Second Generation Biofuel, Analytical tools, Automation... Challenges and Deconstruction Strategies

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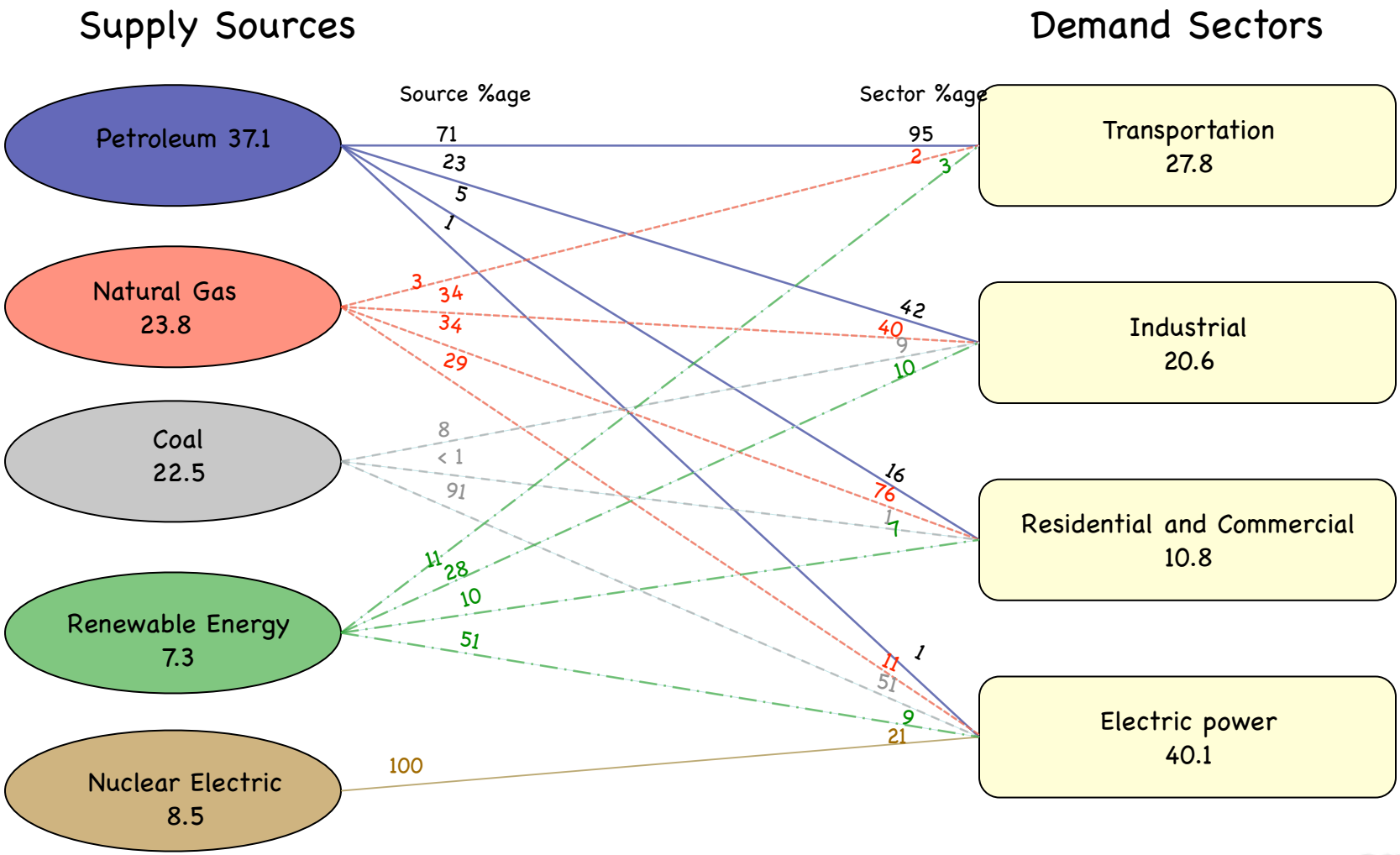
Energy Consumption, Source and Sector



Annual Energy Review 2008 Table 1.3

Rethinking Fuels

- Energy usage is projected to increase by 55% over the next 20 years
- Transportation is the single largest user of petroleum feedstocks
- There will be an additional 2.3 billion cars worldwide on the roads by 2050
  - 1.9 billion will be in developing countries
- Improvements in mileage standards and alternative vehicles will reduce the need but not eliminate the need for liquid transportation fuels
  - Hybrid systems are expected to dominate new vehicle sales by 2030
- Corn ethanol today can reduce emissions by 30-70% compared to gasoline
- Cellulosic fuels (second-generation) will reduce emissions approximately 90% (NREL Report)

World Energy Outlook 2007, International Energy Agency, Energy Information Agency,
International Monetary Fund, US DoE, Merrill Lynch, International Energy Agency, UNICA, Renewable Fuel Association, EU Commission

Corn Ethanol Production Processes

Corn Starch

Enzymatic Conversion

Sugar

Fermentation

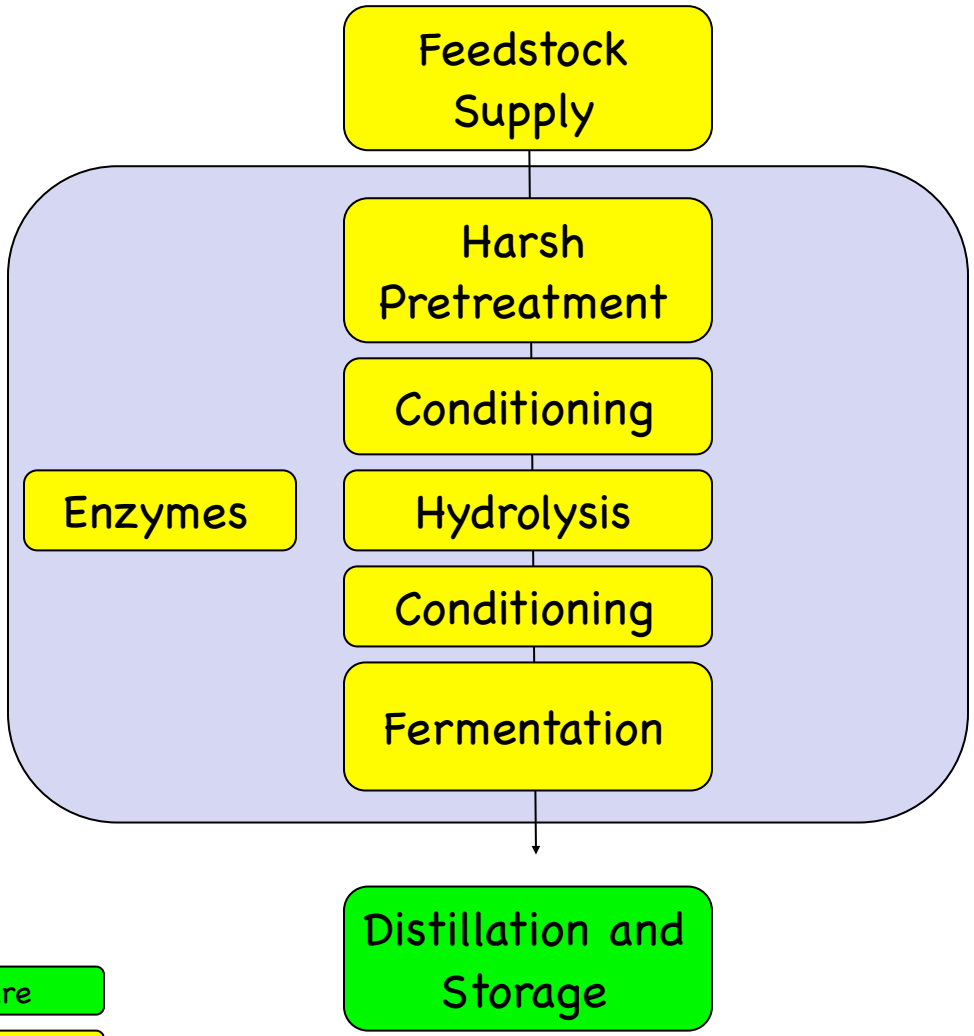
Distillation

Drying

Co-Product Recovery

Ethanol Recovery

Cellulosic Biomass Processing



Most processes are experimental and in development

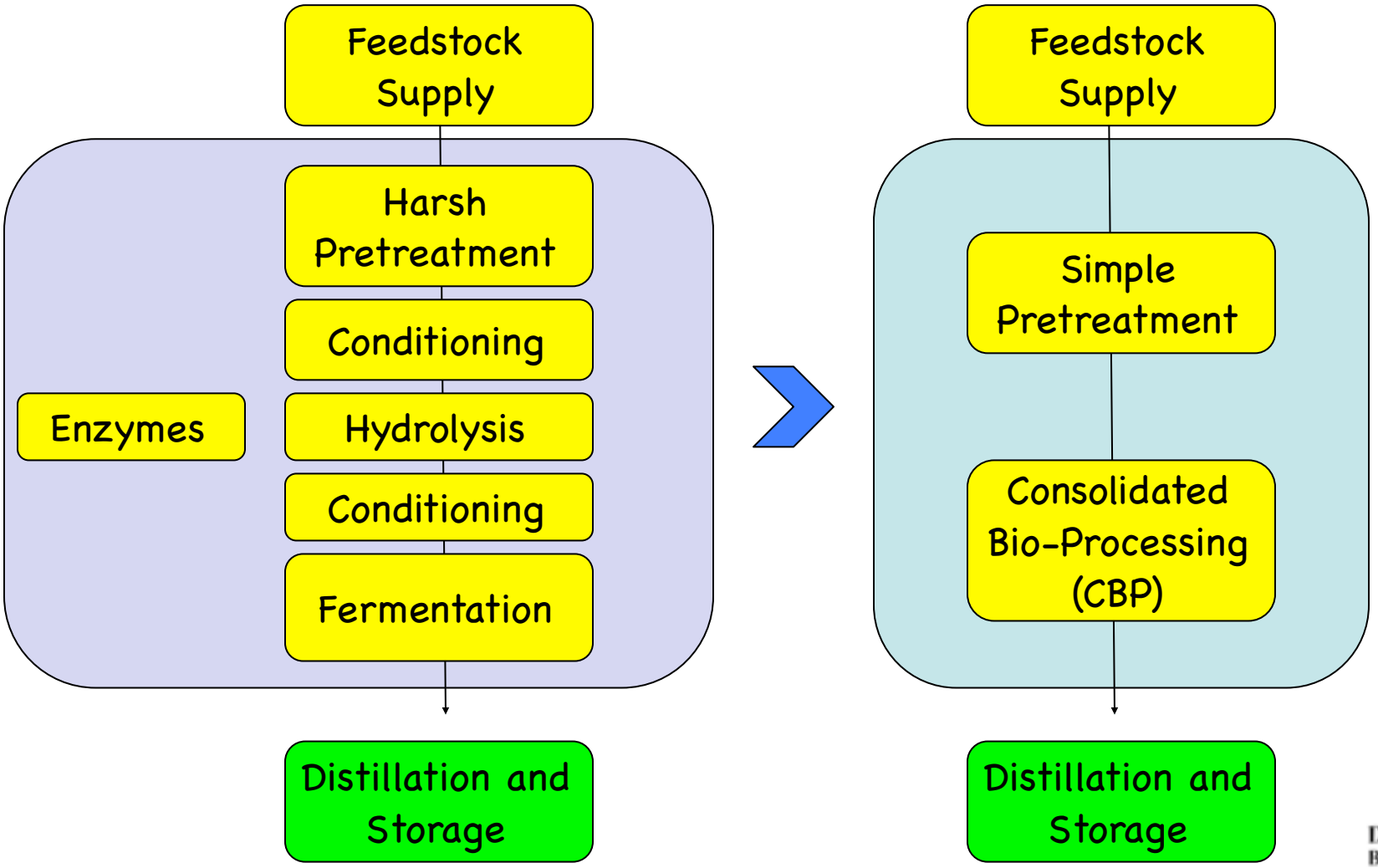
Current approach by companies: POET and Catchlight

Mature

Experimental

Consolidated Bio-processing

Potentially lower costs through process simplification



Industrially Compatible Enzymes

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

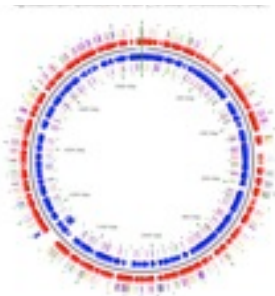
Selected Enzymes

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)

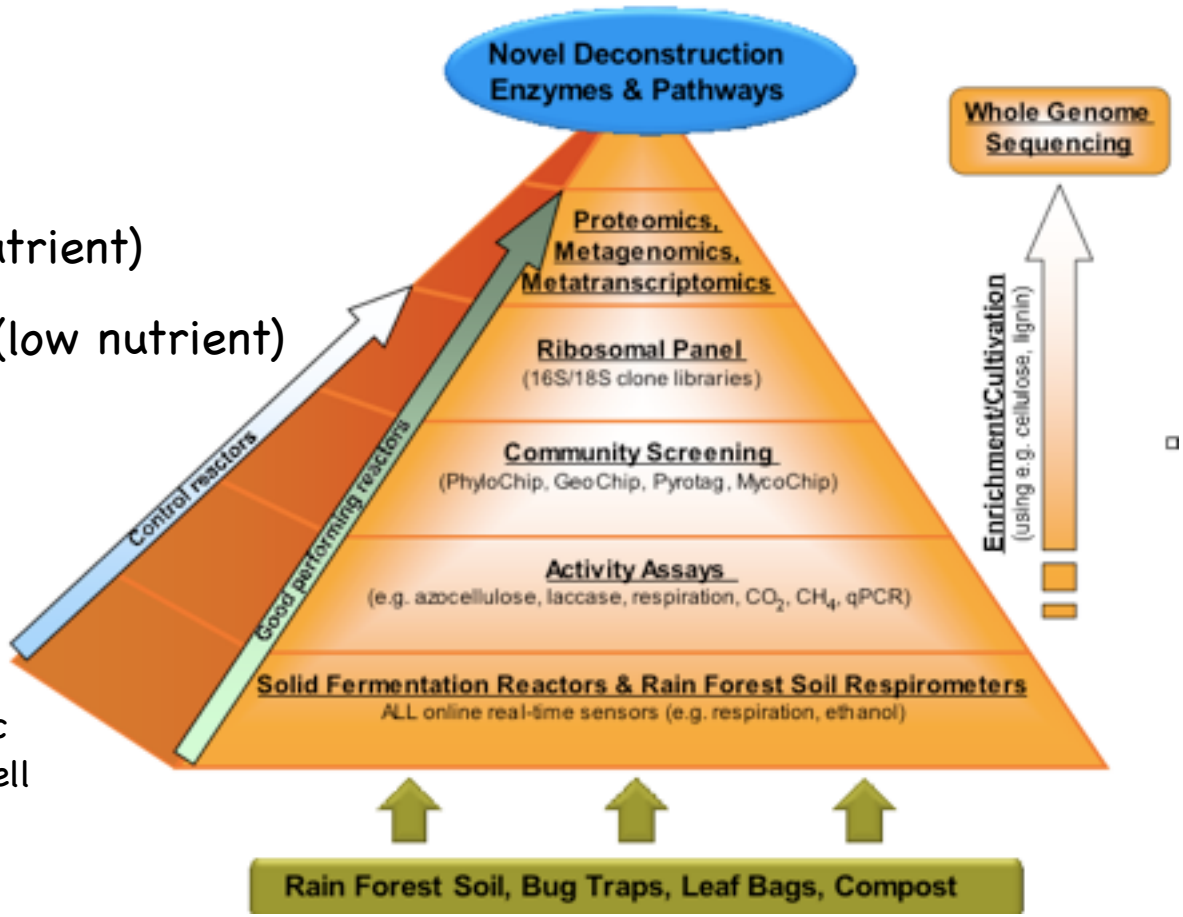


Microbial Communities

Deep and High-Throughput 'Omics' analyses of Microbial Communities in potentially High Return Environments

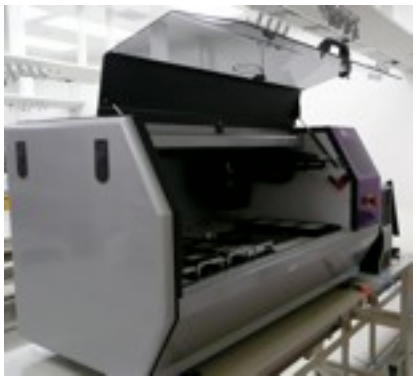
Initial Environments:

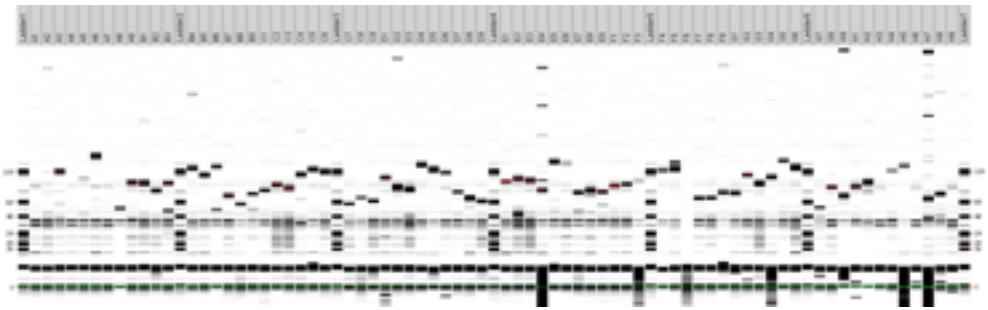
- Green Waste Compost (high nutrient)
- Puerto Rican Rain Forest Soil (low nutrient)



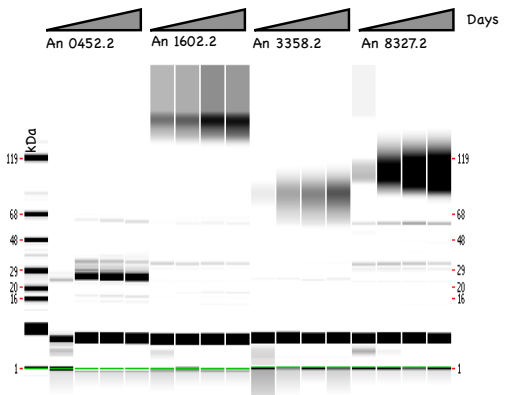
Identified and isolated lignocellulolytic enzymes, metabolic pathways, whole cell biocatalysts, and mixed cultures for deconstruction

Tiered Screening Strategy





E coli Detection



Pichia Optimization

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Biocatalyst Discovery

- hemicellulases from compost metagenome

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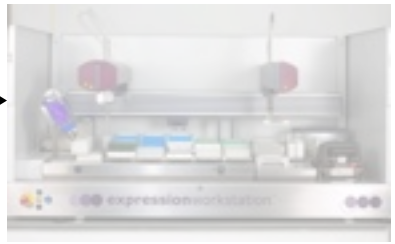
Discovery

Shotgun sequencing

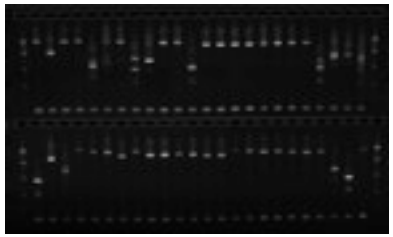
454 Titanium Sequencing:
548K reads, 226Mbp, 8,268 contigs,
22 putative hemicellulases

Gene synthesis

High-throughput cloning



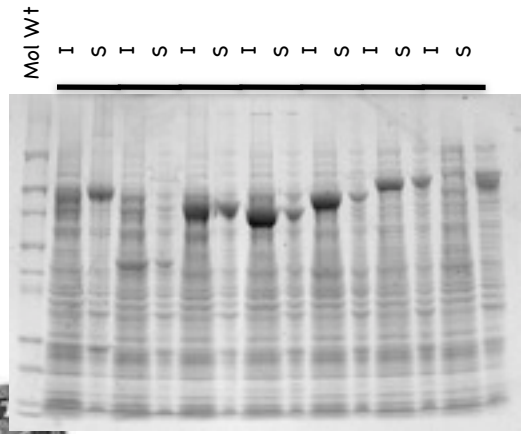
Colony PCR screen



Allgaier et al. PLoS One, 2010

Expression and Activity Screening

Solubility in Whole Cell Extracts

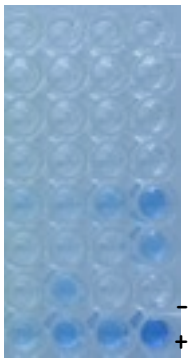


Activity Assays

β-xylosidase

JMC25406

Endoxylanase



JMC37744

Positive hits are being further characterized and optimized for cocktail development

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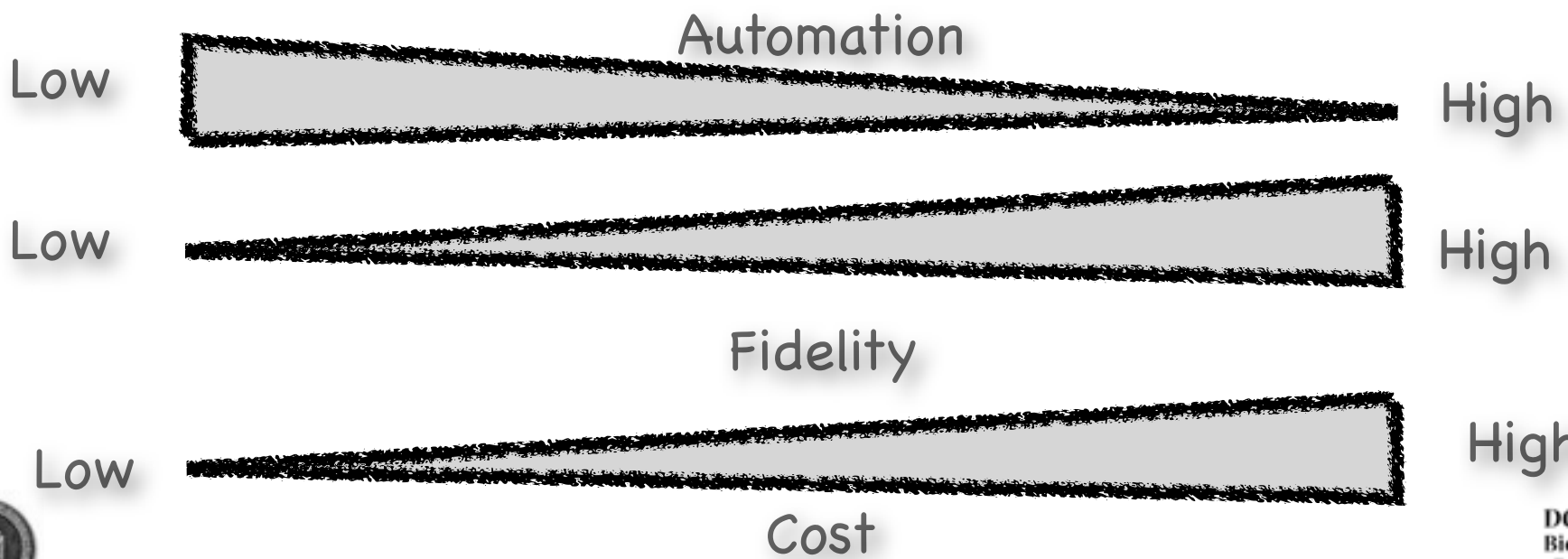
Assay Development

Zone Clearing Assay

DNS Assay

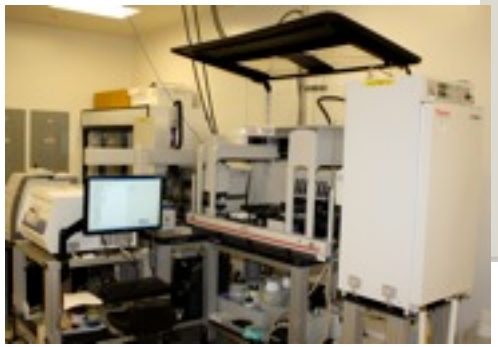
Glucose Assay

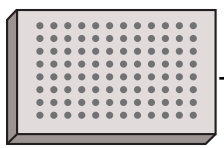
HPLC

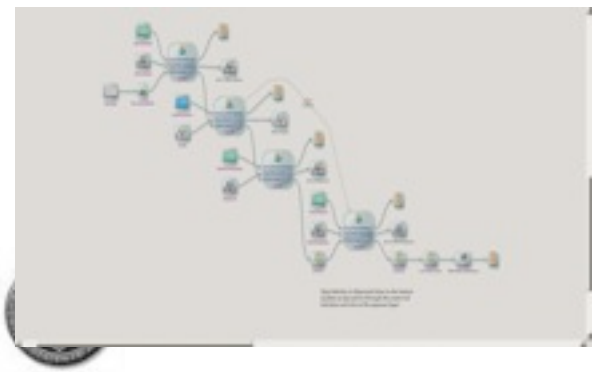


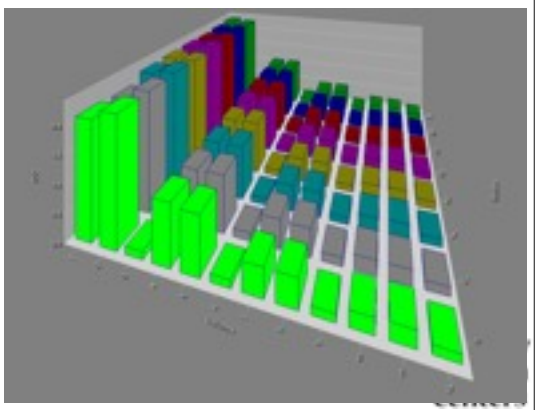
Assay Workstation

- Integrated operations for screening and assays
- Reporting and data management
- Various other liquid handling applications



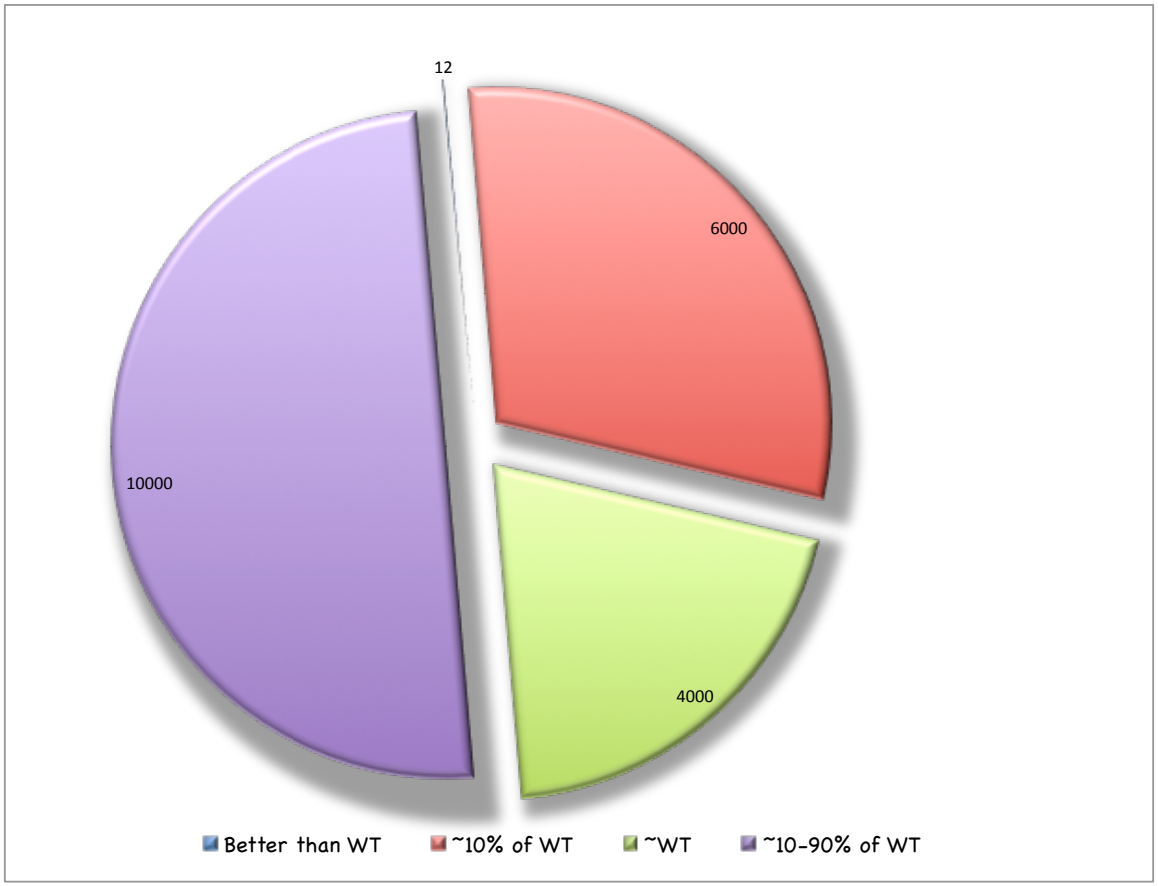






Evolution and HT Screening

- Evolve the ORF
- Screen for activity using CMC and DNS
- Most of them are worse than the "WT" enzyme



Solid Substrate Assays

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Challenges

Biomass dispensing
- High throughput
- Uniformity
- Particle sizes distribution

Enzyme – biomass reaction
- Mass transfer
- High biomass loadings
- Evaporation

Workflow

Substrate Preparations
- Make up 2% biomass slurry in 2% PEG 20K solution
- Dispense 2% biomass slurry to 96-deepwell plate
- Dry plate over night at 70°C
- Store plate at room temperature

Saccharification
- Add 200µL solution containing enzymes (10% w/w enzymes to glucan content per well)
- Seal and incubate plate for 20 – 24 hrs @ 1000 RPM

DNS Assay
- Spin plate for 30 min, 1500 xg
- Aliquot supernatant for DNS reaction in plate reader plate
- Incubate reaction at 70°C for 30min
- Read absorbance at 540nm

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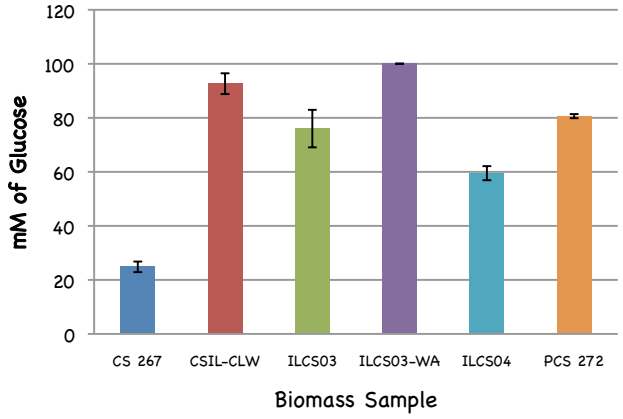
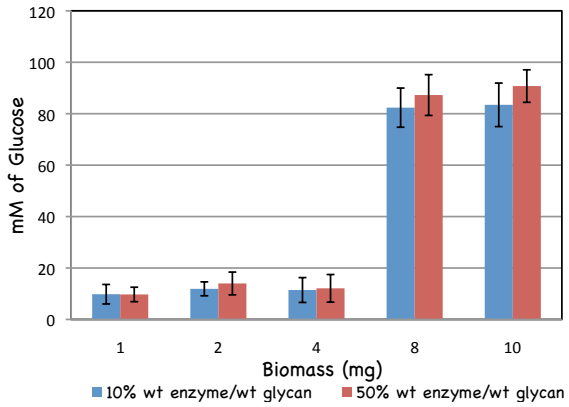
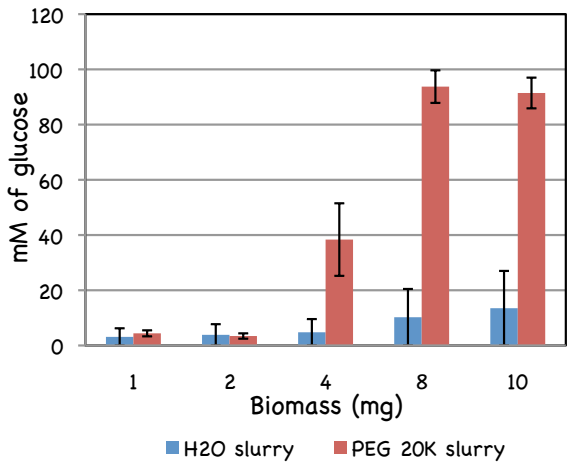
Assaying on "Real World" Substrates



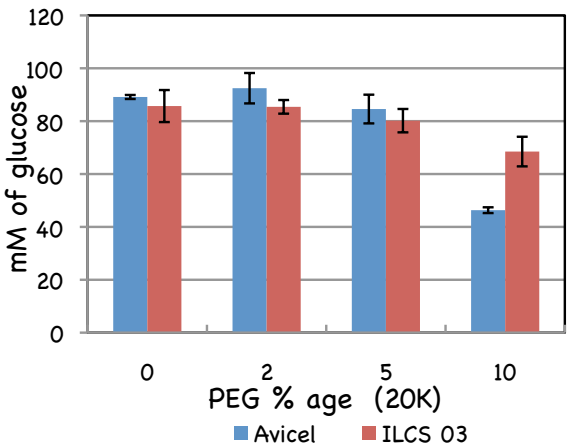
Substrate Source



Enzyme Loading



H_2O vs PEG Slurry



PEG Effects Conversion

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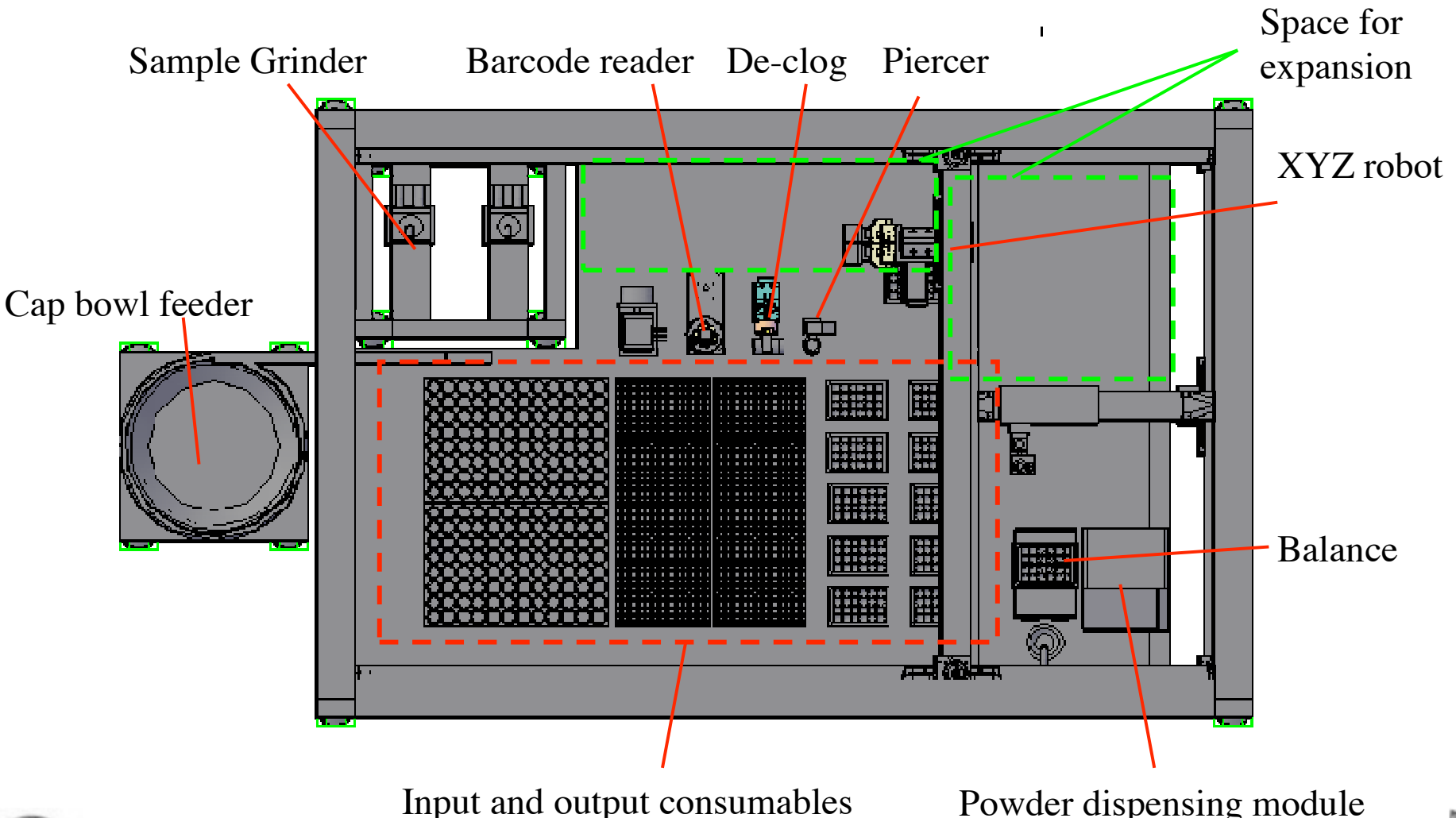
Solid Substrate Dispensing Robot



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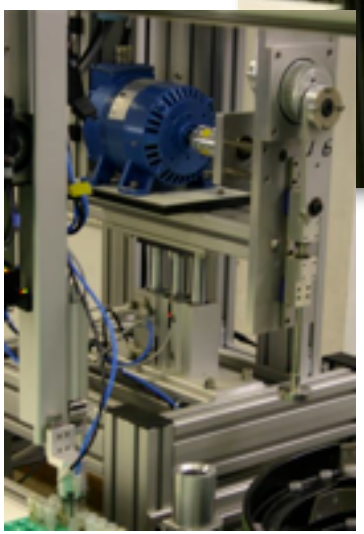
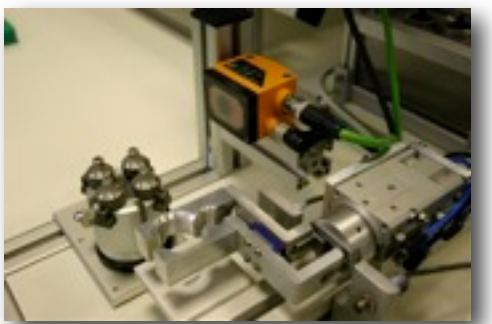
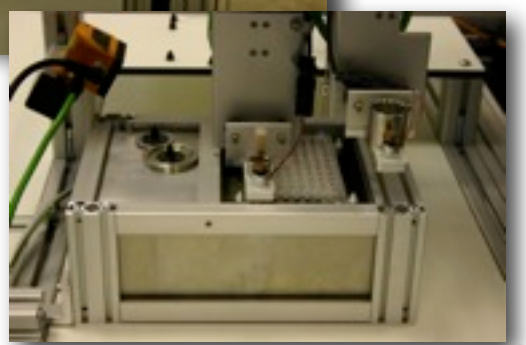
System Image



Large Grinder



Barcode Scanner



Balance

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Large Grinder

Large Grinder

Uniformity Delivery Across Various Biomass

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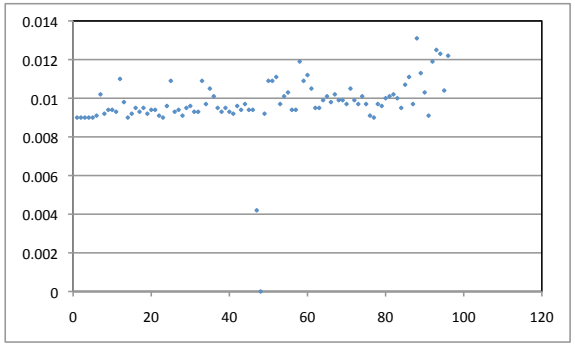
Dispensing Accuracy
2 mg +/- 0.002
5 mg +/- 0.005
10 mg +/- 0.001

Switchgrass

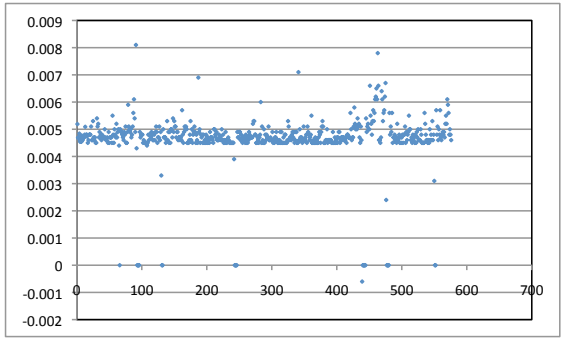
oven dried sample
1 x 96 deep well plate
10 mg target, 1 mg tolerance weight



Deep 96 Well plates
10 mg / well

Corn Stover

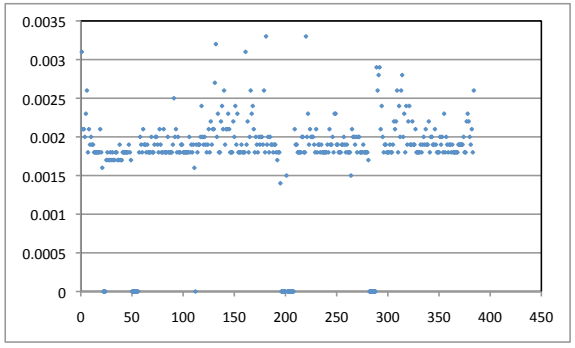
2 X 96 vials to 6 deep well plates
5 mg target, 0.5 mg tolerance



Deep 96 Well plates
5 mg / well

Rice Stem

4 x 96 vials to deep well plates
2 mg target, 0.2 mg tolerance



Shallow 96 Well plates
2 mg / well

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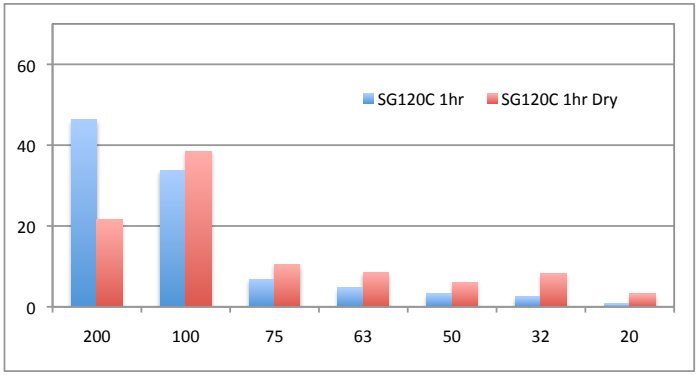
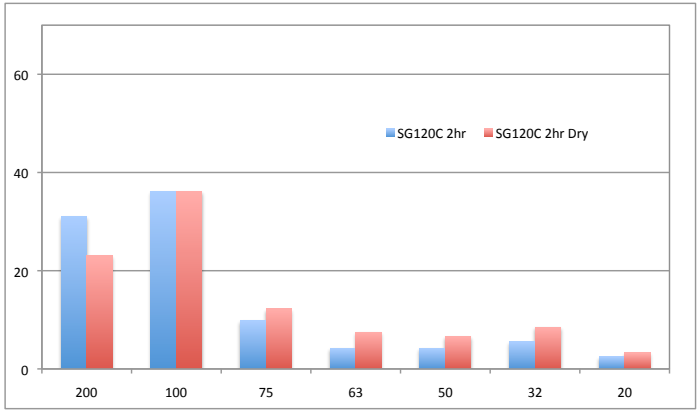
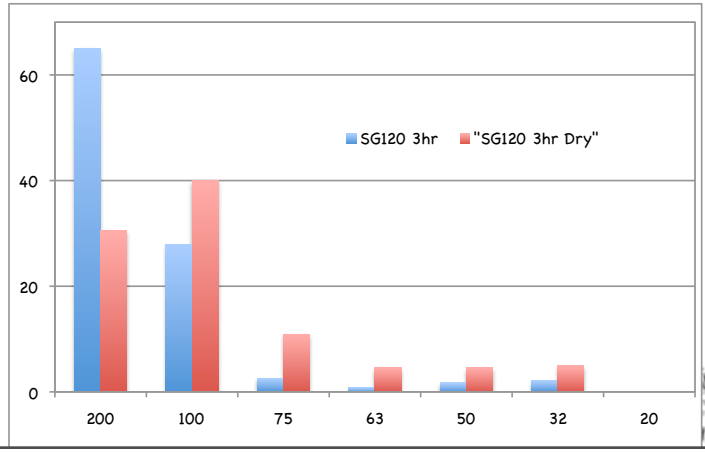
Particle Size Variation

- Particle size varies considerably, depends on source and treatment

- Drying the sample in the oven helps achieve a more uniform grind and significant differences are due to the readily hygroscopic nature of the substrate

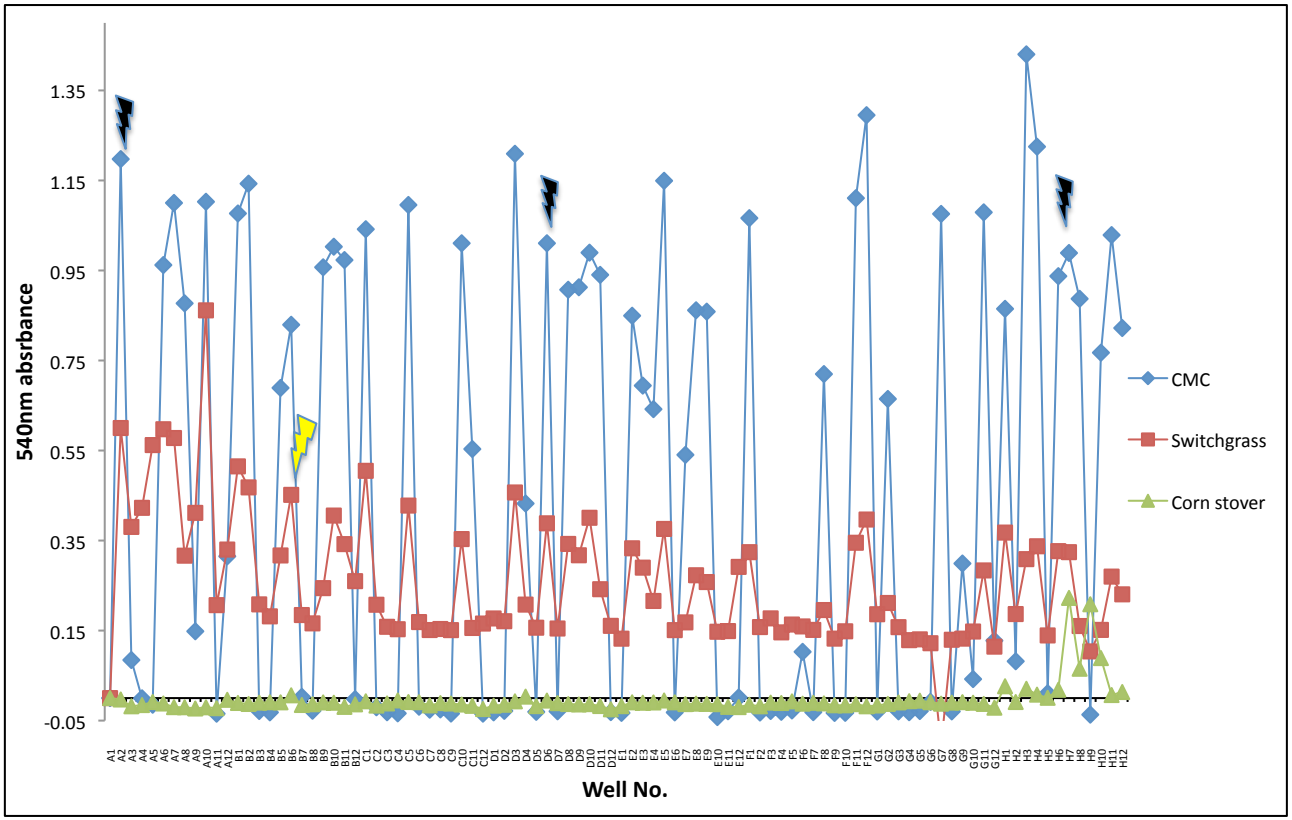






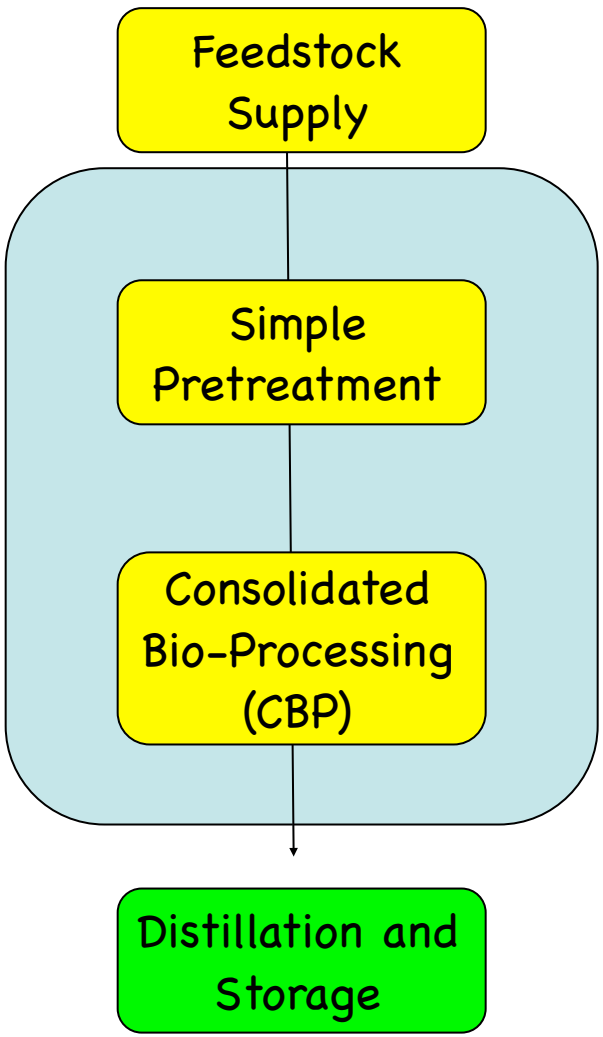
Evaluating on "Real" Substrates

- Re-assay ORFs that were experimentally shown to be active on CMC
- Most of them were dead on real world substrates



Trojan Horse Strategy

Lower costs through process simplification



Engineered Biomass Advantages
- Consolidated bio-processing
- Steps and cost reduction
- Elimination of enzyme costs

About 30-40% of costs are associated with pretreatment

Mature

Experimental

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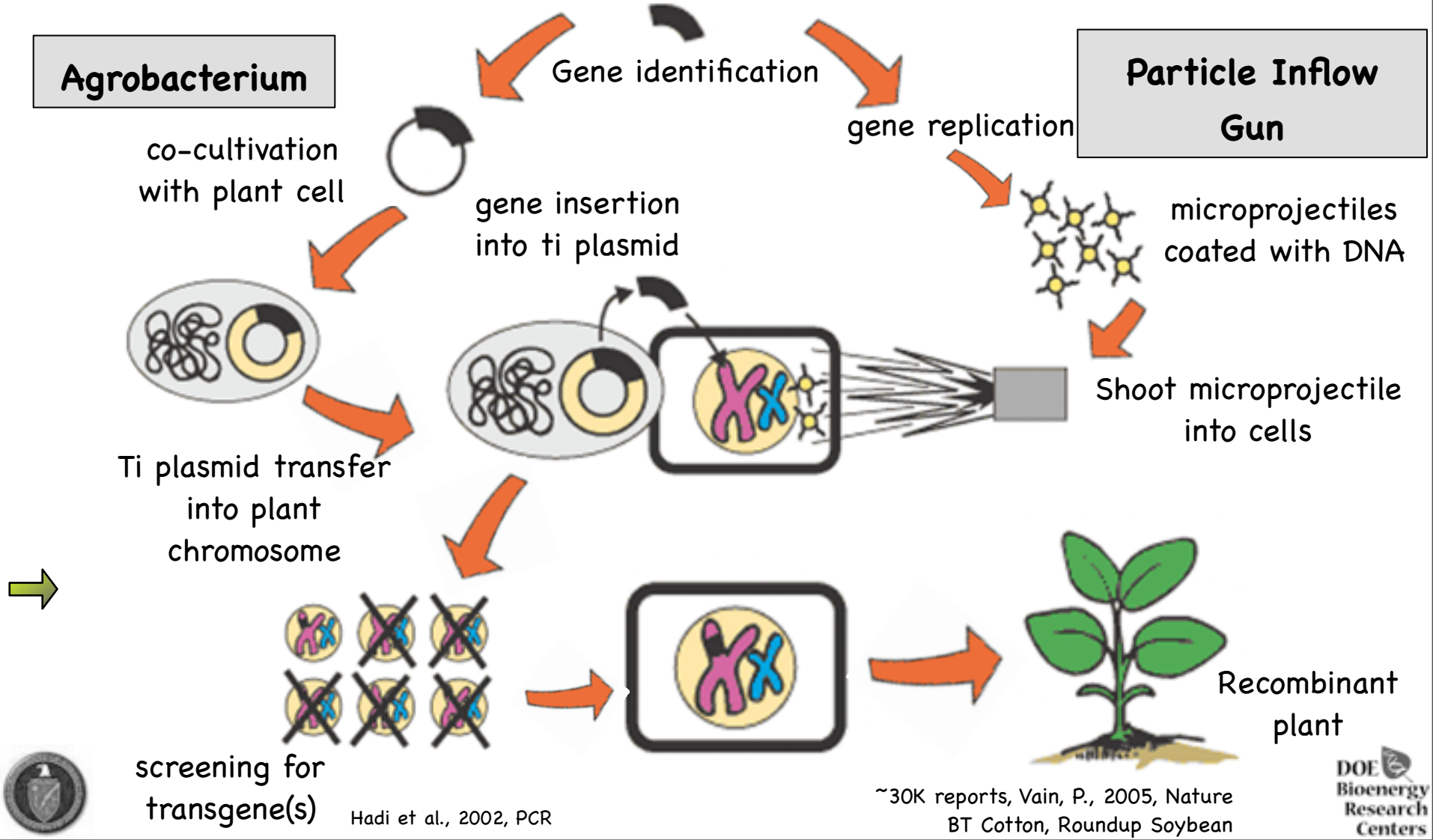
Plant Transformation:

Agrobacterium and Particle Inflow Gun Mediated



Hadi et al., 2002, PCR

~30K reports, Vain, P., 2005, Nature
BT Cotton, Roundup Soybean

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Bioinformatics Analysis

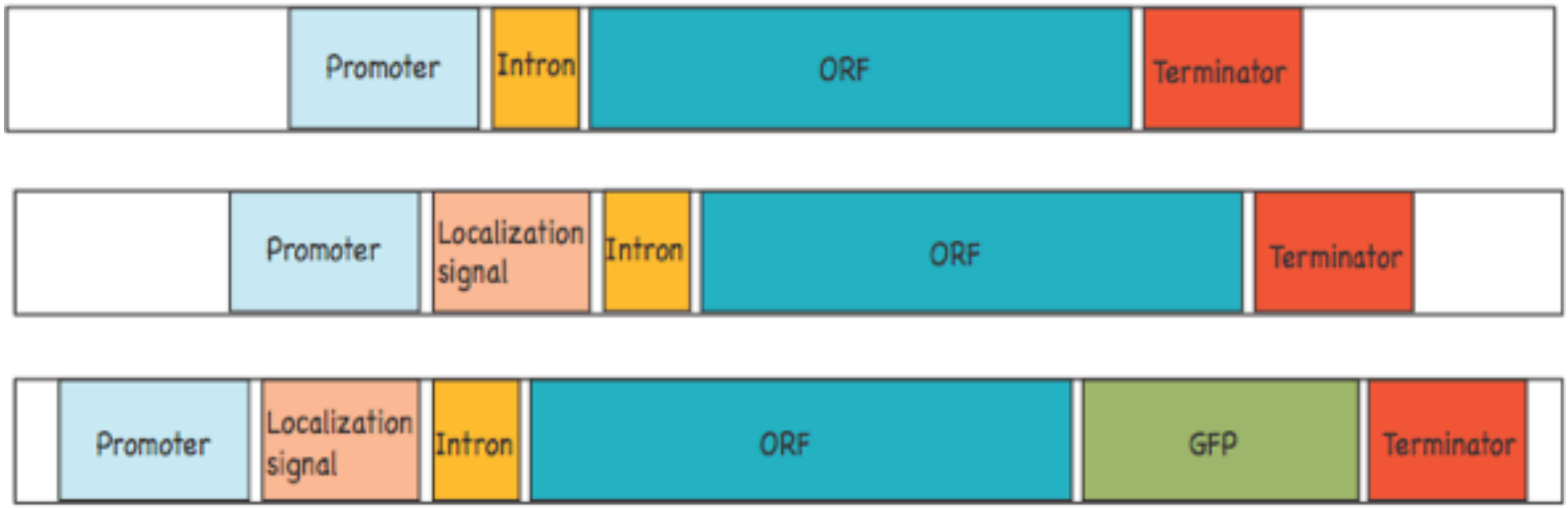
Optimization Gene Synthesis

- CelA
  - CelA from Alicyclobacillus acidocaldarius is optimally active and stable at temperatures above 65°C, belongs to glycoside hydrolase family 1 (GH1) and shows wide substrate specificity on different glycosides and cello-oligosaccharides

- SSO1949
  - SSO1949 gene from thermoacidophilic archae Sulfolobus solfataricus encodes a thermostable (GH 12) carboxymethylcellulose as well as cello-oligomers. This enzyme has a pH optimum of 1.8 and a temperature optimum of approx. 80°C. Furthermore, the enzyme has a half-life of approx. 8 hrs at 80°C and pH 1.8

Actuator Construction

Targeting and Gene Regulatory Sequences



- 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 + intron-SP-Cellulase-GUSPlus-nos cassette in pCAMBIA1305.2 (translational fusion)

Plant Model Systems

Arabidopsis

- Brassicaceae family, are small flowering plants related to cabbage and mustard. This genus is of great interest since it contains Thale Cress (Arabidopsis thaliana), one of the model organisms used for studying plant biology and the first plant to have its entire genome sequenced. Changes in the plant are easily observed, making it a very useful model system

Brachypodium distachyon (L)

- Has many qualities that make it a model for functional genomics studies in temperate grasses, cereals, and dedicated biofuel crops such as Switchgrass. These attributes include small genome diploid accessions, short life cycle, and simple growth requirements

Agro-Infiltration Mediated Transformation

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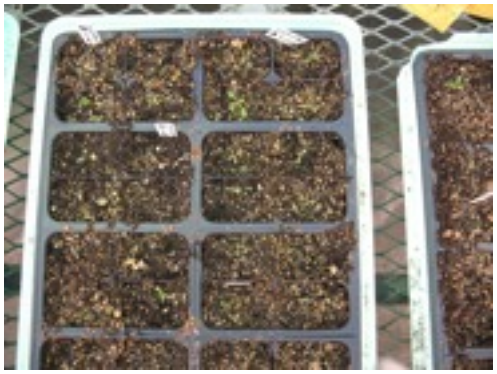
CelA



SSO 1949

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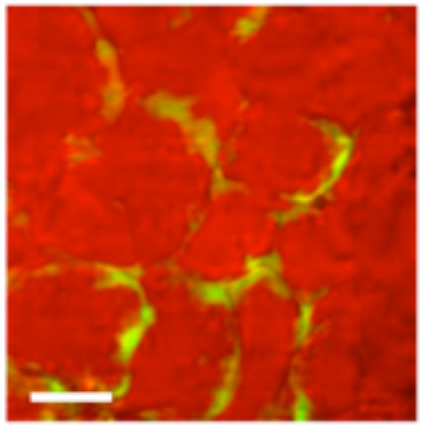
Sub-cellular Protein Localization

Arabidopsis Seedling (Hyperspectral Imaging)

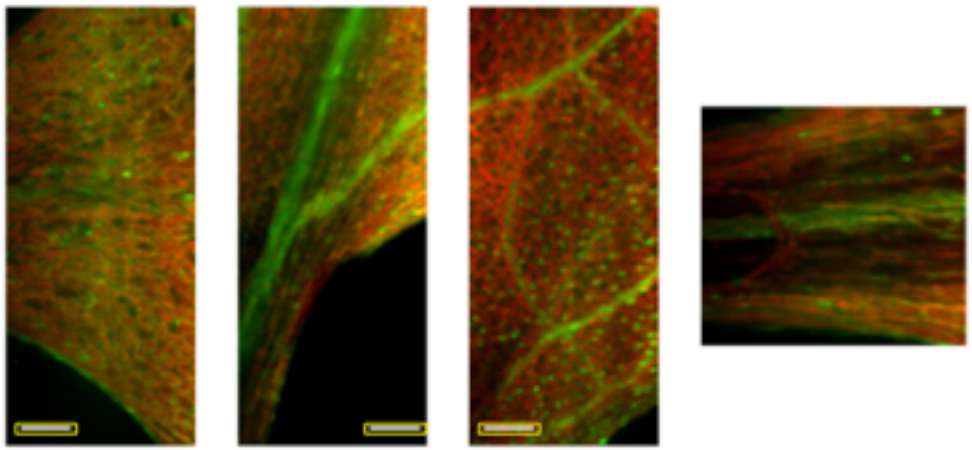
Cytoplasmic

Cajal Body



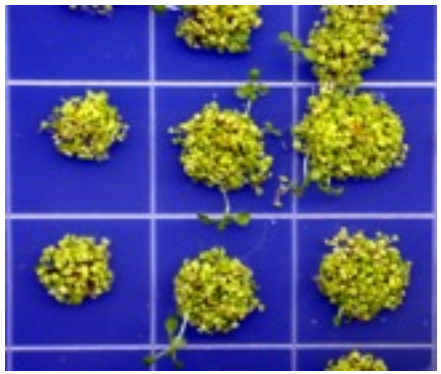
Scale bar is 10 M

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

Arabidopsis Containing the Genetic Circuit Were Selected

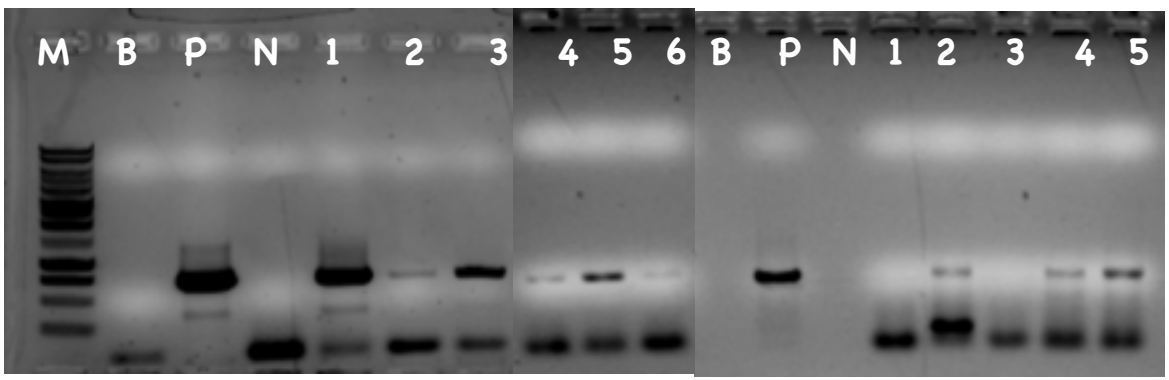


Seed Selection

Transfer to Soil

Grinding & DNA Extraction

SSO 1949

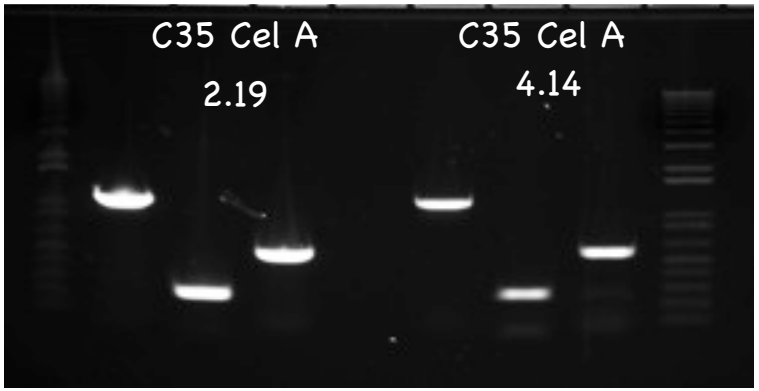


CelA

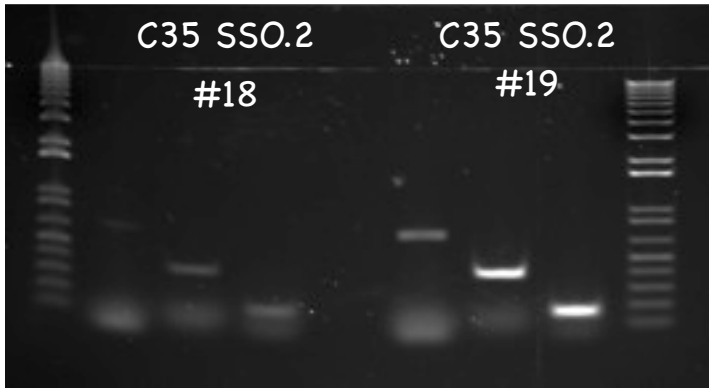
PCR analysis to confirm the presence of actuator in plants

Transcript Analysis

RTPCR in Arabidopsis Transgenics



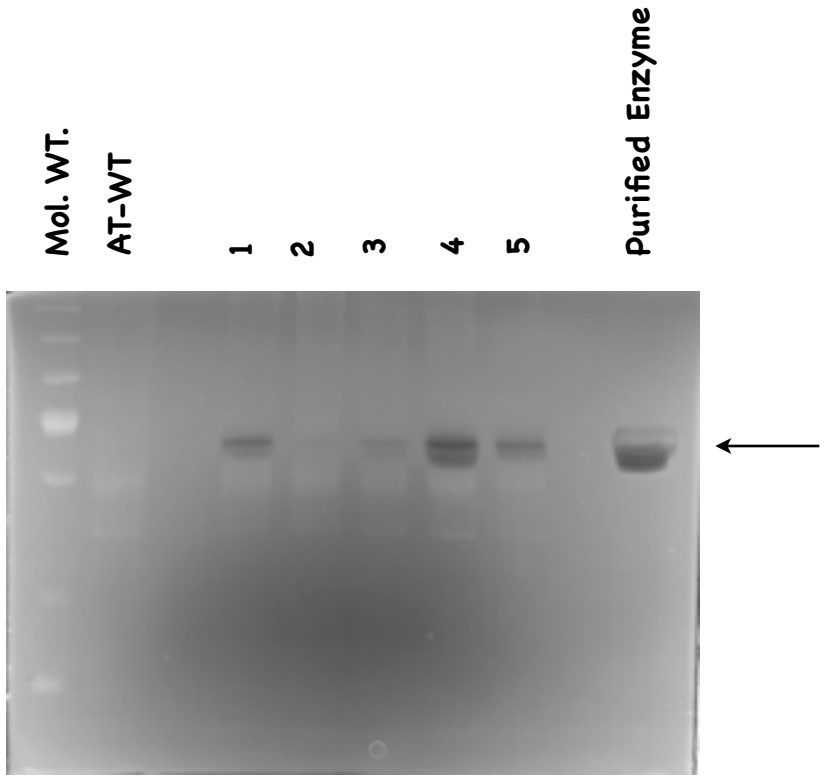
1. CelA F2 + CelA R1 (1194 bp)
2. CelA F1 + CelA R2 (269 bp)
3. CelA F3 + CelA R1 (576 bp)



1. 1949 Plant F1 + 1949 Plant R1 (713 bp)
2. 1949 Plant F2 + 1949 Plant R1 (393 bp)
3. 1949 Plant F3 + 1949 Plant R1 (176 bp)

We were able to confirm expression of both actuators in all trangenics

Zymograms Confirm Enzyme Activity

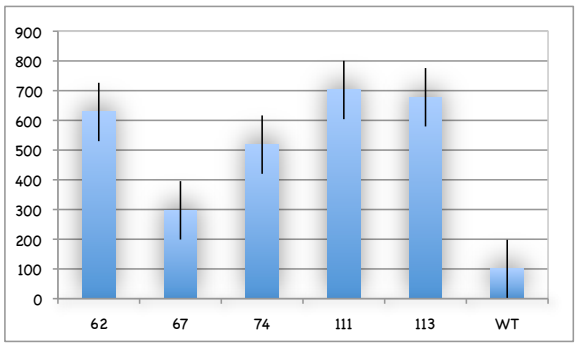


Zymograms were used to assay for protein activity in Arabidopsis extracts.
We were able to confirm activity with whole cell lysates prepared from plants containing CelA

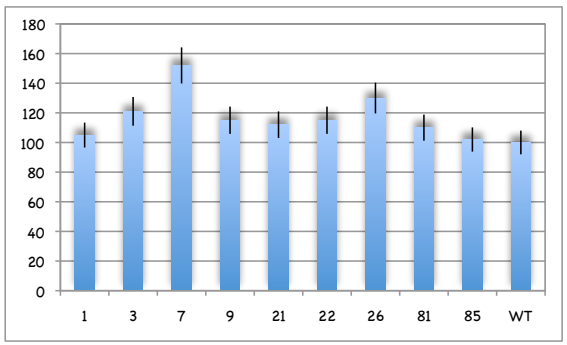
T3 Homozygous Plants

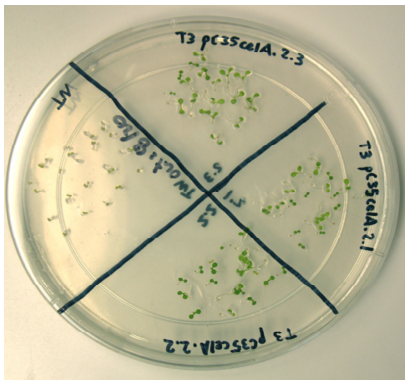
Crossed to Pyramid Genes

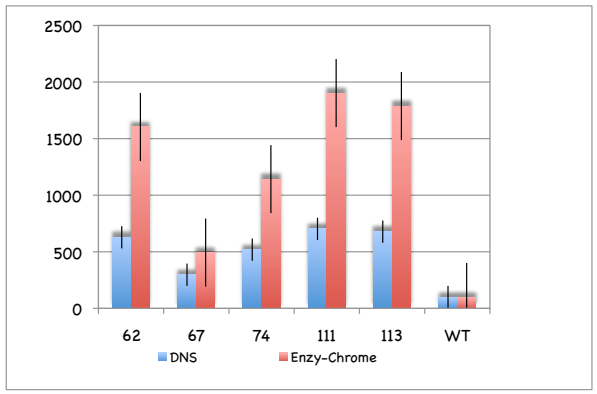
CelA

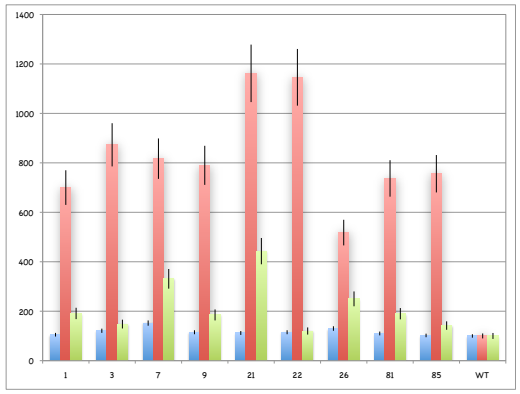


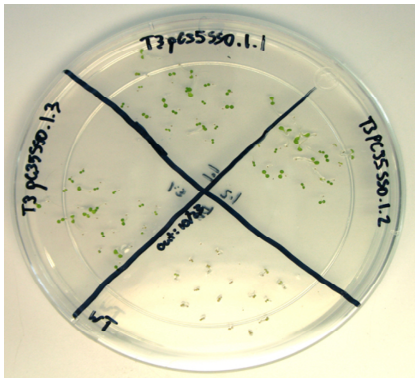
SSO-1949











SSO 1949 containing plants are slower growing compared to wild type and/or CelA harboring

Homozygous SSO 1949 plants have been crossed with homozygous CelA plants in order to pyramid the genes

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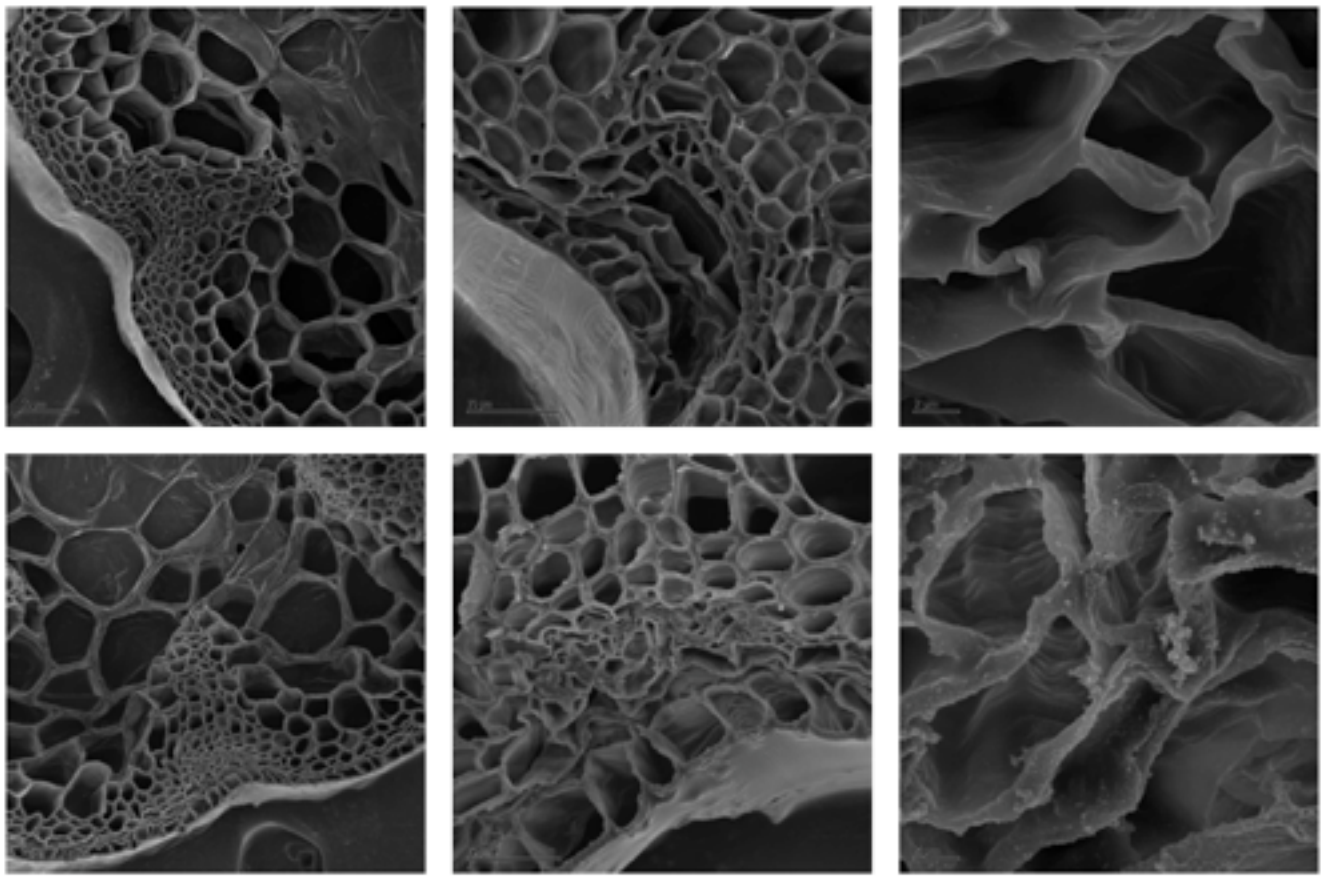
SEM after Hydrothermolysis Pre-treatment



Transgenic Arabidopsis containing Cel A treated under hydrothermolysis conditions for 1 hours

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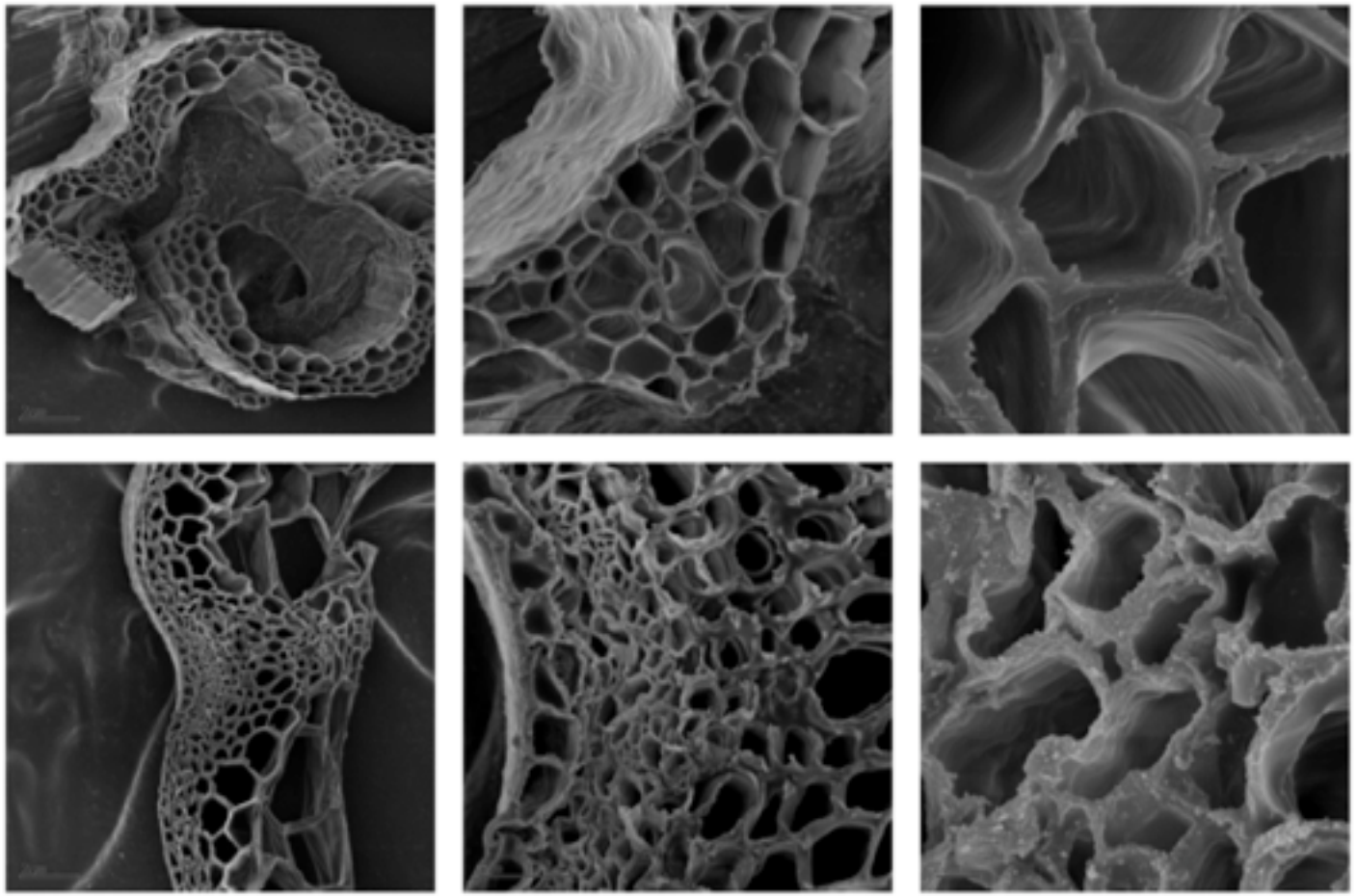
SEM after Dilute Acid Pre-treatment



Transgenic Arabidopsis containing SSO 1949 were pretreated with dilute acid for 2 hours

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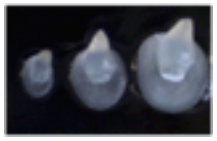
Brachypodium Transformation

Seed to seed 8 months

Green Seeds



Dissected embryos
(<0.3-0.7 mm)

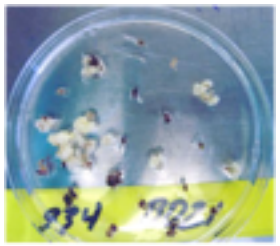
Subculture
Callus 2X
~3 weeks

Callus Induction

Agrobacterium Cocultivation
3 days

Transgenic Plants
12-20 Weeks

Regeneration
2-6 weeks

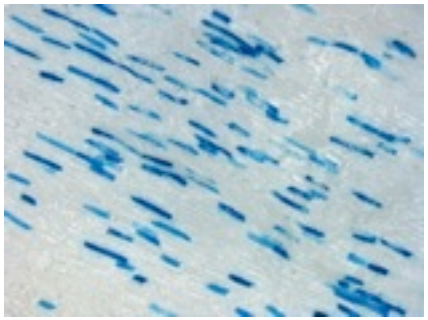


Selection
3-5 weeks

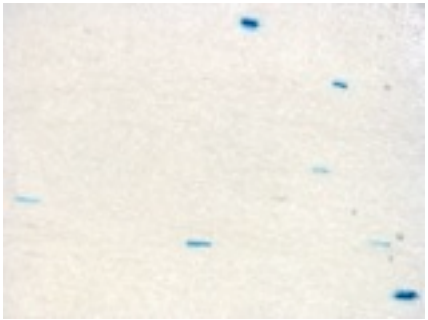
CelA and SSO1949 in Brachypodium

Transient Actuator Testing Using Particle Inflow Gun

Cel A



SSO 1949



Onion cells were transformed using particle inflow gun.
Cells were assayed for reporter protein expression after three days
CelA and SSO 1949 fusion was detected in onion cells.

Brachypodium leaf tissue was transformed using particle inflow gun.
Cells were assayed for reporter protein expression after three days
CelA and SSO 1949 fusions was detected in leaf tissue.

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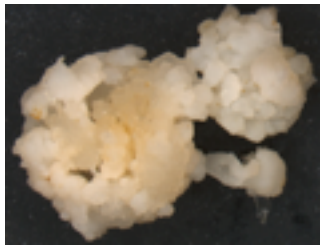
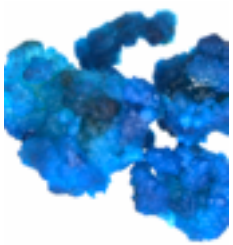
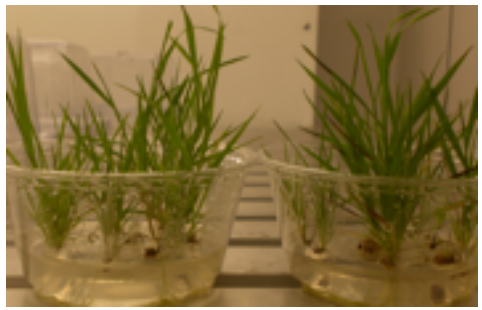
Brachypodium Transformation



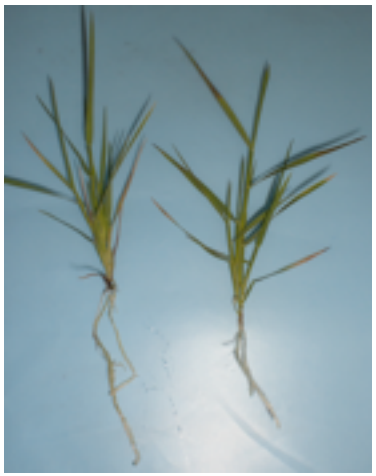


Callus Screening

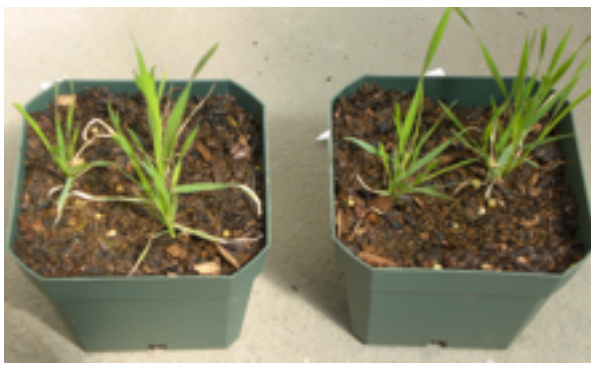


Shoot Regeneration

Shoot-tip Screening



Plantlet Acclimatization



Plantlet Regeneration

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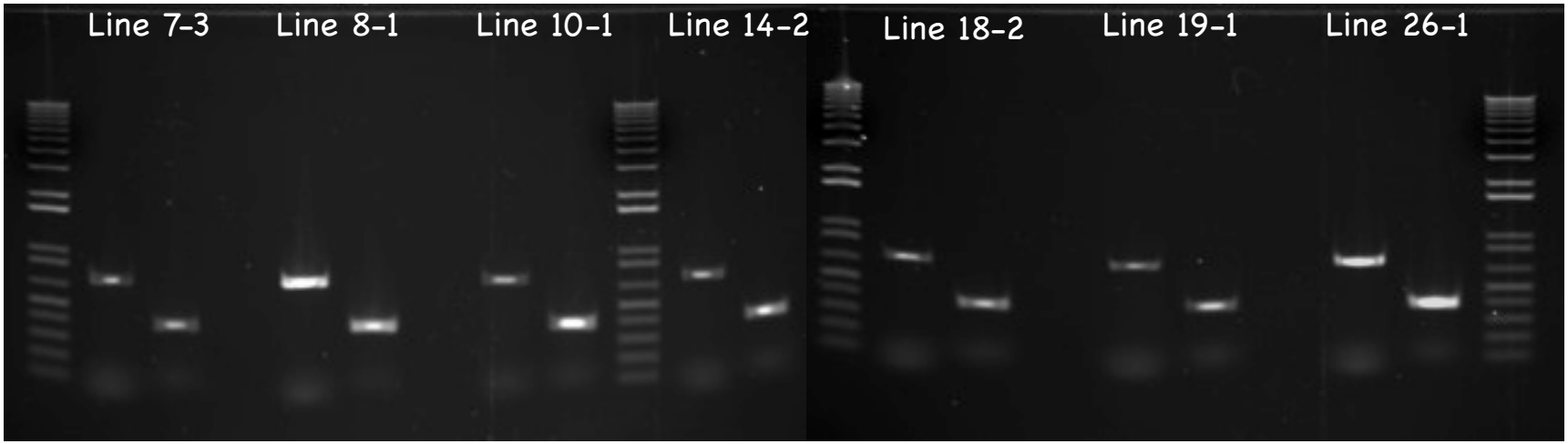
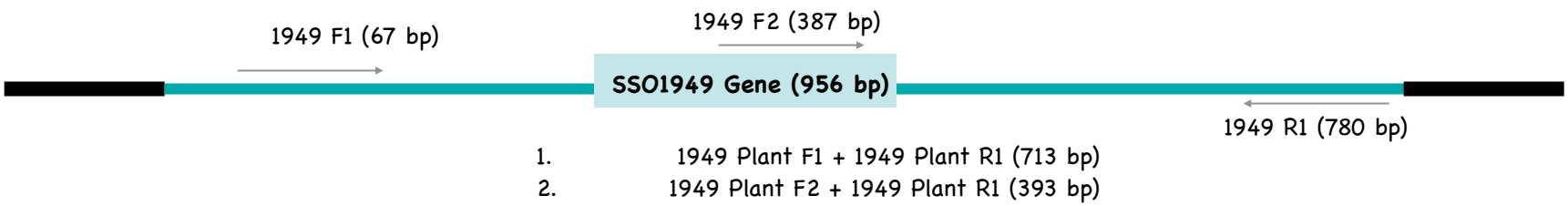
Expression Analysis - RTPCR

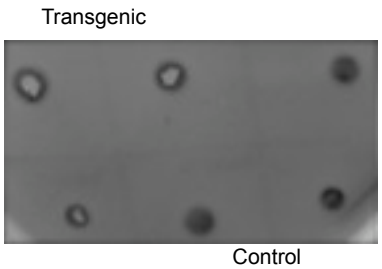




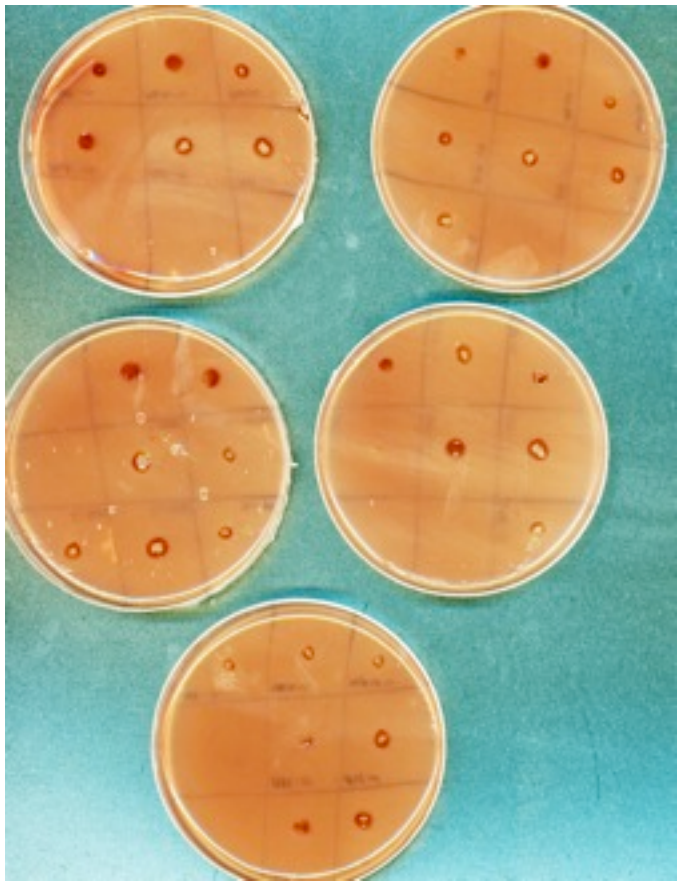
1. 1949 Plant F1 + 1949 Plant R1 (713 bp)
2. 1949 Plant F2 + 1949 Plant R1 (393 bp)

RT-PCR analysis confirms expression of actuators in Brachypodium.

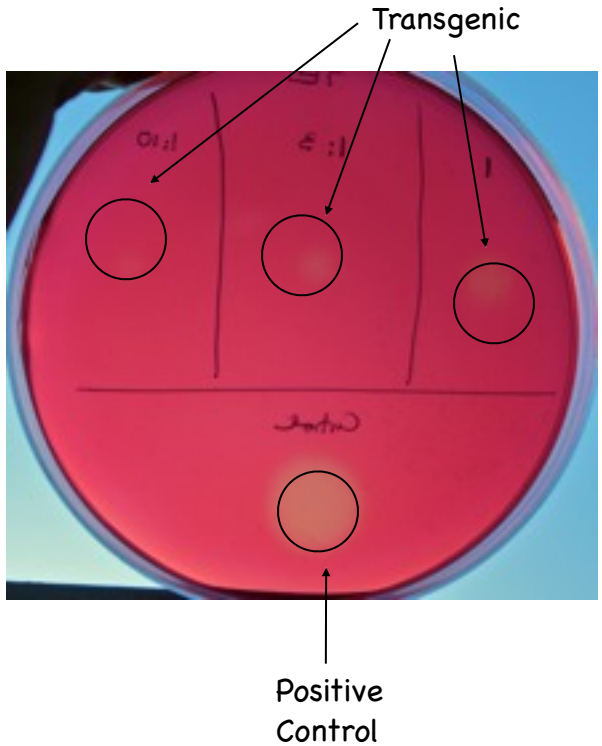
Screening for Enzyme Activity



SSO 1949



Cel A



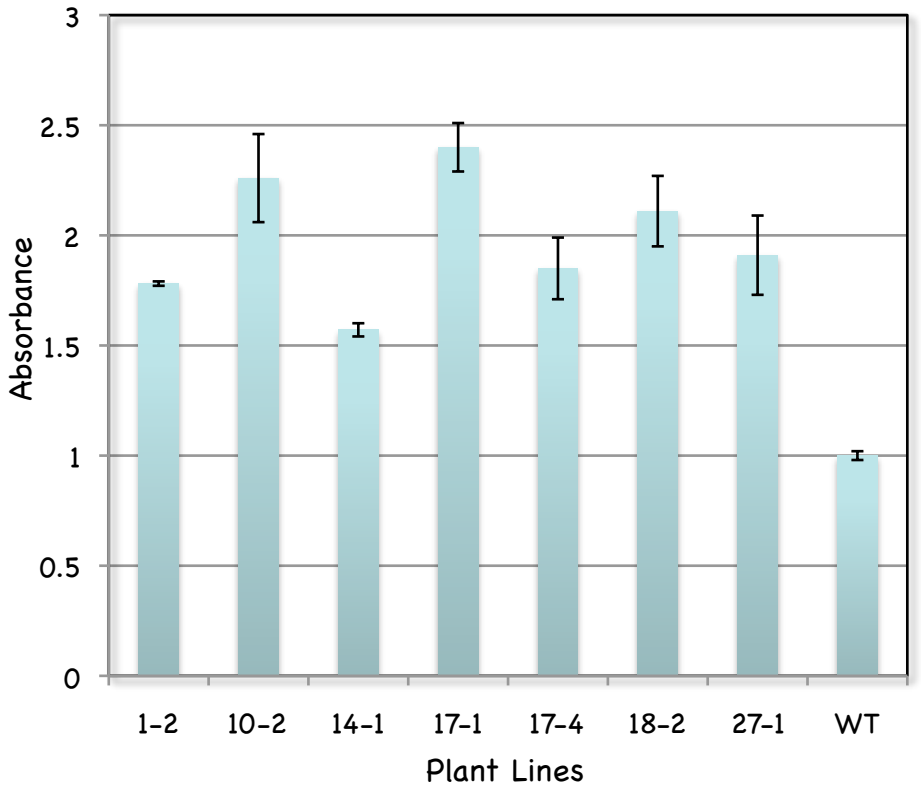
Congo red assay was used to screen for enzyme activity in transgenic callus

Enzyme Activity in Plant Extracts

Extract total protein and quantify

Assay for enzyme activity using CMC

Determine reducing end formation using 3,5-dinitrosalicylic acid (DNS) assay



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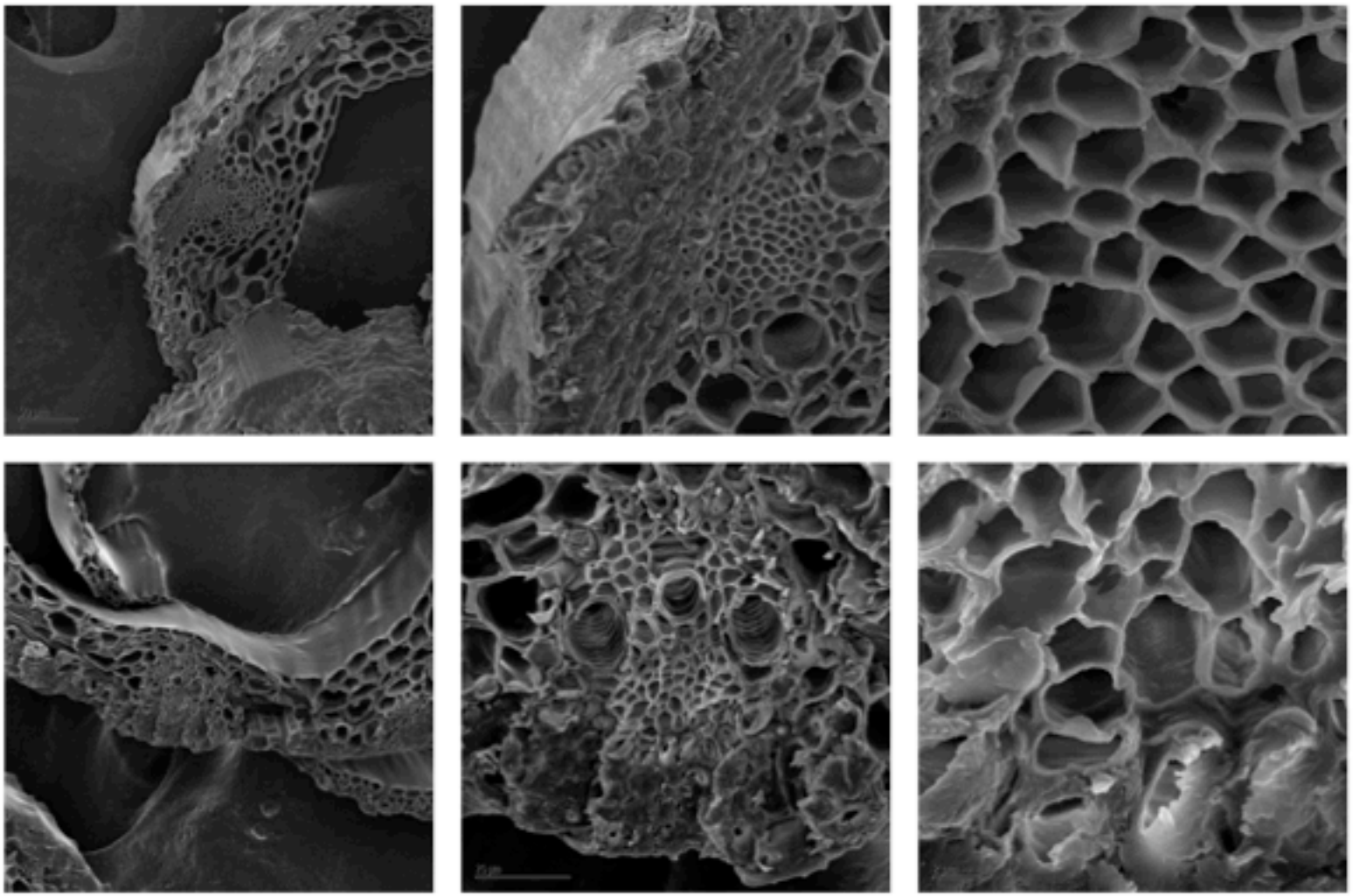
Cel A in Brachypodium after Pretreatment



Brachypodium Cel A construct were incubated under hydrothermolysis conditions for 2 hours

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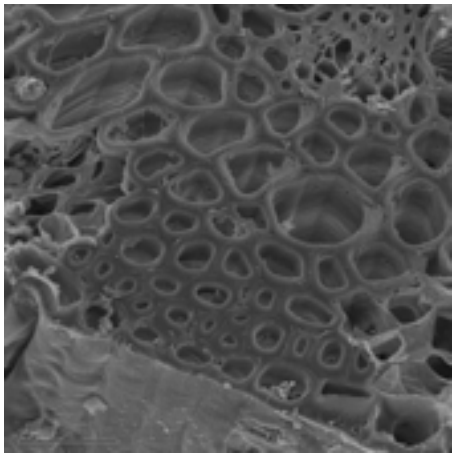
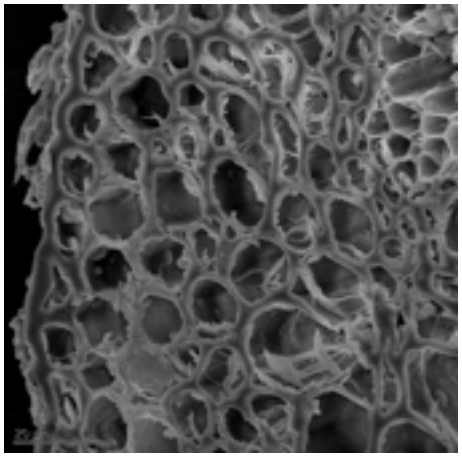
SSO 1949 in Brachypodium

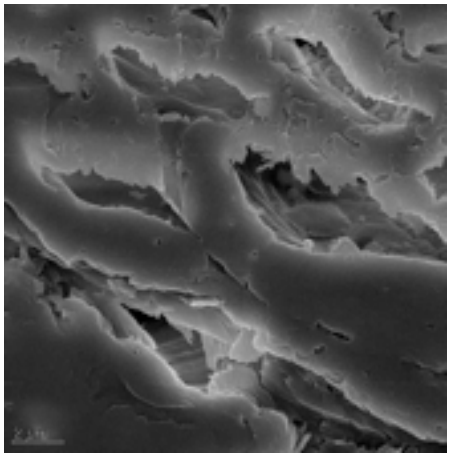
after in situ Enzyme Activation

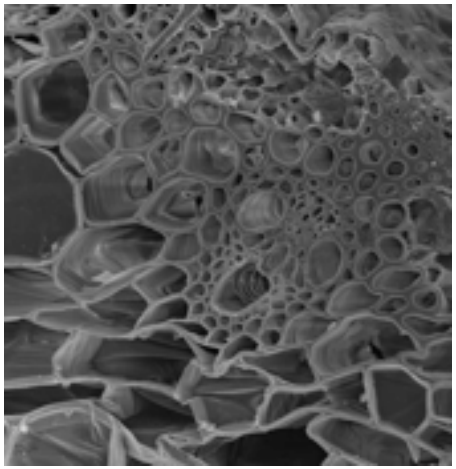
Wild Type

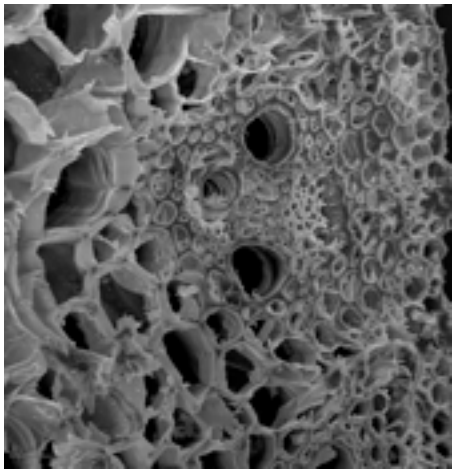


SSO 1949









Cellular integrity is diminished in recombinant plants after mild pretreatment (T= 60 min)

Brachypodium stem tissue was examined under SEM after 1 hour of dilute acid pretreatment

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Summary

- Large Scale purification is the major bottleneck
- "You get what you screen for" remains the dominant truth in the high-throughput screening (HTS) arena
- Biomass substrate source effects results
- Rescreen libraries using these real world substrates
- Evaluate engineered biomass for simultaneous saccharification and fermentation
- Expand genetic toolbox for incorporation into relevant biomass feedstocks (e.g., Panicum virgatum)

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Acknowledgements

- Mary Tran-Gyamfi
- Plant transformation - USDA
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- Anthe George
- Huu Tran
- Eiza Yu
- Blake Simmons
- Harvey Blanch
- Jay Keasling
- Paul Adams

The stone age ended not for a lack of stones, and the oil age will end, but not for the lack of oil
Founding architect of OPEC