2013 DOE Bioenergy Technologies Office (BETO) Project Peer Review

Research and Technology Development for Genetic Improvement of Switchgrass

Albert Kausch and Richard Rhodes, University of Rhode Island

Award # DE-FG-36-08GO88070

Date: Thursday May 23nd 10:30-11 am
Technology Area Review: Feedstock Supply & Logistics
Principal Investigator: Albert Kausch
Organization: University of Rhode Island

Goal Statement

Long Term Goals and Specific Objectives

<u>Long Term Goals</u>

The overarching goal of this project is the development of technology leading to commercial switchgrass hybrid varieties engineered for enhanced, low-cost conversion of cellulosic biomass to liquid biofuels. Another goal is the development of intellectual property that is widely applicable to bioenergy and agricultural crops generally.

<u>Specific Objectives</u>

- ***Development of hybrid plant systems using male and female sterility***
- ***Development of advanced breeding strategies utilizing wide crosses, advanced tissue culture and genomics to produce new Non-GMO hybrids***
- ***Development of robust transgenic and gene confinement strategies***
- ***Enhance education , student training and internship research opportunities in biofuels crop improvement and plant biotechnology***

QUAD CHART OVERVIEW

2013 DOE Technology Area Review: Feedstock Supply & Logistics
Research and Technology Development for Genetic Improvement of Switchgrass
Albert Kausch and Richard Rhodes, University of Rhode Island
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Timelines

Project start date 11/30/08
Project end date (NCE) 11/31/13
Percent complete 95%

Budget

- **Funding for FY11**

(DOE $967,750 / Cost share $125,000)

- **Funding for FY12**

(DOE $1,500,00/ Cost share $250,000)

- **Funding for FY13 (NCE)**
- **Years the project has been funded / average annual funding**

3 yrs (FY10,FY11,FY12)
Ave. Annual Funding $1,500,000

Barriers addressed MYPP 2.1.3

Ft-A Feedstock Availability and Sustainable production and yield in Switchgrass and related species

Ft-C Feedstock Genetics and Development – New varieties; Breeding and genetics; Transgenics and gene confinement

Partners: The URI Bioenergy Consortium

University of Rhode Island
Yale University
Plant Advancements LLC
Ernst Conservation Seeds Inc

Project Management

Dr. Albert Kausch, Director, PBL
Dr. Richard Rhodes, III

PROJECT OVERVIEW

2013 DOE Technology Area Review: Feedstock Supply & Logistics
Research and Technology Development for Genetic Improvement of Switchgrass

Albert Kausch and Richard Rhodes, University of Rhode Island

Award # DE-FG-36-08GO88070

History: *This project was conceived through the collaboration of academic and industry researchers at the University of Rhode Island, Yale University and Ernst Conservations Seeds Inc to meet the need for technology development related to new bioenergy cultivars and gene confinement for GMO trait improved crops.*

Context: *Development of hybrid plant systems is important for both advanced breeding and gene confinement purposes. In this project we have discovered new technologies to develop hybrid plants and non-GMO wide crosses. Also of significance , this project has developed robust transgenic and gene confinement strategies to allow genetically improved varieties to be deregulated through the USDA and eventually released for commercial production on a large scale.*

The High Level Objectives *of this project, dubbed 'Project Golden Switchgrass' are to create hybrid systems, advanced genomics assisted breeding, and gene confinement platforms that are broadly applicable to bioenergy and agricultural crops. This enabling technology aims to improve biofuels crop improvement.*

PROJECT MANAGEMENT PLAN (PMP)

Bioenergy Technologies Office (BETO)

UPDATED PMP

Research and Technology Development for Genetic Improvement of Switchgrass

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UPDATED August 20, 2012

Task M. Technology for New Varietal Development

- **Subtask M.1 Wide cross hybrids**
- **Subtask M.2 Genomic assisted breeding**
- **Subtask M.3 Evaluation of field grown accessions**

Task N. Technology for Hybrid Plant Systems

- **Subtask N1. Evaluation of molecular constructs**
- **Subtask N2. Production and evaluation of transgenic lines**
- **Subtask N3. Screen for Transgenic events**
- **Subtask N4. Greenhouse test of transgenics**

Task O. Development and introduction of new trait genes into switchgrass

- **Subtask O.1 Establish collaborations with industrial and academic partners for access to trait genes for improved biofuels traits.**
- **Subtask O.2 Create the transgenic populations with selected traits.**
- **Subtask O.2.1 Wide cross development relating to Task M**

Task P. Project Management and Reporting

Research and Technology Development for Genetic Improvement of Switchgrass

1- Approach

I. Hybrid Systems:

- ***Development of male (pollen) and female (seed) sterility systems***
- ***Wide crosses recovered through a novel embryo rescue technique***
- ***Recovery of Non-GMO hybrids***

II. Genomics Assisted Breeding

- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***
- ***Development of male (pollen) and female (seed) sterility systems***
- ***Wide crosses recovered through a novel embryo rescue technique***
- ***Recovery of Non-GMO hybrids***

III. Transgenic Trait Improvement

•Gene Confinement and Development of GM Traits for Biofuel Crop Improvement

IV. Patent and other IP development

Seeking industry partnerships for collaboration and introduction of novel transgenic traits

Approach

Research and Technology Development for Genetic Improvement of Switchgrass

2 - Technical Accomplishments/Progress/Results

I. Hybrid Systems:

- ***Development of male (pollen) and female (seed) sterility systems***

Gene	*Target cells*	*Reference*
AMS	developing anthers	Sorensen et al. 2003 [166]
BEL1	developing megaspores	Robinson-Beers et al. 1992 [167]
DDE2	developing anthers	von Malek et al. 2002 [168]
EA1	developing megaspores	NA
EA1	developing megaspores	Marton et al. 2005 [169]
MS1	developing anthers	Wilson et al. 2001 [170]
SIN1	developing megaspores	Robinson-Beers et al. 1992 [167]
TDF1	developing anthers	Zhu et al. 2008 [171]

Table 1. List of target genes for ♂ and ♀ sterility expression cassettes. Promoters from these genes may be operably linked to cytoxic genes or RNAi constructs to direct cell specific ablation leading to the developmental disruption of male or female floral structures.

Research and Technology Development for Genetic Improvement of Switchgrass

2 - Technical Accomplishments/Progress/Results

I. Hybrid Systems:

- ***Development of male (pollen) and female (seed) sterility systems***

Transgenes for Male and Female Sterility
Useful for Breeding and Gene Confinement

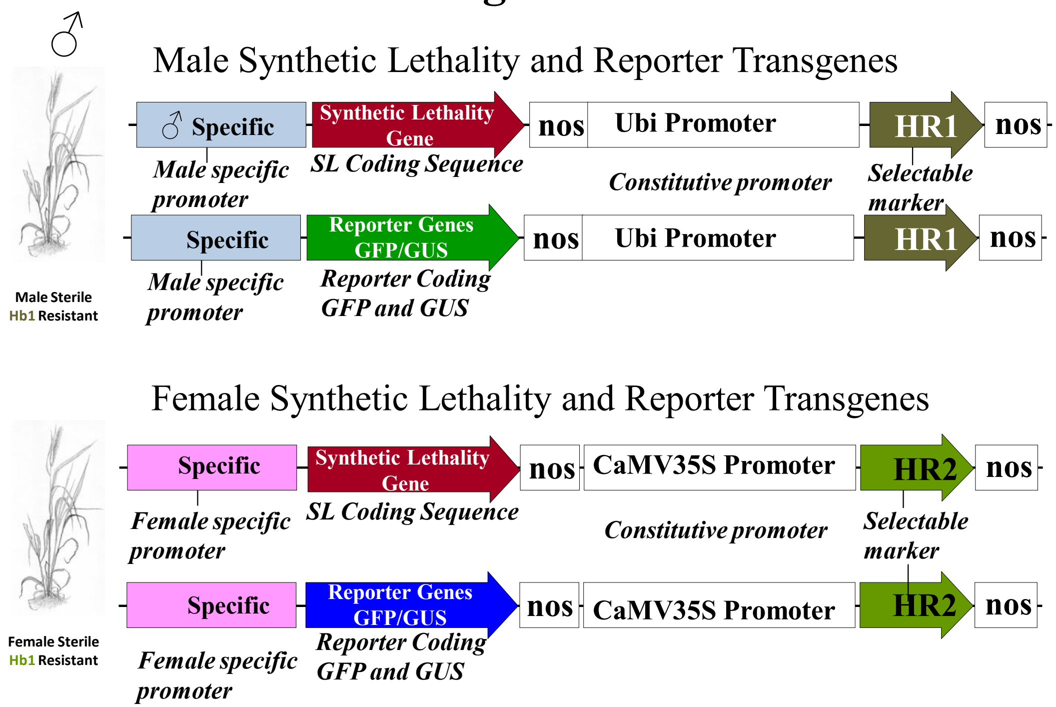

FIGURE 1. Male and female sterile lines useful for hybrid plant breeding or gene confinement. Male and female lines are created through the application of the promoters and/or the coding sequences described in TABLE 1.

Agriculture
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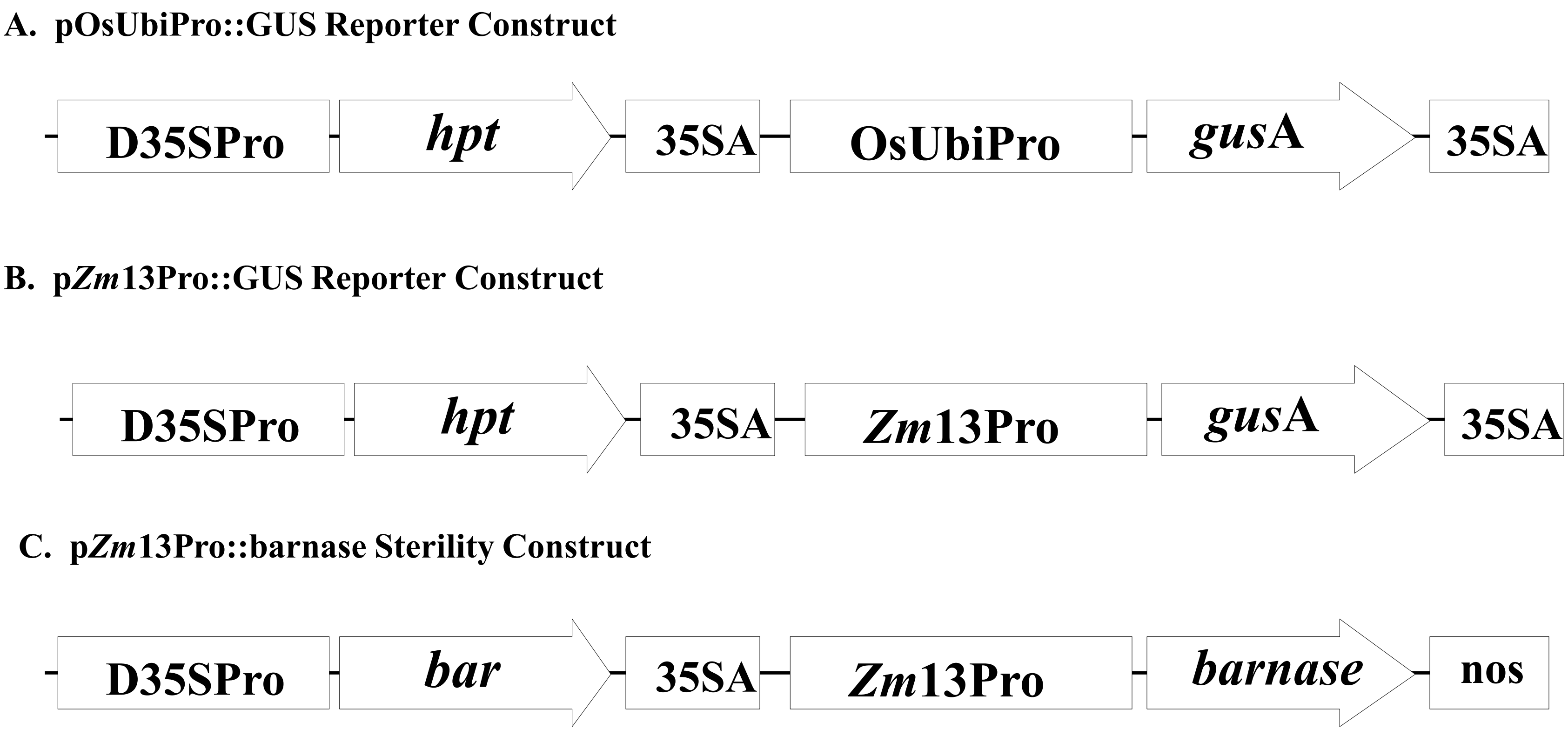
Article

Pollen Sterility—A Promising Approach to Gene Confinement and Breeding for Genetically Modified Bioenergy Crops

Joel P. Hague[1], Stephen L. Dellaporta[2], Maria A. Moreno[2], Chip Longo[1], Kimberly Nelson[1] and Albert P. Kausch[1]*
[1]Department of Cell and Molecular Biology, University of Rhode Island, West Kingston RI 02892: akausch@etal.uri.edu



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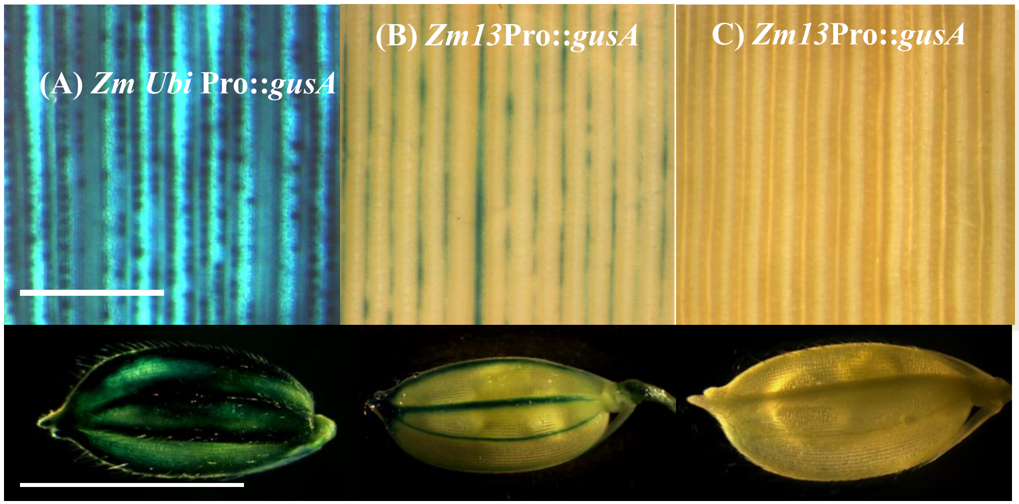
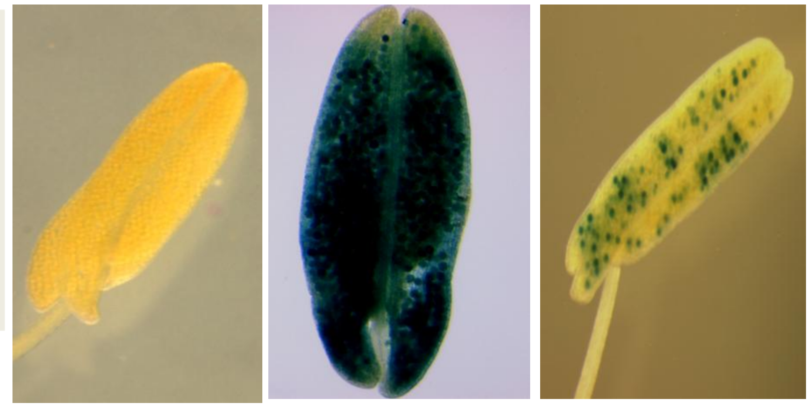
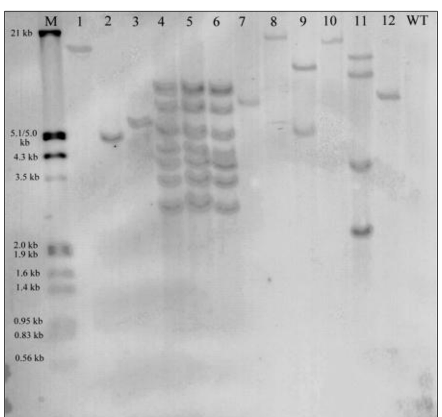
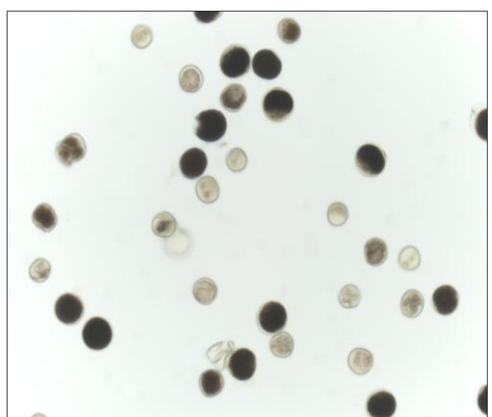


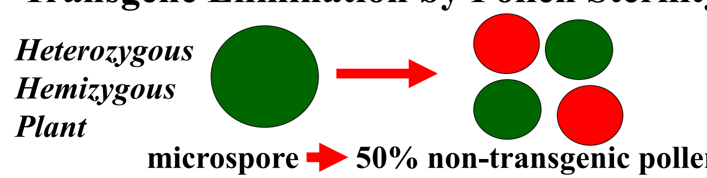


(A) Wild Type (B) Ubi GUS (C) MSP GUS

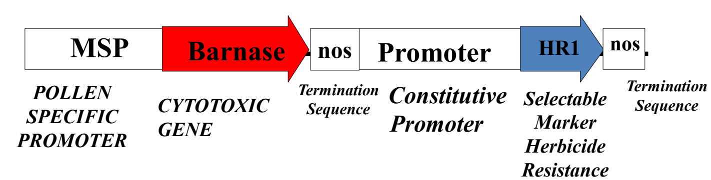




Transgene Elimination by Pollen Sterility

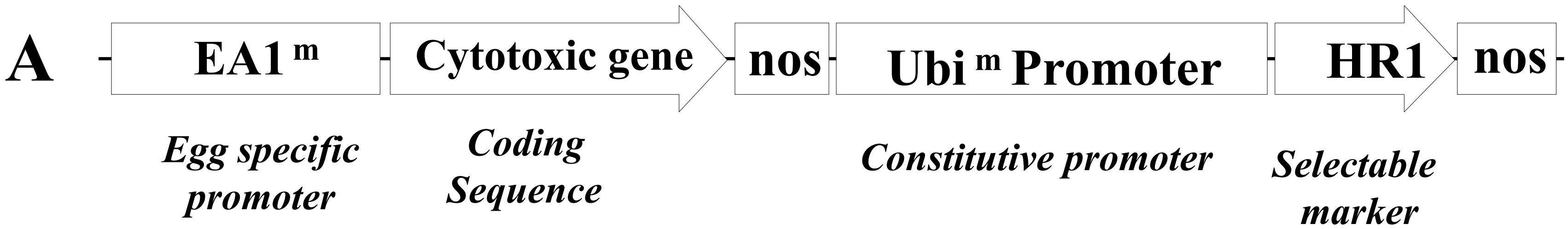


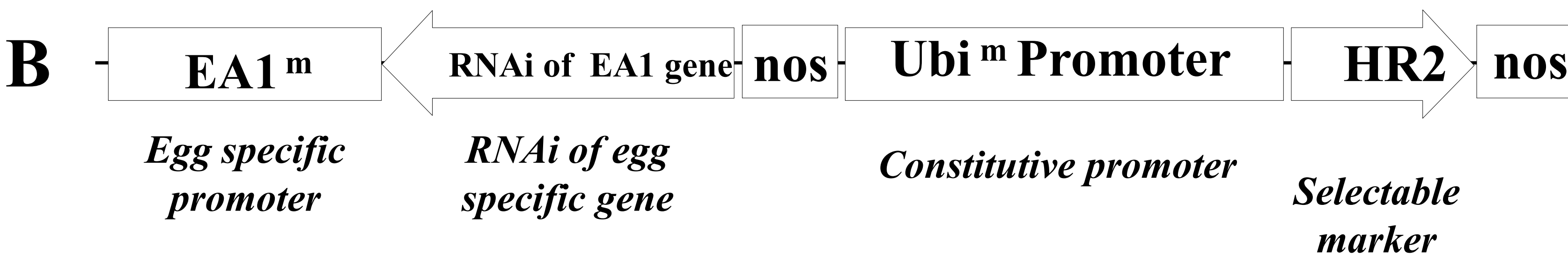
Only Non-GMO Pollen Is Viable



Strategy for Breeding & Gene Confinement

Female (Seed) Sterility





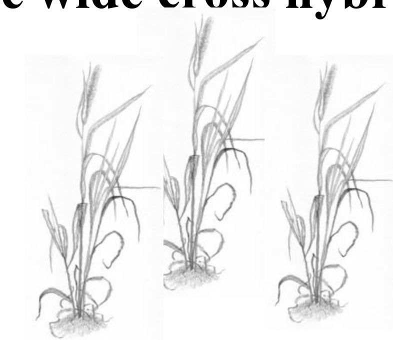
Physical linkage of herbicide resistance (HR1 and HR2) with male- and female-sterility transgenes for creation of bridge intermediate hybrid breeding populations

Male Sterile
Hb1 Resistant

X

Female Sterile
Hb2 Resistant

(Hb2) or (♂ Hb1) used as bridge intermediates to force wide cross hybrids



Total Sterile Hybrid

- **Breeding**
- **Trait Stacking**
- **Bridge Intermediates**
- **Total Gene Confinement**

Fully Sterile
Hb1 & Hb2
Resistant

Research and Technology Development for Genetic Improvement of Switchgrass

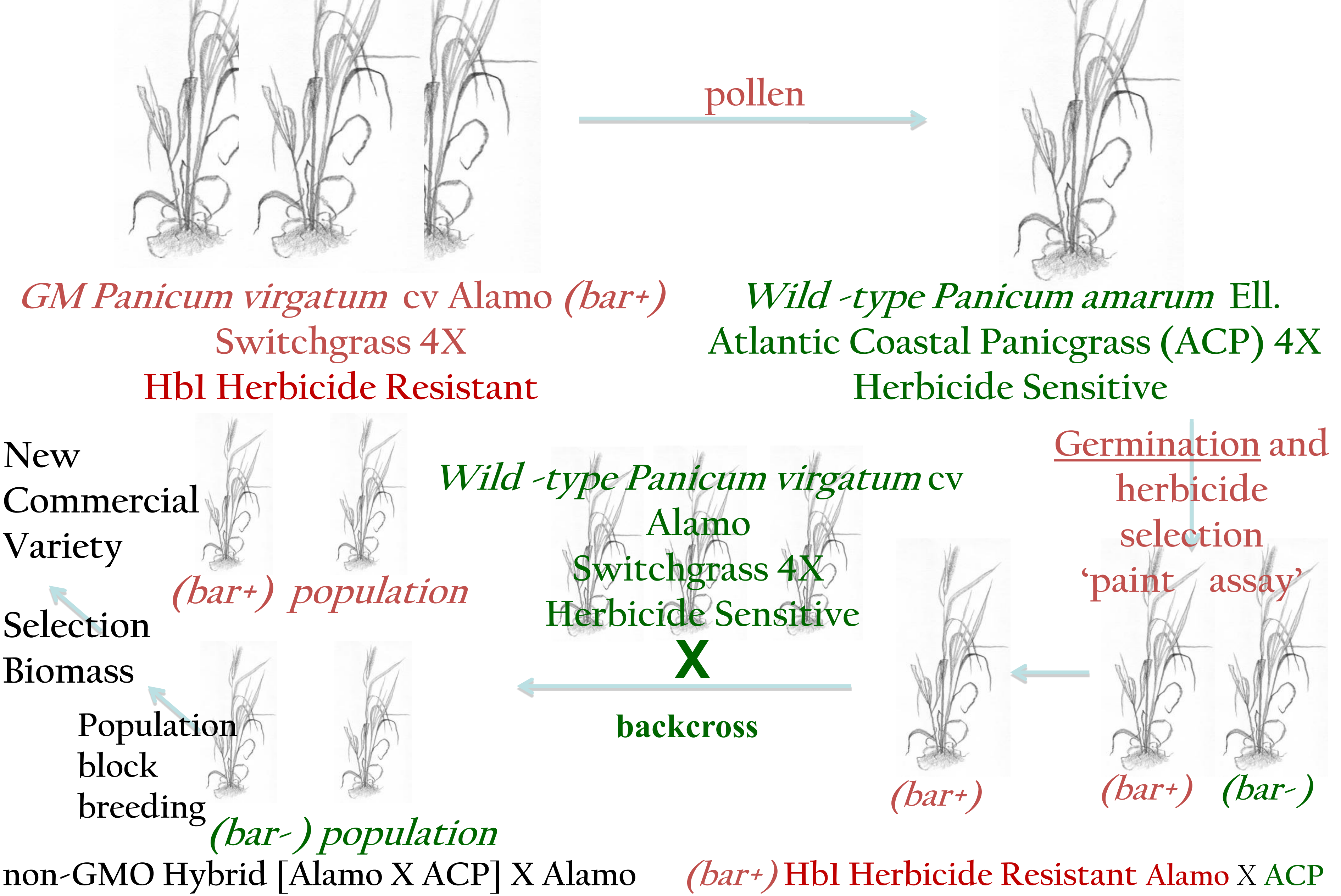
2 - Technical Accomplishments/Progress/Results

I. Hybrid Systems:
- *Wide crosses recovered through a novel embryo rescue technique*
- *Recovery of Non-GMO hybrids*

Can We Use Herbicide Resistant Lines for Recovery of Wide Crosses?

Can We Use Wide Crosses to Create Non-GMO Hybrids?

Intergeneric wide crosses using herbicide selection as a marker



Intergeneric wide crosses using herbicide selection as a marker

2 - Technical Accomplishments/Progress/Results

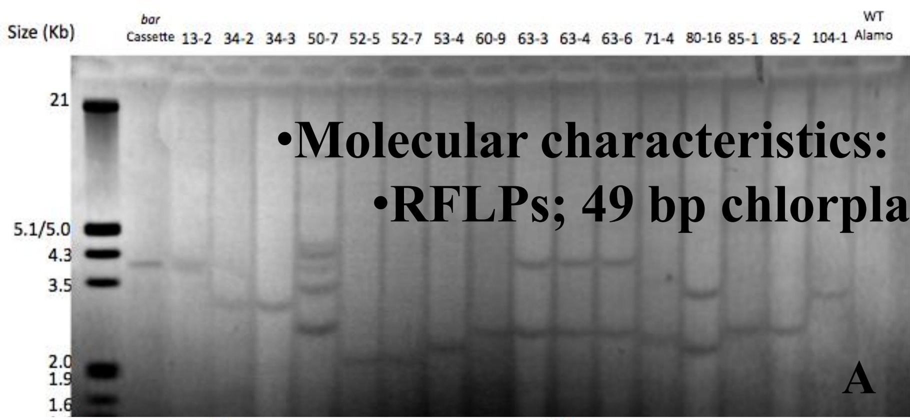
Characterization of Parental and F1 Hybrids

- Parentals (*P. virgatum X P. amarum)* are distinct species
- Phenotypic variation across many characteristics
  - Molecular characteristics:
    - RFLPs; 49 bp chlorplast DNA deletion
  - Leaf characteristics:
    - stomatal density and spacing; interveinal spacing; epicuticular wax
  - Floral characteristics:
    - Floret density and number; stigmatic color
  - Physical characteristics:
    - *P. amarum* drought tolerant

Characterization of F1 Hybrids

- Combination of parental characteristics

- Molecular characteristics:
  - RFLPs; 49 bp chlorplast DNA deletion



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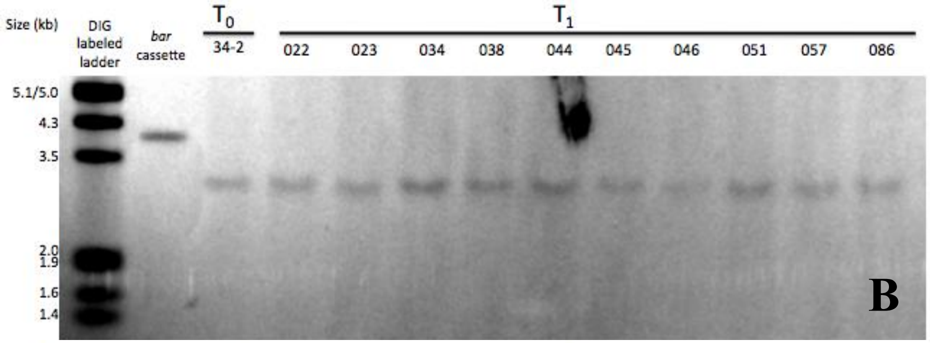

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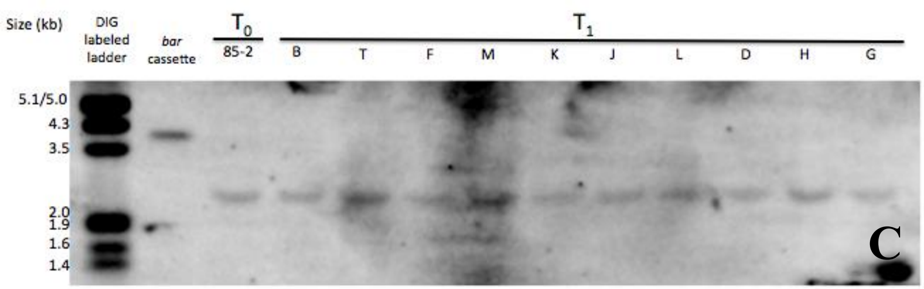

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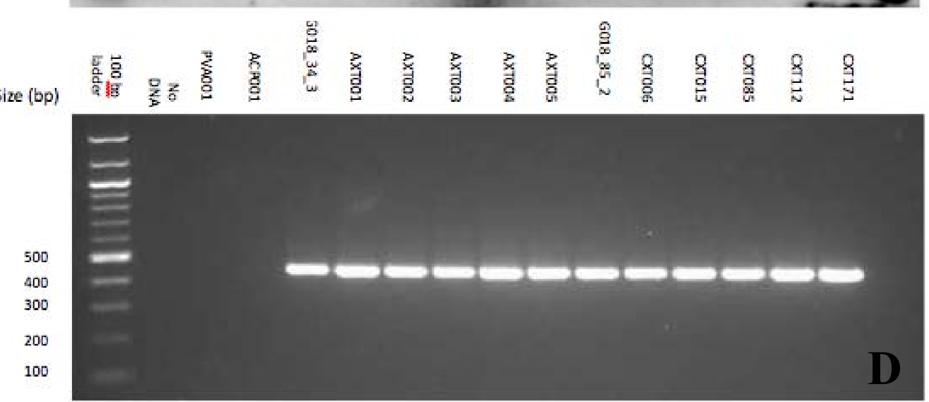
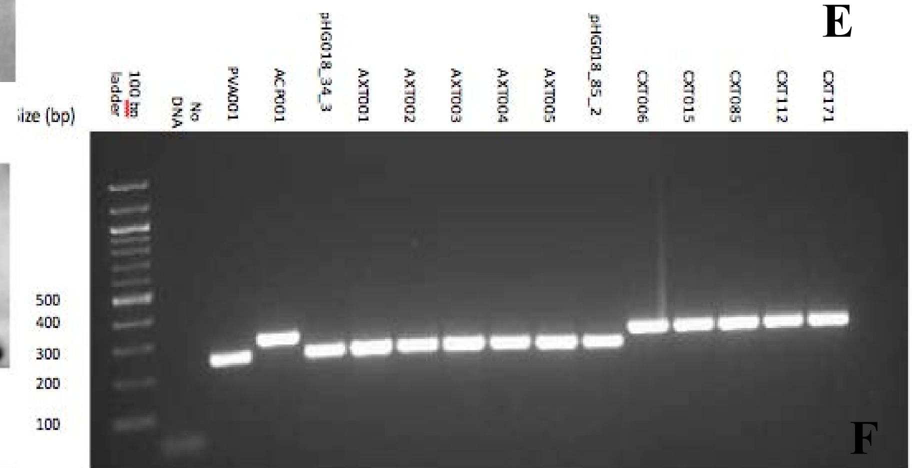


D

```
>gi|24209871|gb|AY142749.1| Panicum virgatum cultivar
Alamo tRNA-Leu (trnL) gene, intron; chloroplast gene
for chloroplast product
----
GGACTTGATATGTATTGAGCCTTGGTACGGAAACCTGCTAAGTGATAACTTCCAA
ATTCAGAGAAACCCTGGAATGAAAAATGGGCAATCCTGAGCCAAATCCCTTTTTT
GAAAAAACAAGTGGTTCTCAAACTAGAACCCAAAGGAAAGGATAGGTGCAGAGA
CTCAATGGAAGCTGTTCTAACGAATCGAAGTAATTACGTTGTGTTGGTAATGGAA
CTCCCTCGAAATTATAGAAAGAAGGGCTTTATACATCTAATACACACGTATAGAT
ACTCAGATAGCAAACGATTAATCATAGAACCCATATCATAATATAGGTTCTTTAT
TTTA-------------------------------------------------
TTTTTAGAATTATTGTGAATCCATTCCAATCGAATATTGAGTAATCAAATCCTT
CAATTCATTGTTTTGAGATCTTCAAAAAAGTGGATTAATCGAACGAGGATAAAGA
GAGAGTCCCATTCTACATGTCAATACTGACAACAATGAAATTTATAGTAAAAGGA
AAATCCGTCGACTTTATAAGTCGTGAG-
>gi|24209837|gb|AY142715.1| Panicum amarum var.
amarulum tRNA-Leu (trnL) gene, intron; chloroplast gene
for chloroplast product
----
GGACTTGATATGTATTGAGCCTTGGTACGGAAACCTGCTAAGTGATAACTTCCAA
ATTCAGAGAAACCCTGGAATGAAAAATGGGCAATCCTGAGCCAAATCCCTTTTTT
GAAAAAACAAGTGGTTCTCAAACTAGAACCCAAAGGAAAGGATAGGTGCAGAGA
CTCAATGGAAGCTGTTCTAACGAATCGAAGTAATTACGTTGTGTTGGTAATGGAA
CTCCCTCGAAATTATAGAAAGAAGGGCTTTATACATCTAATACACACGTATAGAT
ACTCAGATAGCAAACGATTAATCATAGAACCCATATCATAATATAGGTTCTTTAT
TTTATTTTTTAGAATGAAATTAGGAATGATTATGAAATATAAAATTCTGAATTTT
TTTTAGAATTATTGTGAATCCATTCCAATCGAATATTGAGTAATCAAATCCTTCA
ATTCATTGTTTTGAGATCTTCAAAAAAGTGGATTAATCGAACGAGGATAAAGAGA
GAGTCCCATTCTACATGTCAATACTGACAACAATGAAATTTATAGTAAAAGGAAA
ATCCGTCGACTTTATAAGTCGTGA--
```

E

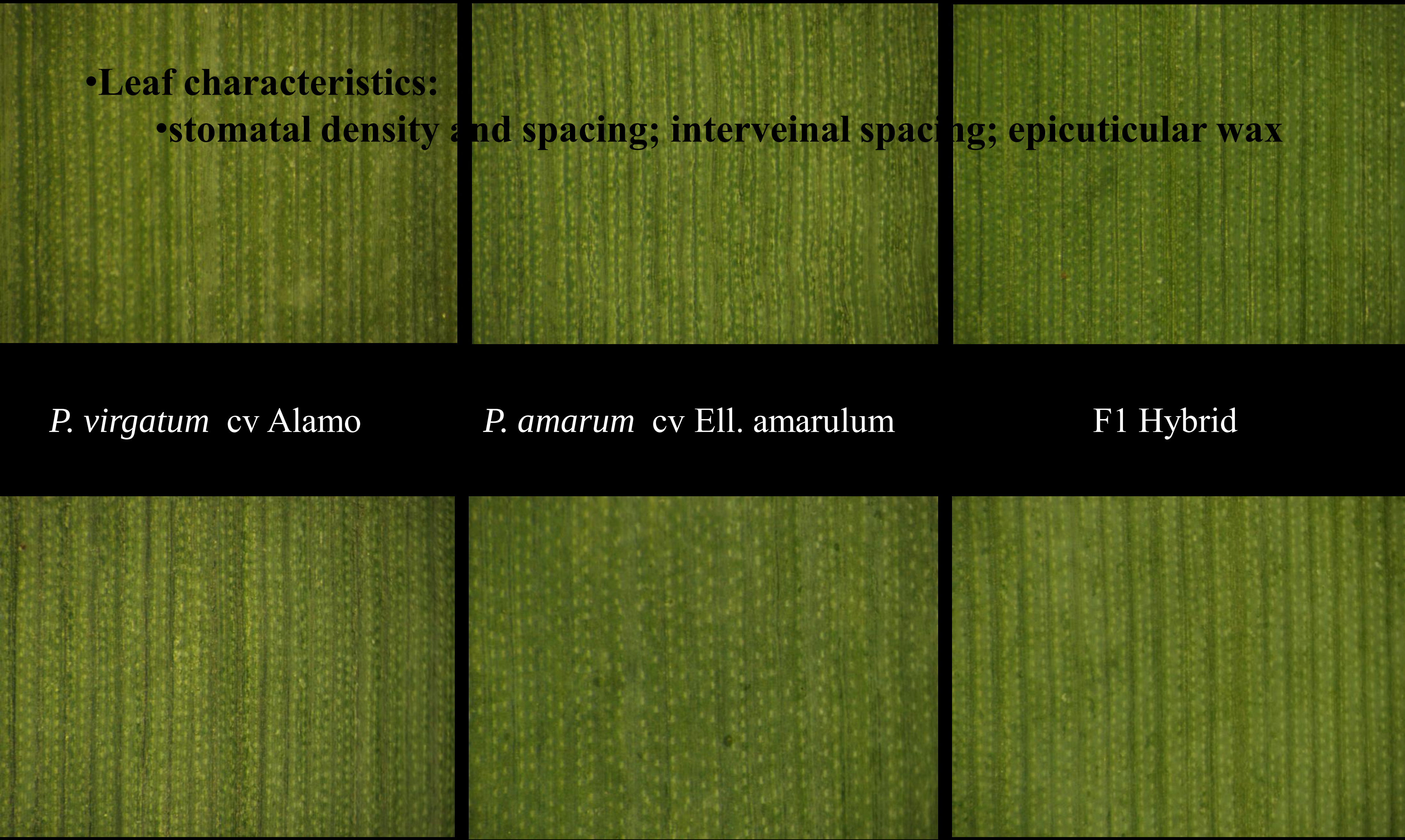


F

These results clearly indicate the recovery of wide intra- and inter-specific crosses by using herbicide resistant *Panicum virgatum* (cv Alamo) as a paternal donor to yield new F1 hybrids

- **Leaf characteristics:**
  - **stomatal density and spacing; interveinal spacing; epicuticular wax**

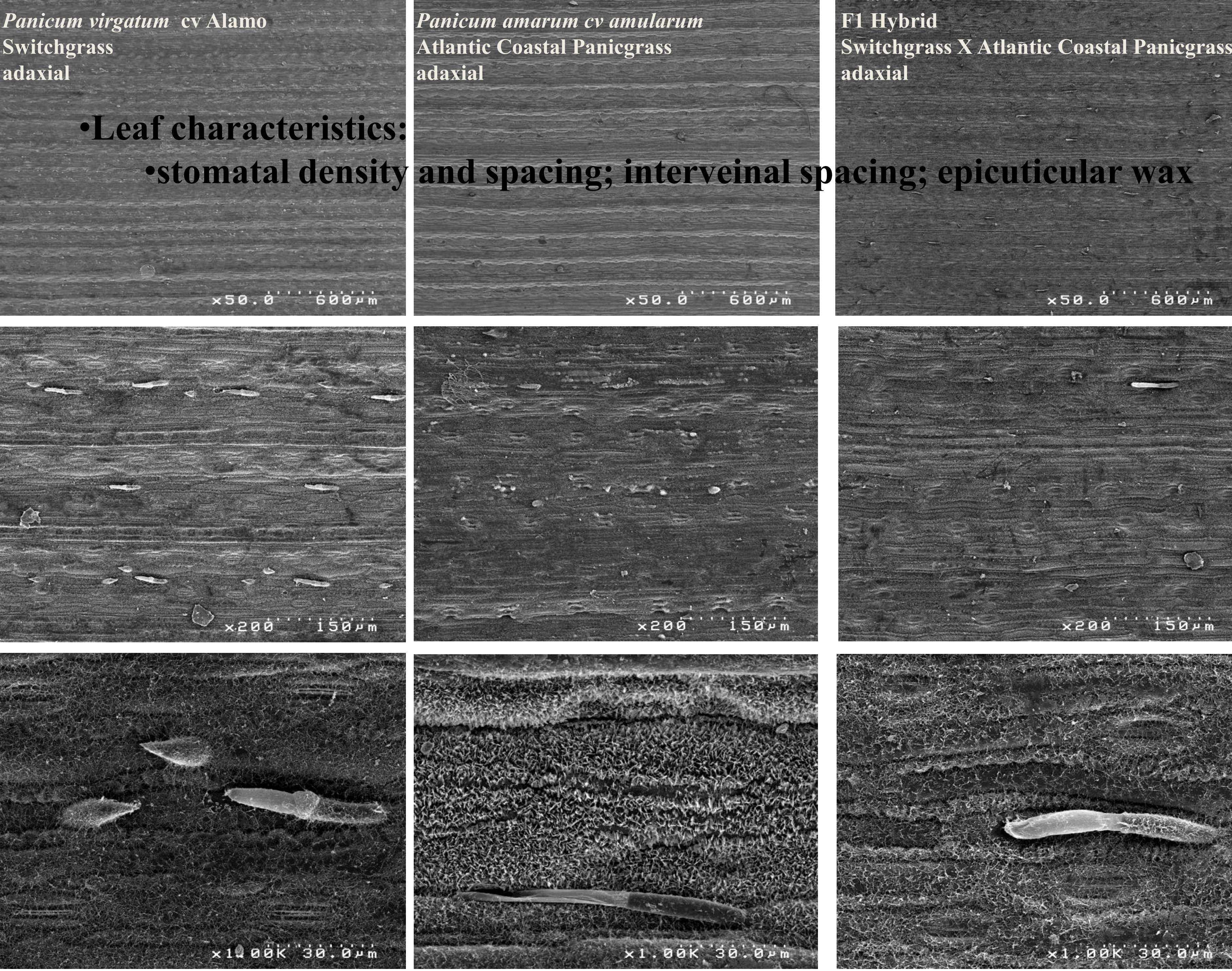
adaxil



abaxil

A

- Leaf characteristics:
  - stomatal density and spacing; interveinal spacing; epicuticular wax



•Floral characteristics:

•Floret density and number; stigmatic color

Inflorescence

P. virgatum cv Alamo | *P. amarum* cv Ell. amarulum | F1 Hybrid

Spikelet

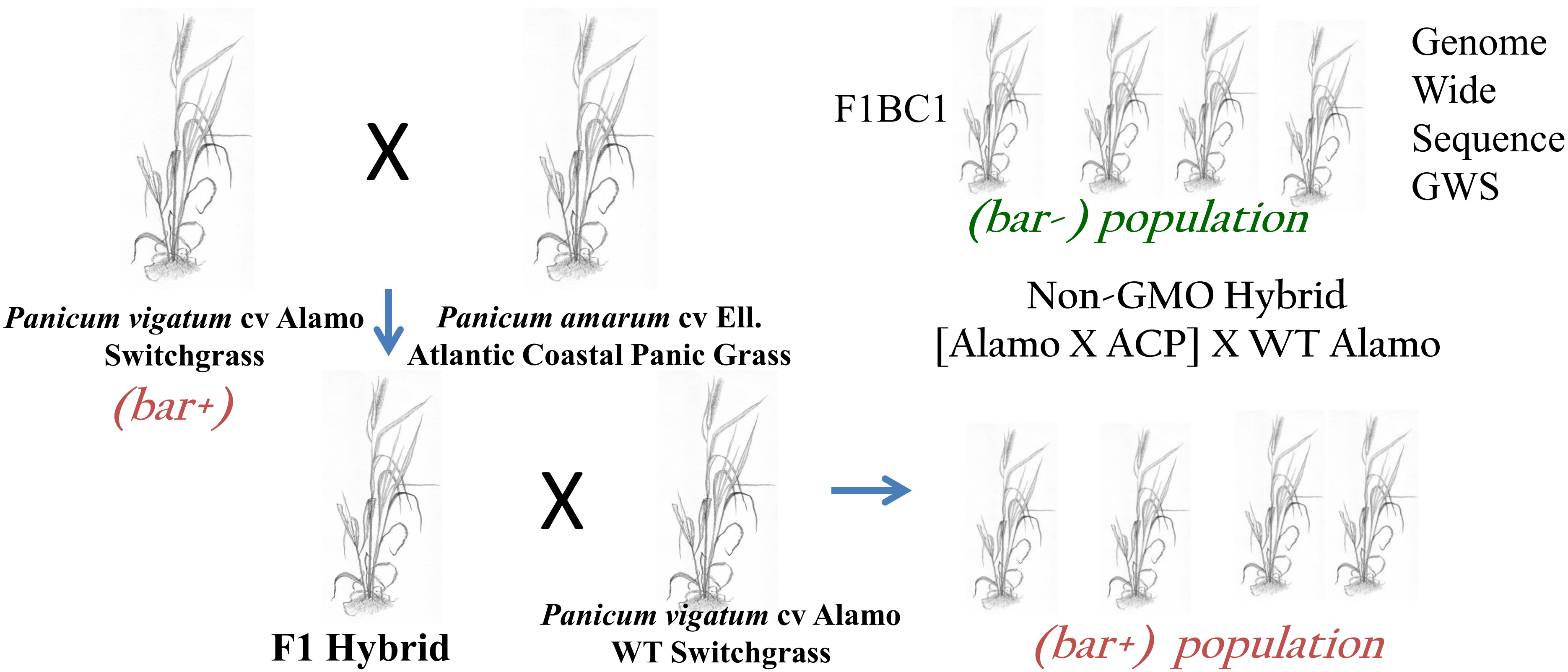
B

Research and Technology Development for Genetic Improvement of Switchgrass

2 - Technical Accomplishments/Progress/Results

II. Genomics Assisted Breeding

- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***
- ***Development of male (pollen) and female (seed) sterility systems***
- ***Wide crosses recovered through a novel embryo rescue technique***
- ***Recovery of Non-GMO hybrids***



Research and Technology Development for Genetic Improvement of Switchgrass

2 - Technical Accomplishments/Progress/Results

II. Genomics Assisted Breeding

- ***Genomic characterization of both parents, the F1 and the Non-GMO F1BC1 population***

Panicum vigatum **cv Alamo**
Switchgrass

Panicum amarum **cv Ell.**
Atlantic Coastal Panic Grass

F1 Hybrid

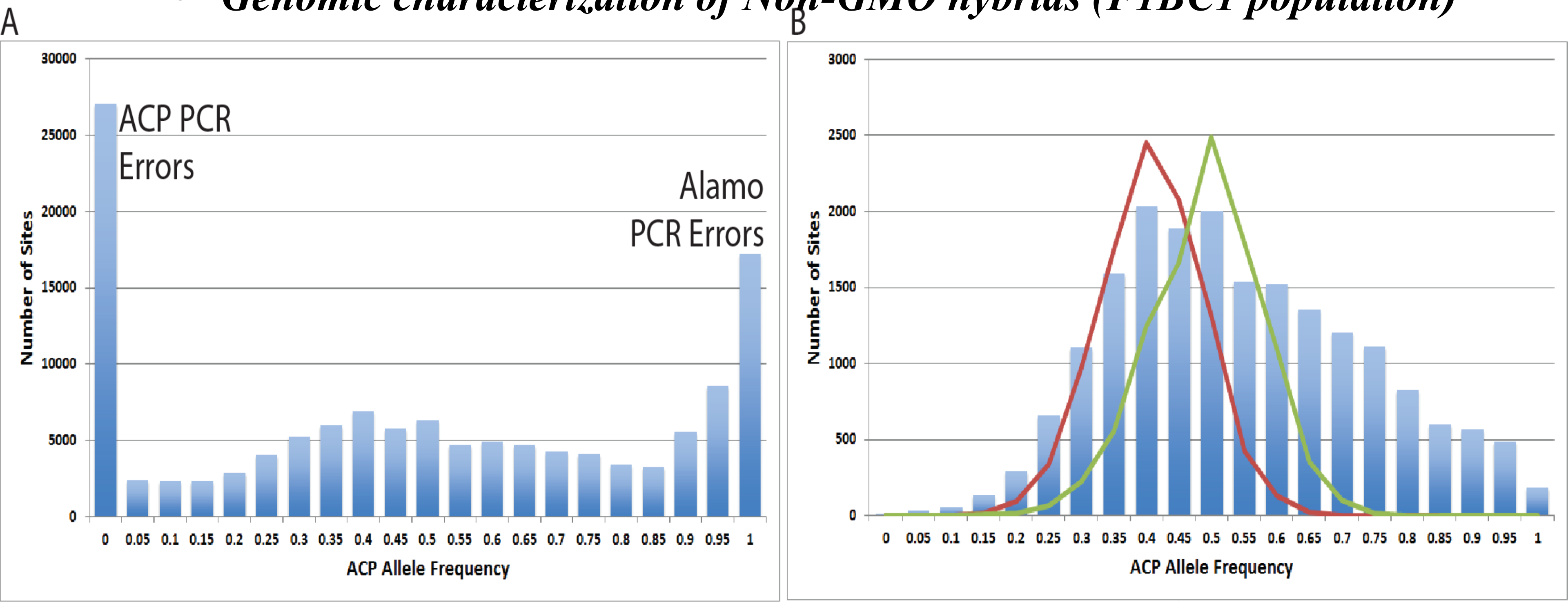
F1BC1 Hybrid
[Alamo X ACP] X WT Alamo

Genome
Wide
Sequencing
GWS

Yale Genome Center

II. Genomics Assisted Breeding

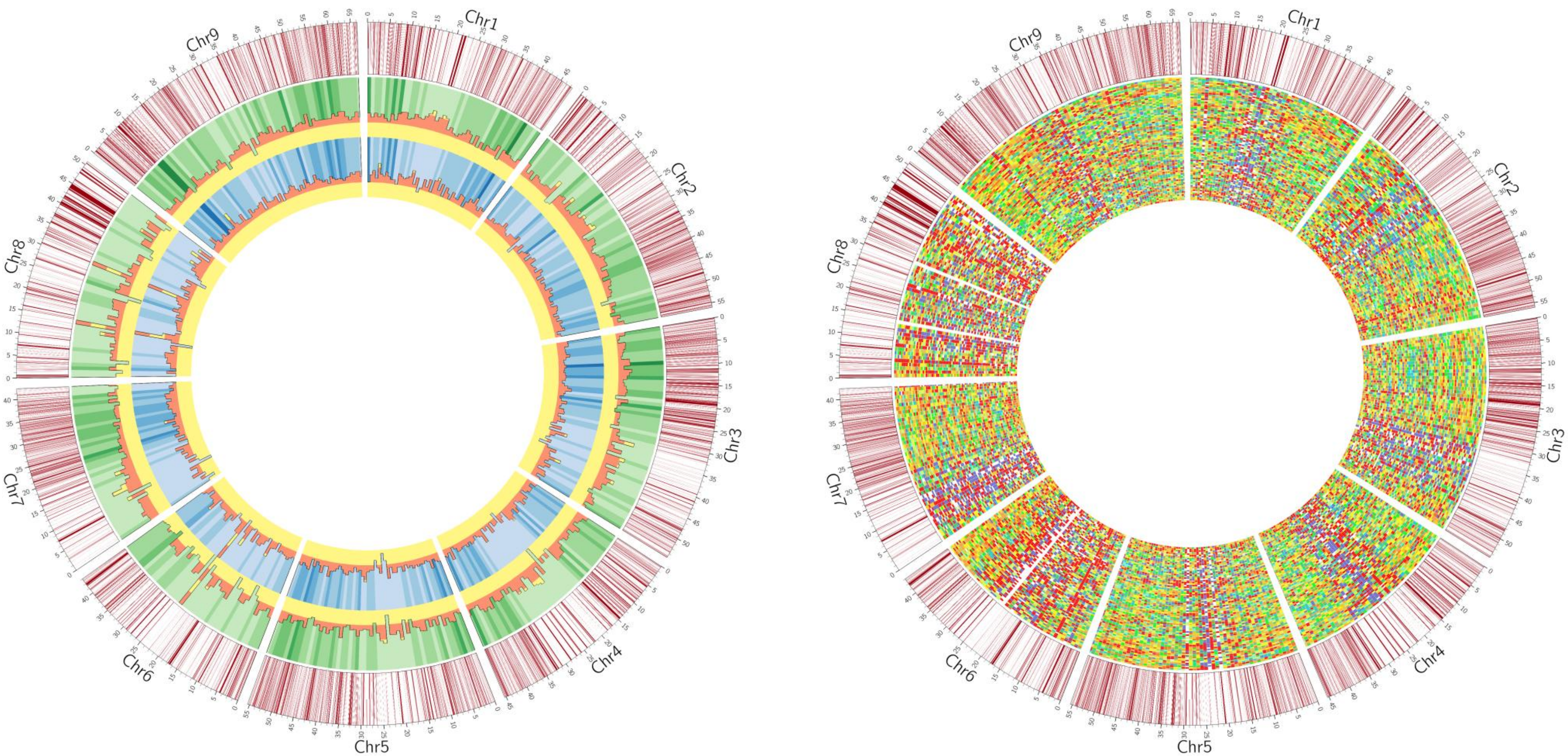
- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***



Distribution of ACP alleles at polymorphic sites. A) Distribution of all sites showing variation between Alamo and ACP parents. The peak at 0.0 is due to ACP PCR errors (e.g. no offspring inherit the ACP variant call), whereas the peak at 1.0 is due to Alamo PCR errors, as all offspring share the reference allele with ACP at the resulting sites. B) Distribution of sites after filtering variants for heterozygosity in the F1 in addition to variation between Alamo and ACP parents. The red line represents the simulated distribution of sites where the recombining pairs of the tetraploid differ enough that the reference genome treats them as two separate diploids, whereas the green line represents the simulated distribution of sites where the recombining pairs of the tetraploid have been collapsed as a single region in the reference.

II. Genomics Assisted Breeding

- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***

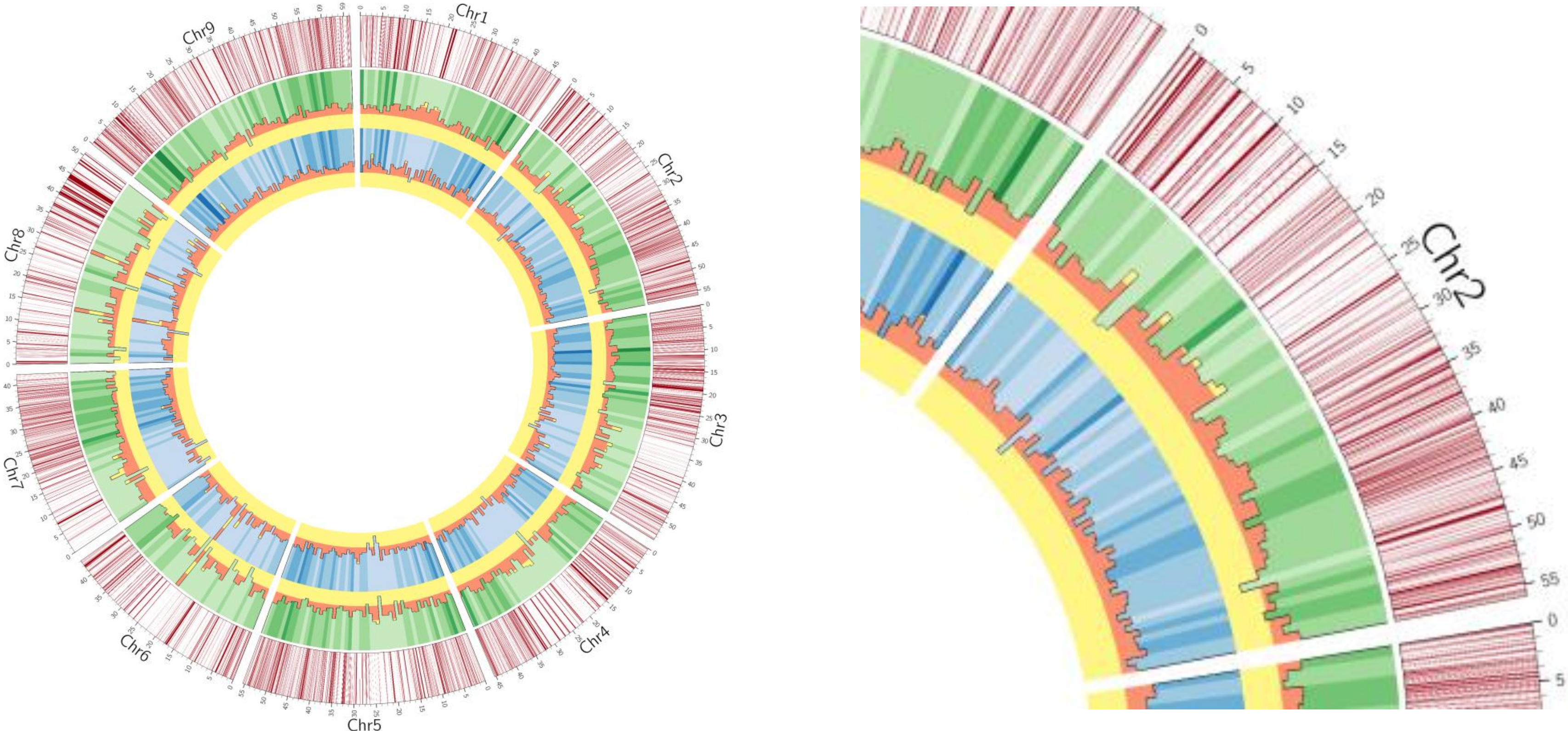


Distribution of variant sites and synteny alignment

Low resolution figure with a summary of the final analysis of all 83 lines. The figure on the left shows the total variant calls (blue histogram = Alamo and green histogram = ACP, total variants red outer circle) on a synteny map of foxtail millet. The right figure shows the ACP (red) and Alamo (blue) variants in each of the 83 F1BC1 lines individually. In both figures, the variants are pooled in 1 MB bins when mapping.

II. *Genomics Assisted Breeding*

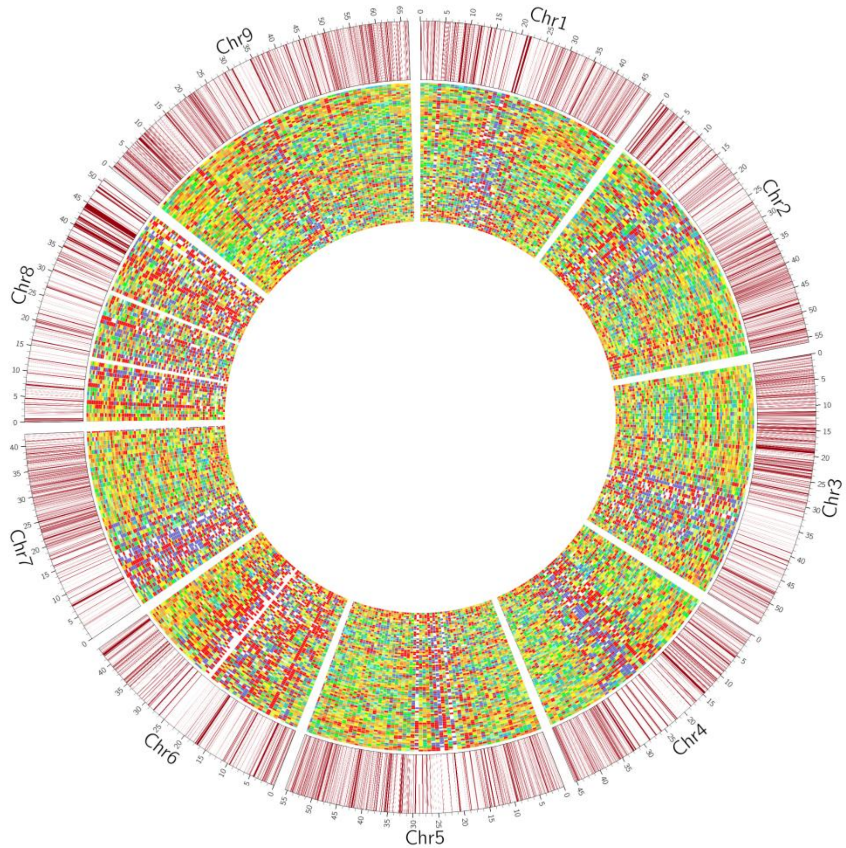
- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***

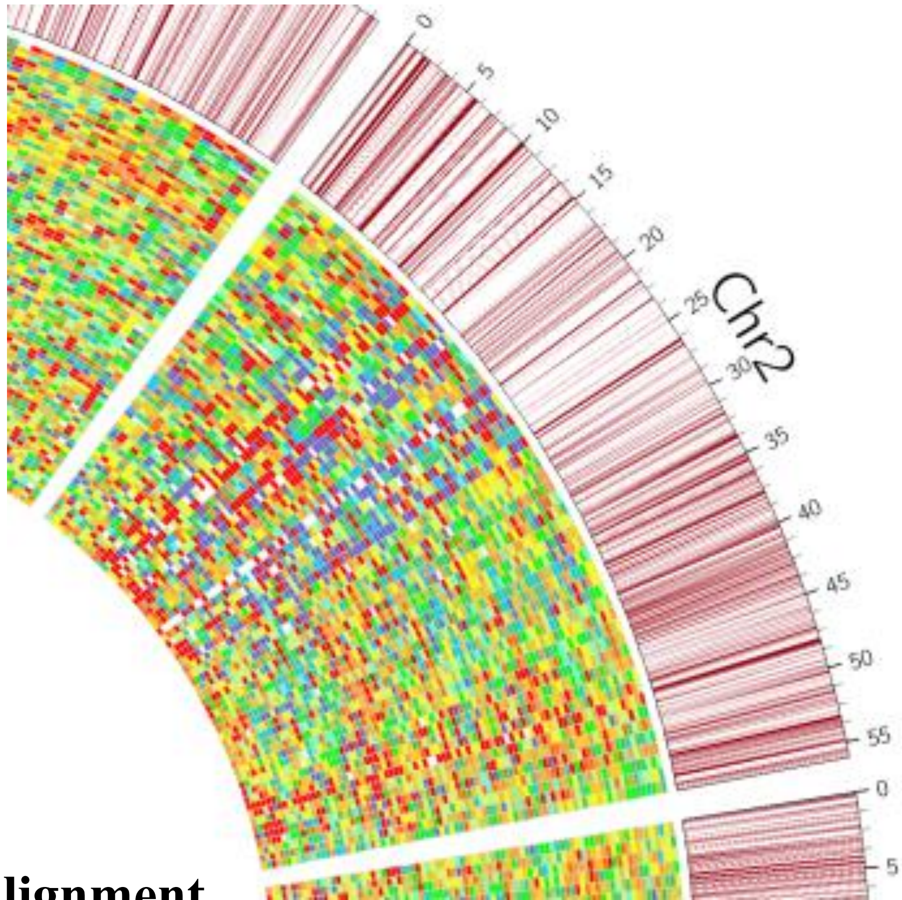


Distribution of variant sites and synteny alignment with the *Setaria italica* genome. The 5130 contigs containing filtered variants identified by both transmission from the ACP and Alamo parents and heterozygosity in the F1 show alignment (dark red lines) to the *Setaria italica* (foxtail millet) genome (white, outer circle). The percent contribution of ACP sites (green histogram) and the percent of F1BC1 samples with coverage at those sites (blue histogram) are shown in 1MB bloc histograms across the *Setaria italica* genome. Percent of covered samples with ACP alleles and overall number of samples showing coverage are between 50-75% for most blocs. Histogram heatmap reflects the numbers of sites within a bloc. Orange and yellow concentric circles under histograms indicate 25% intervals

II. Genomics Assisted Breeding

- ***Genomic characterization of Non-GMO hybrids (F1BC1 population)***





Distribution of variant sites and synteny alignment

Low resolution figure (left) shows the ACP (red) and Alamo (blue) variants in each of the 83 F1BC1 lines individually as a heatmap for the total variant calls; blue = Alamo and green histogram = ACP, total variants red outer circle on a synteny map of foxtail millet. In both figures, the variants are pooled in 1 MB bins when mapping.

A General Method For Hybrid Plant Production Using GMOs as Bridge Intermediates to Create Non-GMO Hybrids

Useful hybrid variants have been identified and characterized

Research and Technology Development for Genetic Improvement of Switchgrass

2 - Technical Accomplishments/Progress/Results

III. Transgenic Trait Improvement

- ***Gene Confinement and Development of GM Traits for Biofuel Crop Improvement***

IV. Patent and other IP development

Seeking industry partnerships for collaboration and introduction of novel transgenic traits

Plant Advancements LLC
Ernst Conservation Seeds Inc

Genetic Improvement of Switchgrass for Bioenergy

The Bioenergy Consortium and The Plant Biotechnology Laboratory

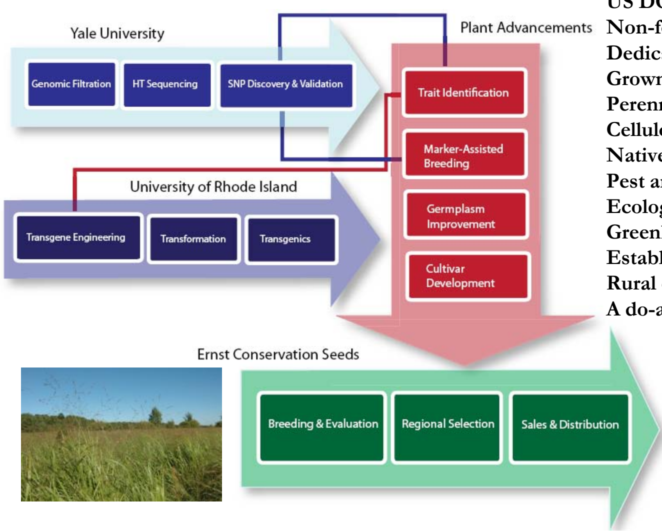
Kimberly Nelson, Joel Hague, Adam Deresienski. Stephen Dellaporta, Maria Moreno Yong Kong, and Albert Kausch

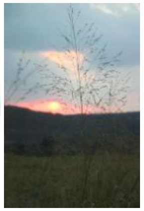
Technology Supporting Clean Energy And Independence from Foreign Oil

Program Synopsis- Energy from biomass, particularly from dedicated energy crops, will have tremendous economic, social and environmental benefits. Biofuels will help alleviate our national dependency on fossil fuels as a renewable resource, with a cost savings of an estimated $20 billion per year based on 2050 estimated fuel costs. Of the available cellulosic energy crops, switchgrass (*Panicum virgatum* L.) is clearly one of the most promising, chosen as a key species by the U.S. DOE Bioenergy Feedstock Development Program. Switchgrass is a native C4 perennial with excellent stand longevity, widely adapted to grow in marginal lands, natural resistance to pests and diseases, and biomass yields in established fields averaging 5-11 Mg.ha-1 tons per acre. Biologists, computer scientists, and engineers from Yale University and the University of Rhode Island and several companies have formed a research consortium to advance technologies for bioenergy production from perennials grasses, such as switchgrass. The Consortium's mission is to develop improved switchgrass varieties through the use of transformation technologies, conventional breeding, genomics, and marker-assisted breeding, and to commercialize these results with industry partners.

URI-Yale-Plant Advancements- Ernst Conservation Seeds

Bioenergy Consortium Overview





Why Switchgrass?

- US DOE Plant of Choice
- Non-food energy resource
- Dedicated energy crop
- Grown on marginal lands
- Perennial biomass production
- Cellulosic bioenergy
- Native to North America
- Pest and drought tolerant
- Ecological advantages
- Greenhouse gas negative
- Established production
- Rural economic growth
- A do-able energy solution

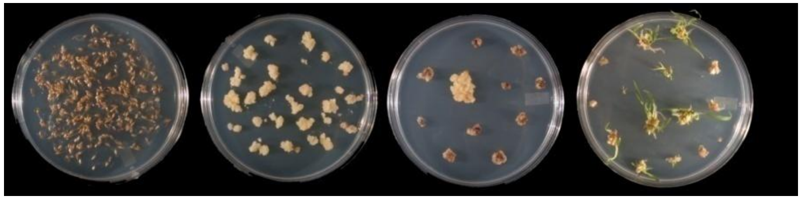
From concept to commercialization with improved dedicated energy crops

Trait Improvement for Bioenergy- Project Golden Switchgrass

Herbicide resistance	Floral sterility
Drought tolerance	Increased shade tolerance
Salt tolerance	Increased biomass/acre
Cold tolerance	Broad fungal resistance
Photosynthetic efficiency	Insect resistance
Enhanced cellulose production	Stay green
Increased root growth	Delayed senescence
Enhanced phosphate uptake	Decreased nitrogen
Increased leaf angle	ncreased free sucrose
Post harvest traits for EtOH	Enhanced endophytes
Decreased lignin	Co-Products

University of Rhode Island

Grass Transformation and Transgenics Technologies



Switchgrass transformation with improved traits and transgenic plant regeneration

Genetic modification of switchgrass
- **Transgenic trait development**

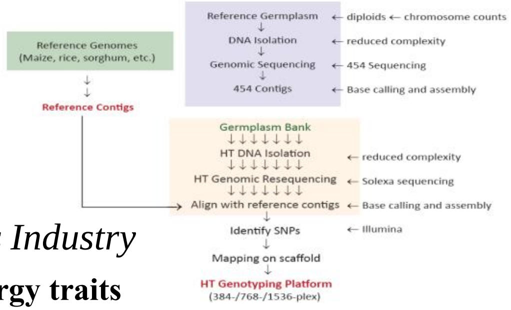
Gene confinement strategies for environmental stewardship

Yale University

Biofuels Genomics and Computational Technologies

Sequencing the switchgrass genome
- **Bioinformatics**

Maker assisted breeding



Plant Advancements, LLC.

Biomass Products and Superior Germplasm for the Biofuels Industry

New variety development for bioenergy traits
- **Regionally selected ecotypes**
- **High yield trait selection**

Conventional breeding and genetics

Transgenic trait commercialization

Ernst Conservation Seeds, Inc.

Biofuels Seed Production and Sales

Foundation field development

Contract growing with biorefineries

Seed processing facility at Ernst Conservation Seeds

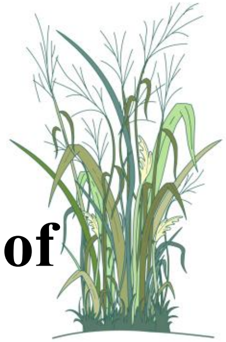
University of Rhode Island

LUX ET VERITAS

Plant Advancements LLC

ERNST Conservation Seeds

Research and Technology Development for Genetic Improvement of Switchgrass



3 - Relevance

- **This project addresses the following goals and objectives of Biomass Program Multi-Year Program Plan (updated November 2012).**

2.1.3 Feedstock Technical Challenges and Barriers

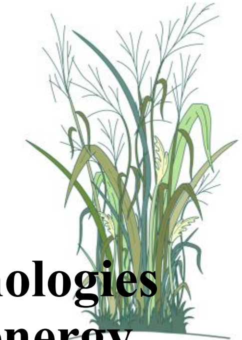
Ft-A Feedstock Availability and Cost **Sustainable production and yield in Switchgrass and related species**

Ft-C. Feedstock Genetics and Development: **The productivity and robustness of terrestrial feedstock crops used for biofuel production improved by selection, screening, breeding, and/or genetic engineering.**

This project considers applications of the expected outputs:

- **New commercial varieties**
- **Technology for development of new varieties**
- **Intellectual property to support commercial development**

Research and Technology Development for Genetic Improvement of Switchgrass



3 - Relevance

Objectives regarding the relevance of this project to the Bioenergy Technologies Office, alignment with MYPP goals, and relevance for the overall bioenergy industry

• ***Development of hybrid plant systems (Ft-A and Ft-C)***

Increased yields, new breeding and gene confinement technology for the future

• ***Development of advanced breeding strategies utilizing wide crosses, advanced tissue culture and genomics to produce new Non-GMO hybrids (Ft-A and Ft-C)***

•Increased yield, new bioenergy specific cultivars, and new technology for the future crop improvements of perennial biofuels crops

• ***Development of robust transgenic and gene confinement strategies (Ft-C)***

"*Any genetically modified organisms deployed commercially will also require prior deregulation by the appropriate federal, state and local government agencies*" and gene confinement will hence be required.

•*Enhance education , student training and internship research opportunities in biofuels crop improvement and plant biotechnology*

Facilitating public education resource development and public perception

Research and Technology Development for Genetic Improvement of Switchgrass

4 - Critical Success Factors

- **Critical success factors (technical, market, business) which will define technical and commercial viability.**

This project has demonstrated the technical success

The market, business and commercial viability is currently dependant on end use for cellulosic biofuels

Patents generated during this project will be broadly applicable to the bioenergy crops field and agricultural biotechnology generally.

There is a large business and commercial viability for the licensing of IP in this area

Research and Technology Development for Genetic Improvement of Switchgrass

4 - Critical Success Factors

Top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results.

1. **End use market to drive biofuels crop production**
2. **Need to commercialize and deregulate transgenic biofuels crops (which will require the IP generated in this project for gene confinement)**

Research and Technology Development for Genetic Improvement of Switchgrass

4 - Critical Success Factors

Demonstrate that the successful project will advance the state of technology and positively impact the commercial viability of biomass and /or biofuels.

- **Hybrid plant systems in this project will affect biofuels crop improvement and other agricultural crops generally**
- **Advanced genomics capabilities in this project will be broadly applied**
- **IP for gene confinement may well be used across many crop species for deregulation.**

Research and Technology Development for Genetic Improvement of Switchgrass

5. Future Work

- **Plans through the end of the project:**

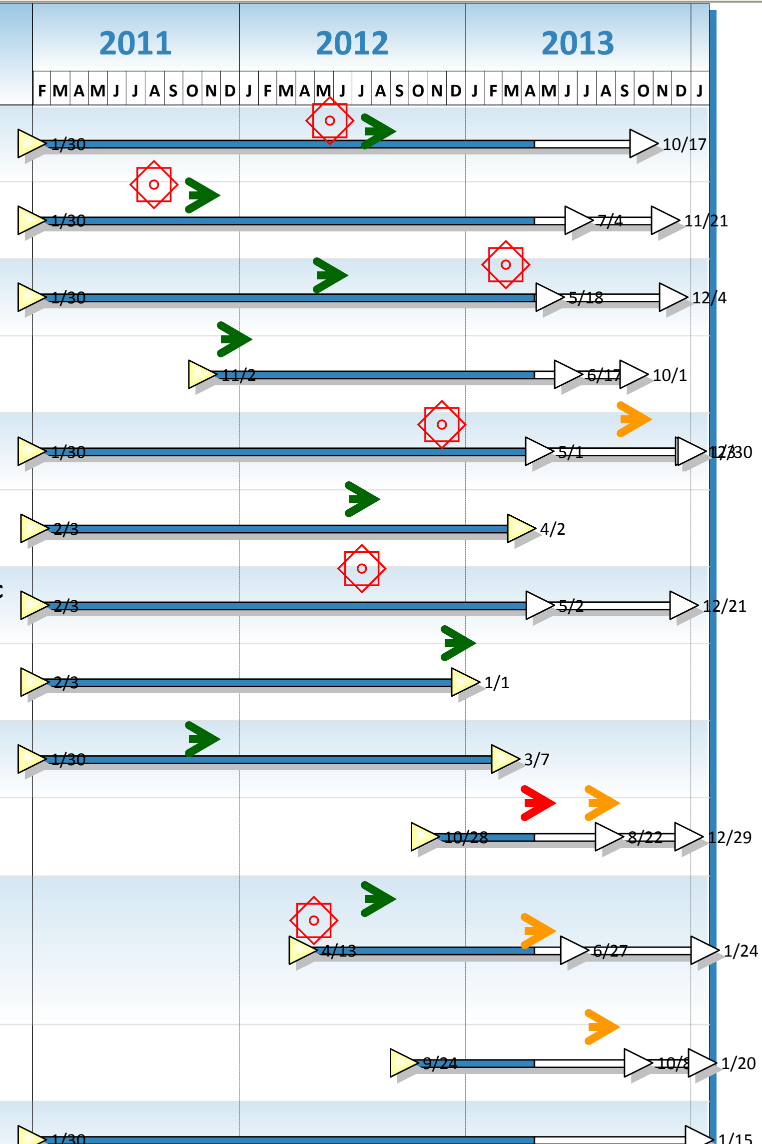
Complete characterization of ♂ and ♀ sterility lines
Complete and submit relevant publications
Complete patent applications
Progress toward commercialization with Plant Advancements LLC

- **Gantt Chart shows highlights of key milestones & Go/No go decision points.**

Gantt Chart for UPDATED PMP Award # DE-FG-36-08GO88070

Research and Technology Development for Genetic Improvement of Switchgrass

Start Date	End Date	TASK
1/30/2011	10/17/2013	Task M. Technology for New Varietal Development
1/30/2011	11/21/2013	Subtask M.1 Wide cross hybrids
1/30/2011	12/4/2013	Subtask M.2 Genomic assisted breeding
11/2/2011	10/1/2013	Subtask M.3 Evaluation of field grown accessions
1/30/2011	1/3/2014	Task N. Technology for Hybrid Plant Systems
2/3/2011	4/2/2013	Subtask N1. Evaluation of molecular constructs
2/3/2011	12/21/2013	Subtask N2. Production and evaluation of transgenic lines
2/3/2011	1/1/2013	Subtask N3. Screen for Transgenic events
1/30/2011	3/7/2013	Subtask N4. Greenhouse test of transgenics
10/28/2012	12/29/2013	Task O. Development and introduction of new trait genes into switchgrass
4/13/2012	1/24/2014	Subtask O.1 Establish collaborations with industrial and academic partners for access to trait genes for improved biofuels traits.
9/24/2012	1/20/2014	Subtask O.2 Create the transgenic populations with selected traits.
1/30/2011	1/15/2014	Task P. Project Management and Reporting



Key Milestones Go/No-Go Yes No Pending

Summary

1) Approach
- Hybrid Plant Systems, Non-GMO hybrids; Gene Confinement; Robust Genomics Platform

1) Technical accomplishments
- Demonstrated the technical success in all major goals

1) Relevance MYPP 2012 *Ft-A and Ft-C*
2) Critical Success factors and challenges
- Dependant on future end use for cellulosic biofuels

1) Future Work
- Commercialization with Plant Advancements LLC

1) Technology transfer
- IP licensing; Publications and Presentations

Thank You

Additional Slides

Publications, Presentations, and Commercialization

Publications:

1. Dellaporta, S. , Moreno, M. Deresienski, A., K. Nelson, J. Hague, and A.P. Kausch. Production and Genomic Analysis of Interspecific Hybrids in Switchgrass *(Panicum virgatum, L.)*. Nature Biotechnology (In preparation).
2. Howard, TP, Tordillos, A, Fragoso, C., Moreno, MA, Mottinger, JP, Kausch, AP, Tohme, J, and Dellaporta, SL (2013) Identification of the maize gravitropism gene *lazy plant1* by a transposon-tagging genome resequencing strategy. *Genetics,* submitted.
3. Nelson, K., A. Deresienski, M. Tilelli, J. Hague, C. Longo and A.P. Kausch. (2013) In situ embryo rescue in Switchgrass (*Panicum virgatum L*.) and 'Atlantic' Coastal Panicgrass (*Panicum virgatum Ell. var. amarulum*) Plant Science (submitted)
4. Kausch, AP, Deresienski A, Hague, J, Tilelli M, Dellaporta SD, Nelson, K and Li,Yi. (2013) Hybrid Plant Systems for Breeding and Gene Confinement in Bioenergy Crops. In: New and Future Developments in Catalysis – Catalytic Biomass Conversion. Steven Suib, Ed. Elsevier Press (in press)
5. Kausch, AP, Hague, J, Deresienski A, Tilelli M, Longo C, and Nelson, K (2013) Issues in Biotechnology: A Massive Open Online Course (MOOC) Covering in Simple Terms Basic Knowledge About DNA and Biotechnology INTED Proceedings
6. Joel P. Hague, Steven L. Dellaporta, Maria Moreno, Chip Longo, Kimberly Nelson, Albert P. Kausch (2012). Pollen Sterility - A Promising Approach to Gene Confinement and Breeding for Genetically Modified Bioenergy Crops. *Agriculture* 2:295-315
7. Kausch, A.P., J. Hague, A. Deresienski, Tilelli M and K. Nelson. (2012) Male Sterility and Hybrid Plant Systems for Gene Confinement. In Plant Gene Containment. M. Oliver and Yi Li. Eds. John Wiley & Sons,. Inc. New York, New York.
8. Albert P. Kausch, Joel P. Hague, Melvin J. Oliver, Yi Li, Henry Daniell, Peter Mascia, Lidia S. Watrud, and C. Neal Stewart Jr (2010). Transgenic perennial biofuel feedstocks and strategies for bioconfinement. *Biofuels* 1:163-176.
9. Albert P. Kausch, Joel Hague, Melvin Oliver, Yi Li, Henry Daniell, Peter Mascia, and C. Neal Stewart Jr. (2010). Genetic Modification in Dedicated Bioenergy Crops and Strategies for Gene Confinement. In: Plant Biotechnology for Sustainable Production of Energy and Co-products, Biotechnology in Agriculture and Forestry 66, P.N. Mascia et al. (eds.), Springer-Verlag Berlin Heidelberg, pp. 299-313.
10. Albert P. Kausch, Joel Hague, Melvin Oliver, Lidia S. Watrud, Carol Mallory-Smith, Virgil Meier, and C. Neal Stewart Jr. (2010). Gene Flow in Genetically Engineered Perennial Grasses: Lessons for Modification of Dedicated Bioenergy Crops. In: Plant Biotechnology for Sustainable Production of Energy and Co-products, Biotechnology in Agriculture and Forestry 66, P.N. Mascia et al. (eds.), Springer-Verlag Berlin Heidelberg. pp. 285-296.
11. Moon, H., J. Abercrombie, A. Kausch, and C. Stewart. 2010. Sustainable Use of Biotechnology for Bioenergy Feedstocks, pp. 1-8 Environmental Management. Springer New York.

Selected Recent Presentations in Meetings, Invited Lectures, Conferences and Symposia

1. A. Kausch, J. Hague, L. Perretta and K. Nelson (2013) Agricultural Biotechnology: A Massive Open Online Course (MOOC) Module Covering in Simple Terms Basic Knowledge About DNA and Plant Biotechnology. Plant Biology 2013, Annual Meetings of the American Society of Plant Biologists, July 20-24, Providence, Rhode Island, USA.
2. J. Hague, M. Tilelli, D. Cunha, K. Nelson and A. Kausch (2013) In Situ Embryo Rescue as a Novel Method for Recovery of Non-GMO Hybrids from Wide Crosses. Plant Biology 2013, Annual Meetings of the American Society of Plant Biologists, July 20-24, Providence, Rhode Island, USA.
3. Kausch, Albert. Invited Speaker. (2012) The use of synthetic male and female sterility for recovery of Non-Genetically Modified Hybrids from Wide Crosses. Department of Horticultural Science, North Carolina State University, Mountain Horticultural Crops Research and Extension Center, October 19, 2012
4. Kausch, Albert (2012) Invited Speaker. Bioenergy: Genetic Improvement of Bioenergy Crops for Biofuels and Prospects for Artificial Photosynthesis. Department of Chemistry. Brown University, Providence Rhode Island. September 14, 2012
5. Kausch, Albert (2012) Invited Speaker. Bioenergy: Genetic Improvement of Bioenergy Crops for Biofuels Department of Botany connecticut College, new London CT. September 21, 2012
6. .A. Kausch, A. Deresienski, J. Hague, M.Tilelli, K. Nelson (2012) Issues in Biotechnology: An Online General Education Undergraduate Course Covering Simple Terms Basic Knowledge About DNA and Biotechnology. Plant Biology 2012, Annual Meetings of the American Society of Plant Biologists, July 20-24, Austin, TX, USA.
7. J. Hague, A. Deresienski, M.Tilelli, K. Nelson, A. Kausch (2012) The Analysis of Expression Characteristics of the Maize Pollen Specific Promoter MPSP Zm13 And A Strategy for Gene Confinement in Transgenic Bioenergy Crops. Plant Biology 2012, Annual Meetings of the American Society of Plant Biologists, July 20-24, Austin, TX, USA.
8. A. Deresienski, K. Nelson, M.Tilelli, J. Hague, A. Kausch (2012) Use of a Herbicide Resistance Selectable Marker for Recovery of Intraspecific and Interspecific Hybrids in Switchgrass. Plant Biology 2012, Annual Meetings of the American Society of Plant Biologists, July 20-24, Austin, TX, USA.
9. K. Nelson, A. Deresienski, M.Tilelli, J. Hague, A. Kausch (2012) A Project-based Undergraduate Internship Program in Agricultural Biotechnology. Plant Biology 2012, Annual Meetings of the American Society of Plant Biologists, July 20-24, Austin, TX, USA.
10. M.Tilelli, K. Nelson, A. Deresienski, J. Hague, A. Kausch (2012) Use of a Selectable Marker for In Situ Embryo Rescue using Transgenic Switchgrass for Recovery of Wide Crosses. Plant Biology 2012, Annual Meetings of the American Society of Plant Biologists, July 20-24, Austin, TX, USA.
11. A. Deresienski, K. Nelson, J. Hague, A.P. Kausch (2009) Male sterility as a method for constructing wide crosses and for gene confinement in switchgrass and other biofuels grasses. Plant Biology 2009, Annual Meetings of the American Society of Plant Biologists, July 18-22, Hawaii, USA.
12. K. Nelson, J. Hague, A. Deresienski and A.P.Kausch. (2009) Improved methods for tissue culture and genetic transformation of switchgrass. Plant Biology 2009, Annual Meetings of the American Society of Plant Biologists, July 18-22, Hawaii, USA.

Publications, Presentations, and Commercialization

Related Patents:

1. Kausch AP, Hague, J, Deresienski, A, Tilelli, M, and Nelson, K., Inventors . 2013 The Use of Genetically Modified Plants for Recovery of Non- genetically Modified Hybrids from Wide Crosses. United States Patent Application. US 2013/004769 Assignee; University of Rhode Island.
2. Kausch AP, Hague, J, Deresienski, A, Tilelli, M, and Nelson, K., Inventors. 2013 In Situ Embryo Rescue as a Method for Recovery of Wide Crosses. United States Patent Application. US 2013/005832 Assignee; University of Rhode Island.
3. Luo; Hong; Chandlee; Joel M.; Kausch; Albert P.; Oliver; Melvin J. , Inventors. 2011. Development of controlled total vegetative growth for prevention of transgene escape from genetically modified plants and for enhancing biomass production.. United States Patent Application Number 20100122366
4. Albert P. Kausch and Stephen Dellaporta , Inventors. 2011 Male and female sterility lines used to make hybrids in genetically modified plants. United States Patent Application. Assignee; University of Rhode Island.

Status of commercialization efforts:

Plant Advancements has acquired significant IP and licensing rights to relevant technologies

Plant Advancements LLC is recent recipient of USDA SBIR grant for commercialization of male and female sterility lines in switchgrass and related species.

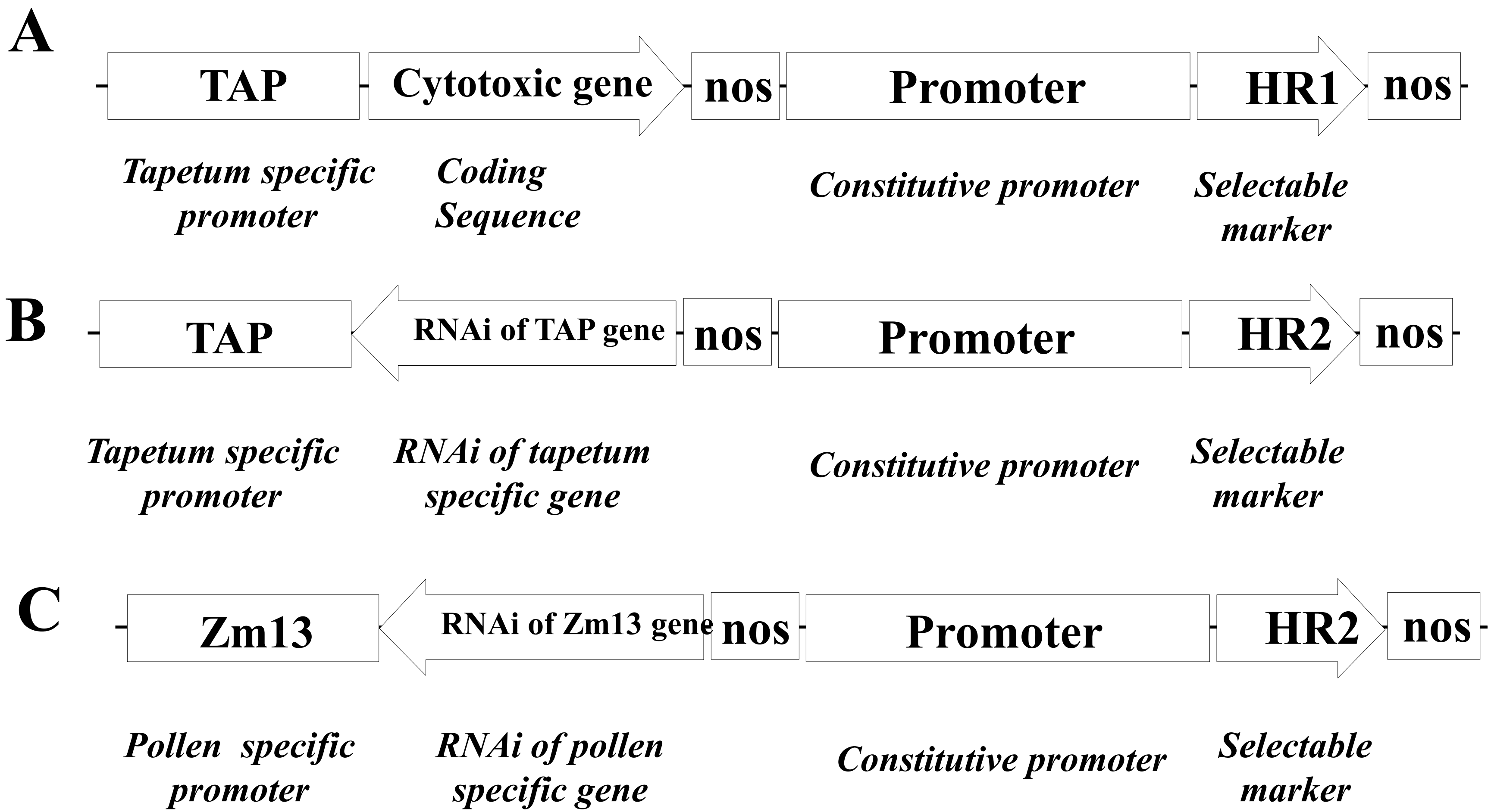
Area: Small Business Innovation Research Program (SBIR)
Program: Plant Production and Protection-Biology
Proposal Number: 2013-00140
Project Director: Thomas K Hodges/Albert P Kausch
Proposal Title: Hybrid Systems for Gene Confinement and Breeding of Perennial Plants Used for Biofuels

Plant Biotechnology Laboratory Staff

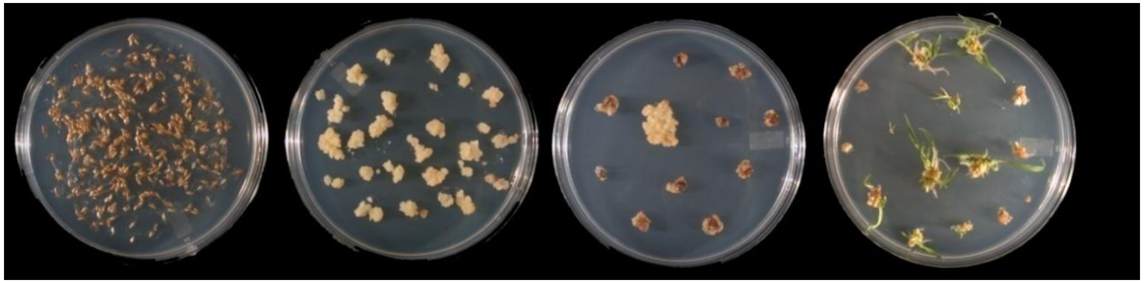
Plant Biotechnology Laboratory
Well Trained Staff

Albert Kausch, Director
Kimberly Nelson, Program Manager
Chip Longo, Research Scientist
Derek Gablineski, Research Scientist
Adam Deresienski, Research Scientist
Joel Hague, Research Scientist
Michael Tilleli, Research Scientist
John Ventura, Graduate Student

Nuclear Male Sterility via Tapetal Or Pollen Ablation



Production of male sterile switchgrass plants



Development of routine transformation in switchgrass (cv Alamo) has been accomplished and improved

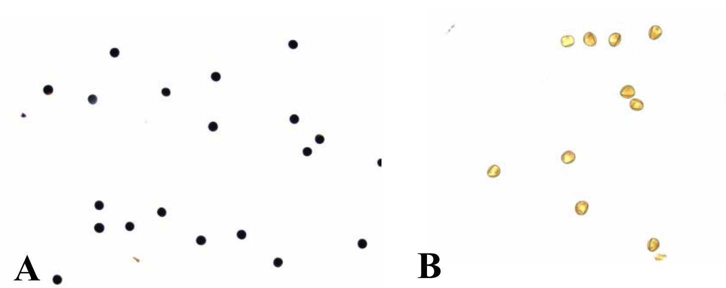
Transformation Efficiency Alamo
male sterile-bar (representative experiments)

Infection Date	# pieces infected	# of events recovered	% transformation efficiency
7/9/2008	577	291	50.03%
7/13/2008	588	463	27.82%
6/9/2008	400	60	11.50%
6/15/2008	600	15	2.50%

Herbicide resistance (glufosinate 10% v/v)

PCR results show both bar and barnase genes

IKI staining for pollen viability



A
Viable pollen stain dark at anthesis

B
Male sterile
Non-viable pollen
T0 Event # 134-07

Growth chamber (right) and greenhouse (above) with transgenic switchgrass plants (157 events)

Intergeneric wide crosses using herbicide selection as a marker

2 - Technical Accomplishments/Progress/Results

Use of an Herbicide Resistance Selectable Marker for Recovery of Intraspecific and Interspecific Hybrids in Switchgrass (*Panicum virgatum L.)*

Adam Deresienski, Kimberly Nelson, Mike Tilelli, Joel Hague, and Albert P. Kausch*
***Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02892**

Characterization of Parental and F1 Hybrids

Use of a Selectable Marker for In Situ Embryo Rescue using Transgenic Switchgrass (*Panicum virgatum* L.) for Recovery of Wide Crosses.

Mike Tilelli , Kimberly Nelson, Adam Deresienski, Joel Hague, and Albert P. Kausch*
***Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02892**

Development of a Novel Embryo Rescue Technique for Recovery of Wide Cross Hybrids

Use of an Herbicide Resistance Selectable Marker for Recovery of Intraspecific and Interspecific Hybrids in Switchgrass (*Panicum virgatum L.)*

Adam Deresienski, Kimberly Nelson, Mike Tilelli, Joel Hague, and Albert P. Kausch*

*Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02892

ABSTRACT **Genetic improvement of perennial grass feedstocks, such as switchgrass and related species, are anticipated by current genomics, bioinformatics, association genetics, marker assisted breeding, conventional genetics and other non-GMO approaches. The role that switchgrass as a bioenergy crop will play for sustainable biofuels development are well understood and widely known. The novel use of a transgenic herbicide resistance for recovery of wide crosses followed by backcrossing to remove the transgene facilitates recovery of non-GMO hybrids that can be rapidly introduced to the agricultural market without costly R&D and deregulation of GM development. Here we demonstrate the use of an herbicide resistance selectable marker (bar) for recovery of intraspecific hybrid offspring of 'Alamo' switchgrass (Panicum virgatum cv. Alamo) and 'Southlow' switchgrass (Panicum virgatum cv. 'Southlow') as well as interspecific hybrid offspring of switchgrass (Panicum virgatum cv. 'Alamo') and 'Atlantic' Coastal panicgrass (Panicum amarum Ell. var. amarulum). Pollen cages were set up enclosing a bar positive transgenic 'Alamo' switchgrass primary transformant that served as a pollen donor to either a wild-type 'Southlow' individual or a wild-type 'Atlantic' Coastal panicgrass individual as a maternal parent. After seed maturation and germination the developing seedlings were assayed for bialaphos resistance by an application of 3% bialaphos. Resistant plantlets were verified for inheritance of the bar gene nuclear marker from the T0 'Alamo' parent through Southern blot. A cytoplasmically inherited cpDNA marker was utilized to verify the maternal inheritance of the 'Atlantic' Coastal panicgrass cytoplasm in the hybrid offspring. Bar positive hybrid offspring were backcrossed to wild-type 'Alamo' plants and bialaphos-sensitive offspring of this cross were isolated as non-transgenic novel hybrid germplasm. This platform could serve as a method for combining desirable characteristics through exploiting additive genetic variation and provide a more timely approach to developing novel switchgrass lines for increased biomass production for biofuel production.**

INTRODUCTION The utilization of dedicated crops as a source of bioenergy from renewable biomass resources is a goal with great relevance to current ecological, economic, and national security issues on a global scale and presents compelling challenges to American agriculture. One concern is whether sufficient amounts of these feedstock materials can be supplied without impacting the cost of agricultural land, competing with food production, and harming the environment. Limitations in the current availability of bioenergy feedstocks are considered a major problem in next-generation non-ethanol liquid biofuels, such as green diesel, and gasoline, biobutanol and aviation fuels. For a variety of reasons, production of fuel from dedicated nonfood crops as cellulosic sources, such as switchgrass, Energy Cane, sorghum, *Miscanthus*, willow, and poplar, is widely understood as a necessary development. Switchgrass (*Panicum virgatum L.*) and related species have become dominant candidates as feedstocks for cellulosic biomass that will be used for the production of various types of biofuels. Switchgrass is native and widespread throughout most of North America (Moser and Vogel 1995) and, importantly, grows on marginal lands that are not competitive with food production resources. The recent genetic improvement of food row crops, such as corn, rice, soybean, canola, and others has been greatly accelerated through advanced applications of the tools of biotechnology, including advanced breeding, genomics and transgenics. Undoubtedly this same model will be useful for improving perennial bioenergy feedstocks). The application of advanced genetics, molecular breeding, association genetics and genetic modification together will provide powerful approaches for improvement of traits important to the development of energy crops. Many transgenic traits are currently being tested for specific biofuel crop applications. While the use of transgenics provides a powerful tool for trait introduction by using genetic material from outside the conventional breeding gene pool, the process is time consuming (requiring up to 6-10 years) requires costly deregulation through the USDA and EPA (in the tens of $ millions) and suffers from a lack of public acceptance. Switchgrass feedstock development can be aided by the creation of hybrid plants, trait selection, association genetics and exploitation of heterosis (Mascia *et al.* 2010, Martinez-Reyna and Vogel, 2002; 2008; Kausch *et al.* 2011). Heterosis can cause dramatic improvements in various aspects of perennial plant performance such as seed yield and size, floral number and size, first year biomass yield, second year biomass yield and other agronomic traits by recombination of genetic variation through intraspecific or interspecific hybrid production (Martinez-Reyna and Vogel 2008, Troyer 2006, Brummer 1999). Switchgrass, a C4 grass species native to North America, is a highly heterozygous, anemophilous obligate outcrosser with both pre-fertilization and post-fertilization self incompatibility systems present (Martinez-Reyna and Vogel 2002; 2008). Self incompatibility demonstrated by Martinez-Reyna and Vogel (2002) show that in controlled crosses between octoploid x octoploid, octoploid x tetraploid, tetraploid x octoploid post-fertilization abortion occurs in many cases 20-40 DAP and maturing caryopses are easily isolate (FIGURE 1). These observations provide a sound background for the present invention. With ample phenotypic and genetic diversity characterized within and among switchigrass cultivars and populations, data on heterosis and hybrid plant development is limited then by the time-consuming and laborious process to recover hybrid plants and fertile alloploids (see Martinez-Reyna and Vogel 2002; 2008). Controlled hybridization techniques, based on floral emasculation and mutual pollination by bagging inflorescences, have been used in recovering both population hybrids and specific hybrids of switchgrass (Martinez-Reyna and Vogel 1998, 2002, 2008). Through these techniques intraspecific crosses between upland and lowland ecotypes, and between spatially separated populations have yielded viable hybrid plants, which do display heterosis (Martinez-Reyna *et al.* 2001, Martinez-Reyna and Vogel, 2000, 2008). Although these methods are accurate and promising, they are tedious, time consuming and produce low numbers of candidate progeny. Additionally, analysis and verification of hybrid plants requires extensive phenotypic observation and measurements based on morphological characteristics before molecular analysis can verify the hybrid genotype. The present paper seeks to address and overcome these problems. Switchgrass has been genetically modified (Richards *et al.* 2001; Somleva *et al.* 2002; Burris *et al.* 2009; Xi *et al.* 2009; Nelson *et al.* 2012) via particle bombardment or *Agrobacterium*-mediated transformation using callus cultures induced from mature caryopses or inflorescence and nodal explants and employed either hygromycin or bialaphos selection. *Agrobacterium*-mediated transformation has been successfully applied to switchgrass with generally low numbers of T-DNA insertions and transmission to progeny as Mendelian loci without rearrangements (Xi *et al.* 2009; Nelson *et al.* 2012). The Kausch lab has demonstrated a high level of proficiency for switchgrass transformation. Significant improvements to previously published procedures have been developed (Nelson *et al.*, 2012), and therefore, high-throughput genetic transformation of switchgrass is now routine. Passage of transgenes to progeny is critically important to immortalization of transgenic lines and incorporation of the transgenic material into useful breeding programs. The first generation of genetically modified transgenic asexually cloned plants derived from tissue culture are known as T0 events, which can be molecularly characterised by the insertion of the transgene cassette. Whereas T1 plants are derived from selfs or crosses using T0 plants to generate progeny designated as T1 plants, and the subsequent generation referred to as T2. However, T1, and T2 generation designations are often also referred to as F1 and F2, respectively. The Nelson *et al.*, 2012 study greatly increased our knowledge of transformation efficiency, extended the genotype range of transformation to various switchgrass cultivars and Atlantic Coastal Panicgrass (*Panicum amarum*, Ell. var. amarulum.) and shows that transgenic (T1) progeny can be readily obtained from crosses with wild-type plants.

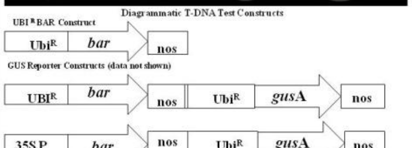
This paper presents the methodology for simple recovery of wide crosses resulting in both intraspecific and interspecific hybrid plants of switchgrass and related species by combining novel applications of transgenics, selection for embryo specific herbicide resistance, tissue culture and classical breeding techniques. At first glance this seems contradictory to previous statements concerning the time consuming and costly application of using transgenics, however in the proposed application this technology aims to use transgenic herbicide resistance as a selectable marker in switchgrass and related species for recovery of rare intra-specific and inter-specific hybrids and crossing out the transgene in the subsequent backcrossed generation. Therefore, in its simplest application, a line of transgenic switchgrass with a dominant herbicide-resistance selectable marker gene can serve as the paternal parent in the proposed intra-specific and inter-specific crosses. Transgenic herbicide resistant switchgrass (cv Alamo) plants have been developed in the Kausch laboratory at the University of Rhode Island and in preliminary studies (Deresienski, 2011) were used to pollinate wild-type individuals of alternate switchgrass varieties or *Panicum* species. By isolating entire flowering switchgrass plants in crosses within individual pollen cages, as opposed to bagging inflorescences, the chances of recovering hybrid plantlets is increased dramatically. A simple herbicide treatment of seedlings from the maternal wild-type plant verifies the hybrid nature of the offspring. The transgenic traits can then be selected against in the F2 population to recover herbicide sensitive hybrids that are essentially non-GMO. These hybrids can be verified as non-GMO using our genomics and sequencing approaches and thus can be rapidly introduced to the commercial market without the costly and time consuming process of deregulation.

The opportunity addressed here is to provide the technology that will rapidly accelerate new cultivar development that are non-GMO resulting in varieties with improved biofuels traits which can be introduced into the market without the process of deregulation. In addition, the use of a linked transgenic male sterility trait with herbicide resistance used in conjunction with advanced tissue culture embryo rescue techniques (Nelson *et al.* 2012) will be exploited to force recovery of rare wide crosses. When the herbicide resistance marker is linked to a dominant male sterility trait and used as the maternal parent, this will serve as: 1) an ample filter to facilitate forcing and recovery of rare wide cross progeny; but more importantly, (2) when pollinated by wild type pollen, the immature caryopses can be used as explants onto embryogenic callus initiation medium containing bialaphos for herbicide resistance selection, whereby only callus derived from a fertilized embryo will be recovered. The embryogenic callus, derived from a wide cross can then easily be regenerated to whole plants and further characterized. The benefit of this novel wide cross recovery method is that it will dramatically increase the numbers of wide crosses that can be recovered and the numbers of clones of each wide cross (Nelson *et al.*, 2012).

MATERIALS AND METHODS

FIGURE 1. Switchgrass transformation sequence. (A) Embryogenic callus initiation from mature caryopses; (B) followed by transfection of embryogenic callus with *Agrobacterium* carrying vectors; (C) selection of transformants for herbicide resistance; and (D) transgenic plant regeneration. Note: the same embryogenic callus induction media used here will be deployed for induction of callus from immature caryopses from the wide crosses.

Diagrammatic T-DNA Test Constructs



RESULTS

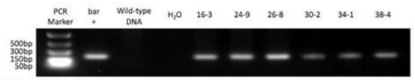


FIGURE 2. PCR and herbicide resistance correlate with the introduced bar gene construct. Top panel: Results in the viable 'paint-assay' shown left to right wild-type *Panicum virgatum* L. cv Alamo leaf response to treatment with 3% finale followed by the responses of the corresponding primary *Panicum virgatum* L. cv Alamo T0 transformants, respectively, wild-type *Panicum virgatum* L. cv Alamo, H_2O control and T0 event numbers, 16-3, 24-9, 26-8, 30-2, 34-1, and 38-4 correspond to the PCR results shown in the bottom panel. Bottom Panel: PCR amplifications of 202 bp bar fragment fragment from Switchgrass cv Alamo primary T0 transformants corresponding to those in the top panel. Shown left to right PCR marker; *bar* + positive control plasmid DNA; wild-type -: negative control, wild-type Alamo DNA; H_2O: water control. Transformation event numbers, 16-3, 24-9, 26-8, 30-2, 34-1, and 38-4 are listed above each lane corresponding to the leaf samples in the top panel.

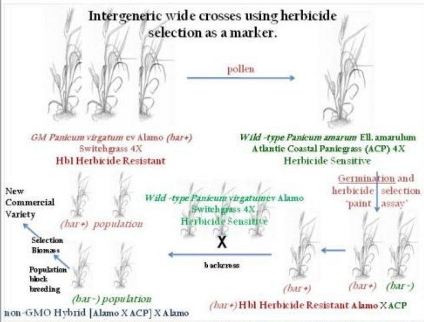
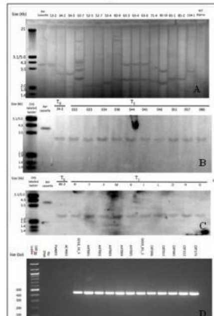
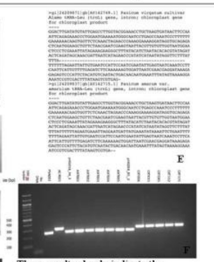


FIGURE 3. Diagrammatic scheme illustrating the recovery of intergeneric crosses using herbicide selection as a marker. This diagrammatic scheme illustrates, as an example, the recovery of wide inter-specific crosses using herbicide selection as a marker, however, this same or similar scheme also applies to wide intra- and inter-varietal, intra- and inter-specific, inter-generic and distant relative crosses. In this example, genetically modified (GMO) *Panicum virgatum* L. cv Alamo Switchgrass (4x), (at upper left, shown in dark red) is herbicide resistant (Hb1, bar+, containing the bar gene, resistant to bialphos and 3% Finale or Liberty) and is used as the paternal pollen donor in a wide cross. These plants may be hemizygous T0, or contain at least one copy of the transgene in T1, T2, or … generations. The maternal pollen recipient is wild-type *Panicum amarum Ell.* amarulum Atlantic Coastal Panicgrass (ACP) (4x, at upper right) which in non-genetically modified (non-GMO) and hence (shown in dark green) herbicide sensitive to bialaphos and 3% Finale or Liberty. Pollinations may be most conveniently accomplished in pollen cages using one several clones of an event herbicide sensitive as a pollen donor and a single wild type plant as pollen recipient. After pollination and seed maturation, seed is harvested only from the wild-type maternal parent and germinated (center, right). Seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar+, red) and herbicide sensitive (bar-, green) populations (lower right). At floral maturity the (bar+) Hb1 Herbicide Resistant Alamo X ACP hybrid plant(s) are used preferably as paternal pollen donor(s) in a backcross to wild-type *Panicum virgatum* cv Alamo Switchgrass (4x) non-GMO herbicide sensitive plants (lower center) and the resultant seed is recovered and germinated. The resultant seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scoring for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar+, red) and herbicide sensitive (bar-, green) and populations (lower left). Seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scoring for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar+, red) and herbicide sensitive (bar-, green) and populations (lower right). The non-GMO hybrid plants contain Alamo X ACP X Alamo (blue, lower left) genomic contributions and are subsequently analyzed and scored for desirable traits correlated with genomic markers. These plants, can also serve as bridge intermediates to cross with other compatible or incompatible parents. Desirable plants may enter into population block breeding plots, and mass selection and subsequent commercial development can proceed.

Cultivar	State of Origin	Plant Form
Alamo	TX[1]	Lowland
Blackwell	OK[1]	Upland
Bowmaster	AR, NC[2]	Lowland
Carthage	NC[1]	Upland
Cave In Rock	IL[1]	Upland
Dacotah	ND[1]	Upland
Forestburg	SD[1]	Upland
High Tide	MD[6]	Lowland
Kanlow	OK[1]	Lowland
Miami	FL[3]	Lowland
Performer	AR,OK,NC[2]	Lowland
Shawnee	IL[4]	Upland
Shelter	WV[1]	Upland
Southlow	MI[7]	Upland
Stuart	FL[3]	Lowland
Sunburst	SD[5]	Upland
Timber	AR?, NC?[4]	Lowland

FIGURE 4. List of seventeen public switchgrass (*Panicum virgatum L.*) cultivars and their corresponding State of Origin, and Plant Form (i.e. Upland or Lowland). Corresponding sources shown in superscript. [1]USDA Soil Conservation Service Agricultural Handbook No. 170. Grass Varieties in the United States. [2] Plant Patent Application. [3]USDA-NRCS Release documents from Brooksville, FL Plant Materials Center. [4] Personal communication to Calvin Ernst. [5]USDA NRCS Bismarck, ND. Switchgrass Biomass Trials in North Dakota, South Dakota, and Minnesota. [6]USDA-NRCS, Cape May Plant Materials Center "High Tide Switchgrass" release brochure. [7] USDA-NRCS, Rose Lake Plant Materials Center "Southlow Switchgrass" release brochure.





These results clearly indicate the recovery of wide intra- and inter-specific crosses by using herbicide resistant *Panicum virgatum* (cv Alamo) as a paternal donor to yield new F1 hybrids

FIGURE 5 Molecular analysis shows interspecific nature of the Panicum virgatum L. x *Panicum amarum* Ell. var. amarulum, hybrids. FIGURE 5A. T0 transgenic events of *P. virgatum* cv Alamo. A simple Southern blot analysis of NcoI-digested DNA from cv Alamo T0 paternal parents using *bar* probe shows 7 independent single gene insertion events. Clones of events show identical results. FIGURE 5B. Intra-specific hybrids from crosses between wild-type cv 'Southlow' (♀) and T0 cv 'Alamo' (♂). A simple Southern blot analysis of NcoI-digested DNA from paternal parent T0 cv Alamo transformant #34-2 alongside 10 randomly selected herbicide resistant T1 plantlets from the intra-specific cross of wild type cv 'Southlow' switchgrass (♀) X cv 'Alamo' transformant #65-2 (♂) using the *bar* probe. All sampled T1 progeny show stable inheritance of the transgene. FIGURE 5C. *Interspecific hybrids* from crosses between T0 cv Alamo (♂) and wild-type 'Atlantic' coastal panicgrass (♀). Southern blot analysis of NcoI-digested DNA from paternal parent T0 cv 'Alamo' transformant #65-2 alongside 10 randomly selected herbicide resistant T1 plantlets from the wide hybrid cross wild-type 'Atlantic' coastal panicgrass (♀) X cv 'Alamo' transformant #65-2 (♂) using the *bar* probe. All sampled T1 progeny show stable inheritance of the transgene. FIGURE 5D. PCR amplifications Wild-type (non-transgenic control) X herbicide resistant transgenic and interspecific cross with Atlantic Coastal Panicgrass. PCR Results for the nuclear *bar* marker in (controls) wild-type *Panicum virgatum* cv Alamo, wild-type *Panicum amarum* Ell. var. amarulum, and five randomly selected herbicide-resistant T1 plantlets from the cross wild-type *Panicum virgatum* cv. Alamo X PVA-34_3 (AXT001-AXT005) and five randomly selected herbicide-resistant T1 plantlets from the cross wild-type *Panicum amarum* Ell. var. amarulum X PVA (CXT006, CXT015, etc.). FIGURE 5E. Chloroplast deletions in Alamo but not Atlantic Coastal Panicgrass and can be used as a diagnostic for hybrids. The tRNA-Leu (trnL) gene of the chloroplast contains an intron with a 49 bp deletion present in cv Alamo (that is not present in 'Atlantic' Coastal Panicgrass. MUSCLE results of the amplified fragment containing this deletion. FIGURE 5F. Chloroplast deletion detected and verifies interspecific hybrid. PCR amplifications of chloroplast DNA tRNA-Leu (trnL) 49 bp deletion site in wild-type *Panicum virgatum* cv. Alamo, wild-type *Panicum amarum* Ell. var. amarulum, PVA-34-3, PVA-65-2, five randomly selected herbicide-resistant T1 plantlets from the cross wild-type *Panicum virgatum* cv. Alamo X PVA-34_3 (AXT001-AXT005) and five randomly selected herbicide-resistant T1 plantlets from the cross wild-type *Panicum amarum* Ell. var. amarulum X PVA (CXT006, CXT015, etc.).

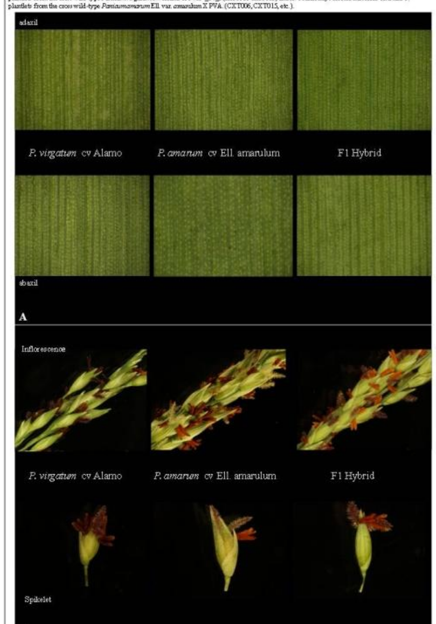


FIGURE 6 Characteristics of the wild-type Panicum virgatum cv. 'Alamo', and wild-type Panicum amarum Ell. var. amarulum, and T1 Panicum virgatum cv. 'Alamo' X Panicum amarum Ell. var. amarulum hybrid. FIGURE 6 TOP AND BOTTOM ; Characteristics of adaxial Abaxial (respectively) sides of the mature leaf were sampled from the second leaf lower than a mature inflorescence and photographed 2 cm from the axial all with consistent lighting parameters and exposures. Left to Right: the wild-type Panicum virgatum cv. 'Alamo', and wild-type Panicum amarum Ell. var. amarulum, and T1 Panicum virgatum cv. 'Alamo' X Panicum amarum Ell. var. amarulum hybrid. FIGURE 6 TOP AND BOTTOM Characteristics of inflorescence sampled from the main axis 3 cm from the terminal apex and spikelets (respectively) photographed with consistent lighting parameters and exposures. Left to Right: the wild-type Panicum virgatum cv. 'Alamo', and wild-type Panicum amarum Ell. var. amarulum, and T1 Panicum virgatum cv. 'Alamo' X Panicum amarum Ell. var. amarulum hybrid. Note the clusters of Panicum virgatum cv. 'Alamo' spikelets have red pistiles and are fewer than that of Panicum amarum Ell. var. amarulum which has white pistles, and is more dense, and that the T1 Panicum virgatum cv. 'Alamo' X Panicum amarum Ell. var. amarulum hybrid (left) is intermediate.

Transgenic Nuclear Male Sterility as a maternal parent for recovery of herbicide resistant wide crosses via

(A) Tapetal ablation; (B) Microspore abortion; and, (C) Pollen sterility

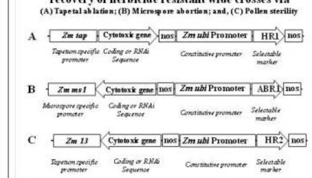


FIGURE 7 Diagrammatic representations of transgenic cassettes for induction of male sterility. Nuclear male sterility is induced by: (A) tapetal ablation using a tapetum specific promoter from maize (Zm tap) driving expression of either (Coding), a cytoxic gene (i.e. *barnase*) or (RNAi) the antisense of the native gene with selection via herbicide resistance; or using a similar strategy driven by (B) a microspore specific promoter (*Zm ms1*); or, (C) a pollen specific promoter (*Zm 13*). Transgenic lines from both (B) and (C) can be subjected to colchicine treatments to recover homozygous lines. Orthologues of these genes could also be the target for ZFN or TAL modifications to direct sterility functions.

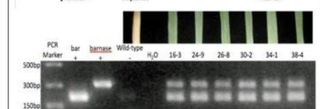


FIGURE 8. PCR and herbicide resistance correlate with the introduced bar gene construct. Top panel: Results in the viable 'paint assay' shown left to right wild-type *Panicum virgatum* L. cv Alamo leaf response to treatment with 3% finale followed by the responses of the corresponding primary *Panicum* 701 *virgatum* L. cv Alamo T0 transformants; respective Wild-type, H2O and T0 event numbers, 16-3, 24-9, 26-8, 30-2, 34-1, and 38-4 correspond to the PCR results shown in the bottom panel. Bottom Panel: T0 transformants after herbicide application and their representative *bar* and *barnase* fragment amplifications. PCR amplifications of 202 bp *bar* fragment and 303 bp *barnase* fragment from Switchgrass cv Alamo primary T0 transformants. Shown left to right PCR marker; *bar* +: *bar* positive control, plasmid DNA; barnase +: barnase positive control, plasmid DNA; wild-type -: negative control, wild-type Alamo DNA; H20: water control. Transformation event numbers, 16-3, 24-9, 26-8, 30-2, 34-1, and 38-4 are listed above each lane corresponding to the leaf samples in the top panel. This shows that co-transformation of a male sterility gene co-integrates with the selectable marker gene.

Strategies for Plant Sterility

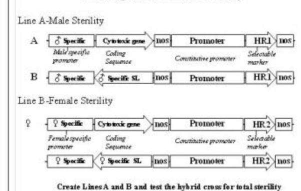


FIGURE 9 Male and female lines are created through the application of the promoters and/or the coding sequences described in FIGURE 10. Male sterile lines (top, line A-Male Sterility) are generated through the introduction specific promoters are used to drive (A) cytotoxic genes such as barnase or (B) specific synthetic lethality genes, such as RNAi. These genes or their orthologues, could be the target for ZFN or TAL modifications to direct sterility functions. Female sterile lines (bottom , line B-Female Sterility) are generated through the introduction specific promoters are used to drive (A) cytotoxic genes such as barnase or (B) specific synthetic lethality genes, such as RNAi. These genes or their orthologues, could also be the target for ZFN or TAL modifications to direct sterility functions.

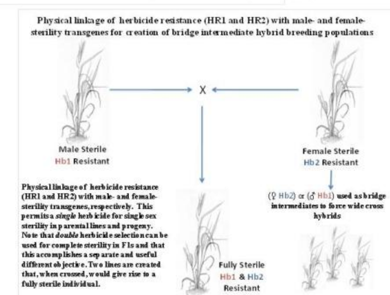


FIGURE 10 Physical linkage of herbicide resistance (HR1 and HR2) with male- and female-sterility transgenes can be used for creation of bridge intermediate hybrid breeding populations. Physical linkage of herbicide resistance (HR1 and HR2) with male- and female-sterility transgenes, respectively, can also be used to create total sterile outcomes. This permits a *single* herbicide for single sex sterility in parental lines and progeny. Note that *double* herbicide selection can be used for complete sterility in T1 (F1)s and that this accomplishes a gene confinement strategy. Two lines are created that, when crossed, would give rise to a fully sterile individual.

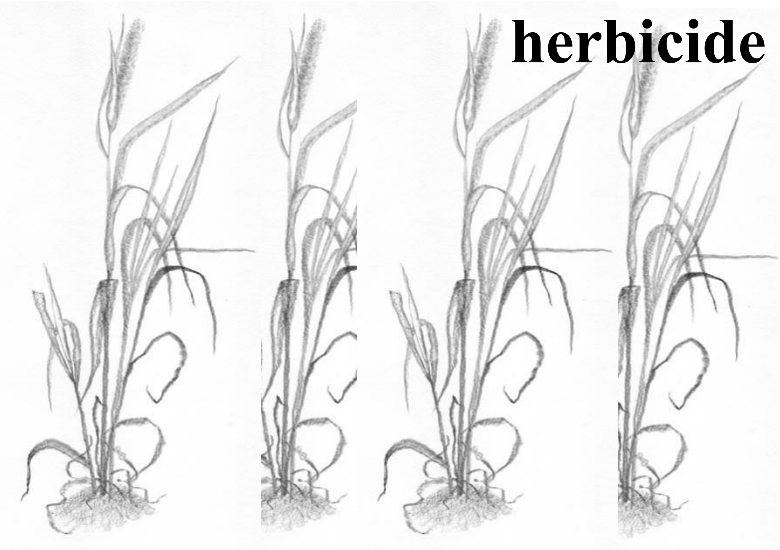
CONCLUSION

Hybrids can be generated via wide crosses and back crossed to wild type parents to segregate the transgene. This strtategy generates new non-GMO hybrids for breeding purposes. Hybrids can be selected for desirable phenotypes contributed by either parent; including bioenergy traits, such as carbon allocation characteristics in root vs. shoot mass, cellulose content, low lignin, sugar content, photosynthetic efficiency, enhanced biomass yield acre, reduction of perception of nearest neighboring plant or tiller, biomass value added compounds, changes in photomorphogenic responses, including phytochrome red/far-red light perception and crypotchrome perception, optimized photoperiod, floral sterility, regulated dormancy, input requirements, such as fertilizers and pesticides, stratification characteristics, crown size, leaf phenotypes (including size, color, length width and angle), root mass and depth, tillering, stand development characteristics, seed set, inflorescence number, height and width, floral development; as well as biotic and abiotic stresses including water use efficiency, cold and freeze tolerance, pest resistance (including insect, nematode, fungus, bacterial, virus). Genomic and marker assisted breeding is deployed characterize parental genomic contribution and to follow traits in subsequent downstream breeding for varietal development. Hybrids can be sexually crossed and/or vegtetatively propagated.

The benefit of sterility lines is the ability to force efficient hybrid production. In this embodiment, the scheme for generating and recovery of rare wide crosses and bridge intermediates relies on the use of pistillate *Panicum virgatum* L. cv Alamo plants as a pollen recipient in wide-crosses as an example. [Note: that staminate reference plants can be used in reciprocal crosses as needed]. The floral ablation phenotype, physically linked to an herbicide selectable marker, can be used in embryo rescue experiments to select and recover F1 hybrids. Interestingly, in both strategies only 50% of the hybrid offspring produced by these methods are transgenic due to the hemizygous nature of the transgene. Therefore, non-transgenic hybrids can be recovered readily by screening for herbicide sensitivity in the T1 (F1) or advanced generations. The simple non-destructive leaf painting assay has been used for this purpose. Herbicide-resistant F1 hybrids are unisexual and backcrossed with non-transgenic reference Alamo plants. The first generation resultant from the backcross (BC1) progeny can be assayed and selected for segregation of the transgene. Both non-transgenic (herbicide-sensitive) and transgenic, unisexuals (herbicide-resistant) BC1 progeny will be identified.

Supported by DOE Award Number: DE-FG-36-08GO88070 Project Title: Research and Technology Development for Genetic Improvement of Switchgrass, Albert P. Kausch and Richard Rhodes

In situ embryo rescue of wide crosses using herbicide selection as a marker



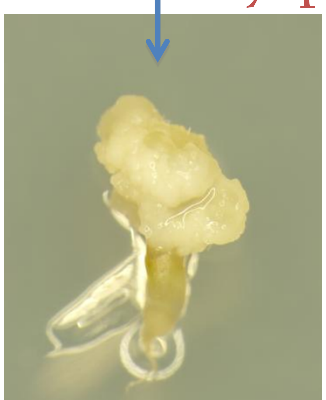
pollen →

T0 (bar+)Panicum virgatum
cv Alamo n=4
Hbl Herbicide Resistant

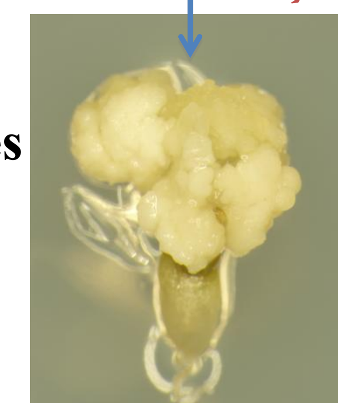
Wild Type Panicum virgatum
cv Cave –in-Rock n=8
Herbicide Sensitive

Immature caryopsis

Immature caryopsis



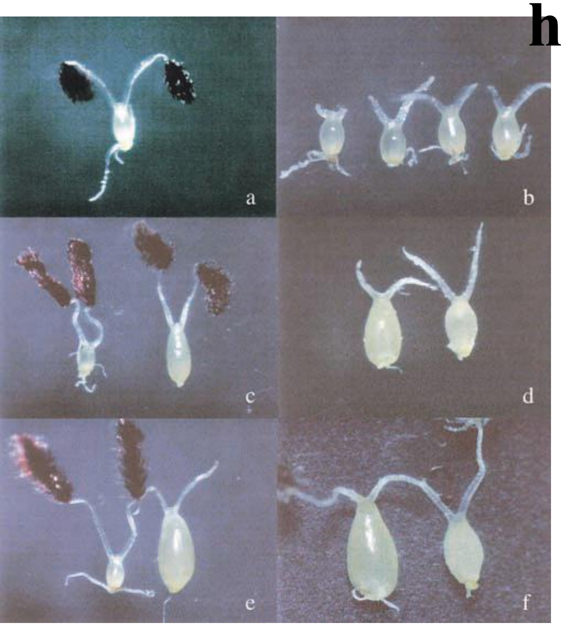
Demonstrates
Embryo rescue from wide crosses



Hbl Herbicide Resistant control

(bar+) Hbl Herbicide Resistant Alamo X Kanlov

In situ embryo rescue of wide crosses using herbicide selection as a marker



Caryopsis development of tetraploid by octaploid switchgrass cross n days after pollination.

GM (bar+)Panicum virgatum cv Alamo
Switchgrass 4x
Hbl Herbicide Resistant

Wild Type Panicum virgatum cv Cave-in-Rock
Switchgrass 8x
Herbicide Sensitive

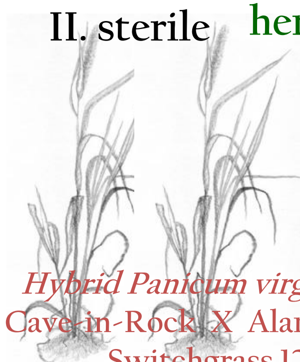
Chromosome doubling treatments

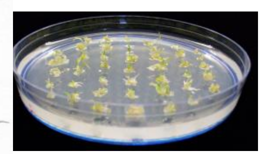
Caryopsis isolation & Bar selection on embryogenic callus induction medium

In situ embryo rescue and herbicide selection

II. sterile

I. fertile



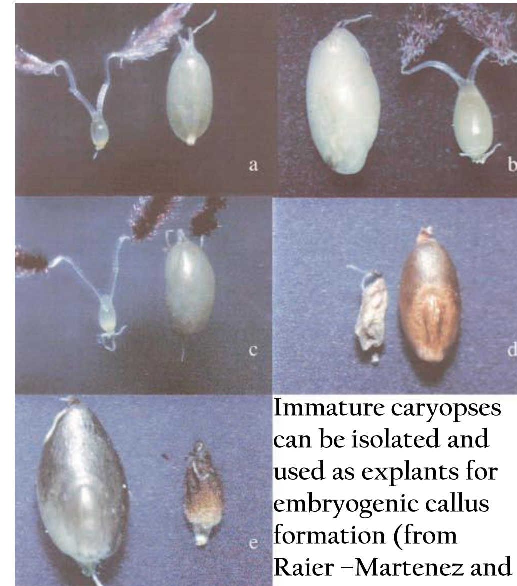


colchicine

Hybrid Panicum virgatum cv Cave-in-Rock X Alamo hybrid Switchgrass 12x
Herbicide Resistant Sterile
Total Transgene Confinement
[vegetative only]

Cave-in-Rock X Alamo hybrid Switchgrass 12x
(bar+) (bar-)

Backcross w/ WT Alamo select for bar negative
New Non-GMO Commercial Variety
[Cave-in-Rock X Alamo] X Alamo hybrid Switchgrass 12x



Immature caryopses can be isolated and used as explants for embryogenic callus formation (from Raier –Martenez and Vogel 2002

Examples of caryopsis development of tetraploid by octaploid crosses 15 d after pollination (right)

Use of a Selectable Marker for In Situ Embryo Rescue using Transgenic Switchgrass (*Panicum virgatum* L.) for Recovery of Wide Crosses.

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***Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02892**

ABSTRACT **The need for the US to increase its efforts on production of renewable biofuels and the role that perennial grasses as bioenergy feedstocks will play are well understood and widely known. This project aims to create and characterize new breeding lines in publicly available breeding programs of switchgrass (Panicum virgatum L.) and related species with a focus on selection for useful bioenergy traits. To accomplish this goal we will generate a series of intra- and inter-specific wide crosses in switchgrass and related species. We have utilized transgenic switchgrass variety, cv. 'Alamo' and herbicide or antibiotic selection for recovery of wide intra- and inter-specific F1 crosses by embryo rescue. We describe a protocol for "in situ embryo rescue" from inter-varietal, inter-specific and intraspecific crosses between switchgrass (Panicum virgatum) and its relatives through utilization of a transgenic selectable marker (bar or hpt). Pollen cages were established with bar or hpt positive transgenic switchgrass T1 plants serving as pollen donors to wild-type individuals. Approximately 15-20 days post pollination, wild-type inflorescences were removed and surface sterilized, and immature developing embryos and entire ovules were excised and plated on embryogenic callus induction medium containing 3mg/L bialaphos or 150 mg/L hygromycin. We demonstrate the recovery and selection of resistant hybrid embryogenic callus through both the culture of isolated embryos and in-ovulo embryo culture. Resistant callus cultures derived from these embryos were regenerated into entire plantlets and verified by PCR to contain either the bar or hpt gene. F1 hybrids are backcrossed to the reference Alamo cultivar to segregate away the transgene generating a non-GMO BC1 mapping population for genomic analysis. This tool will be valuable to overcome post-fertilization barriers that exist between ecotypes, ploidy levels, and closely-related non-switchgrass species and aid in the development of novel hybrid switchgrass lines for biomass production or other potentially beneficial characteristics.**

INTRODUCTION Intervarietal, interspecific and intergeneric, or more distantly related crosses collectively are referred to as 'wide crosses'. Breeding of wide crosses are not often prevented through either pre- and post-fertilization incompatibility mechanisms. For example, Martinez-Reyna and Vogel (2002) demonstrated a pre-fertilization system similar to the S-Z incompatibility system previously characterized in the Poaceae family (Hayman 1956; Lundqvist 1956) exists in *Panicum sp.* (Martinez-Reyna and Vogel 2002) and has been described in many other plants. Incompatibility through this system involves the interactions of two multiallelic gene loci, S and Z. If the same alleles are present at these loci in both the pollen grain and the style of the female pollen recipient, fertilization is inhibited (de Nettancourt 1997). The S-Z incompatibility system has been observed in many monocotyledonous including *Secale cereale* (Lundqvist 1954), *Festuca pratensis* (Lundqvist 1955), *Phalaris coerulescens* (Hayman 1956), *Hordeum bulbosum*, *Dactylis aschersoniana* (Lundqvist 1965), *Briza media* (Murray 1974), *Lolium perenne* (Cornish, Hayward et al. 1979), and *Lolium multiforum* (Fearon, Hayward et al. 1983). However, these pre fertilization self-incompatibility systems have been shown to be ineffective or non-existent in wide crosses. instead post-fertilization incompatibility is the main obstacle to successful seed development.

Post-fertilization incompatibility was also observed in interploid crosses within switchgrass (Martinez-Reyna and Vogel 2002). In the studies of Martinez-Reyna and Vogel, tetraploid (4x) x octaploid (8x) crosses yielded small, shriveled seed while the reciprocal octaploid (8x) x tetraploid (4x) crosses produced small seed with floury endosperm. Caryopses from the 4n X 8n crosses developed abnormally and formed an aqueous endosperm at about 15 days post-pollination. Roughly 20 days after pollination, as these seeds ripened, the endosperm began to shrivel and was completely shriveled 16 days later. The 8n X 4n crosses yielded caryopses that developed sluggishly in comparison to control crosses but had solid endosperm at 15 days post-pollination. At 30 days post-pollination these seeds were about one-third the size of those obtained from control crosses and had developed a floury endosperm quite unlike the seeds from the reciprocal cross. These results indicate that fertilization and embryo development does occur following pollination but an abortive process is induced some days later (20-36 days after pollination). Results from this study were similar to those obtained by earlier studies addressing crossability of octaploid and tetraploid switchgrass cultivars (Taliaferro, Vogel et al. 1999). Therefore, the technique of embryo rescue could be useful for recovery of wide crosses particularly where post-fertilization mechanisms are the predominant barrier to successful F1 seed set.

In 1947, Brink and Cooper proposed the idea that endosperm degeneration was the chief mechanism behind the failure of interspecific and intraspecific interploid crosses in many plants which exhibit post-fertilization incompatibility (Brink and Cooper 1947). The endosperm is important in proper seed development through physiological and genetic relationships to the developing embryo (Johnston, Nijs et al. 1980). Johnston et al. proposed that normal endosperm development relies on achieving a 2:1 maternal to paternal ratio of whole genomes in the endosperm (Johnston, Nijs et al. 1980). They termed this ratio as the Endosperm Balance Number (EBN) and further went on to suggest that any ratio that deviates from this EBN will cause abnormal endosperm development and will often result in seed abortion before the embryo can fully develop (Shen, Gmitter et al. 2011). In wide crosses which are not prevented by pre-fertilization incompatibility the technique of embryo rescue overcomes seed abortion that develops through abnormal endosperm development by surgically excising the immature embryo and germinating or culturing it on artificial media, independent of the endosperm.

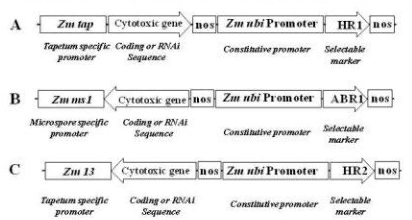
Since its inception, plant embryo culture has become a highly valued tool for plant breeding and is widely used for a diverse range of applications including further understanding requirements for development of the embryo, shortening the breeding cycle, overcoming seed dormancy, testing the viability of seeds, providing explants for micropropagation, and rescuing immature embryos from incompatible crosses (Bridgen 1994). The successful in vitro germination of an excised mature embryo was first demonstrated in 1904 by Hannig using embryos excised aseptically from two cruciferous plants and a medium containing basic mineral salts and sugar (Bridgen 1994).

Embryo rescue is defined as a tool frequently used in plant breeding to recover an immature embryo arising usually from an interploid hybrid cross by excising and culturing the embryo in vitro (Monnier 1990) and subsequently culturing the embryo to a whole plant (fertile or infertile). Typical the post-excision embryo is germinated directly on an appropriate medium. In some species it may not be technically feasible to surgically excise embryos out of fertilized ovules and in these cases the whole ovule or entire ovary can be cultured. This technique is encumbered because it is tedious and time consuming resulting in low yields and inefficient recovery of rare wide crosses. In addition, effects of the maternal tissue (especially the ovular wall) may be deleterious to embryo rescue, further contributing to low yields. The outcome of both surgical removal embryo rescue and immature ovule or caryopsis culture techniques is usually a single plantlet. In many (most) wide cross embryo rescue crosses, the recovered F1 plants, especially between crosses involving different ploidy levels, the outcome is in not often sterile. This sterile outcome may in some cases be desirable, especially where such plants can be vegetatively propagated by cuttings or grafting techniques, producing 'seedless' plants. Many types of seedless plants that are the result of triploidy or other increased ploidy levels have been commercially produced. However in some other cases it may be desirable for hybrid outcomes to be entered into a normal breeding program. The resultant plants or tissue cultured explants from those outcomes generated from conventional embryo rescue techniques may be treated with compounds (such as colchicines) that result in chromosomal doubling or loss, to restore fertility so that these outcomes can be incorporated into normal breeding programs. This technique is also encumbered because it is tedious and time consuming resulting in low yields and inefficient recovery of rare wide crosses to conventional breeding programs where fertile plants are desirable. Therefore, while wide crosses have proven as valuable to breeding hybrids, the method of conventional embryo rescue is encumbered for a variety of reasons, which limit its application to certain plants and breeding schemes.

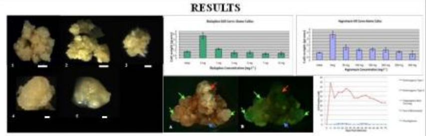
Through the application of a selectable marker, such as herbicide resistance, it would possible to selectively culture a fertilized embryo into embryogenic culture as an intermediate away from maternal tissue of the ovule. If the selectable marker were a transgene, present in the genome as a either a hemizygous (T0) homozygous (selfed T1,T2,...) of heterozygous (outcrossed T1,T2,...the embryogenic callus could then be proliferated and regenerated under selection to produce clonal herbicide resistant wide-hybrid individual plantlets. After crossing of the putative hybrid with a wild-type parent, it would be possible to select for sensitive individuals and recover non-GMO hybrid individuals with potentially desirable hybrid characteristics.

Transgenic Nuclear Male Sterility as a maternal parent for recovery of herbicide resistant wide crosses via

(A) Tapetal ablation; (B) Microspore abortion; and, (C) Pollen sterility



RESULTS



Optimization of Embryogenic Callus Growth in Tissue Culture of Switchgrass

FIGURE 1. **Selection and Optimization of Growth of Embryogenic Callus in Switchgrass** Figure 1 A Description of various callus morphotypes in switchgrass cv 'Alamo' cultures. (1) embryogenic (Type I) organized, compact embryogenic structures with an epidermal surface cell layer, light yellow (2) embryogenic (Type II), friable callus masses with white somatic embryoids, (3) organogenic (root forming) compact callus forming roots with root hairs and non-regenerable to shoots and (5) mucilaginous. Embryogenic callus visual identification and selection is important to the practice of present invention. Bar = 0.5 mm. FIGURE 1B. Kill curve data on increasing amount of bialaphos (top) and hygromycin (bottom) for cv 'Alamo' shows adequate selection for bialaphos is at 3 mg l^{-1} and 300 mg l^{-1} hygromycin. Killing levels of selective agent are required for practice of the invention. FIGURE 1C. Expression of pJLU13 16 days after co-cultivation shows a non-uniform distribution of GFP expression. Positive expression is seen associated with embryogenic Type II (green arrows) but not in embryogenic Type I (red arrow), mucilaginous (blue arrow) and nondifferentiated callus morphotypes. Embryogenic cultures derived from in situ recovered embryos are most useful for practice of the invention. FIGURE 1D. Transfection results after 70 days post-transfection and 71 days hygromycin selection on 100 pieces of each of the five basic morphotypes consisting of (1) embryogenic (Type I), (2) embryogenic (Type II), (3) organogenic (root forming), (4) nondifferentiated, and (5) mucilaginous, using the 35S hph selectable marker cassette in the EHA105 *Agrobacterium* strain. This data shows that embryogenic calli are preferentially recovered by the medium used in the in situ embryo rescue procedure.

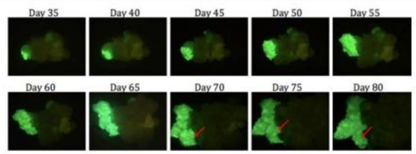
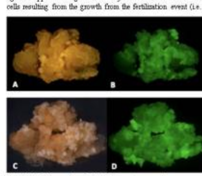


FIGURE 2. Identification of embryogenic stem cells in switchgrass calli under selection. GFP imaging of a single hygromycin resistant colony is shown sequentially over 45 day period. Doubling time is approximately 5 days. By day 70 intense green foci (arrows) appear as cytoplasmically dense cells of somatic embryos characteristic of embryogenic Type II callus. These results demonstrate that embryogenic callus can be selected from nontransgenic cells in the presence of killing levels of the selective agent as applied during in situ embryo rescue. All maternal non-transgenic tissue is eliminated allowing only the proliferation of cells resulting from the growth from the fertilization event (i.e. from the contribution of the transgene from the GMO parent)



FFIGURE 3. Expression of the GFP reporter gene in switchgrass (cv 'Alamo') in a single representative stable transgenic event. Left brightfield microscopy. Right: 470nm UV GFP microscopy. Micrographs (A,B) of GFP expression in a stable transgenic callus prior to regeneration. Note intense GFP fluorescence associated with meristematic and somatic embryogenic tissues. The same transgenic event as (A,B) 7 days on regeneration media (C,D). Morphological changes in development noted as well as early leaf development (arrow) from germinated embryos prior to light regime. These results show that under these selection criteria a callus selected from in situ embryo rescue will uniformly be transgenic.

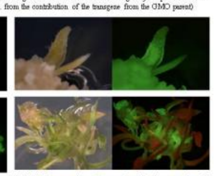
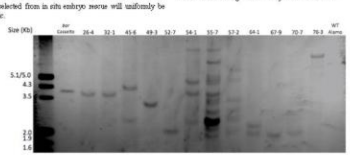


FIGURE 4. Regeneration of transgenic calli with expression of the GFP reporter gene. The GFP marker is retained in visible in regenerating plantlets and not in non transgenic controls (not shown). Transgenic events grow normally and have normal chlorophyll development. Autofluorescence of chlorophyll masks expression. While chlorophyll autoflorescence may mask the detection of GFP under these conditions, subsequent molecular analysis demonstrates that these plants are stable transgenic events. All plants are clonal as determined by Southern blot analyses without escapes. This quality will prove useful for following in situ embryo rescue results.



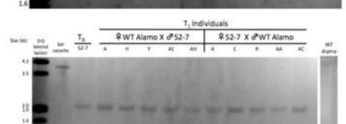


FIGURE 5. Southern blot hybridization of NcoI-digested genomic DNA extracted from cv 'Alamo' switchgrass pHG018 primary transformants with DIG-oxigenin labeled *bar* probe. EcoRI-digested *bar* cassette was included as positive hybridization control followed by 12 independent transgenic events and finally an NcoI-digested genomic DNA sample of wild-type cv 'Alamo' DNA. These data demonstrate that plantlets derived from embryogenic calli under these conditions are all clonal as determined by Southern blot analyses without escapes and supports the use of these approaches for in situ embryo rescue for transgenic cells derived from crosses. This data supports the contention that stable integration of transgenes are inherited as T1 plants taht can be used for donor plants during in situ embryo rescue.

FIGURE 6. Southern blot hybridization of NcoI-digested genomic DNA extracted from cv 'Alamo' switchgrass pHG018 primary transformant #52-7 and 10 T_1 individuals with DIG-oxigenin labeled *bar* probe. Included in blot is EcoRI-digested *bar* cassette included as positive hybridization control, followed by T_0 parent #52-7, five individuals from cross where wild-type cv 'Alamo' served as pistillate parent and five individuals from reciprocal cross where primary transformant #52-7 served as the pistillate parent.

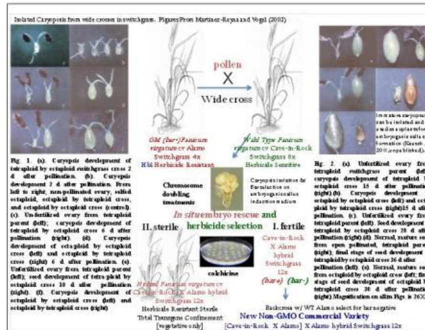


FIGURE 7. Strategy for recovery of wide crosses useful as bridge intermediates via *in situ* embryo rescue using embryogenic callus induction medium with herbicide selection. This diagrammatic scheme illustrates, as an example, the recovery of wide inter-specific crosses using herbicide selection as a marker, however, this same or similar scheme also applies to wide intra- and inter-varietal, intra-and inter-specific, inter-generic and distant relative crosses. In this example, genetically modified (GMO) *Panicum virgatum L.* cv Alamo switchgrass (4x), (at middle left, shown in dark red) is herbicide resistant (Hb1, bar+, containing the bar gene, resistant to bialphos and 3% Finale or Liberty) and is used as the paternal pollen donor in a wide cross. These plants may be hemizygous T0, or contain at least one copy of the transgene in T1,T2, or .... generations. The maternal pollen recipient is wild -type *Panicum virgatum* cv Cave-In-Rock (CIR) (8x, at middle upper right) which in non-genetically modified (non-GMO) and hence (shown in dark green) herbicide sensitive to bialaphos and 3% Finale or Liberty. Pollinations may be most conveniently accomplished in pollen cages using one several clones of an event herbicide sensitive as a pollen donor and a single wild type plant as pollen recipient. After pollination immature caryopses are harvested only from the wild type maternal parent and plated onto embryogenic caluus induction medium (center, left). Caryopses can be selected from various stages of development, as shown by in the accompanying micrographs of isolated caryopses from various wide crosses in switchgrass, from the figures of Martinez-Reyna and Vogel (2002) as a guide. Some of the caryopses form embryogenic calli. In this embodiment chromosome 'doubling' treatments can be applied at this stage. While the treatments may not result in precise chromosomal doubling, they can, in some cases restore fertility. Treatments may include but are not limited to colchicines and/or temperature shocks. Seedlings are regenerated from the calli in the presence of bialaphos for selection of the paternally inherited bar gene. Regenerated seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar +, red) and herbicide sensitive (bar-, green) populations (middle lower right). Seedlings may also be analyzed for chromosomal number, content and/or integrity. At floral maturity the (bar+) Hb1 Herbicide Resistant Alamo X CIR hybrid plant (s) are scored for fertility and if fertile (I. fertile) used preferably as paternal pollen donor (s) in a backcross to either wild -type *Panicum virgatum cv* Alamo switchgrass (4x) non-GMO herbicide sensitive plants (lower center) or CIR wild type plants. These plants, can also serve as bridge intermediates to cross with other compatible or incompatible parents. The resultant seed from each population is recovered and germinated. The resultant seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar +, red) and herbicide sensitive (bar-, green) and populations (left). The non-GMO hybrid plants contain Alamo X CIR X Alamo (12x or lower, blue, lower left) or Alamo X CIR X CIR genomic contributions and are subsequently analyzed and scored for desirable traits correlated with genomic markers. Desirable plants may enter into population block breeding plots, and using genomic assisted breeding and mass selection can enter subsequent commercial development. If sterile (lower left; II. sterile) seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for verification of resulting resistant plants after 21 days to reveal herbicide resistant (bar +, red) populations (lower right). These plants have a robust gene confinement phenotype for deregulation of transgenic traits in hybrid backgrounds. These GMO hybrid plants contain Alamo X CIR (red, lower left) ,with 12x or lower genomic contributions and are subsequently analyzed and scored for desirable traits correlated with genomic markers. Desirable plants may enter into population block breeding plots, and using genomic assisted breeding and mass selection can enter subsequent commercial development.

FIGURE 8. Bottom Panel (A-D): Switchgrass transformation sequence from seed. (A) Embryogenic callus initiation from mature caryopses; (B) followed by transfection of embryogenic callus with *Agrobacterium* carrying vectors; (C) selection of transformants for herbicide resistance; and (D) transgenic plant regeneration. Note: the same embryogenic callus induction media used here is deployed for induction of callus from immature caryopses from in situ embryo rescue. Panel (E-K): Various stages of ovule and embryo development suitable for in situ embryo rescue from *Panicum viragtum* cv Alamo. (E) Ovule approximately 4 dpf (F) Ovule approximately 6 dpf (G) Ovule approximately 9 dpf (H) Ovule approximately 12 dpf (I) Immature excised embryo (J) Immature embryo within spikelet (K) Nearly mature excised embryo

In situ embryo rescue of wide crosses using herbicide selection as a marker.

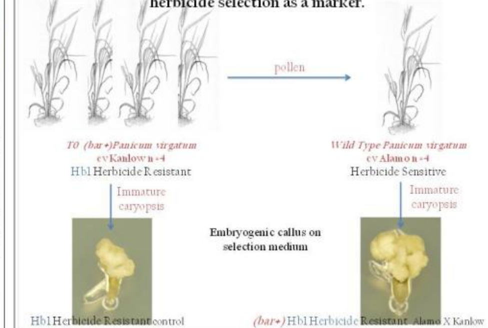


FIGURE 9. Strategy for recovery of wide crosses via *in situ* embryo rescue using embryogenic callus induction medium with herbicide selection.

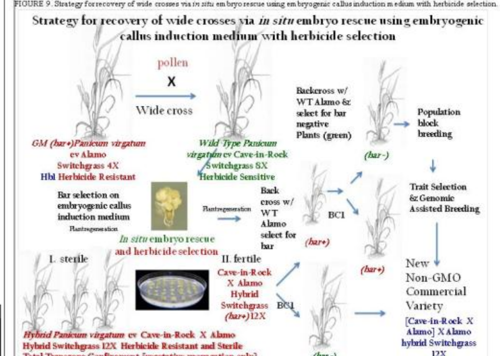


FIGURE 10. Strategy for recovery of wide crosses via *in situ* embryo rescue using embryogenic callus induction medium with herbicide selection. This diagrammatic scheme illustrates, as an example, the recovery of wide inter-specific crosses using herbicide selection as a marker, however, this same or similar scheme also applies to wide inter-varietal, inter-specific, inter-generic and distant relative crosses. However, in this example, genetically modified (GMO) *Panicum virgatum L. cv* Alamo switchgrass (4x), (at upper left, shown in dark red) is herbicide resistant (Hb1, bar+, containing the bar gene, resistant to bialphos and 3% Finale or Liberty) and is used as the paternal pollen donor in a wide cross. These plants may be hemizygous T0, or contain at least one copy of the transgene in T1,T2, or .... generations. The maternal pollen recipient is wild -type *Panicum virgatum cv* Cave-In-Rock (CIR) (8x, at middle upper right) which in non-genetically modified (non-GMO) and hence (shown in dark green) herbicide sensitive to bialaphos and 3% Finale or Liberty. Pollinations may be most conveniently accomplished in pollen cages using one several clones of an event herbicide sensitive as a pollen donor and a single wild type plant as pollen recipient. After pollination immature caryopses are harvested only from the wild type maternal parent and plated onto embryogenic caluus induction medium (center, left). Some of the caryopses form embryogenic calli. Seedlings are regenerated from the calli in the presence of bialaphos for selection of the paternally inherited bar gene. Regenerated seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar +, red) and herbicide sensitive (bar-, green) populations (middle lower right). At floral maturity the (bar+) Hb1 Herbicide Resistant Alamo X CIR hybrid plant (s) are scored for fertility and if fertile (I. fertile) used preferably as paternal pollen donor (s) in a backcross to either wild -type *Panicum virgatum cv* Alamo switchgrass (4x) non-GMO herbicide sensitive plants (lower center) or CIR wild type plants. The resultant seed from each population is recovered and germinated. The resultant seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for resulting resistant and sensitive plants after 21 days to reveal herbicide resistant (bar +, red) and herbicide sensitive (bar-, green) and populations (left). The non-GMO hybrid plants contain Alamo X CIR X Alamo (12x or lower, blue, lower left) or Alamo X CIR X CIR genomic contributions and are subsequently analyzed and scored for desirable traits correlated with genomic markers. Desirable plants may enter into population block breeding plots, and using genomic assisted breeding and mass selection can enter subsequent commercial development. These plants, can also serve as bridge intermediates to cross with other compatible or incompatible parents. If sterile (lower left, II. sterile) seedlings are treated with 3% Finale using one-several leaves in the viable paint assay and scored for verification of resulting resistant plants after 21 days to reveal herbicide resistant (bar +, red) populations (lower right). These plants have a robust gene confinement phenotype for deregulation of transgenic traits in hybrid backgrounds. These GMO hybrid plants contain Alamo X CIR (red, lower left) ,with 12x or lower genomic contributions and are subsequently analyzed and scored for desirable traits correlated with genomic markers. Desirable plants may enter into population block breeding plots, and using genomic assisted breeding and mass selection can enter subsequent commercial development.

CONCLUSIONS

IN SITU EMBRYO RESCUE AND RECOVERY ON NON-GENETICIALLY MODIFIED HYBRIDS FROM WIDE CROSSES

This paper demonstrates the basis for a new technique for the recovery of wide crosses. The technique of embryo rescue overcomes seed abortion that occurs through abnormal development caused by various incompatibility mechanisms. Conventional embryo rescue techniques are accomplished by surgical excision of the immature embryo resulting from incompatible plant crosses and germinating or culturing the developing embryo on artificial media, independent of the endosperm. These techniques are tedious, time consuming and have low yields especially with low fertilization frequencies. The present paper overcomes the problems of conventional embryo rescue by utilizing a transgenic selectable marker in the paternal parent and culturing the immature embryo *in situ* in the developing ovule on embryogenic culture initiation media which includes the selective agent. This novel method is coined here as 'in situ embryo rescue' since it does not involve surgical removal of the embryo according to traditional procedures. The resulting embryogenic culture can be regenerated to whole plants. The ability to combine genomes within and between distant plant taxonomic groups including varieties, species, genera and more distant relatives offers the opportunity to rapidly accelerate the development of new varieties, cultivars, species, and crops. The utilization of these novel techniques and the exploitation of the recovery of fertile or infertile progeny from wide crosses increases genetic diversity and can be used to introduce novel genetic and useful traits. This process is advantageous since the outcomes can include genetically modified organisms (GMO) traits when appropriate, or these can be segregated to produce plants that do not contain transgenes and hence, are non-GMO.

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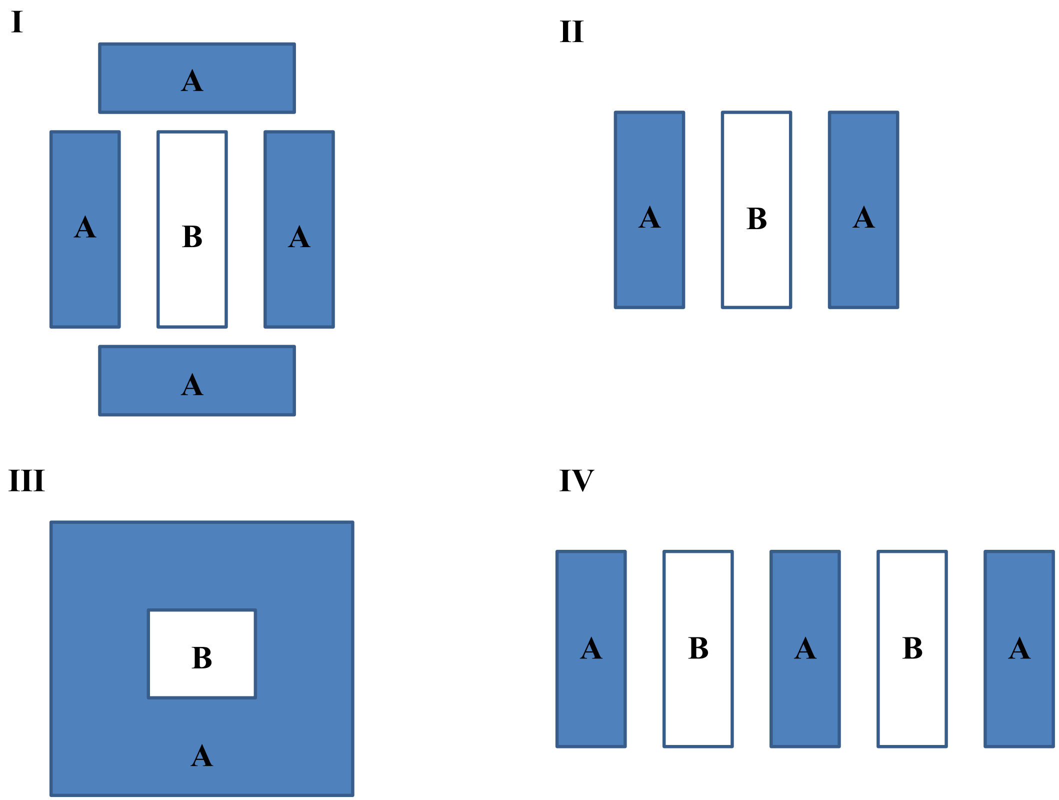
List of seventeen public switchgrass (*Panicum virgatum L*) cultivars and their corresponding State of Origin, and Plant Form (i.e. Upland or Lowland).

Cultivar	*State of Origin*	*Plant Form*
Alamo	TX[1]	Lowland
Blackwell	OK[1]	Upland
Bowmaster	AR, NC[2]	Lowland
Carthage	NC[1]	Upland
Cave In Rock	IL[1]	Upland
Dacotah	ND[1]	Upland
Forestburg	SD[1]	Upland
High Tide	MD[6]	Lowland
Kanlow	OK[1]	Lowland
Miami	FL[3]	Lowland
Performer	AR,OK,NC[2]	Lowland
Shawnee	IL[4]	Upland
Shelter	WV[1]	Upland
Southlow	MI[7]	Upland
Stuart	FL[3]	Lowland
Sunburst	SD[5]	Upland
Timber	AR?, NC?[4]	Lowland

Corresponding sources shown in superscript. [1]USDA Soil Conservation Service Agricultural Handbook No. 170. Grass Varieties in the United States. [2] Plant Patent Application.[3]USDA-NRCS.Release documents from Brooksville, FL Plant Materials Center. [4] Personal communication to Calvin Ernst. [5] USDA NRCS Bismarck, ND. Switchgrass Biomass Trials in North Dakota, South Dakota, and Minnesota. [6] USDA-NRCS, Cape May Plant Materials Center "High Tide Switchgrass" release brochure. [7] USDA-NRCS, Rose Lake Plant Materials Center "Southlow Switchgrass" release brochure.



Breeding schemes to produce population hybrids in Switchgrass

I. Parent A produces a pollen cloud sufficient to swamp out pollen from B to force the hybrid production. II. Parent A is selected to as a genotype that out-produces that of parent B. III. Use of male sterility, CMS, nuclear, or transgenic as parent B surrounded by a pollen donor A parent. IV. Use of transgenic female sterile herbicide resistant parent A in alternating rows with male sterile parent B resistant to a second herbicide for totally sterile hybrid production.

Ernst Conservation Seeds Inc