Cereal Crop Plant Transformation and Genome Editing Training Workshop

University of Rhode Island Plant Biotechnology Laboratory

And

The New England Biophilia Institute

April 10-20, 2022

The Kausch Laboratory

University of Rhode Island

April 10-20, 2022

Supported by:

NSF Plant Genome Research Program
Division of Integrative Organismal System

and

The Department of Energy
The Biological and Environmental Research (BER) Program

"You should leave here and be able to do it"

Cereal Crop Plant Transformation and Genome Editing Training Workshop

WELCOME TO THE KAUSCH LAB

NSF PLANT WORKSHOP

AGENDA

Sunday

12:00 The Higgins Center University of Rhode Island MAIN CAMPUS

Introductions and Orientation

1:00-1:45pm

Lecture 1. Introduction to Plant Transformation and Genome Editing for Advanced Plant Breeding, Crop Improvement and Basic Plant Science

Break

2:00-3:00 pm

Lecture 2. Essential Components of Successful Plant Transformation and Genome Editing Systems

Tissue Culture System

- Regeneration of whole plants from single cells-Totipotency

Molecular Gene Construction Technology

- Molecular vector construction using foreign DNA-

Method for DNA Delivery

- *Agrobacterium tumefaciens*
- Microprojectile Bombardment
- Direct DNA Uptake
- Whiskers
- Sonication

Efficient Selection Strategy

Selectable markers are an indispensable part of successful integrative transformation.

- Positive vs Negative Selection
- Kill Curves
- Antibiotic Resistance
- Herbicide Resistance
- Metabolic Selection

Reporter gene systems- GUS, GFP, LUX, DS RED, Anthocyanin markers, etc

Regeneration of Transgenic Plants to Seed-The Immortalization of Events

Break

3:15-4:30 pm

Lecture 3. Advanced Breeding of Transgenic Events- Where do they go from here?

Transformability- What is it?

Genotype Dependence- What determines it?

Dicot vs Monocot Systems

Arabidopsis (why it doesn't really count)

Tobacco, Alfalfa, Tomato, and Cannabis

Maize, Rice, Sorghum, and *Zostera**

**Zostera* is included here as a part of the Special Projects section for this Workshop

5:00-6:30 Social Hour at The Hathaway's

7:00 pm Dinner at The Hathaway's

Monday

8:00-9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Lecture 4. Sorghum Transformation Biology and Practice: Experiential Laboratory Training on the Techniques in the Plant Transformation Lab

This lecture corresponds DIRECTLY with the Hands-on Experiential component of the Workshop. The Goal of this part of the Workshop is for the participant to be able to transform Sorghum on their own, in their own lab, as a outcome of the Workshop.

10:30- 4:00pm (with breaks)

Session 1. The Kausch Lab-Experiential Laboratory Training– Rapid transformation and plant regeneration of sorghum (Sorghum bicolor L.) mediated by altruistic Baby boom and Wuschel2

Sorghum Transformation-from Agro-infection through tissue culture and selection to plant regeneration to T1 seed

Growing of donor plants and transgenics in the Greenhouse- The importance of healthy vigorous donor plants as a source for immature embryo explants

Staging of the immature embryo and surface sterilization of the Caryopses

Preparation of *Agrobacterium* cultures

Preparation of Co-infection Media

Embryo isolation and infection

Embryo Co-cultivation

The "RESTING" phase

Selection of Transgenic Events-Subculturing
Plant regeneration and transition to soil
Morphogenic regulator mediated transformation (MRMT)

5:00-6:30 Social Hour at The Hathaway's

7:00 pm Enjoy your Evening!

Tuesday

8:00-9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Lecture 5. - Microscopy and Imaging for Publication

Introduction to the Special Publication Components of this Workshop.* We have been invited to contribute a special White Paper as an outcome of this Workshop which will be submitted for publication as a Review Paper in In Vitro Cell & Devel Biol Plant 2022 Plant Springer publ.

Title: ***"The Democratization of Genome Editing and Advanced Plant Breeding for Agricultural crop Improvement: Moving it from the Silo to the Field"***

10:30-4:00 (with breaks)

Session 2. The Kausch Lab-Experiential Laboratory Training– Microscopy of Biological Specimens Required for Transformation Technology Development Research and Publication

Stereo Light Microscopy- Introduction, optics and operation of the Leica THUNDER MODEL ORGANISM Imaging System M165FC w/GFP and CHER

Scanning Electron Microscopy (SEM) -Introduction, sample collection, chemical fixation and dehydration, critical point drying specimen mounting, sputter coating and imaging in the SEM (visit to the Leduc Imaging facility, Brown University)

We will be conducting the experiments with the stable Cas9/GFP plants as a platform for genome editing in this Session. We will also be conducting our imaging analysis of the stages of somatic embryogenesis from altruistic *Agro* infections and taking corresponding samples for SEM analysis.

5:00-6:30 Social Hour at The Hathaway's

7:00 pm Enjoy your Evening!

Wednesday

8:00-9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Lecture 6. Wednesday- Analysis of Transgenic Events- Phenotypic and Molecular Analysis of Transgenic and Genome Edited Events

Phenotypic analysis-

The bar gene 'paint assay', phenotype by transgene function

Data Analysis of Reporter gene systems- GUS, GFP, LUX, DS RED, Anthocyanin markers,

Imaging and Data analysis for Transformation Technology

Molecular Analysis-

Genomic DNA isolation

PCR, and qPCR

Southern Analysis and Southern by Sequencing (SBS)

10:30- 4:00pm (with breaks)

Session 3. The Kausch Lab-Experiential Laboratory Training– Subculturing from regeneration to the greenhouse, special considerations, Sampling for analysis and processing.

5:00-6:30 Social Hour at The Hathaway's

7:00 pm Enjoy your Evening!

Thursday

8:00-9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Lecture 7. Thursday- Cereal Crop Plant Transformation: Generalities and Peculiarities

This lecture will cover the Maize and Rice Systems in a step-by-step fashion with technique enablement in mind, with every effort to share all of the special laboratory techniques and 'secrets' that do not often show up in publications. This is how to actually do it. This lecture as well as the accompany one on Rice is then in a format similar to the presentation on Sorghum in Lecture 2**.**

The Maize Systems

The Rice Systems

10:30- 12:00

Session 4. The Kausch Lab-Experiential Laboratory Training–

Subculture Transformation immature embryos to resting medium, imaging of transient expression GFP and DSRED, and working on the participants projects.

Session 5. The Kausch Lab-Experiential Laboratory Training–

Media Preparation and Fundamentals of a Tissue Culture Faciltiy.

2:00- Explore New England

Friday

7:00-9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Lecture 8. Friday- Understanding Plant Morphogenesis and Tissue Culture

The influence of plant developmental biology and the ability to manipulate plant morphogenesis by using tissue is an essential component to the design and optimization of all currently used protocols for plant transformation (with the exception of *Arabidopsis*). This lecture will dove-tail with the Special project assignment o to develop a strategy for transformation of Zostera Marina. Zostera (eelgrass) is a significant plant regrading global carbon capture and marine habitat in parallel importance to the coral reef and as fragile to climate change. Zostera has never been transformed. It is a monocot with terrestrial plants as ancestors. Its genome has been sequenced. The participant is expected to be able to design how to approach the development of a transformation protocol for a plant that has never been transformed.

The importance of directed morphogenesis to successful plant transformation protocol development.

Somatic embryogenesis

Organogenesis

Session 4. The Kausch Lab-Experiential Laboratory Training– Participants Projects on going. Tissue culture-Processing samples for microcopy and imaging for scanning electron Microscopy, A visit to the Leduc Bioimaging Facility Brown University. Providence Rhode Island.

10:30- 2:00pm (with breaks)

7:00 pm SPECIAL EVENT The New England Biophilia Institute Gallery, is Hosting the Premier Opening for the Exhibit The Desert Nanoscape of Saguache Colorado by Geoff Williams, in Westerly Rhode Island. All Workshop Participants are Welcome to Attend.

Session 4. The Kausch Lab-Experiential Laboratory Training–

Saturday and Sunday No Lectures - All Day will be spent in the Lab on the Special Projects and Manuscripts

"The Democratization of Genome Editing and Advanced Plant Breeding for Agricultural crop Improvement: Moving it from the Silo to the Field"

Monday

8:00-9:30 am

Lecture 9. Cereal Transformation Laboratory Basics and Set Up- UltraSmall, Small, Medium and Large: How to build a functional genome editing lab.

How to set up a Plant Transformation Facility: Small, medium and large. This description will include consideration of space and separation of associated tasks, aseptic conditions, Facilities and Equipment from soup to nuts. This is another topic which is publication worthy as an outcome of this Workshop.

Tuesday:

Lecture 10. Conclusions, Workshop Publications, and Next Steps for the manuscripts and technology transfer after the Workshop. What happens next.

Tuesday: Session 4. The Kausch Lab-Experiential Laboratory Training– Participants Projects on going

Wednesday: 9:00 am 9:30 am The Higgins Center University of Rhode Island MAIN CAMPUS

Conclusions, Farewells (For now) and Planning for the Future.

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ACKNOWLEDEMENTS

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and

The Department of Energy
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Wednesday April 20, 12:00-	**Lunch and Departures**

Emergency Contacts During the Workshop

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Special Laboratory Projects and Deliverables for the Participants

In addition to the hands-on participation in all phases of the sorghum transformation process (staged ahead of time for this Workshop. You will see all phases of the six-month procedure in the 10-day Workshop.

***1. Imaging Workshop for Publication**-Stereo microscopy imaging of live tissue culture samples while maintain sterile cultures over time*

Specimen preparation of sorghum embryogenic samples for Scanning electron microscopy (SEM)

Sample selection and chemical fixation, dehydration through an ascending ethanol series to critical point drying specimen mounting, sputter coating and imaging in a SEM at the Leduc Bioimaging Center at Brown University (near the University of Rhode Island)

Light microcopy imaging of living aseptic cultures using the Leica THUNDER MODEL ORGANISM System.

Imaging of developmental sequence of somatic embryogenesis in sorghum immature embryos as initiated by BBM/WUS

2. Imaging of dissected Sorghum T1 seeds (BAR-Cas9-GFP) and observational analysis of GFP expression over 48 hrs.

3. Surface sterilization/disinfestation of difficult samples to introduce into aseptic tissue culture.

Sorghum immature embryo preparation

The garlic bulbil challenge- Can you devise a method to eliminate microbes from outdoor grown garlic bulbils for introduction into tissue culture for transformation and genome editing purposes. Why garlic bulbils you ask?

4. Giving a prepared lecture on an Agricultural Biotechnology related topic, geared to a general audience.

The lecture will be recorded as a part of this Workshop. This will add to a compilation already in production with Dr. Albert Kausch and Dr. David Songstad which we are producing for a new Online course on Agricultural Biotechnology. We want you to join us as co-authors in this series. Your contribution and participation will be a requirement in this part of the Workshop.

NSF Plant Genome Sorghum Transformation Workshop

"Learn it like you need to use it"

The Kausch Lab

University of Rhode Island

Introduction

The Poaceae, formerly known as the Gramineae, is a large family of monocotyledonous flowering plants known collectively as the grasses, contains over 9,000 species distributed nearly throughout the world. From these, 35 species have been domesticated and cultivated as the cereal crops that feed the world. The major cereal crops ranked by worldwide production are maize at 1,125 million tonnes (megatonne, Mt), wheat (775.8 Mt), rice (505 Mt), barley (159.74 Mt) sorghum (62.05 Mt), oats (22.53 Mt), and rye (14.3 MT) harvested in 2020-2021 (Shahbandeh 2021). Recent reviews of plant transformation and genome editing in the cereal crops underscore the importance of this technology to global agriculture (Borisjuk *et al.* 2019; Hensel 2020; Kausch *et al.* 2021a; Kausch *et al.* 2021b; Nishimura 2020).

The significance of cereal crops to global agriculture, economy, food security and international stability is well documented and widely understood. The functional development of a genome-level knowledge base linking genes to phenotypes through the use of transgenics in cereal species is critical to understanding fundamental physiological functions important to crop improvement. The capability to create, test and cultivate transgenics has enabled some of the most innovative and important scientific discoveries and agricultural achievements for over the last three decades. Transgenics are integral to basic modern agricultural practices and functional studies in plants including the ability to knock-out (down) gene expression, conduct promoter expression analyses, make specific adjustments in protein structure and function, and observe over-expression and ecotopic characteristics. Emerging technologies such as plant synthetic biology and metabolic engineering depend heavily on transgenic technologies.

Plant transformation has enabled fundamental insights into plant biology and revolutionized the seed industry. Unfortunately, for most crop plants, transformation and

regeneration remain arduous processes, even after over thirty years of technological advances. Plant genome editing promises to enable a step change in our ability to enhance crop plant productivity but typically relies on genetic transformation and regeneration of plants with altered DNA sequences. Thus, reaping the benefits of new genome editing technologies for crops will require more efficient plant transformation. In light of our current knowledge, we identify key research needs to enable genome editing in crops. First, tissue culture is suboptimal and clumsy for most crops, and time in culture should be streamlined and minimized. The manipulation of plant genes can aid in programming cell and tissue development that could boost recovery of transformed plants. Second, *Agrobacterium*- and biolistics-mediated transformation are powerful tools that are still far from efficient in crops. Third, synthetic biology has been underutilized in crop improvement and plant genomics but offers innovative approaches.

Plant transformation encompasses two distinct and consecutive steps: (1) DNA introduction into plant cells (sometimes known as transient transformation, in which transgenes have not yet integrated into the genome), and (2) integration of the introduced DNA into the plant genome (stable transformation). Each step is useful in basic plant research and biotechnology, but the second step is necessary to produce transgenic plants with heritable traits of interest. For most crops, transgenic plant production requires the ability to regenerate plants from transformed tissues. Although considered part of the transformation process, the plant regeneration step is often a greater bottleneck than is the stable integration of DNA sequences in crops. In this workshop, we will review current knowledge and bottlenecks to plant transformation and implementation of high throughput genome editing in the context of the various associated techniques for maize, sorghum and rice. As we look to the future, we will discuss strategies to address the shortcomings.

The ability to link genomic information to functional and translational studies renders robust transgenic capabilities an imperative genomics technology in cereals vital to worldwide food security. Transgenics are vital to advancing our understanding of basic plant processes from development, metabolomics, signaling networks, and to plant-microbe interactions. A more widespread adoption of cereal transformation will increase innovation and basic knowledge of genome functioning thereby benefiting the entire community of plant sciences**.** Transgenics have increasingly become essential to modern agricultural production and genome analysis. The ability to use transgenics will become progressively more important for further characterization of the cereal genome, trait identification and germplasm improvement. Improved transgenic approaches will have a significant benefit to society by providing superior germplasm with improved yields, lower input resources, and increase resistance to biotic and abiotic stresses. The widespread

utility of transgenic technology in cereals requires the development of training programs outlined in this proposal to empower a new generation of plant scientists to use this technology for the benefit of society and scientific research.

Sorghum Transformation and Genome Editing: An Essential and Enabling Technology for functional Genomics Research and Trait Gene Analysis

Sorghum is the fifth most important cereal crop worldwide after wheat, rice, maize and barley and the third most important cereal crop grown in the U.S (Shahbandeh 2021). It is highly drought-tolerant, tolerates soil toxicities, and can grow in a wide range of climate and temperature conditions on marginal lands not suitable for the other cereals. Grown for multiple purposes, sorghum is important globally as a food, forage, feed, and fiber source. Sorghum is an important food resource especially in India and Africa and more widely used for grain and stover for animal feed in the Americas and Australia (Belton and Taylor 2004; Xin *et al.* 2009; Xin *et al.* 2019; Xiong *et al.* 2019). Sorghum has been identified as a prospective bioenergy crop for renewable energy (Belton and Taylor 2004; Nishimura 2020; Xin *et al.* 2009; Xin *et al.* 2019) and serves as a genetic and genomic model for the design of other C4 grass bioenergy crops (Mullet *et al.* 2014).

Genomics resources available for sorghum are extensive (Goodstein *et al.* 2011) since the completion of the approximately 730-megabase *Sorghum bicolor* (L.) Moench diploid genome (Cooper *et al.* 2019; Paterson *et al.* 2009). Over 98% of sorghum genes have been placed in their chromosomal context by genomic analysis and using whole-genome shotgun sequencing were validated by physical, genetic, and syntenic information (Cooper *et al.* 2019; Hao *et al.* 2021). Additional resources supporting sorghum functional genomics studies are available including the USDA sorghum germplasm system with 42,614 accessions and EMS mutation stocks developed by the USDA Plant Stress and Germplasm Development Unit in Lubbock, TX (Xin *et al.* 2009; Xin *et al.* 2019). Genomic information and computational analysis provide the basis for genetic manipulations by transgenics, cisgenics and genome editing (Kausch *et al.* 2019; Songstad *et al.* 2017). An efficient genetic transformation and or genome editing system is therefore central to applications of molecular breeding and for understanding the basic

biological processes of genetic control of plant physiology and development (Altpeter *et al.* 2016).

Advanced molecular breeding efforts (Che *et al.* 2018; Hao *et al.* 2021) for sorghum crop improvement will focus on target input and output traits (Duodu *et al.* 2003; Kumar *et al.* 2013; Li *et al.* 2018; Lin and Eudes 2020; Lin *et al.* 2021; Yang *et al.* 2020). Input traits addressing yield include approaches to crop protection through control of biotic and abiotic stresses such as insect resistance (Bt technologies) are already being applied through transgenic approaches (Casas *et al.* 1993; Zhao *et al.* 2000). Additional targets include improved nutrition for humans by editing of the alpha-kafirin gene family (Li *et al.* 2018), grain iron and zinc concentration (Kumar *et al.* 2013), bioenergy traits affecting lignocellulose ratios, and engineering of polyermers for biodegradable bioplastics (Lin and Eudes 2020; Lin *et al.* 2021; Yang *et al.* 2020), gene containment, synthetic hybrid plant strategies for breeding purposes, and nitrogen use efficiencies (Lin and Eudes 2020; Lin *et al.* 2021; Yang *et al.* 2020). The recent advent of applied genome editing affords the potential to affect more nuanced traits affecting yield (Hao *et al.* 2021). New capabilities for genome editing of trait genes in sorghum increases the need for improved and streamlined transformation, and recent advances in genomics, plant transformation, and genome editing (Duodu *et al.* 2003; Kausch *et al.* 2021b; Li *et al.* 2018) will come to bear on sorghum crop improvement.

Sorghum has been widely reported as a recalcitrant crop for genetic transformation (Aregawi *et al.* 2020; Casas *et al.* 1993; Che *et al.* 2018; Che *et al.* 2021; Grootboom *et al.* 2010; Visarada and Kishore 2015; Zhao *et al.* 2000) because of a lack of adequately responsive model genotypes, serious tissue culture limitations (accumulation of phenolic compounds), low plant regeneration frequency and loss of fertility through repeated sub-culture (Casas *et al.* 1993; Che *et al.* 2018; Che *et al.* 2021; Grootboom *et al.* 2010; Visarada and Kishore 2015; Zhao *et al.* 2000). The first report of successful sorghum transformation (Casas *et al.* 1993) was by particle bombardment with a low transformation efficiency of just 0.286%. The first *Agrobacterium*-mediated transformation of sorghum was first described by Zhao *et al.* (Zhao *et al.* 2000) with a 2.12% transformation efficiency. Until recently, sorghum transformation technologies (Aregawi *et al.* 2020; Visarada and Kishore 2015) lagged behind those of other major

cereals including rice (Hiei and Komari 2008), barley (Ibrahim *et al.* 2010) and maize (Ishida *et al.* 2007). However, several reports published incremental improvements through optimized media compositions and transformation parameters (Able *et al.* 2001; Belide *et al.* 2017; Do *et al.* 2018; Gao *et al.* 2005; Gurel *et al.* 2009; Nguyen *et al.* 2007; Williams *et al.* 2004; Zhao and Tomes 2003). The standard stalwart *Agrobacterium*-mediated transformation protocols for sorghum use differentiating embryogenic callus induced from immature embryos in the genotype BTx430 (Belide *et al.* 2017; Do *et al.* 2018). However, while the Standard protocols for sorghum transformation are reliable, they are lengthy, as well as time and resource expensive. With increased sorghum genomic resources and opportunities afforded by CRISPR/Cas9 editing technology, genotype dependent and labor-intensive transformation has remained as a significant bottleneck to functional genomics studies, genome editing and applied molecular breeding programs (Altpeter *et al.* 2016; Borisjuk *et al.* 2019; Hensel 2020; Kausch *et al.* 2021b; Nishimura 2020). To reach the full potential of CRISPR/Cas editing for breeding programs, the mutually inclusive goals of delivery of the reagents into an appropriate competent plant cell and the ability to recover an edited seed must be routinely achievable.

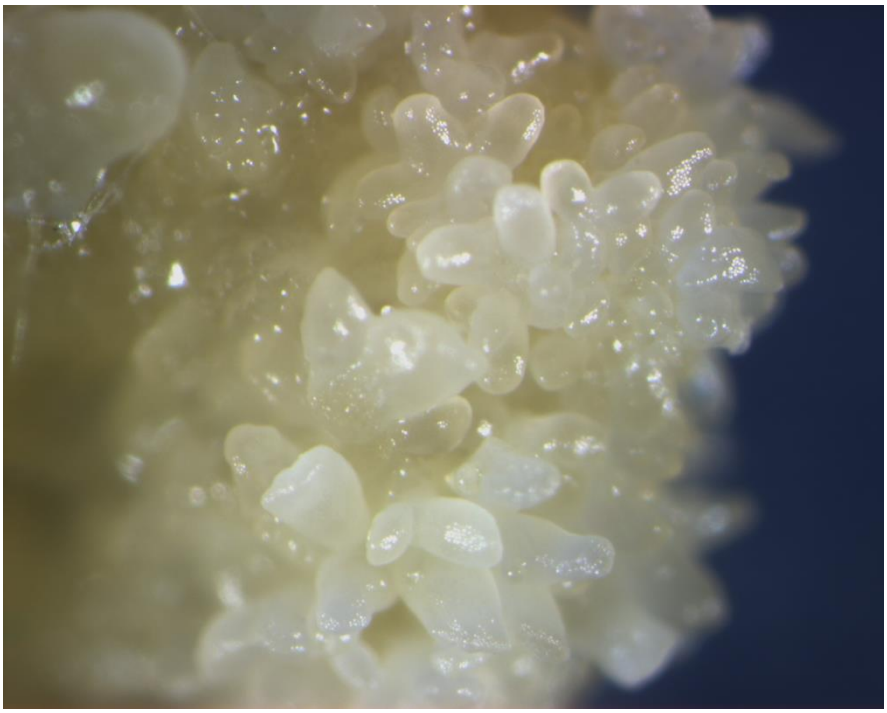
A breakthrough development by Lowe *et al.* (Lowe *et al.* 2016) described using controlled expression of developmental transcription factors as morphogenic regulators to initiate somatic embryogenesis. Morphogenic regulator mediated transformation (MRMT) technology was both confirmed and extended to recalcitrant maize and sorghum varieties by Mookkan *et al.* (2018). MRMT has now been accomplished through the recovery of transgenic and edited plants in a wide range of recalcitrant species and genotypes, including maize and sorghum (Hoerster *et al.* 2020; Lowe *et al.* 2018; Mookkan *et al.* 2018; Nelson-Vasilchik *et al.* 2018). Differential expression of morphogenic genes *Baby Boom* (*Bbm*) and *Wuschel2* (*Wus2*), in conjunction with new ternary constructs and a novel transformation system, designated as “altruistic” transformation, have increased the genotype range with various explant sources, that can be used for transformation and genome editing in maize and sorghum (Aregawi *et al.* 2020; Che *et al.* 2021; Hoerster *et al.* 2020).

Altruistic transformation uses differential co-infection with two *Agrobacterium* strains, one delivering a T-DNA conferring *BBM/Wus2* expression, and another delivering a trait-containing T-DNA into neighboring cells (Aregawi *et al.* 2020; Che *et al.* 2021). Somatic embryogenesis in maize is initiated through either non-cell autonomous WUS protein movement to the trait gene-containing cells and/or transient expression of *Wus2*, with no stable integration (Mookkan *et al.* 2018). The cells integrating the trait gene/selectable marker form somatic embryos that regenerate without any integration of the *Wus2* T-DNA (Hoerster *et al.* 2020). Recently, two excellent papers by Aregawi *et al.* (2020) and Che *et al.* (2021) have demonstrated the use of BBM/WUS for MRMT in sorghum.

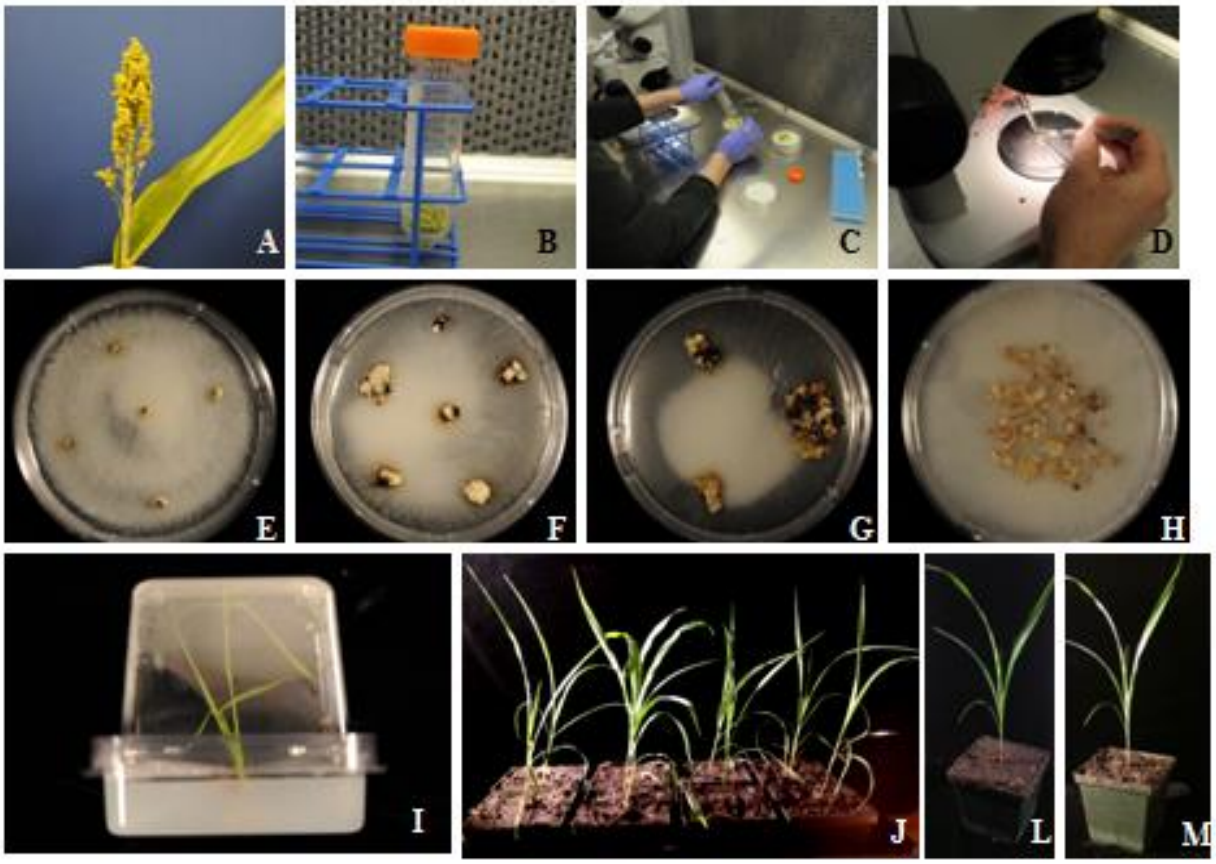
Che *et al.* (2021) demonstrated that controlled expression of *Wuschel2 (Wus2)* enabled sorghum (*Sorghum bicolor* L.) transformation and increased the efficiency of CRISPR/Cas-targeted genome editing. Using *Agrobacterium*-mediated altruistic transformation of immature embryo explants, Che *et al.* (Che *et al.* 2021) demonstrated efficient, genotype-independent transformation for generating high-quality morphogenic gene-free and/or selectable marker-free sorghum events. In all cases, CRISPR/Cas editing frequency across various targeted loci in different sorghum genotypes using *Wus2*-enabled transformation was significantly higher compared to conventional transformation.

Aregawi *et al.* (2020) created a complete MRMT pathway from *Agrobacterium* infection to high-throughput molecular genotyping to facilitate functional genomics in sorghum. After demonstrating success with the co-transformation approach, BBM/WUS were included in a non-altruistic construct containing the large CRISPR/Cas9 gene editing cassette. The non-altruistic method was used to demonstrate editing of another exemplary gene of interest, phytoene desaturase.

The main aim of This Workshop in The Kausch Lab is to transfer the skill set to enable the participants to be fully informed and competent for successful sorghum transformation and genome editing-independently in their own lab.



SORGHUM TRANSFORMATION



12:00-	**Lunch and Departures**

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