2009 Annual Report

PI: Anireddy S.N. Reddy
Organization: Colorado State University
ONR Award Number: N000140810470
Award Title: Metabolic Engineering of Plants to Produce Precursors (Phloroglucinol and 1,2,4-butanetriol) of Energetic Materials

Technical Report

A. Scientific and Technical Objectives

The chemical-based synthesis of energetic materials uses toxic ingredients and produces many environmentally undesirable wastes. In addition, chemical-based production is not sustainable because many of the chemicals used in making these precursors are petroleum-based. An alternative strategy is to produce precursors of energetic materials using a bio-based approach. Recently, significant progress has been made in identifying the genes and enzymes in microbes that can produce precursors of energetic materials. This knowledge can now be exploited to use plants for producing precursors of energetic materials in a sustainable manner. The focus of our project is to use bacterial enzymes to engineer plant metabolism to produce precursors of energetic materials. The specific objectives of this proposal are:

1) Metabolic engineering of plants to produce high levels of phloroglucinol by introducing a bacterial gene that converts malonyl Co-A to phloroglucinol into plants.
2) Metabolic engineering of plants to produce butanetriol. This will be accomplished by introducing bacterial genes involved in butanetriol synthesis from xylose and arabinose.
3) To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

B. Approach

Most plants naturally produce the precursors but not the enzymes to convert them to the desired precursors of energetic materials, whereas bacteria have the enzymes that convert these precursors into end product. Our approach is to express bacterial genes under constitutive and/or tissue-specific promoters to produce phloroglucinol and 1,2,4-butanetriol. The genes to be introduced are: xylose dehydrogenase, xylonate dehydratase, arabinose dehydrogenase, L-arabinonante dehydratase, benzoylformate decarboxylase, dehydrogenase and *PhlD*. Because of many available resources for Arabidopsis, we will use this plant as a model system to test this concept. Once proven, these constructs will be introduced into Miscanthus, a non-food crop that is known to grow well on marginal soils with minimum or no inputs. Transformation of plants will be done using Agrobacterium-based disarmed binary vectors using floral-dip method (in case of Arabidopsis) and regeneration of plants in tissue culture after co-cultivation with Agrobacterium (in case of Miscanthus). Most of our initial work will be performed in Arabidopsis and the best gene constructs will be transferred to Miscanthus, a grass species. We will be using a sterile hybrid of Miscanthus for these studies. Propagation of this variety is done through rhizomes.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2009	**Annual**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Metabolic Engineering of Plants to Produce Precursors (Phloroglucinol and 1,2,4-butanetriol) of Energetic Materials	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Colorado State University**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

C. Concise Accomplishments

We have cloned and sequence verified and/or synthesized six genes (*PhlD*, xylose dehydrogenase, xylonate dehydratase, arabinose dehydrogenase, L-arabinonate dehydratase and benzoylformate decarboxylase) that are necessary to produce these chemicals in plants. We have moved *PhlD* to a plant transformation vector and transformed Arabidopsis plants for phloroglucinol production. We obtained several independent transgenic plants expressing *PhlD*. In addition, we are making two transformation constructs, each with three genes under three different promoters, for butanetriol production. Once we establish that we can produce these chemicals in Arabidopsis, these genes will be then introduced into *Miscanthus*, a non-food crop that is known to grow well on marginal soils with minimum or no inputs thus providing a renewable and less costly resource for the production of precursors of energetic material. Towards this goal, we are developing transformation methods for *Miscanthus*. We have done over three hundred hormonal combinations to optimize callus induction from Miscanthus organs. Of all the organs tested, roots from tissue culture grown plants and inflorescence yielded good callus induction. We are now using the root and inflorescence calli to develop regeneration and transformation methods for Miscanthus.

D. Expanded Accomplishments

Objective 1: Metabolic engineering of plants to produce high levels of phloroglucinol by introducing a bacterial gene that converts malonyl Co-A to phloroglucinol into plants.

We have cloned *PhlD* from *Pseudomonas fluorescens* and introduced into two entry vectors Impact vector 1.1 and pSAT4A. The entry vectors contain promoters and terminators for expression of the genes in plants. The promoter, gene, terminator cassette was digested from the entry vectors and cloned into binary vectors pRCS2-nptii and pBin. Both binary vectors were used to transform *Agrobacterium* and those transformants were then used to transform Arabidopsis plants. Seeds were collected from the transformed plants and were grown on kanamycin containing media. Transformants were obtained from the *PhlD* pRCS2-nptii construct (See Figure 1) and plants were transferred to soil (See Figure 2). Tissue from the leaves of transformants was analyzed for expression of the mRNA for *PhlD*. Expression was confirmed in varying degrees in the different plants (Figure 3). These plants are ready to be tested for the presence of phloroglucinol.

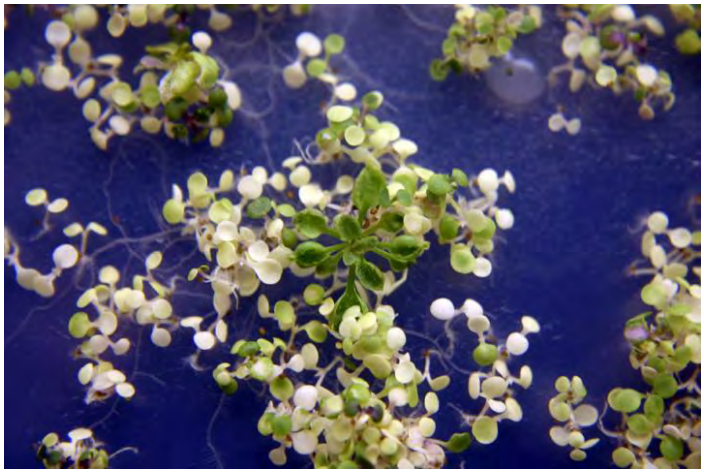


Figure 1. Left: Selection of transformants from PhlD transformation. **Right:** Transformants obtained with PhlD driven by 35S promoter are grown on kanamycin plates.

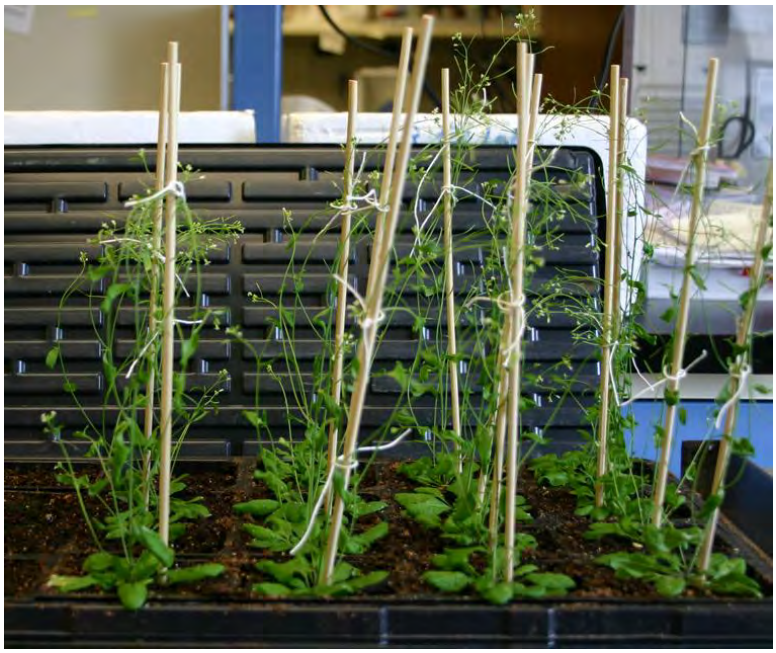
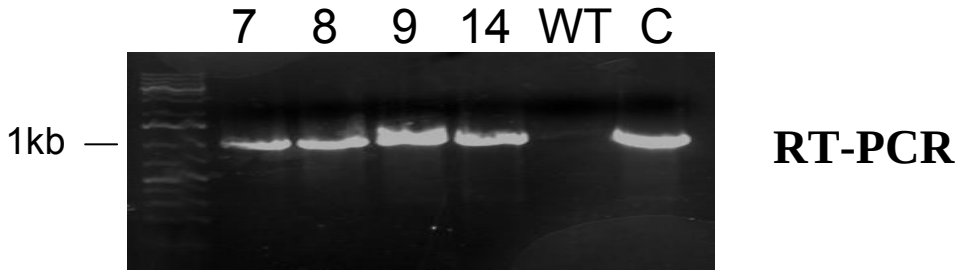
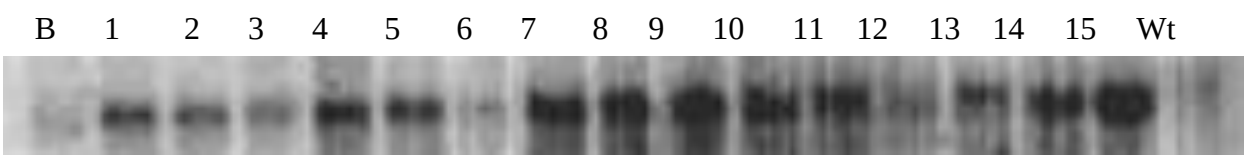


Figure 2. Transgenic Arabidopsis lines carrying PhlD gene driven by 35S promoter.



RT-PCR



Northern blot

Figure 3. Expression of *PhlD* in transgenic lines. **Top:** RT-PCR analysis showing the levels of *PhlD* transcript in four transgenic lines and wild type (WT). C, positive control. **Bottom:** Northern blot. Numbers 1 to 15 are transgenic lines in which *PhlD* is driven by 35S promoter, B, transgenic line in which *PhlD* is driven by rubisco promoter. Wt, wild type.

Objective 2: Metabolic engineering of plants to produce butanetriol. This will be accomplished by introducing bacterial genes involved in butanetriol synthesis from xylose and arabinose.

Xylose dehydrogenase (XDG) was cloned from *Calobacter crescentus* and cloned into pSAT3A, xylonate dehydratase (XDT) was cloned from *E. coli* and cloned into pSAT4A, benzoylformate decarboxylase (BFD) was cloned from *Pseudomonas putida* and cloned into pSAT6A. Arabinose dehydrogenase (AD) and arabinonate dehydratase (ADT) were constructed synthetically based on the genes from *Burkholderia vietnamiensis* G4 and *Pseudomonas fluorescens* and were cloned into pSAT3A and pSAT4A, respectively. The synthetic genes have altered codons that are optimized for expression in Arabidopsis. The pSAT vectors have the promoters and terminators for expression of the genes in plants.

To construct the binary vectors with XDG XDT, BFD or AD, ADT, BFD, each gene is cloned in sequentially. The XDT cassette was digested from the pSAT4A vector and cloned into pRSC2-bar and then the BFD cassette was digested from the pSAT6A vector and cloned into pRSC2-bar-XDT and finally the XDG cassette was digested from the pSAT3A vector and cloned into pRSC2-bar-XDT-BFD (Fig. 4) giving the construct pRSC2-bar-XDG-XDT-BFD. This construct is ready to be introduced into Arabidopsis plants for the production of butanetriol.

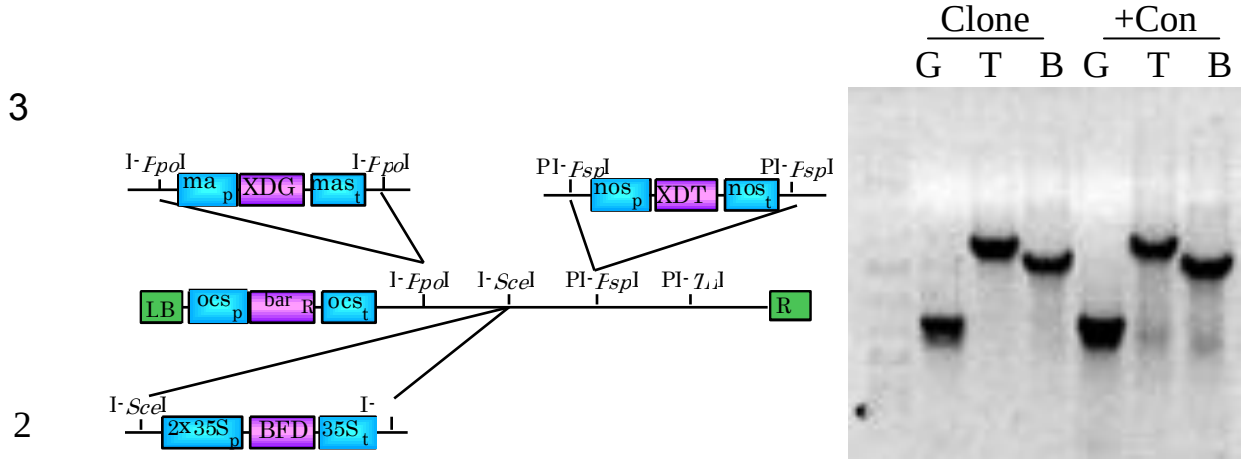


Figure 4. Left: Schematic diagram showing cloning of three genes into a binary vector. 1. Inserted XDT into pSAT4A. Transferred cassette to pRCS2-ocs-bar; 2. Inserted BFD into pSAT6A. Transferred cassette to pRCS2-ocs-bar/XDT; 3. Inserted XDG into pSAT3A. Transferred to XDT/BFD/pRCS2-ocs-bar. **Right:** PCR verification of XDG, XDT and BFD in pRCS2-ocs-bar (Clone); +Cont, PCR from plasmids; G- XDG, T- XDT, B- BFD,

Objective 3. To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

Different plant parts (roots, leaves and inflorescence) from Miscanthus (*Miscanthus x giganteus*, a triploid species) were used for callus induction (Figure 5). We have tried 126 hormonal combinations for each tissue. Leaves did not produce callus in any hormonal combinations. Roots and inflorescence produce good calli under a few hormonal combinations (Figure 6).

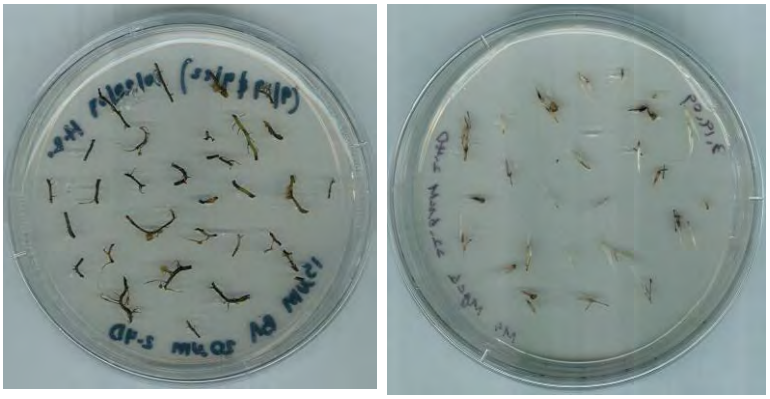


Figure 5. Induction of callus from leaves (left), roots (middle) and inflorescence (right)

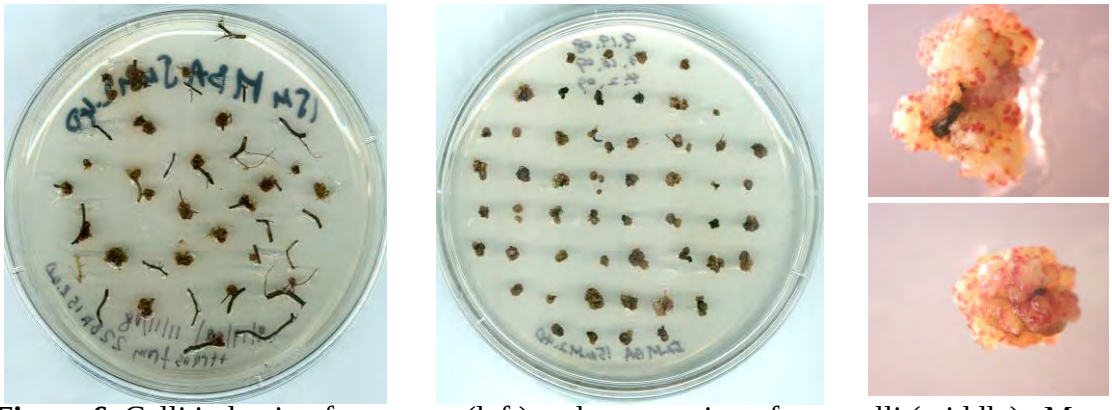


Figure 6. Calli induction from roots (left) and propagation of root calli (middle). Magnified view of two calli (right).

We have constructed a fluorescent reporter fused nuclear protein (SR1) for transformation of Miscanthus (Fig. 7).

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

YFP5 Light

Figure 7. Verification SR1-YFP construct. The fusion protein expression is verified in transgenic Arabidopsis plants (left, YFP). Bright field image is on the right (light).

E. Work Plan:

The transformed PhlD plants we have already obtained will be tested to identify homozygous plants for the *PhlD* gene. Homozygous plants will then be grown and analyzed for the presence of phloroglucinol. We also plan to transform additional plants with the PhlD construct with chloroplast targeting signal since the chloroplast is where malonyl-CoA is likely to be more abundant. A third *PhlD* construct will be made using a synthetic gene constructed using codons optimized for expression in Arabidopsis. Arabidopsis will be transformed with this gene and the plants produced will be analyzed and the production of phloroglucinol will be compared with production of phloroglucinol in the earlier constructs.

The pRCS2-XD,XDT,BFD construct will be used to transform *Agrobacterium,* which will then be used to transform Arabidopsis plants. Plants produced from this transformation will be tested for production of butanetriol.

We will also obtain synthetic genes for XD, XDT, BFD, AD, and ADT using the Arabidopsis preferred codons. We will then put them into the pSAT vectors and ultimately into the pRCS2 vectors as XDsyn, XDTsyn, BFDsyn and ADsyn, ADTsyn,BFDsyn constructs. We will transform Arabidopsis with *Agrobacterium* containing these constructs, obtain transformed plants and analyze them for butanetriol production.

To develop a plant regeneration system, we will use calli from roots and inflorescence and optimize conditions for shoot and root induction to generate whole plants. We will then use the optimized regeneration conditions for transformation using fluorescent reporter fused constructs. Transformation with different strains of Agrobacterium as well as direct gene transfer using gene gun method will be used.

F. Major Problems/Issues

Although no major problems were encountered, there were several minor problems. We were unable to clone the arabinose dehydrogenase and arabinonate dehydratase genes from the bacteria by PCR. We then turned to having the genes synthetically constructed.

The cloning of the genes from the pSAT vectors into the pRCS2 vectors has proven to be a challenge. Due to the large size of the vectors, especially when they contained the cloned genes, cloning has been difficult. Different enzyme strategies have had to be developed and optimization of cloning has taken a longer time than expected.

Callus induction from inflorescence took a while as it took 8 months for Miscanthus plants to flower. Because of this it has taken a long time to obtain calli from flowers.

G. Technology Transfer

We are in the early stages of this project. No technology transfer as of now.

H. Foreign Collaborators and Supported Foreign National

Dr. Gul Shad Ali