

Tool Development for Transformational Biotechnology Advances

*October 6-7, 2011
Arlington, VA*



Summary Report

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ARPA-E Background

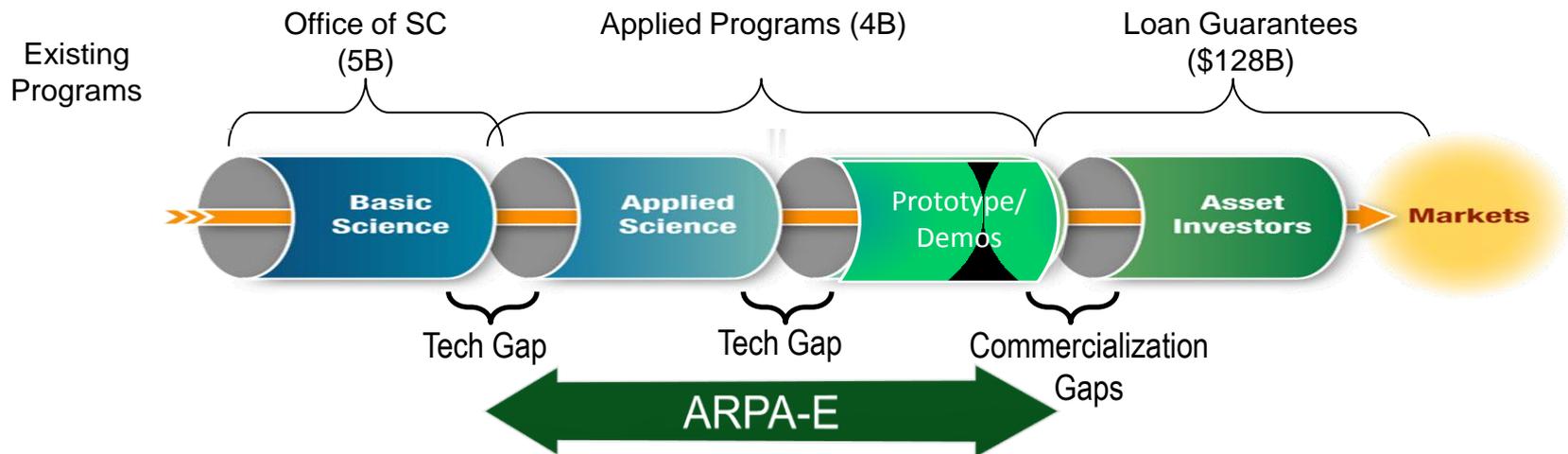


ARPA-E's Mission



- Find and fund high-risk, high-impact projects
- Identify and promote revolutionary advances in fundamental sciences
- Accelerate transformational technologies or create new technologies where none currently exist
- Translate scientific discoveries and cutting-edge inventions into technological innovations
- Bridge gaps in the energy innovation pipeline

ARPA-E was created with a vision to bridge gaps in the energy innovation pipeline



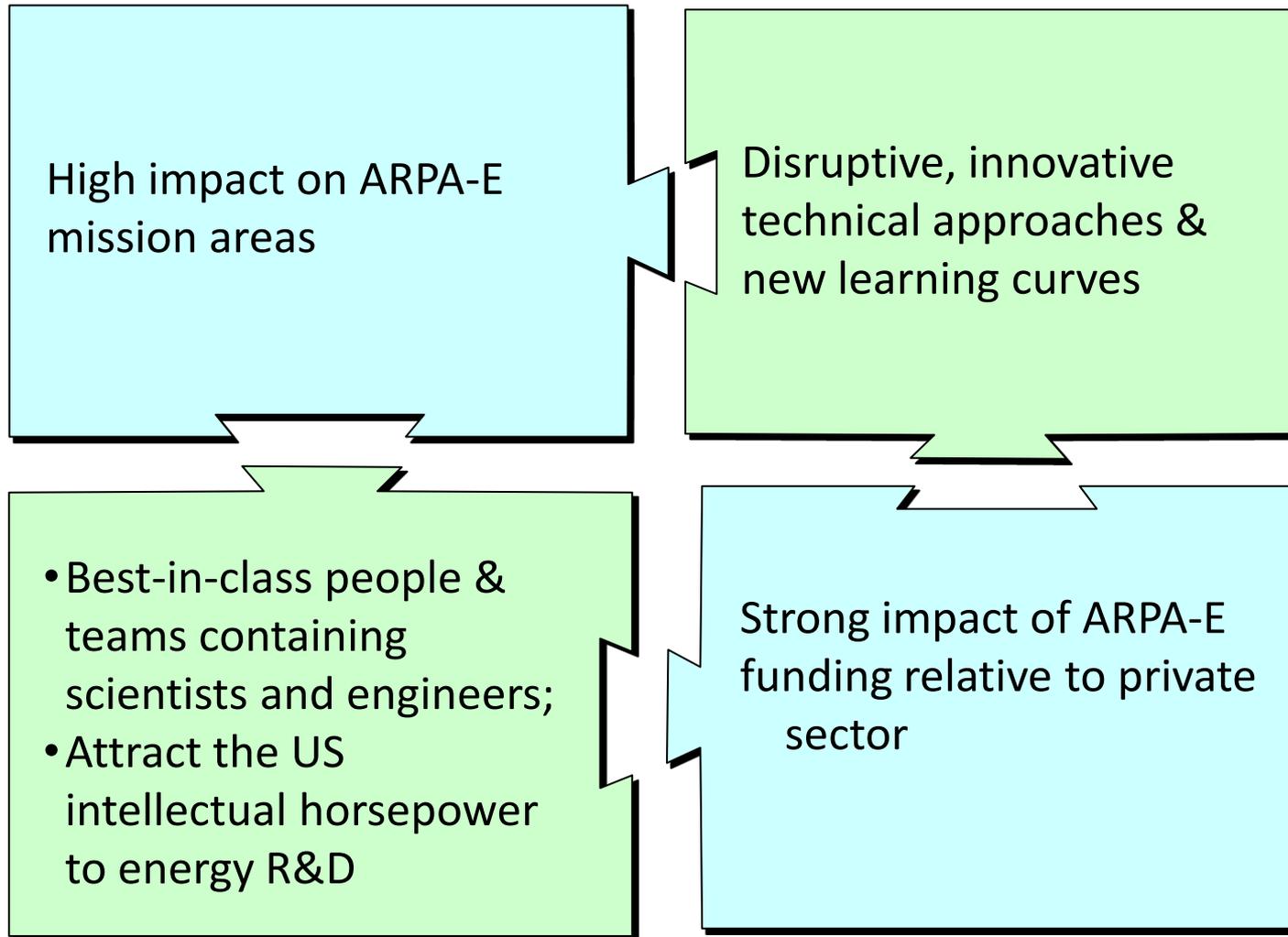
What ARPA-E will do

- Seek high impact science and engineering projects
- Invest in the best ideas and teams
- Will tolerate and manage high technical risk
- Accelerate translation from science to markets
- Proof of concept and prototyping

What ARPA-E will NOT do

- Incremental improvements
- Basic research
- Long term projects or block grants
- Large-scale demonstration projects

What is an ARPA-E project?



Technology Readiness Levels



TRL 9 : Actual technology system qualified through successful mission operations.

TRL 8 : Actual technology system completed and qualified through test and demonstration.

TRL 7 : Technology prototype demonstration in an operational environment.

TRL 6 : Technology demonstration in a relevant environment.

TRL 5 : Technology validation in relevant environment.

TRL 4 : Technology validation in laboratory.

TRL 3 : Analytical and experimental critical function and/or characteristic proof-of-concept.

TRL 2 : Technology concept and/or application formulated.

TRL 1 : Basic principles observed.

WORKSHOP CONTEXT



Premise of the Workshop

- Agriculture, as process engineering, is far from optimized,
 - Inefficient energy transduction ($\eta < 6\%$)
 - Wasteful carbon capture and processing (yield $< 25\%$)
 - Sequestration in difficult-to-process forms (e.g., cellulose)
- New tools lead to transformational changes, and
- There are opportunities for significant advances.
 - “Plant Biotechnology” lags other biotechnologies
 - Is it able to progress more rapidly as a consequence??
 - “Genetic Engineering” remains aspirational
 - “Art”, at best, for all but a few well-understood organisms...
 - ...but progress \approx data throughput.
 - “Synthetic Biology” is in its infancy
 - “...new biological parts, devices and systems...” [syntheticbiology.org]
 - “... novel artificial biological pathways, organisms or devices...” [Royal Society]
 - “... combines science & engineering [for] novel biological functions & systems...” [SynBERC]
 - “... the engineering of biology...” [European Commission]

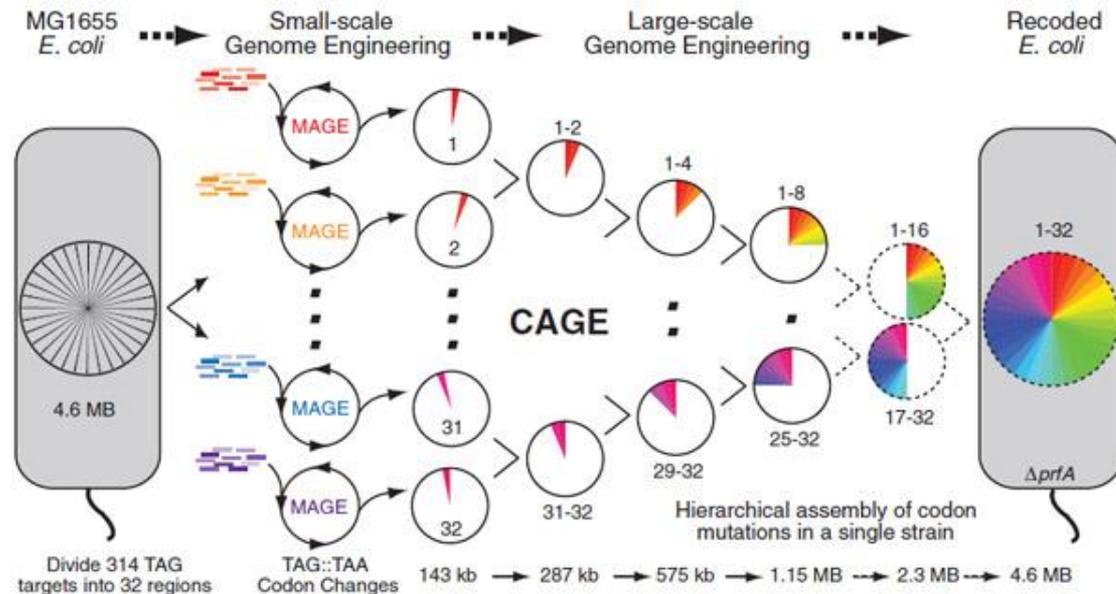


VS.



Examples of Recent Advances in Science

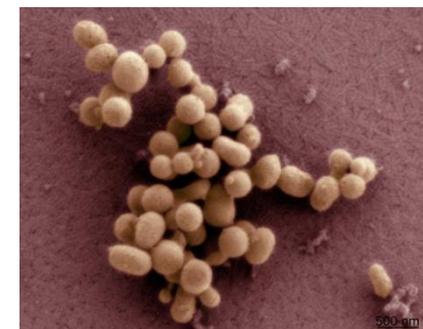
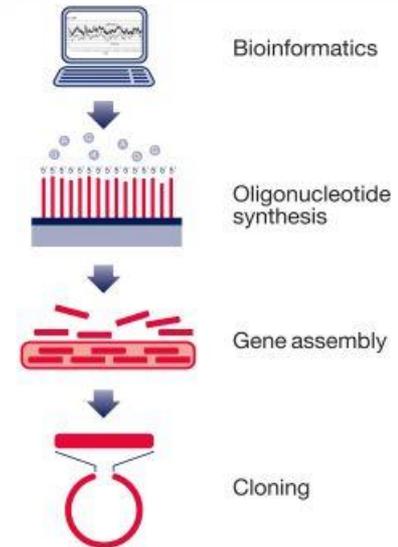
- Large scale genome wide modifications in microbes using techniques such as Multiplex Automated Genome Engineering (MAGE).



- Conjugative Assembly Genome Engineering (CAGE) converts all TAG codons in *E. coli* to TAA [Isaacs et al., *Science* **333**, 348 (2011)]

Examples of Recent Advances in Science

- Large scale gene synthesis capabilities now allow the routine production of plasmids containing entire gene pathways.
- This technology can be scaled up to synthesize entire genomes *de novo* and incorporated into microbial cells.



M. mycoides, JCVI-syn1.0, Venter Institute

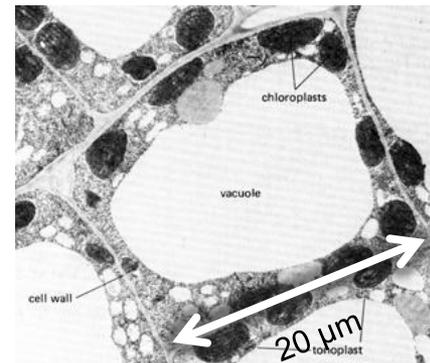
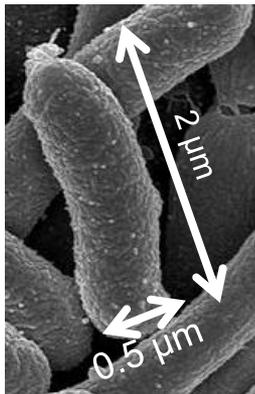
Agriculture provides a low cost production platform at scale

- Fuels = Commodity: Cost is the major determinant
- Low capital costs for large area
- Low operational costs: Can use a variety of inputs



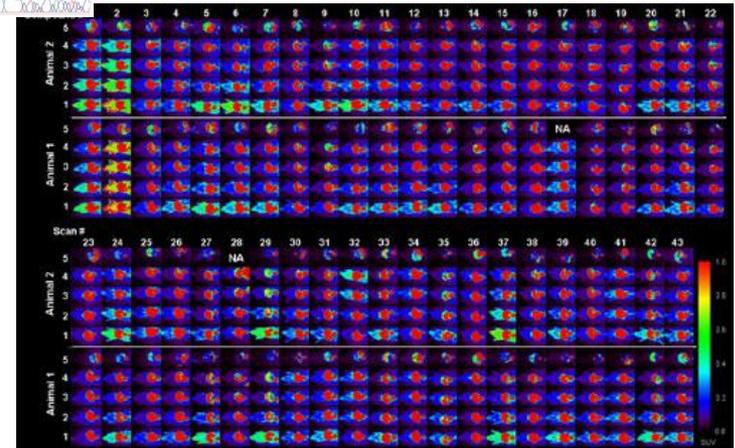
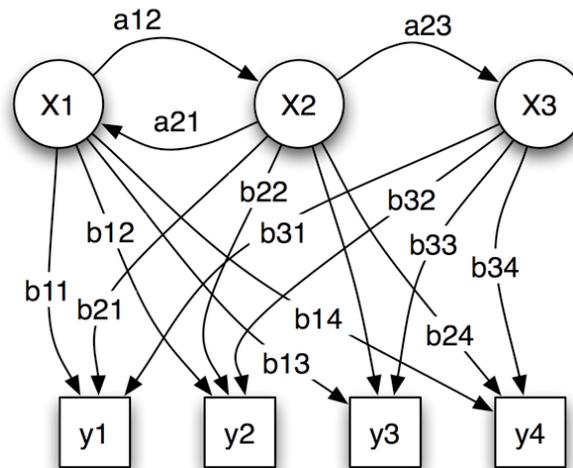
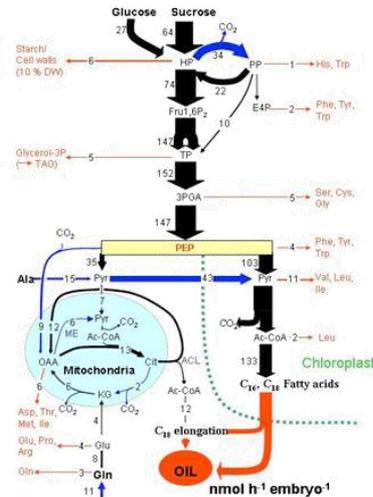
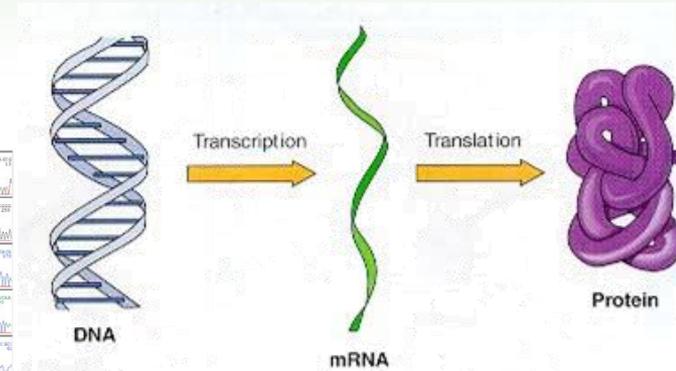
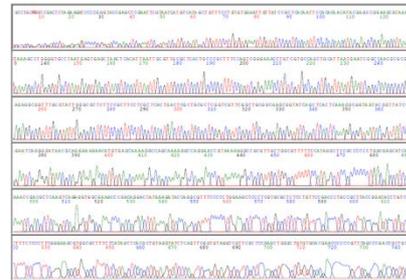
But genetic engineering of plants is *much* more difficult

<i>E. Coli</i>	Agricultural Crops
<ul style="list-style-type: none">• Single type of cell	<ul style="list-style-type: none">• Differentiated cells
<ul style="list-style-type: none">• One compartment	<ul style="list-style-type: none">• Multiple compartments/organelles
<ul style="list-style-type: none">• Single membrane	<ul style="list-style-type: none">• Multiple membranes/cell walls
<ul style="list-style-type: none">• Single genome	<ul style="list-style-type: none">• Multiple genomes
<ul style="list-style-type: none">• Reproduce every 20'	<ul style="list-style-type: none">• Reproduce annually
<ul style="list-style-type: none">• Haploid	<ul style="list-style-type: none">• Diploid/polyploid
<ul style="list-style-type: none">• Heterotrophic (fixed food)	<ul style="list-style-type: none">• Autotrophic (diurnal, CO₂)



What has biology + engineering done already?

- DNA Technologies
 - Synthesis
 - Analysis
- ‘High-throughput’ analysis
 - More data/time
 - Fewer human errors
- Computational Algorithms

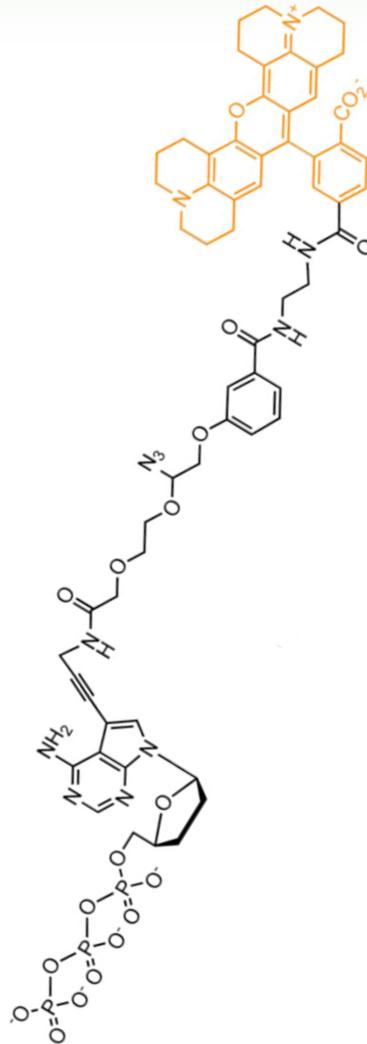
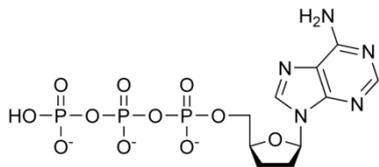
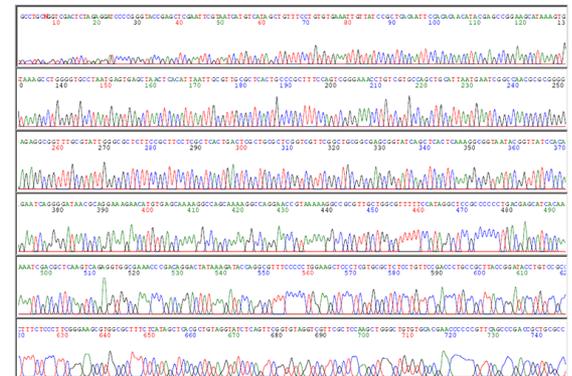


Example 1: Automated DNA Sequencing



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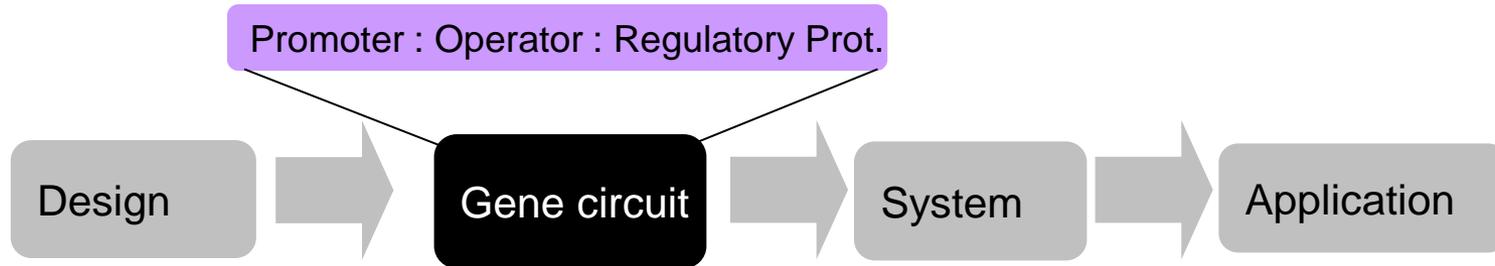
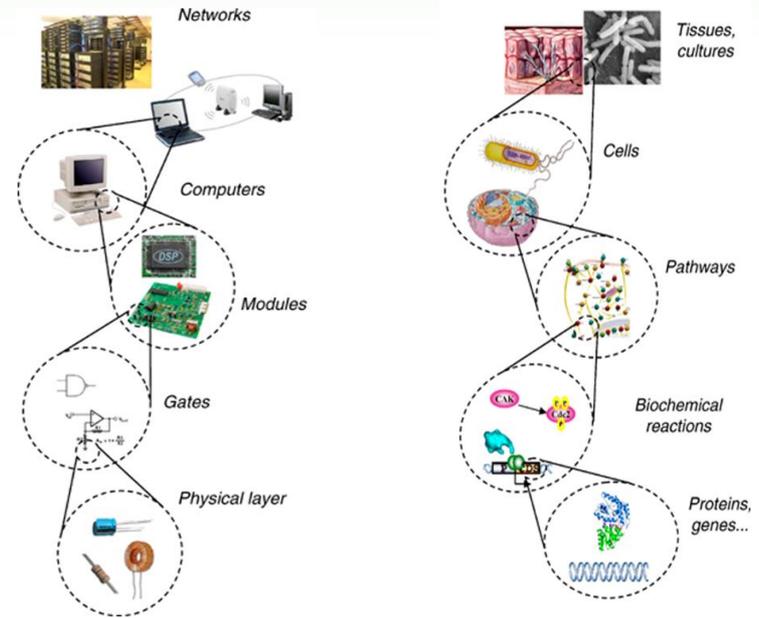
PRESENTATION SUMMARIES



Plant Synthetic Biology

June Medford (CSU)

- A goal is to engineer biological systems as routinely as electronic systems.
- Key for both systems are standard, reliable components.
- These tools are being assembled for bacterial systems, but are lacking in plants.



Schematic of a designed biological process.

Making Plant Transformation Easier and Faster

Neal Stewart (University of TN)

- Advances in cloning tools allows high-throughput generation of plant transformation vectors, but transformation tools are not available to utilize this capability.
- Most species have not been transformed.
- There is genotype specificity (genotype media) and in most cases tissue culture with plant regeneration from single-to few cells is required.
- It is unclear why certain species and genotypes are more transformable, but metabolomics may provide clues (significant differences observed in levels of 2-pyrrolidinone and ethylphosphate between two switchgrass clones).



Type II callus of *Panicum virgatum*



A high regeneration *P. virgatum* clone

There's no silver bullet for enabling an undergraduate to achieve high transformation efficiency in a novel crop/genotype, and expects it would take 5 years of work to develop this capability in a new crop.

MORNING BREAKOUT SESSIONS

Plant Systems

Genetic Tools

Engineering Tools

General questions for the breakout sessions

- **What are the quantitative (theoretical) limits of the technology?**
 - If the technology improvement works at 100%, what would there be an impact on energy use, the production of bioenergy, or agriculture in general?
 - What factors might reduce this impact, and how might they be quantified?
- **How can we reduce these ideas to practice?**
 - What is the TRL (technology readiness level)?
 - Is the idea science, engineering, or both?
- **Is a technology breakthrough in a 3-5 year timeframe realistic?**
- **Are there advances in related fields that could shorten the timeline?**
- **Please remember that ARPA-E is not looking to fund basic research into better understanding the technology.**

MORNING BREAKOUT SESSIONS

Plant Systems

Genetic Tools

Engineering Tools

Goals

The focus of this session is to:

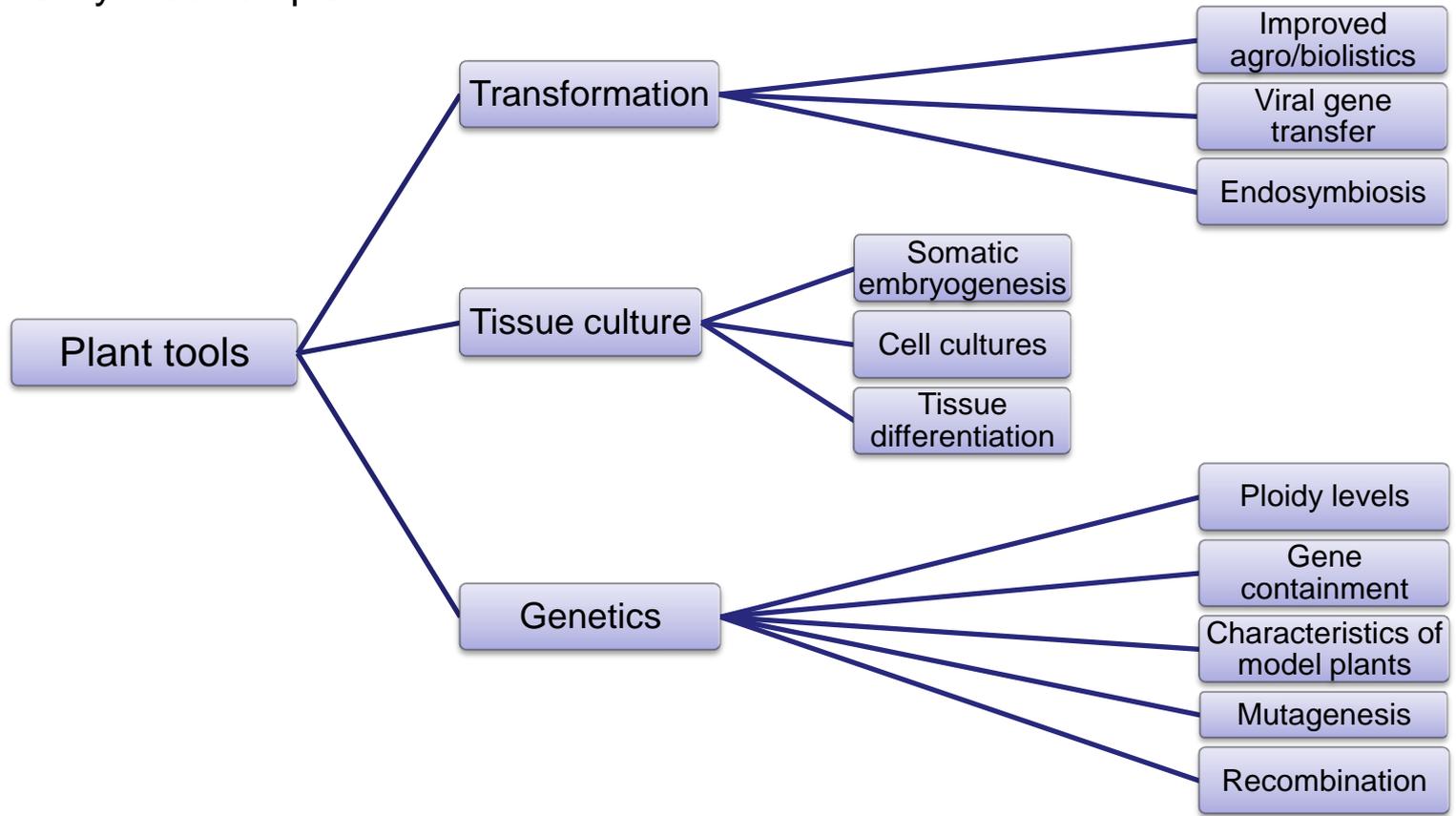
- 1) Discuss briefly the most commonly used techniques used for plant transformation and manipulating their genomes.
- 2) Clarify to ARPA-E what techniques have the greatest potential for high efficiency and ease of use over a wide range of plant species, and what challenges are involved in fully developing them. Identify the recent technical advances made that support the expectation that significant improvements can be made in plant transformation.
- 3) Identify multiple tools that could be utilized together to dramatically increase the ability of researchers to manipulate plant's genome or control its metabolism.

Challenges

- How to improve the efficiency of transformation?
- How to shorten the time necessary to produce a plant for characterization?
- How to simplify the process?

Plant Systems Tools

Potentially promising tools from to improve production of genetically modified plants:



Questions to Address on Plant Tools

- 1) Besides Arabidopsis what are the best established plant model systems? (and why?) How relevant are unicellular photosynthetic organisms or plant tissue cultures? What tools are available in these systems and what would it take to develop them in crop plants such as sorghum, or an undomesticated plant?
- 2) What is needed to produce a completely synthetic plant chromosome (> 250 kb) routinely and get it incorporated into a plant cell? How close to deployment is this capability, both for specific plant species and for general plants? Would it be a significant advantage if 1 Mb chromosomes could be produced?
- 3) What biological properties are common throughout crop plants that could be utilized for developing novel tools? Is there a transformation approach that would be likely to be effective across a wide variety of plant species? For example, could the totipotency of plant cells be utilized to create highly transformable cell lines similar to DH5- α E. coli?

Questions to Address on Plant Tools

- 4) What aspects of plant transformation could be automated? Describe the capabilities that would be needed in a transformation robot.
- 5) What modifications can be made to plants themselves to make them significantly more amenable to genetic manipulation? List all of the traits that would be desired in an elite transformable line. Would this approach be more promising than focusing on agrobacteria or other delivery systems?
- 6) Is enough known about endosymbiosis and mechanisms of plant pathogens to allow the stable incorporation of microbial cells into plant cells? If so, what would a engineered microbe designed as a trait delivery system look like, how would it function, and what advantages and disadvantages would its use have over stable plant transformation?
- 7) What is the earliest point following transformation that you could screen modified plants for a phenotype? What are the various stages of transformation and regeneration in which you can assay genotypes/phenotypes and which ones have the greatest potential to be automated? Which have the greatest potential to be predictive of a mature plant?

Responses to Questions about Plant Tools

- In identifying new model organisms for biofuel development, it could be useful to think more broadly than just plants. For example liverworts and moss are both simpler photosynthetic organisms than Arabidopsis with well developed genetic tools, optical analysis, and simple growing conditions.
- Plastid transformation is a powerful tool, but so far has not been successfully developed in monocot plants and is a need ARPA-E could address.
 - Plastids can move from cell to cell, allowing the possibility of producing synthetic plastids that could be incorporated into plant cells and spread naturally.
 - Possibly argobacteria could be retargeted to the chloroplast instead of the nucleus to allow plastid transformation.

MORNING BREAKOUT SESSIONS

Plant Systems

Genetic Tools

Engineering Tools

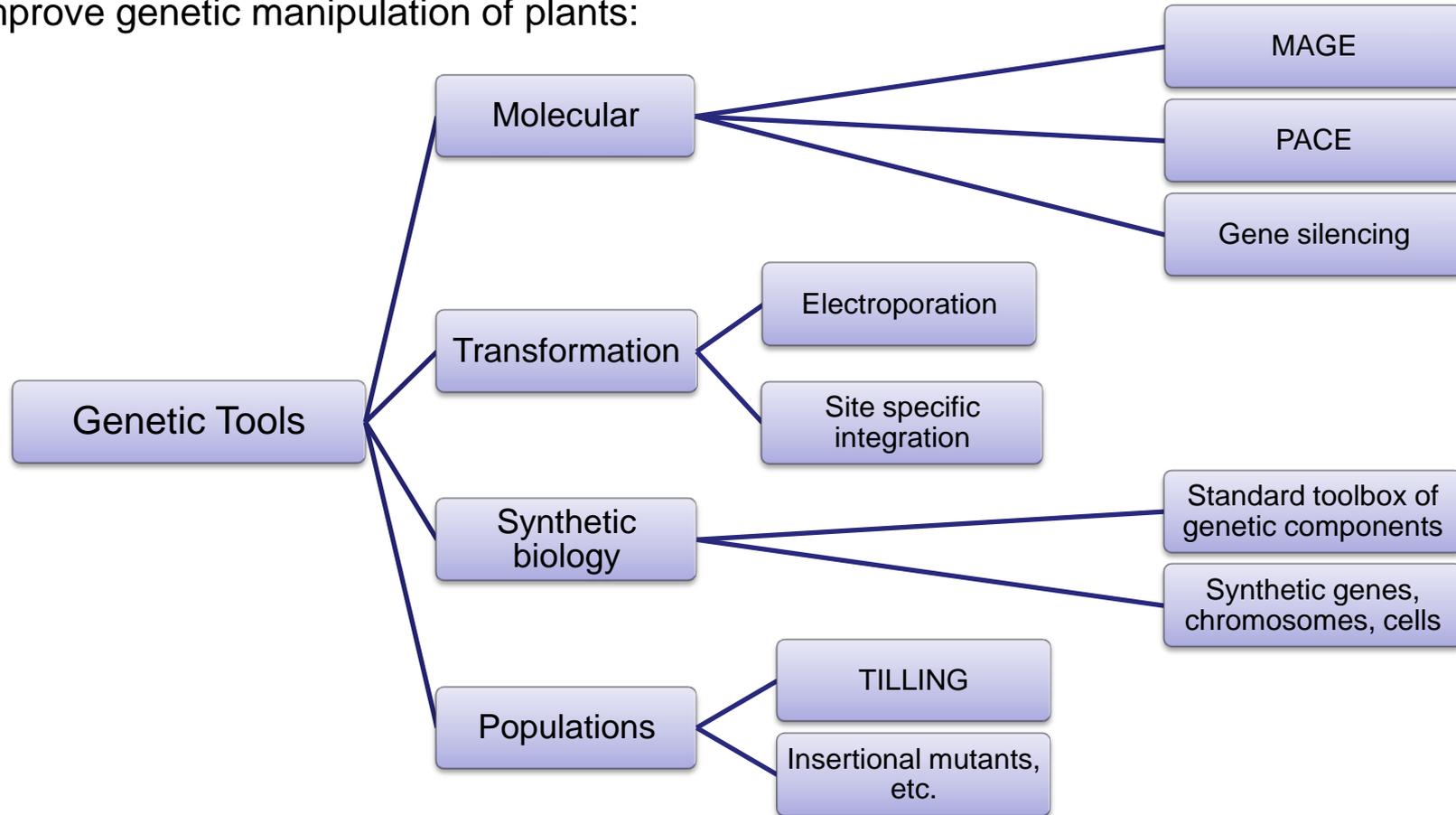
Goals

The focus of this session is to:

- 1) Discuss briefly the most powerful genetic tools that have been developed in model systems for transformation and manipulating their genomes.
- 2) Clarify to ARPA-E what are the most valuable genetic tools from model systems for plant manipulations, and what challenges are involved in translating them for use in plants.
- 3) Identify multiple tools that could be utilized together to dramatically increase the ability of researchers to manipulate plant's genome or control its metabolism.

Genetic Tools in Model Systems

Potentially promising tools from model systems to improve genetic manipulation of plants:



Questions to Address on Genetic Tools

- 1) Identify some of the most powerful tools available to model organisms (*E. coli*, *S. cerevisiae*, *Arabidopsis*) and select the three that would be most desirable to have in crop plants. How likely are these tools able to be adapted for plants?
- 2) What is needed to produce a completely synthetic plant chromosome (> 250 kb) routinely and ensure that it stably persists in plant cells? How close to deployment is this capability, both for specific plant species and for general plants? Would it be a significant advantage if 1 Mb chromosomes could be produced?
- 3) Are there approaches that would allow easy identification of transformed cells at the single cell level or assess gene expression?
- 4) What capabilities will cheap, large scale sequencing allow? Would being able to readily generate large quantities of sequence data allow novel high throughput methods of genotyping?

Questions to Address on Genetic Tools

- 5) What biological factors in plant cells prevent homologous recombination from occurring? Can an efficient system be developed so that genes or regions of chromosomes can be deleted or replaced?
- 6) How could you make multiple modifications to specific genes throughout the plant genome? Are there non-transformation methods to control gene expression?
- 7) What would be necessary to produce a synthetic cell/virus/?? that could persist symbiotically in a plant? How many of these components are well characterized now, or demonstrated in a plant? What sorts of applications would this capability be preferable over stable plant transformation.
- 8) What are the key tools necessary to achieve predictable incorporation, expression, and function of a foreign gene/protein in a plant? Discuss both in terms of specific genetic components and modifications to the plant host.

Responses to Questions on Genetic Tools

- The most useful genetic tools identified for plants included:
 - Homologous recombination
 - Methods to increase doubling time of plant cells
 - Large scale genome modification
- Many companies have tried small oligo-based approaches for genome modifications in plants, but these have been largely unsuccessful so far as is known. Homologous recombination exists in chloroplasts, so there is the potential to perform MAGE on chloroplasts.
- The chloroplast is also small enough to consider synthesizing *in vitro* to allow customization, and there is evidence that plastids travel from cell to cell, opening up the possibility that engineered chloroplasts could be used as large scale trait delivery systems.
- Large scale sequencing could identify good transgene integration sites through recombination.

MORNING BREAKOUT SESSIONS

Plant Systems

Genetic Tools

Engineering Tools

Goals

The focus of this session is to:

- 1) Discuss briefly the state of the art in engineering approaches used to dramatically increase the throughput and efficiency of formerly time-consuming, delicate, and manpower-intensive tasks.
- 2) Clarify to ARPA-E what are the most promising approaches that can be applied for the characterization of plants and what challenges are involved in developing these technologies.
- 3) Identify how we can leverage the engineering approaches (identified above) to dramatically increase the throughput and efficiency of plant transformations and phenotype analysis.

Transformation/Analytical Tools

Gene-delivery methods

Agrobacteria

Viral

Biolistics

Electrotransfection

Polyfection

Lipofection

Injection-based
methods

Wave/beam mediated

Dessication

Analytical methods

Mass spectrometry

Radiolabeling

Fluorescence-labeling

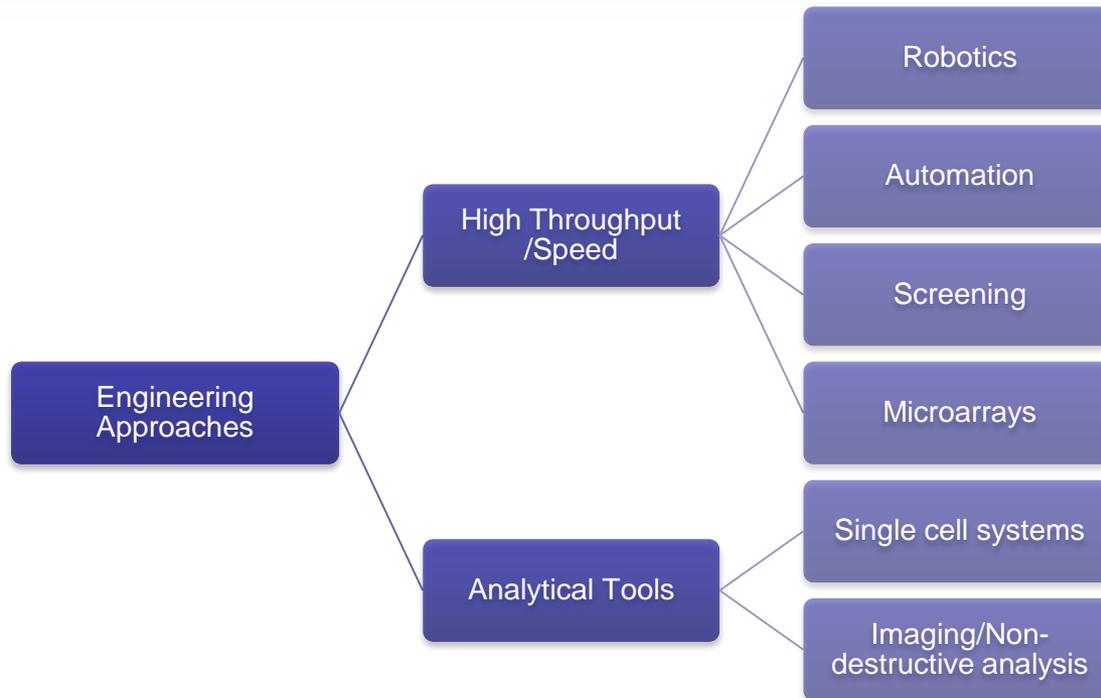
PCR

'omics

Visual analysis

Engineering & Analytical Tools

- Transformation
- Phenotyping



Questions on Transformation and Analysis

- What aspects of plant transformation (including transient expression in tobacco or tissue culture) can be automated that reduces manpower and increases reliability? What capabilities would be needed?
- Are there roadblocks that stand in the way of automating the sequencing and analysis of plant genomes?
- Are there reliable methods of massive parallel metabolite analysis that might be useful to increase throughput? What would it take to develop these methods?
- Can we leverage our experience from semiconductor development for the analysis of plants?
- What non-invasive/non-destructive methods are available for manipulation and analysis (e.g. assay metabolite/protein levels) of single cells in an automated fashion? In contrast, what are the capabilities of systems that can analyze larger tissue samples or complete organisms?

Summary Points

- It is hard to program automated machinery to identify what makes a “good” versus “bad” callus, therefore a more metrics driven definition of callus viability may enable automated analysis.
- There may be opportunities to use smaller callus samples in an array that can be analyzed
- Utilizing robotics to maintain a plant during maturation after callus development would help to speed up some of the labor-intensive processes
- Biomarkers for metabolic, genetic, and morphological changes were identified as needs as well as predictive modeling for how plants may develop out of callus tissue
- The ability to shrink down the culture needed to develop a plant may accelerate overall transgenic plant development time
- Uniform samples are needed for analysis in high-throughput tools. Is there a method to grow plants to present a uniform sample for robotic systems?

AFTERNOON TECHNICAL EXERCISE

Design the ideal system to rapidly transform and screen plants

Host Modification

Transformation

Analysis

The following is the output presented by the group to all workshop attendees for discussion and debate

Host modification challenge

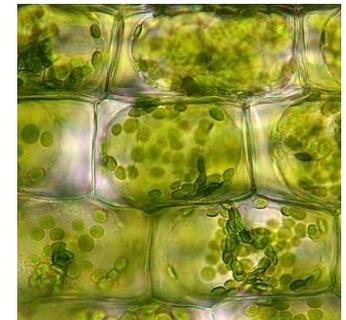
Goal: Develop a generic plant system that can serve as a platform for rapid genetic manipulation. It allows a similar level of capability for modifications as *E. coli* and *S. cerevisiae* does today.

- The key capabilities of the system are...
- The key biological aspects are...
- This approach was attractive because...
- The expertise needed to develop the technology was...
- We thought the biggest problem would be...

Features/Benefits of the technology approach

Developing a model bioenergy plant system:

- Using simpler model organisms such as moss would allow the use of genetic tools that could meet the desired metrics for transformation and characterization. Alternatively an *in silico* model could be developed to predict outcomes.
- However, to be a model for a bioenergy crop a higher plant, probably a C4 grass should be optimized for genetic manipulation.
- Options for a C4 model system were to develop a tissue culture system that is transformable, or a high throughput transient expression system.
- Setaria and sorghum were identified as some of the most promising C4 grasses that could be used to develop this model. It would be optimal to use an inbred line to generate the model system.



Features/Benefits of the technology approach

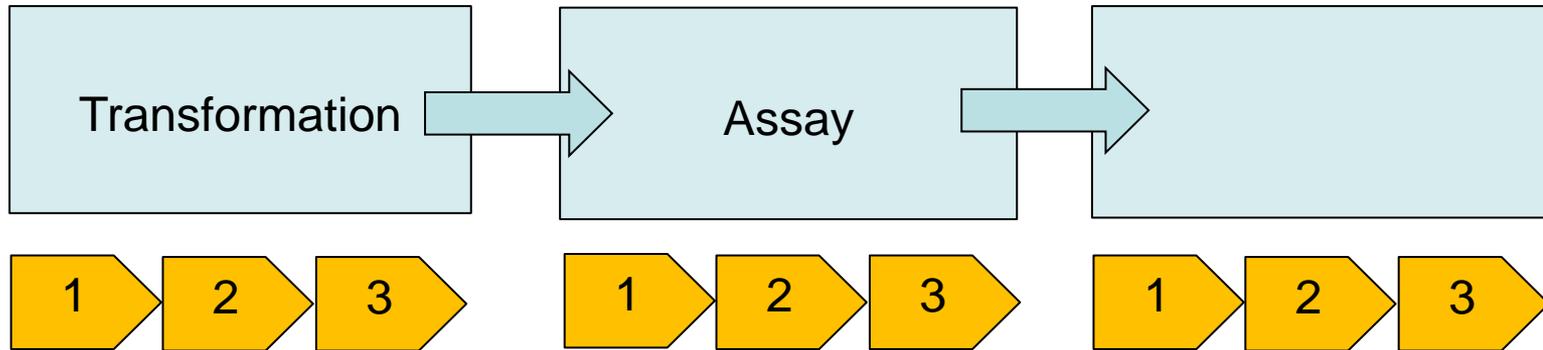
Desired genetic characteristics of the model C4 system:

- Synthetic meristem gene circuit in combination with a circuit that allows DNA uptake.
 - Variable by plant species? Maybe by dicot vs monocot
 - Would not be a large number of genes
 - Photosynthetic system
- Groups of synthetic genetic components (promoters...) functionally characterized in the C4 plant.
- Synthetic switches to induce pathways in plants.
- Add genes known to improve transformation identified in other systems, either modifying host plants or transfer the factors with the agro.
- Site specific integrase, specific good genomic sites.

Optimized plant system

BLOCK DIAGRAM:

Diagram what your proposed system looks like



- Starting with a C4 inbred grass line.
- Individual cells programmed with meristem traits to maintain as single cells.
- 2nd egg cell circuit for DNA uptake.
- Incorporate known host factors for improved transformation in the agro.
- Incorporate targeted integration site.

- After assay (either at a single cell or juvenile stage depending on assay) can be triggered to transition to mature plants

Throughput
10³-10⁹ single cell samples/day
10¹⁰ transformants/month

AFTERNOON TECHNICAL EXERCISE

Design the ideal system to rapidly transform and screen plants

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Transformation

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The following is the output presented by the group to all workshop attendees for discussion and debate

Transformation challenge

Goal: Develop a transformation system that has a broad plant range that increases efficiency (lowering labor needs and raising # of transgenic events generated) at least 5X and has the potential to be automated.

- Target transformation efficiency...
- Target throughput/time to generate a transgenic plant...
- Level of operator expertise...
- This approach was attractive because...
- The expertise needed to develop the technology was...
- We thought the biggest problem would be...

Features/Benefits of the technology approach

Developing an optimized transformation system:

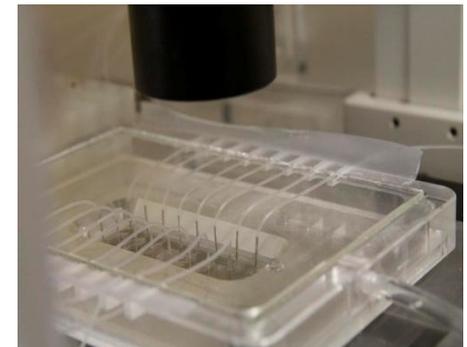
- Improved regeneration from tissue culture is the key technological advance to improve plant transformation.
- By better understanding the genetic mechanisms behind regeneration (embryogenesis), an extract based on those factors could be applied to cells for transformation.
- Will screen different cell types that give rise to differentiated tissues using genomics and proteomics to identify these factors.
- Gene clusters have been identified in Arabidopsis that give rise to embryogenesis, but not yet clear if they are the same in crop plants.



Features/Benefits of the technology approach

Producing an automated transformation instrument:

- The instrument would handle plant tissue, apply the transformation factors, then ready them for agrobacterium mediated or biolistic transformation.
- The instrument would also be able to optimize tissue culture conditions for specific plant varieties. Normally 20-30 variables need to be optimized such as media composition and growth conditions.
- The instrument could be designed to handle single cells such as protoplasts, or larger clusters of cells using micro-fluidics.
- Once transformed cells have regenerated to plantlets automated visual analysis could be performed.



AFTERNOON TECHNICAL EXERCISE

Design the ideal system to rapidly transform and screen plants

Host Modification

Transformation

Analysis

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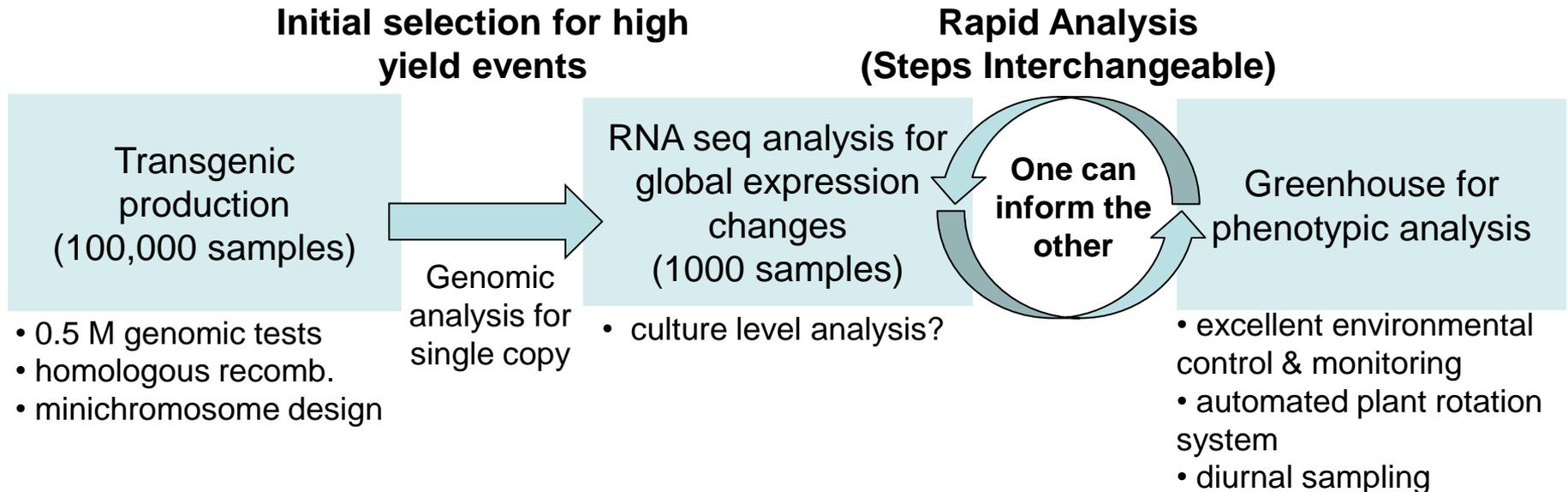
Analysis technique challenge

Goal: Develop an analysis system capable of high throughput analysis (> 100 crop plants/day/person) for transgenic plants to screen for a desired phenotype (i.e. specific metabolites or transgene expression) under greenhouse or field conditions.

- The ideal plant growth stage for analysis...
- Level of operator expertise...
- This approach was attractive because...
- The expertise needed to develop the technology was...
- We thought the biggest problem would be...

Features/Benefits of the approach

- RNA expression analysis that is 100x cheaper and 100x the throughput compared to today
- Analysis of 100 statistically significant samples per day
- Approach starts with many transformants all going through a genomic filter, followed by analysis of a subset, potentially through a culture level analysis that allows selection of a smaller population for phenotypic analysis



Needs to enable this technology

- A way to grow plants uniformly taking into account different environmental conditions and developmental rates
- A very controlled way from seed to plant of watching the sample to make sure that variations occur systematically
- Very well controlled greenhouse design that gives the operator the ability to move plants around automatically to account for the unknown variations that inevitably occur
- Data management to handle analysis of extremely large quantities of sequencing data
- Combinatorial approach to testing variability of transgenic plants in different environments (temperature, humidity, sunlight, etc.) in different greenhouses
- A better way to establish correlations between genotypes and phenotypes

Challenges

- Understanding the biology better will translate into more efficient (more statistically significant) sampling- there is a tradeoff between how well you need to know a system and how many samples will be adequate to understand the system
 - The greater rate at which we learn enables us to more quickly identify models, rules, etc. that will be applicable to developing a more rapid analysis system
- High throughput tools will enable us to learn more about how we can move closer to an approach that allows many (e.g. 15) transgenes to be expressed consistently and reliably which should be a goal for the field.
- Would it be possible to design an analysis system that could mount on a combine to rapidly examine plants in the field?
- Is it possible to use only one tool to look at a plant and get as much genetic and phenotypic information as possible?

WRAP-UP SESSION

October 7

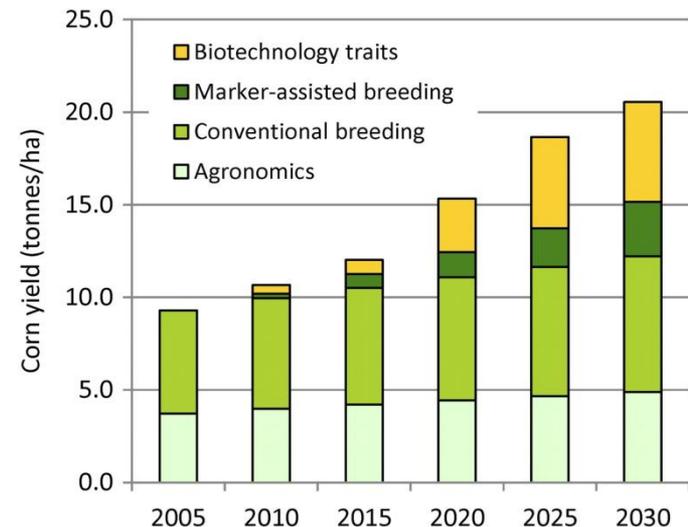
What have we learned?

What can we do that will be revolutionary?

How will we tell that we've succeeded? (metrics)

State of the world

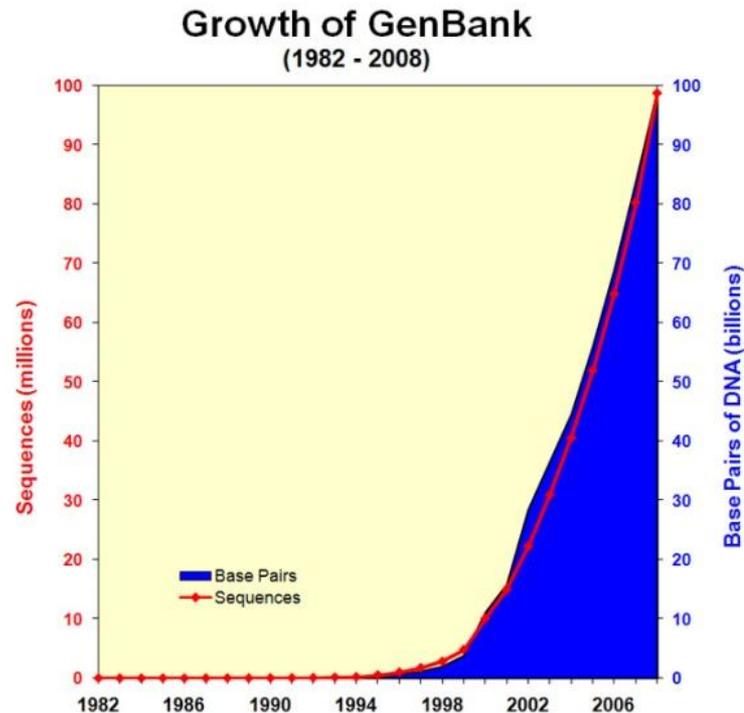
- The green revolution during the 1960s and 1970s provided dramatic yield increases and prevented expected food shortages, but has been dependent on large inputs of fertilizers and pesticides that are not sustainable.
- There is now a call for a blue revolution, to achieve the same level of improvement in agricultural productivity but in a sustainable way. Biotechnology will be key to achieving this target.



Edgerton M D Plant Physiol. 2009;149:7-13

Current state of plant genomics

- High throughput sequencing capability has increased exponentially over the past decade with technological advances.



- Today, multiple plant genomes can be completed each year.

The issue

- The promise of “genetic engineering” in plants has not been reduced to practice fast enough.
- Plant engineering is much more difficult than *E. coli*, because the tools are not adequate.
- In general, the focus has been on increasing the rate of data collection without commensurate increases in converting that data into insight that makes a difference: ARPA-E?

Promising technologies

- There is a high need for additional genetic or analytical tools for plant biotechnology, as the capability to produce transgenic crop plants outside of large industry is severely limited. These tools could include instruments that could perform high throughput handling or analysis of plant samples.
- There is also a potential to develop transformation tools that would not lead to regulated plants. Options for this include generating an artificial T-DNA based on fragments from plant genomes or isolating natural endophyte populations that confer desired traits.
- However, significant variation from plant to plant (and genotype to genotype, environment to environment) makes development of a universal tool to improve transformation efficiency unlikely.

Promising technologies

- Will need to differentiate between the technical needs for tools that themselves are truly transformative and high-risk (ARPA-E focus) and high throughput tools that might lead to novel technologies.
- While there were some important enabling tools identified that are proprietary to companies, there were still many needs identified that are sufficiently cutting edge or high-risk that would not likely be supported by current sources of funding.
- There does not appear to be any funding programs for the development specifically of plant biotechnology tools, though there were programs identified at NSF and NASA on related topics. Additionally, DOE's Biological and Environmental Research recently held a workshop on Biosystems Design, which covered aspects of synthetic biology and molecular toolkits.

Program metrics

- Potential metrics for biotechnology tool development will need to be tied to specified plants because of the large biological variation between crops (i.e. transformation efficiency of sorghum vs. tobacco).
- It will be challenging to define metrics for tool development that directly address ARPA-E's missions to:
 - Reduce Energy-Related Emissions
 - Reduce Energy Imports
 - Improve Energy Efficiency