC TGA ACTAGT

Optimized sequence for the SSO Truncated PLANT DicotT7 CODON SEQ

GGC GCG CCG CCA CCA TGG CAA TTT ATC TTC ACC ATC AGA GTC CAA
ACG TCA AGA CCA GTA GTA TTA CCG TTA CAA CGA ACG AGA CCA CTA
CTC TTA TGA GCA TTA CAA CAA ACA CTG TTC CAA CCA CTG TGA CGC
CCA CGA CTT CCT CTA TTC CCC AAC TAA TCT ATG TTA CTA GCA GCG
CCA GTT CAC CGA CGC CGG TTT ATC TGA ATA ATA GTA CTG TGC CAT
CTT TTT ATT TGG AAG TTA ACA TGT GGA ACG CAA AAA CAT GGA ATG
GAA ATT ATA CGA TGG TGT TTA ATC CAC TTA CTC GTA CTT TAA GCG
TGT CAT TCA ATT TGA CGC AGG TCA ACC CTC TCC AGT GGA CCA ACG
GAT ATC CAG AAA TCT ATG TGG GTA GAA AGC CAT GGG ACA CCT CAT
ACG CAG GAA ATA TAT TTC CAA TGA GGA TTG GAA ATA TGA CTC CAT
TTA TGG TGT CTT TTT ACA TAA ACT TAA CAA AAC TCG ACC CTA GTA
TCA ATT TTG ATA TCG CCT CAG ACG CCT GGA TTG TTC GTC CCC AAA
TTG CAT TTT CAC CCG GTA CAG CGC CCG GTA ATG GAG ATA TCG AGA
TAA TGG TTT GGC TGT TTA GCC AGA ATC TTC AGC CAG CAG GTC AGC
AAG TTG GTG AGG TTG TTA TAC CAA TAT ATA TCA ATC ACA CAC TGG
TGA ATG CGA CAT TTC AGG TCT GGA AAA TGA AAA ATG TTC CAT GGG
GGG GAT GGG AAT ATA TTG CTT TTC GTC CTG ACG GCT GGA AGG TTA
CGA ACG GCT ACG TGG CCT ATG AAC CTA ACT TGT TTA TTA AGG CCC
TTA ATA ATT TTG CGT CTT ATA ACA TAA CTA ACT ACT ATC TCA CTG
ACT GGA AAT TCG GTA CTG AAT GGG GAA CCA TGA CCA GTA ATG GAA
CGG CGT ATT TTT CAT GGA CGA TTT CTA ATT TTT ATG AGA CAT TGT
TGT AAA CTA GT

SSO TRUNC POMBE SEQ

GGCGCGCCGCCACC ATG GCT ATC TAC CTC CAT CAT CAG TCT CCT AAC
GTG AAG ACA TCA TCA ATA ACA GTC ACA ACA AAT GAA ACA ACA ACT
CTG ATG TCT ATT ACT ACT AAT ACT GTG CCC ACA ACC GTC ACT CCA
ACC ACT TCA TCA ATT CCC CAA TTA ATT TAT GTA ACA AGC TCT GCC
TCT TCT CCA ACT CCC GTC TAC TTA AAC AAT TCC ACT GTT CCT TCC
TTT TAT TTA GAA GTT AAC ATG TGG AAC GCA AAA ACA TGG AAT GGT
AAC TAT ACC ATG GTC TTT AAC CCA TTA ACC CGT ACC TTG AGC GTT
TCT TTC AAC TTA ACC CAA GTT AAT CCT CTT CAA TGG ACT AAC GGG
TAT CCT GAA ATT TAT GTA GGC CGT AAA CCT TGG GAT ACC TCC TAC
GCA GGT AAT ATT TTT CCT ATG CGT ATC GGT AAT ATG ACT CCC TTT
ATG GTT TCT TTT TAT ATT AAC TTG ACC AAA TTA GAC CCG TCC ATT
AAC TTC GAT ATT GCT TCG GAT GCT TGG ATT GTG CGC CCA CAA ATT
GCA TTT TCA CCT GGC ACA GCT CCG GGC AAT GGT GAT ATT GAA ATT
ATG GTC TGG CTG TTT TCT CAA AAC TTA CAA CCT GCT GGA CAA CAA
GTT GGA GAA GTT GTT ATC CCT ATC TAC ATT AAT CAC ACT CTC GTG
AAT GCT ACC TTT CAA GTG TGG AAG ATG AAG AAT GTT CCT TGG GGA
GGT TGG GAA TAT ATT GCT TTT AGG CCT GAC GGA TGG AAG GTT ACT
AAT GGC TAC GTT GCT TAT GAA CCT AAC TTG TTT ATT AAA GCC CTG
AAT AAT TTT GCA AGT TAC AAT ATC ACT AAT TAT TAC TTG ACG GAT
TGG GAG TTT GGT ACT GAA TGG GGT ACT ATG ACC TCC AAT GGG ACA
GCT TAC TTC AGT TGG ACC ATC TCA AAC TTT TAT GAA ACG CTT CTT
TAA ACTAGT

Construction of Actuator:

We started by established collaborations with USDA facility in Albany, Cambia in Australia, JIC in UK and the MPIZ in Germany to obtain expertise in vectors and plant gene expression/transformation. We constructed four expression vectors for actuator testing (cytoplasmic-ORF, apoplast targeted-ORF and apoplast targeted-ORF-fusion with plant optimized beta-glucuronidase (GUSplus) protein and yeast expression) (Fig 4 and 5). All moncot vectors are driven by a maize ubiquitin promoter containing an intron while the dicot vector under the control of the 35S promoter. We PCR amplified the optimized synthetic ORFs and cloned them into the vector backbones (Fig 6). Green fluorescent protein (GFP) was also cloned into the monocot and dicot vectors.

Testing the Actuator:

Agrobacterium mediated transformants were selected and alloeed to grow to 4-6 week plantlets and analyzed visually using a hand held VU lamp for over all GFP signal. Positives were allowed to grow for another 1-2 weeks and harvested and imaged using a hyperspectral imaging facility in New Mexico (Fig 7). Imaging experiments confiormed the expression of as well as its localization to the appropriate compartment in the cells.

Transient Testing of the Actuator in Brachypodium and Allium Cells:

Transient expression was tested by the introduction into *Allium sativa* epidermis cells and *Brachypodium sylvaticum* leaf tissue and expression of a co-segregating beta-D- glucuronidase (GUS). This determines actuator compatibility as well as expression levels and effects of surrogate gene on the host system. The celA actuator appears to express robustly both in onion and Brachypodium cells. However, the expression of sso1949 was as robust as expected and this could be due to cellular toxicity (Figure 8). These toxicity effects gave us clues on handling transgenic materials downstream, similar trend was observed in yeast as well as Arabidopsis lines.

Introduction of Actuator into Arabidopsis:

Agrobacterium were transformed using the dicot actuator constructs selected on antibiotic. Actuator containing agrobacterium were used for floral dip transformation of Arabidopsis. Transformants were selected and after 4-6 week plants were selected using antibiotics and positive plants were tested for encoding genes by PCR (Fig. 10). To date we have 6 confirmed lines of p35S-SSO and 3 of p35S-celA. It required screening ~20,000 seeds to identify the 6 SSO transgenics and only ~2000 seeds to find the 3 celA. Given the difference it is was quite obvious that there were toxicity related issues with SSO being driven by a strong 35S promoter. We followed it up by screening more celA seeds to identify the minimum ten lines of CelA. We've also constructed the pNOS-SSO

construct for transformation. The NOS is a much weaker promoter and allowed us to make the sso1949 protein more tolerable in planta. This construct gave us ten stably transformed clones which were then characterized enzymatically.

Introduction of Actuator into Brachypodium:

Immature Brachypodium embryos were excised and used to introduce the actuators into Brachypodium cells. Agrobacterium was used for this transformation (typically a 6-8 month cycle). Several hundered stably transformed putative plantlets encoding sso1949 and CelA were selected and sampled through various selection schema developed during this process (Fig 9). These were then acclimated to the greenhouse. During this process we developed several QA/QC steps in order to reduce the number of false positives/ escapes during the length transformation and regeneration (Fig. 9). Putative transformants for celA and SSO 1949 were evaluated by PCR, and RT PCR for expression of the transgenes (Fig 16). Shoot generation from the tissue appeared to be the easiest part of the regeneration process. Most of the shoots were unable to set seed and were lost in culture. The next highest losses were observed in the aclimitazition of tissue to greenhouse conditions. In total we characterized twenty four SSO 1949 containing plant and seventy seven Cel A containing plants both for gene expression and enzymatic activity.

Expression of Actuator in Yeast cells:

During the attempted express sso1949 ORF we observed toxicity issues similar to the ones we have encountered during transient expression in onion cell and Brachypodium cells as well as during screening of transgenics in Arabidopsis seeds. We were able to recover a very small number colonies when selected using induction media, which could not be grown in subcultures. However, using a two stage selection process we were able to select six yeast colonies that contained the intact sso1949 open reading frame. Yeast cells were cultured at one liter scale and following growth in induction media were aliquoted in 50 ml fractions. Pellets were washed with PBS frozen till ready for use. Pellets were resuspended in PBS buffer and conditions optimized for efficient cells lysis. Briefly, a combination of mechanical (bead beating) and chemical treatment appears to give the best cell lysis. Fractions of the lysate extracts were separated on an SDS page acrylamide gel. We observed a protein band migrating at the expected molecular weight in lanes from extracts prepared from sso1949 ORF containing cells. Preliminary qualitative results indicate that the extracts from sso1949 containing yeast cells have a higher affinity for Azo-CMC compared to extracts from WT yeast cells. Detailed analysis is on-going and once established we will use these extracts to spike extracts prepared from wild-type Arabidopsis and Brachypodium biomass in order to establish a baseline for the lowest detectable activity for transgenic plants and develop an assay for enhanced degradation of the biomass.

DNS Reducing Sugar Assay:

DNS assay was used as a first pass to assay for enzyme activity. This assay is based on the presence of free carbonyl group (C=O), the so-called reducing sugars (aldehyde functional group present in glucose and the ketone functional group in fructose). In their presence and simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions.
It is suspected that there are many side reactions, and the actual reaction stoichiometry is quite complicated. The type of side reaction dependent on the nature of the reducing sugars. Different sugar type result in varying color intensities; thus, the assay has to be calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color. Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar is added to this sample. Because of the above issues we deligently ran a number of blank as well as recombinant enzyme purified control reactions. Both for CelA as well as SSO1949 we observed varying level of enzyme activity. This variation in activity had a strong correlation with the RT PCR results. The combnined data indicated that the enzyme expression was a function of message abundance and the position of integration into the host genome.

EnzyChrom Glucose Assay:

This is a simple, direct and high-throughput assays for measuring glucose concentrations. BioAssay glucose assay kit uses a single working reagent that combines the glucose oxidase reaction and color reaction in one step. The assay can be ruin in a absorbance or fluorescence format. The color intensity of the reaction product at 570nm or fluorescence intensity at Xem/ex = 585/530nm is directly proportional to glucose concentration in the sample. The assay utilizes as little as 20 ul sample and the linear detection range in 96-well plate: 5 to 600 RM glucose for colorimetric assays and 1 to 30 RM for fluorimetric assays. This assay is quite specific and does not suffer form the background issues like the DNS assay. We rescreened all the samples run by DNS with the Enzychrome assay and observed much higher concentrations of glucose in the finished reactions (Fig 13 and 17).

Scanning Electron Microscopy:

The biggest challenge in the SEM analysis was to get reproducible sections without crushing the tissue and without dehydrating the tissue which could

potentially inactivate the enzyme. We developed an embedding system that allows for actively growing stem tissues to be sectioned. Tissue was harvested from the greenhouse and first embedded into 8 % agarose in water and then sliced into 100 µm thick sections using a Leica VT1000S vibratome. The sections were picked up from the water and directly transferred onto brass sample stubs and pretreated to activate the enzyme with a few drops of either 1.2% (w/w) sulfuric acid (for SSO samples), a buffer or Cel-A samples or water (as a control for both). Samples were incubated in an oven for 1 and 3 h at 70 °C, keeping them under a moist atmosphere using wet filter paper to avoid evaporation of the drops. Both the Cel A and the SSO 1949 plants appear to loose cellular integrity once the enzymes are activated but no gross structural changes were observed in the control samples (Fig 15, 15, 18 and 19).

Sandia is a recognized leader in the alternative energies, especially hydrogen storage and fuels cells. The production of integrated systems that can reduce the economic costs of biomass conversion into fermentable sugars is of interest not only to DOE but also an important national security priority. It would also impact biotechnology ad E&IA community and establish Sandia's professional standing in the fields of synthetic biology.

4. REFERENCES

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HUANG Y, KRAUSS G, COTTAZ S, DRIGUEZ H, LIPPS G (2005) A highly acid-stable and thermostable endo-*β*-glucanase from the thermoacidophilic archaeon Sulfolobus solfataricus Biochem. J. (2005) 385, 581–588.

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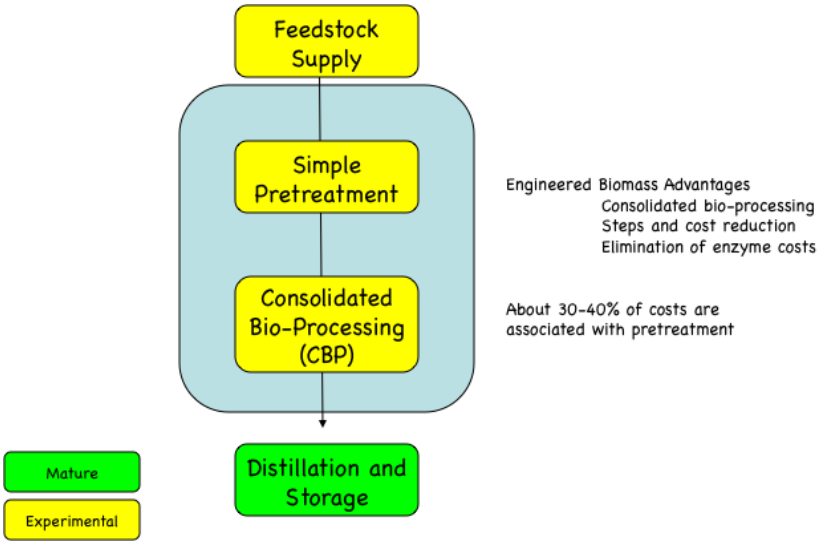
FIGURES:

Figure 1:

Trojan horse strategy is to reduce costs by consolidating the process and eliminating the costs of recombinant enzymes.



Figures 2:

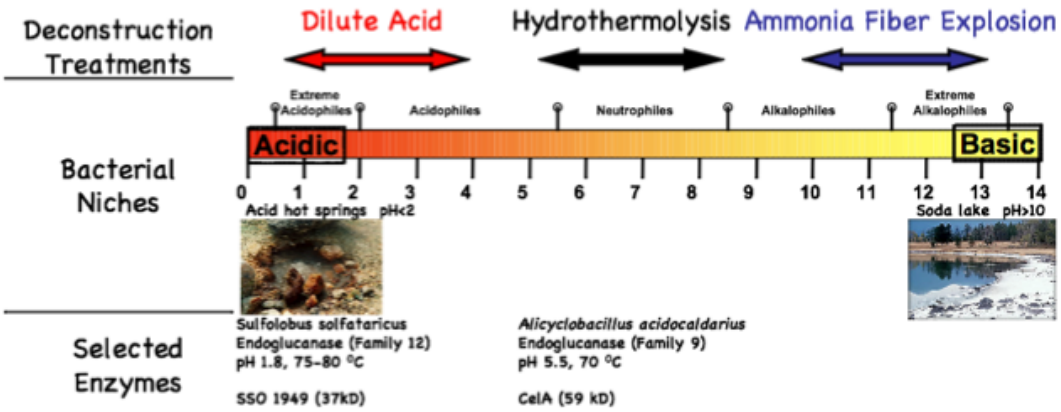

Deconstruction Treatments
Dilute Acid
Hydrothermolysis
Ammonia Fiber Explosion
Bacterial Niches
Extreme Acidophiles
Acidophiles
Neutrophiles
Alkalophiles
Extreme Alkalophiles
Acidic
Basic
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Acid hot springs pH<2
Soda lake pH>10
Selected Enzymes
Sulfolobus solfataricus
Endoglucanase (Family 12)
pH 1.8, 75-80 °C
SSO 1949 (37kD)
Alicyclobacillus acidocaldarius
Endoglucanase (Family 9)
pH 5.5, 70 °C
CelA (59 kD)

Figure 3:

Genetic Transformation of Plant Cells.

There are two common methods of plant transformation. One uses Agrobacterium as a vehicle for gene introduction while the other required the particle inflow gun. In both cases the transformed tissue are selected for a co-segregating marker.

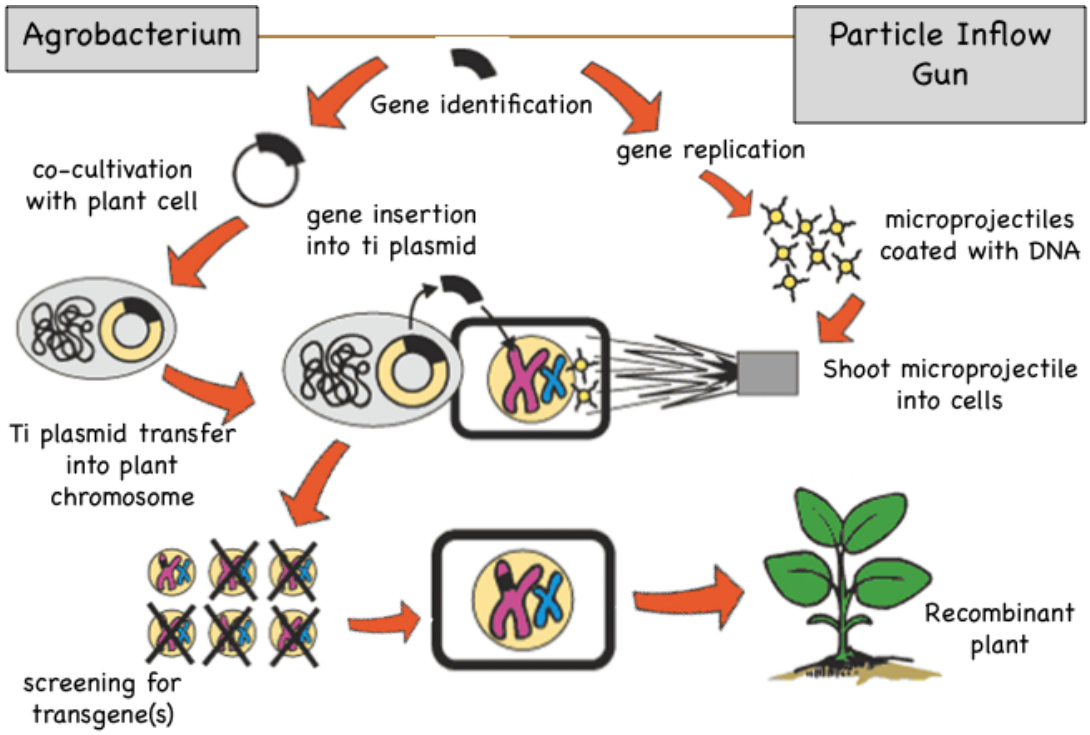
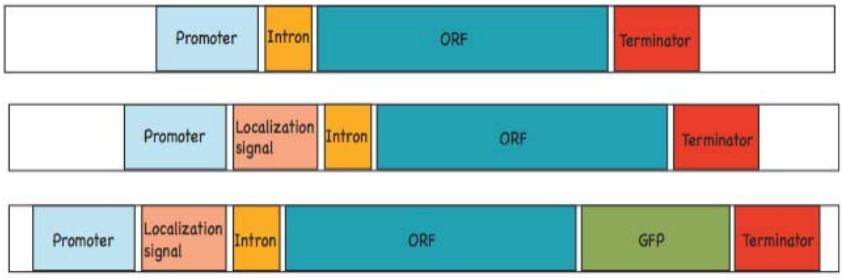


Figure 4:

Bioinformatics for targeting and other regulatory signal sequences.
Gene construct for all the brachypodium transformation.



Cytoplasmic Expression. Maize Ubiquitin promoter + intron Cellulase-nos cassette inserted into pCAMBIA 1305.1.
Apoplast Expression. Maize Ubiquitin promoter + intron-SP-Cellulase-nos cassette in pCAMBIA1305.2 (translational fusion)
Apoplast Expression of GUSPlus Fusion Protein. Maize Ubiquitin promoter plus intron-SP-Cellulase-GUSPlus-nos cassette in pCAMBIA1305.2 (translational fusion).

Figure 5:

Map of pC35s-CelA vector into with the SSO 1949 and CelA open reading frame was cloned and used for transformation of Arabaidopsis plant tissue. The genes are under the control of CMV promoter and expression resulting in ubiquitous expression.

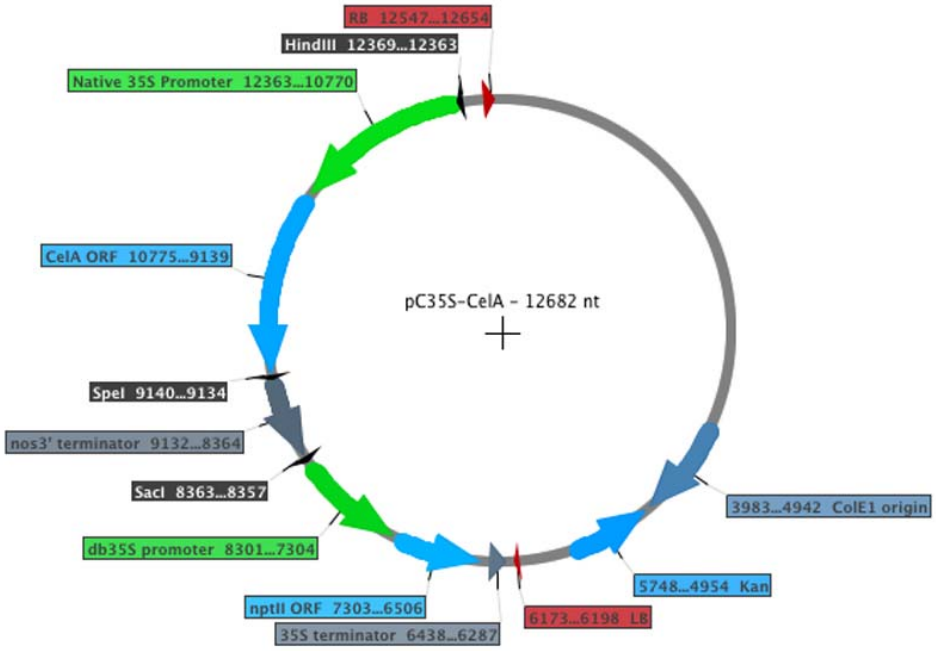
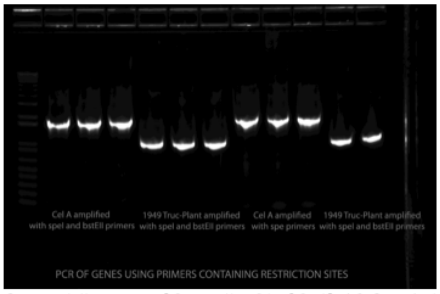
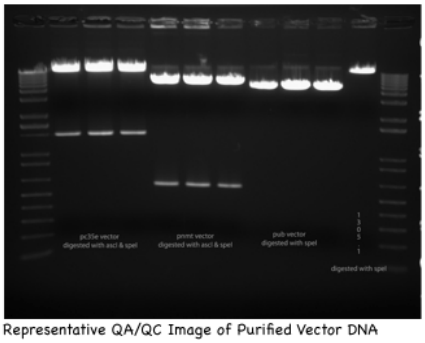


Figure 6:
Actuator Construction and various QA/QC during the construction process.



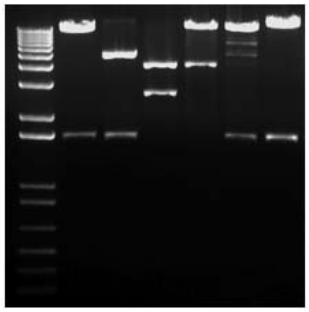
A). Representative Image of Preparative QA of Purified PCR products.

Lane 1-3. Cel A ORFs amplified with specific primer pairs.
Lane 4-6. Plant optimized SSO1949 amplified with specific primer pairs.
Lane 5-9. Cel A ORFs amplified with specific primer pairs.
Lane 10-12. Plant optimized SSO1949 amplified with specific primer pairs.



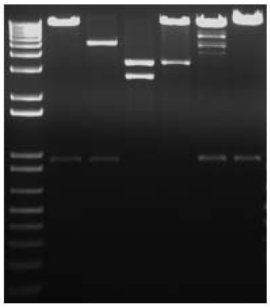
B). Representative QA/QC Image of Purified Vector DNA

Lane 1-3. Monocot. expression vector.
Lane 4-6. Yeast expression vector.
Lane 7-9. pBASK cassette containing vector.
Lane 10. Dicot expression vector.



C). Representative Image of Large scale DNA preps for CelA prior to introduction into Agrobacterium

Lane 1 Molecular weight marker.
Lane 2 pc35e (AscI/SpeI),
Lane 3 pUbi BaskNos (AscI/SpeI),
Lane 4 pUbi BaskNos (EcoRI/HindIII),
Lane 5 pCambia 1305.1 (EcoRI/HindIII),
Lane 6 pCambia 1305.2 Uni-sp-GusPlus (SpeI/BstEII)
Lane 7 pCambia 1305.2 Uni-sp-Gus Fusion (SpeI)

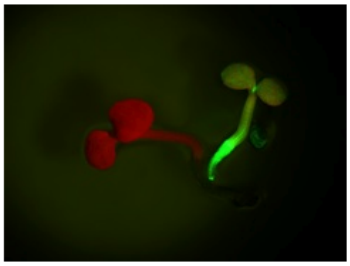


D). Representative Image of Large scale DNA preps for SSO1949 prior to introduction into Agrobacterium

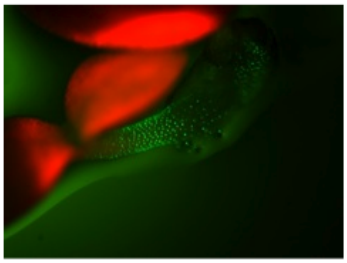
Lane 1 Molecular weight marker.
Lane 2 pc35e (AscI/SpeI),
Lane 3 pUbi BaskNos (AscI/SpeI),
Lane 4 pUbi BaskNos (EcoRI/HindIII),
Lane 5 pCambia 1305.1 (EcoRI/HindIII),
Lane 6 pCambia 1305.2 Uni-sp-GusPlus (SpeI/BstEII)
Lane 7 pCambia 1305.2 Uni-sp-Gus Fusion (SpeI)

Figure 7:

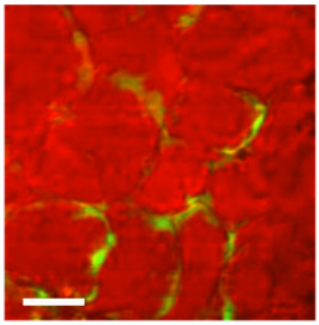
GFP protein signal was analyzed in whole seedling (top panel) and positive plants grown for another 4-6 weeks and were sectioned and analyzed by hyperspectral imaging.
GFP protein is seen localizing to the apoplastic space when fused to the targeting signal for apolastic space (lower left). Free expression of GFP result signals from cytoplasm (bottom right).



Cytoplasmic

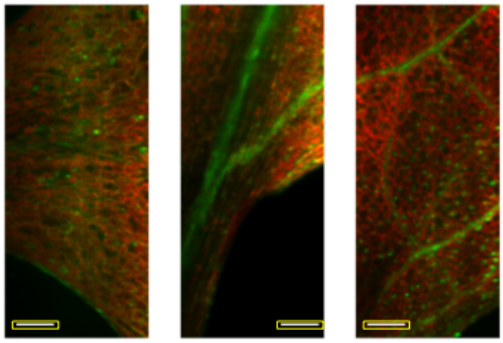


Cajal Body

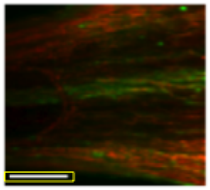


Scale bar is 10 M

GFP fused to the apoplast targeting signal is localized to the apoplast in leaf tissue.



Images scanned with 5 um pixels resulting in image size of 515 um X 1.0 mm
All color channels have been adjusted independently for contrast.
All scale bars 100 um



Images scanned with 5 um pixels resulting in image size of 515 um X 400 um

Figure 8:

Transient Expression Testing of Actuators Using Particle Inflow Gun

A). celA encoding actuator was transformed into Allium sativa cells and after three days the cells were stained for activity for a co-segregating marker. Robust expression of the segregating marker was observed.
B). celA encoding actuator was transformed into Brachypodium cells and after three days the cells were stained for activity for a co-segregating marker. Robust expression of the segregating marker was observed.
C). Actuator encoding sso1949 was transformed into Allium sativa cells and after three days the cells were stained for activity for a co-segregating marker. Lack of robust expression of a co-segregating marker was observed which indicate cellular toxicity.
D). sso1949 encoding actuator was transformed into Brachypodium cells and after three days the cells were stained for activity for a co-segregating marker. Robust expression of the segregating marker was not observed which might indicate cellular toxicity.

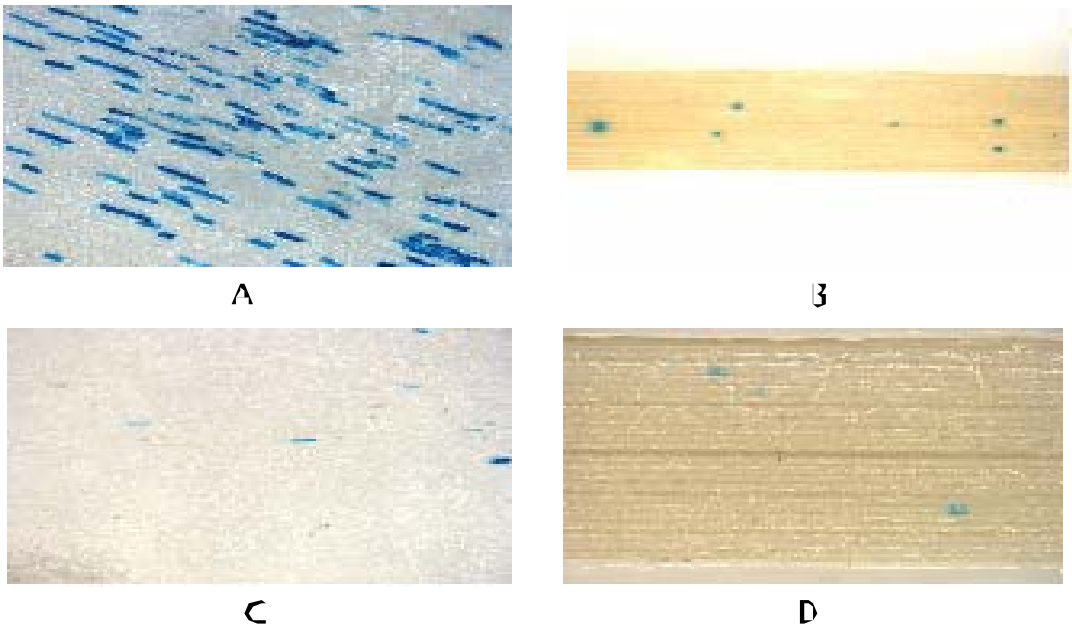


Figure 9:

Representative Images From Various Steps During Brachypodium Transformation and Regeneration.

A). Shoot generation from transformed Brachypodium embryos.
B) Root generation from transformed Brachypodium embryos.
C). Acclimated transformed plants prior to being moved to greenhouse for growth.
D). Transformed plants being grown in greenhouse for biomass increase.
E). Control callus tissue after being assayed for a co-segregating marker.
F). Transformed callus after being assayed for co-segregating marker. Robust expression was observed.
G). Leaf tissue from transformed plants growing in the green house when assayed for the activity of a co-segregating marker protein exhibit robust expression.
H). Leaf tissue from control plants growing in the green house when assayed for the activity of a co-segregating marker protein exhibit no expression.

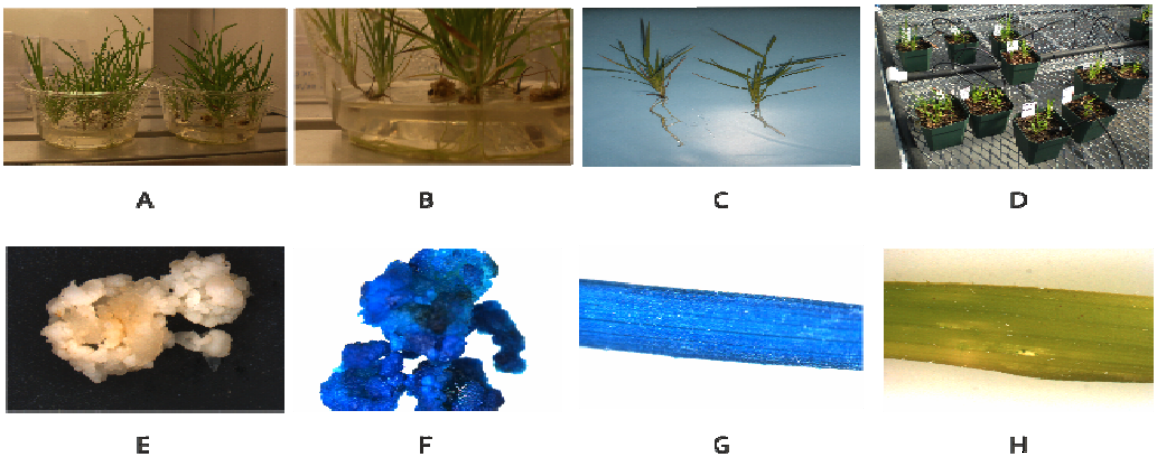
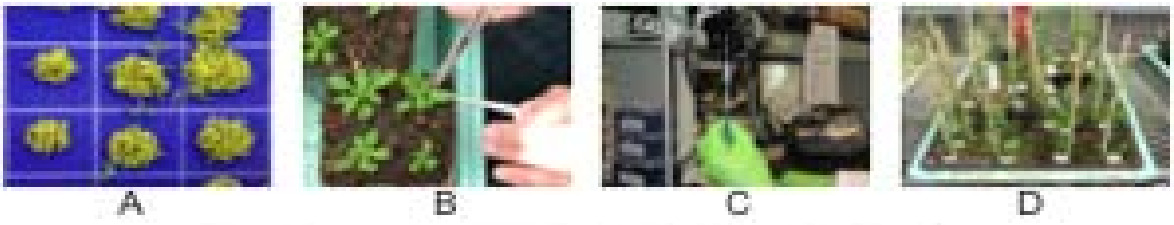
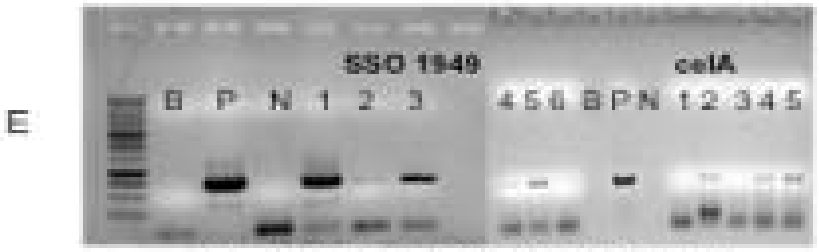


Figure 10:

Representative images during transformation, selection, growth and analysis.

A). Seeds from transformed plants being selected in vitro for a co-segregating marker (antibiotic resistance).
B). Antibiotic resistant seedlings being transferred to the greenhouse for growth.
C). DNA being extracted from leaf tissue of antibiotic resistant plants for analysis.
D). Transgenic plant's seeds drying before analysis for homozygosity analysis.
E). To date we have 6 confirmed lines of p35S-SSO and 3 of p35S-celA. It required screening ~20,000 seeds to find the 6 SSO and only ~2000 to find the 3 celA. Given the difference it is quite obvious that there are toxicity related issues with SSO being driven by a strong promoter. We are in the process of screening more of the celA seeds to find the minimum 10 lines of celA. We've made the pNOS-SSO construct for transformation also. The NOS is a weaker promoter and will make the protein more tolerable in planta.

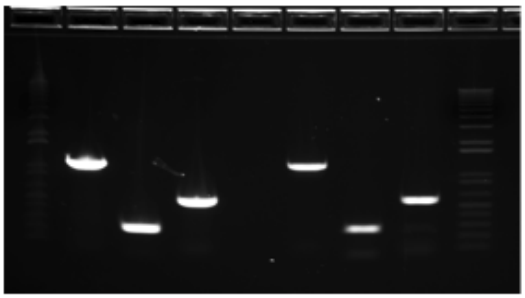




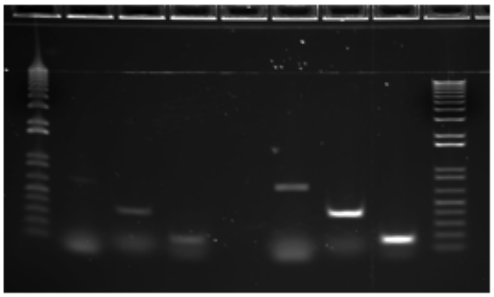
B= Blank, P= Positive, Clone #

Figure 11:

RT PCR analysis of Arabidopsis transgenic plants results in robust expression of recombinant mRNA.



1. CelA F2 + CelA R1 (1194 bp)
2. CelA F1 + CelA R2 (269 bp)
- CelA F3 + CelA R1 (576 bp)



1. 1949 Plant F1 + 1949 Plant R1 (713 bp)
2. 1949 Plant F2 + 1949 Plant R1 (393 bp)
- 1949 Plant F3 + 1949 Plant R1 (176 bp)

Figure 12:
Zymagram confirms activity of recombinant protein.

Zymograms were used to assay for protein activity in Arabidopsis extracts. We were able to confirm activity with whole cell lysates prepared from plants containing CelA. However, zymogram experiments with SSO1949 were not successful.

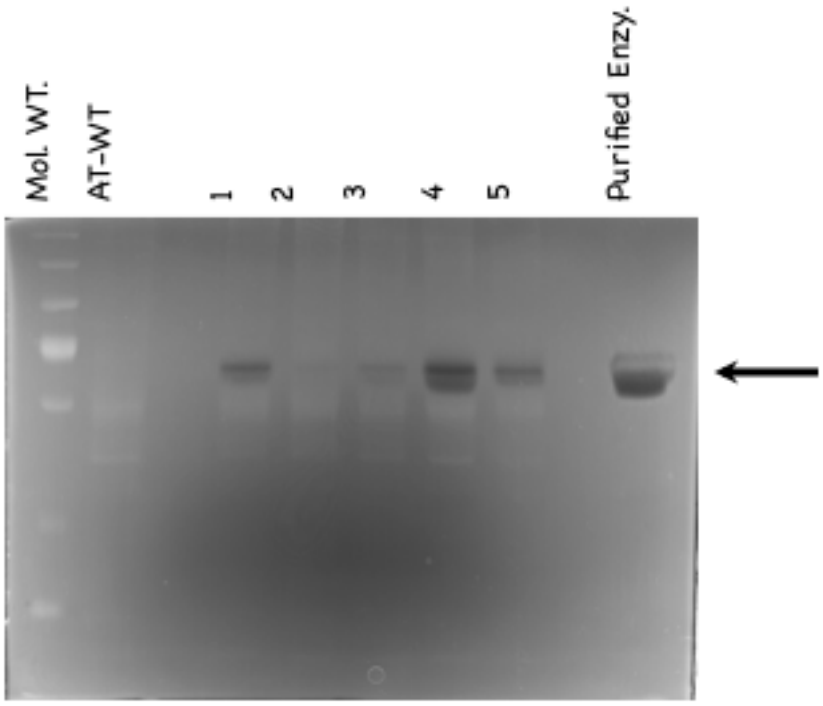


Figure 13:

Enzymatic Activity of Arabidopsis Trojan Horse Trangenics.
Total protein was extracted from transgenic plants and concentrated as described in materials and methods. This concntrate was then evaluated to sugar release. Results shown are from five independent experiments.

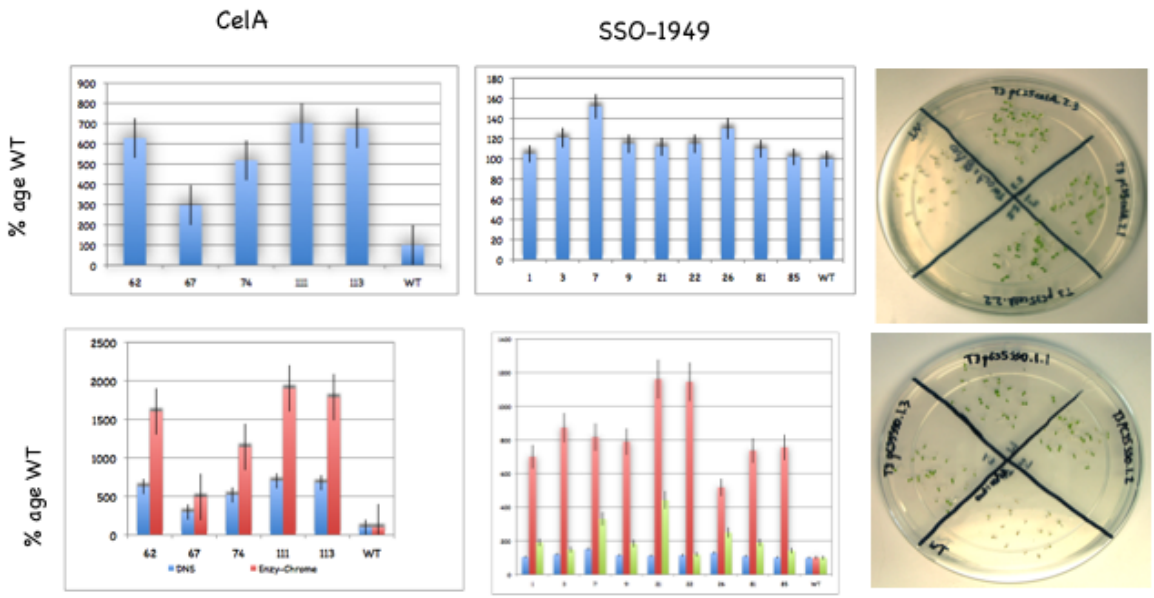


Figure 14:
SEM Analysis of Arabidopsis Wild-type and Cel A containing Transgenic Tissue.

Plant tissue was prepared for SEM analysis and incubated under conditions for enzyme activation. Wild-type tissue appears to have no change while the transgenic tissue looses its structural integrity upon enzyme activation.

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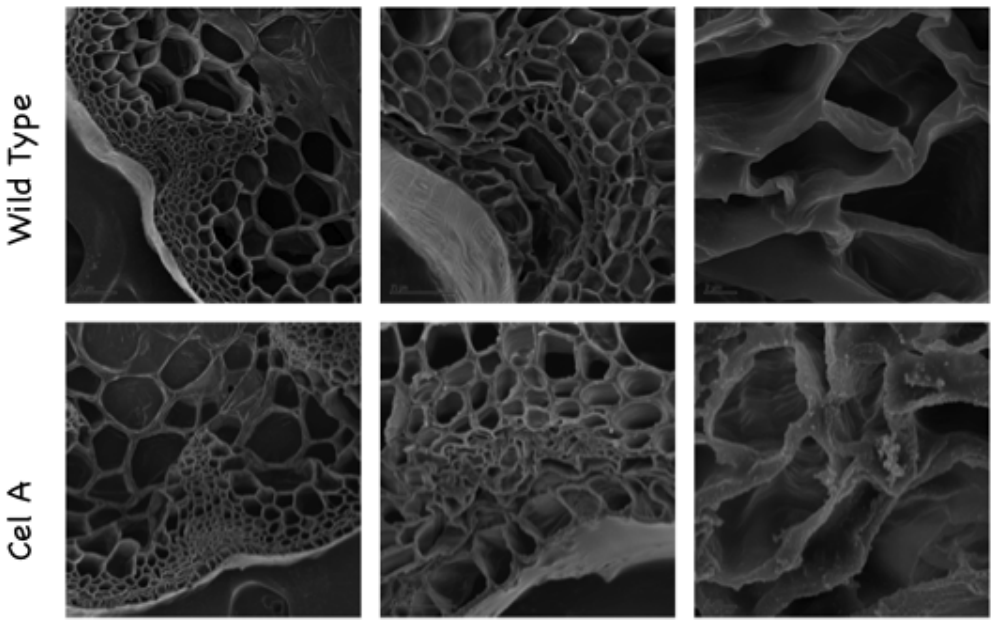


Figure 15:
SEM Analysis of Arabidopsis Wild-type and SSO 1949 containing Transgenic Tissue.

Plant tissue was prepared for SEM analysis and incubated under conditions for enzyme activation. Wild-type tissue appears to have no change while the transgenic tissue looses its structural integrity upon enzyme activation.

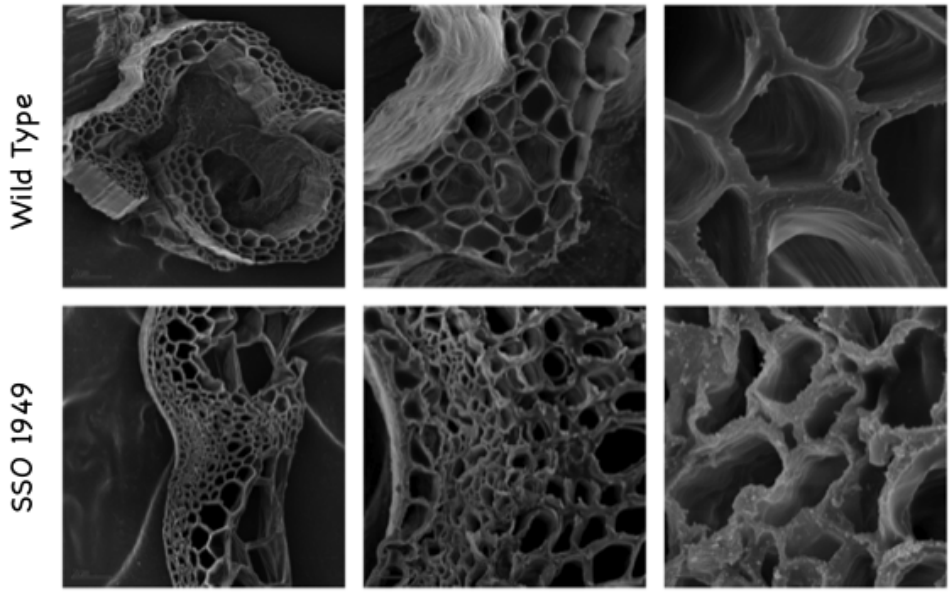


Figure 16:

RT PCR analysis of Brachypodium transgenic plants results in robust expression of recombinant mRNA. We were able to detect expression using multiple primer pairs.

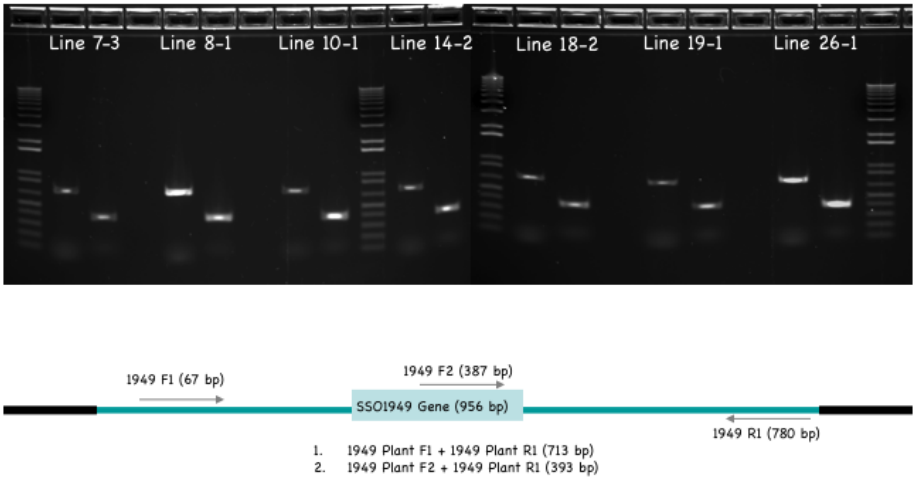


Figure 17:

Enzymatic Activity Measurements Using Extracts Prepared from Brachypodium Wild-type and Transgenic CelA plants
Total protein extracts were prepared from green house grown brachypodium plants and protein was quantified using micro-BCA. Assay for enzyme activity was performed using CMC. We then determined reducing end formation using 3,5-dinitrosalicylic acid (DNS) assay

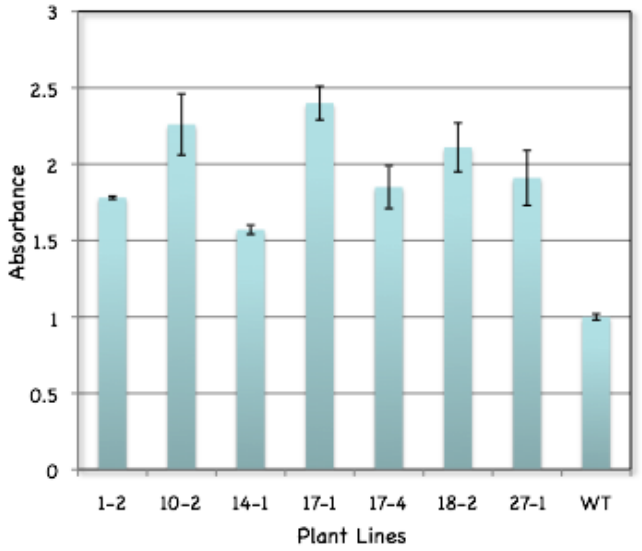


Figure 18:

SEM Analysis of Brachypodium Wild-type and Cel A Containing Transgenic Tissue.

Plant tissue was prepared for SEM analysis and incubated under conditions for enzyme activation. Wild-type tissue appears to have no change while the transgenic tissue looses its structural integrity upon enzyme activation.

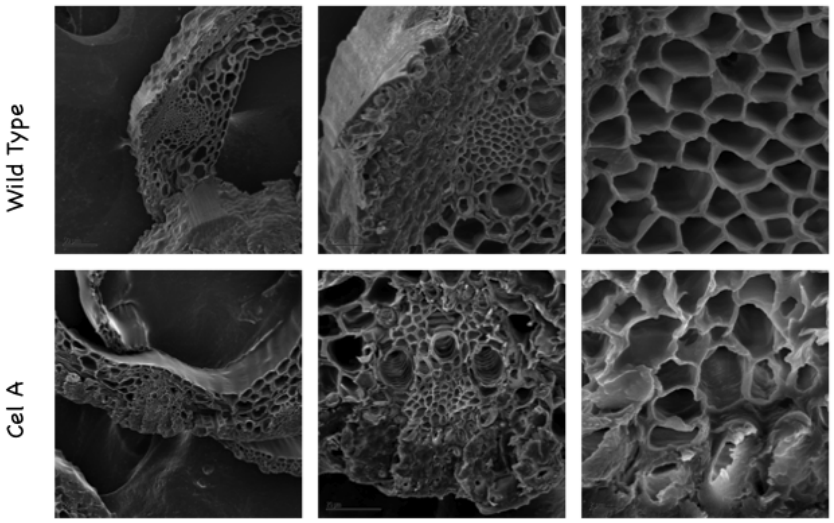
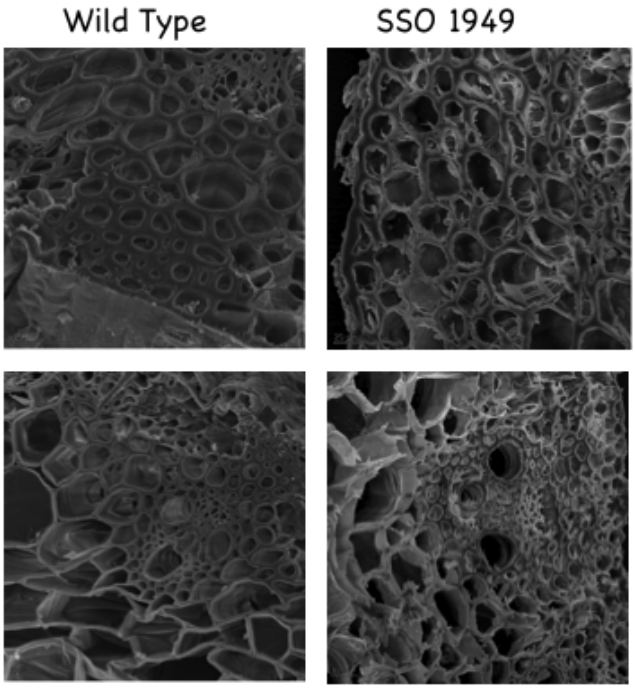


Figure 19:

SEM Analysis of Brachypodium Wild-type and SSO 1949 containing Transgenic Tissue.

Plant tissue was prepared for SEM analysis and incubated under conditions for enzyme activation. Wild-type tissue appears to have no change while the transgenic tissue looses its structural integrity upon enzyme activation.



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APPENDIX:

Presentations and publications resulting from this project are listed below:

•Invited Speaker:

•Invited talk on synthetic biology for bio-fuels at european science foundation conference (the only other us speaker)
•Chi bio-fuels conference 2008
•Scientific advisory panel of the chi bio-fuels conferences 2008-09
•National doe ldrd day 2008
•Biosciences forum 2008
•Bsap review 2010
•Business of biofuels 2009
•Biofuels feedstocks 2009
•Agrigenomics 2010
•Invited speaker at the lab-automation conference 2008
•Southwest biofuels conference 2010
•Session chair and invited speaker lab-automation conference 2009 and 2010

•Poster presentations:

•Synthetic biology meeting 2008
•Plant biology meeting 2009
•Protein society meeting 2009
•Arabidopsis meeting 2010
•Biocatalysis 2010
•Biosymposium 2008

•Invited paper:

Hyperspectral imaging of endogenous pigments in the bioenergy sciences. Microscopy and microanalysis submitted

Collaborations and Partnerships Established During this Project:

•Ceres inc. (ken feldman, the biofuels company)
•Arcadia biosciences (jos van boxtel, vic knauff)
•USDA-ARS, Albany
•Cambia, Australia
•Dr Manfred Auer at Lawrence Berkeley National Labs
•UC Davis plant sciences department
•Dr Philip Vain at BBSRC JIC center for crop genetics
•Dr Bernd Reiss Max Planck Institute fur Zuchtungsforschung

•Follow-up Joint Funding Opportunities

•Pre-proposal submitted in response to plant feedstock genomics for bio-energy rfp
•Pre-proposal submitted to NIFA rfp - DOE
•Mohar Davidow ventures
•George Antos, NSF (chemical, bioengineering, environment & transportation systems)
•Bill Otrs, usda - Jeffrey Steiner president's interagency biofuels working group

•Richard Flavell (FRS, CBE - commander of the british empire) founder of JIC

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