Final Report ER64667

Title of Grant: DEVELOPMENT OF GENOMIC AND GENETIC TOOLS FOR FOXTAIL MILLET, AND USE OF THESE TOOLS IN THE IMPROVEMENT OF BIOMASS PRODUCTION FOR BIO-ENERGY CROPS -TRANSFORMATION TECHNOLOGIES

Objectives: The objectives of this grant are to optimize foxtail millet transformation technologies using a tissue culture and *in planta* approach, and to generate transgenics with altered expression of candidate biomass genes.

Proceedings and publications:

1. Baxter H., Equi R., Chen X, Berk K. and Zale J. **Establishing Efficient *in vitro* Protocols For Foxtail Millet (*Setaria italica* L. cv. Yugi 1)**. Plant & Animal Genomes XVIII Conference XVIII, San Diego, California, January 2010

2. Chen X, Zale J and Chen F. **The Regeneration and Transformation of Foxtail Millet (*Setaria italica*), A Model Biofuel Crop**. Genomic Science Awardee Meeting IX and USDA-DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting, Crystal City, Virginia, April 2011

3. Chen, F., Tholl, D., Bohlmann, J., and Pichersky, E. (2011) **The family of terpene synthases in plants: A mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom**. *Plant J.* 66: 212–229.

Personnel development:

This project has provided training for the following personnel: Dr. Xinlu Chen (postdoc), Xiaomei Liu (postdoc), Jayashree Desai (postdoc) and Kyle Berk (Undergraduate researcher)

We have made significant progress optimizing *in vitro* protocols for the foxtail millet genotype Yugu1, increasing transient gene expression. The results will be divided into four sections:

1. We optimized protocol for tissue culture and regeneration of foxtail millet and plantlets via organogenesis from explants of immature inflorescences and seed, and via somatic embryogenesis from explants of immature inflorescences were produced. This is the first report describing the production of somatic embryos on a simple, solidified tissue culture medium, and SEM images were taken to definitely identify/characterize the embryos at the various stages of embryoid development.
2. Transient *Agrobacterium*-mediated GUSPlus™ expression *in vitro* after pre-treatment of calli, immature embryos designed to increase the efficiency of expression and genetic transformation. Germinating seedlings were used for transformation.
3. Background information on the *tb1* and *moc1* genes and sub-cloning these cDNAs into *Agrobacterium* and biolistics vectors, and their use in transformation experiments.

4. Results from *in planta* transformation experiments, similar to the floral dip in Arabidopsis [1], utilizing a red fluorescence protein reporter (*rfp*) and the hygromycin selectable marker (growth on selection media, red fluorescence protein expression, and PCR screening).

Results:

1. Protocols have been developed for callus initiation, somatic embryo formation, and plantlet regeneration from explants of immature inflorescences and seeds of foxtail millet, genotype Yugu1. The optimal media for calli induction from explants of immature inflorescences and seed was Murashige and Skoog (MS) containing 2.5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.6 mgl^{-1} 6-benzyl aminopurine (BAP), and 3% sucrose. Calli induction from explants of immature inflorescences was significantly higher (76%) in comparison to the other two media tested (Table 1), whereas calli induction from seed explants was also relatively high on this medium compared to six others tested (68.5%; data not shown).

There were no significant differences in the numbers of plants regenerated on three different media using calli induced from immature inflorescences; however the greatest absolute number of plantlets (97.5%) were regenerated on MS medium with 2 mgl^{-1} kinetin (KN) and 3% sucrose (Table 2). For seed explants, this was also the most effective medium compared to six others tested, resulting in a mean percentage of 30.2% in plantlet regeneration (data not shown). Overall, calli derived from immature inflorescences of foxtail millet proved to be the most capable of plant regeneration, with the callus induction phase being the key factor.

Table 1. Media Comparison for Induction of Calli From Immature Inflorescences

Media	**Explants Plated**	**Calli Induced**	**Percentage of Calli Induced**	**Mean Percentage[a] ± S.E.[b]**
MS, 2.5 mgl^{-1} 2,4-D, 0.6 mgl^{-1} BAP	560	426	76.1	76.1 ± 5.3 a
MS, 5.0 mgl^{-1} 2,4-D, 1.1 mgl^{-1} BAP	210	0	0.0	0.0 ± 0.0 b
N6E	210	8	3.8	4.0 ± 0.0 b

[a]Mean percentages followed by the same letter are not significantly different at the 5% level as determined by Tukey's Multiple Comparison; there were four replicates of each treatment.
[b]S.E. = standard error.

Table 2. Plantlet Regeneration of Inflorescence Calli Induced on MS, 2.5 mgl^{-1} 2,4-D, 0.6 mgl-1 BAP

Media	Calli Plated	Plantlets Regenerated	Percentage of Plantlets Regenerated	Mean Percentage ± S.E.[a]
MS, 2 mgl^{-1} KN	117	113	96.6	97.5 ± 1.6
MS, 1.1 mgl^{-1} BAP	121	108	89.2	85.6 ± 8.1
MS, 2 mgl^{-1} KN, 0.3 mgl^{-1} IAA	82	61	74.4	80.0 ± 10.8

[a]S.E. = Standard error; there were three replicates of each treatment.

Somatic embryogenesis is the formation of non-zygotic embryos in tissue culture, and the stages of embryo development (e.g. proembryo, globular, torpedo, heart-shape) resemble zygotic embryo development; however, somatic embryos lack a seed coat and an endosperm. Calli induced on a medium with an auxin may form embryos and these will mature, germinate and grow into plantlets on a medium without hormones. Somatic embryogenesis is a desirable step in a tissue culture approach to genetic transformation because it increases the likelihood of regenerating plants from transformed cells.

Some foxtail millet calli that were induced on a solidified medium of 2.5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.6 mgl^{-1} 6-benzyl aminopurine (BAP), and 3% sucrose produced somatic embryos at relatively high frequency (Fig 1). These embryos matured, germinated and grew into plantlets on MS medium without hormones (33% germination rate), however, the addition of 2.0mg l^{-1}kinetin (KN) significantly increased the number of plants regenerated (97.5%). Plants developed from in vitro generated plantlets grow well in greenhouse (Fig.2).

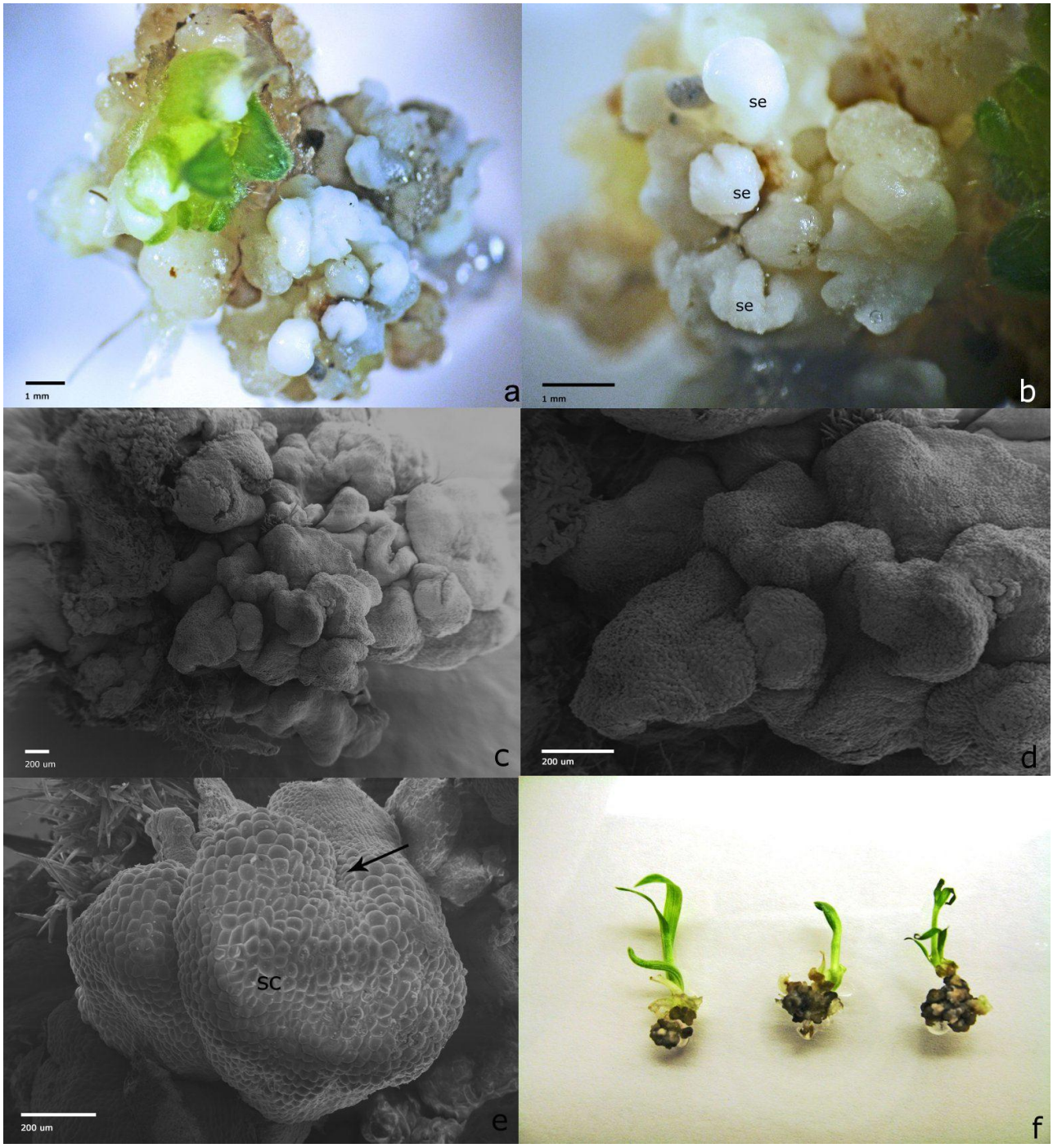

Fig 1 - Somatic embryogenesis of inflorescence-derived callus cultures of foxtail millet. (A) Callus after three weeks on regeneration media with shoot emerging from mature embryo. (B) Somatic embryos (se) in a cluster among callus tissue. (C) (D) Scanning electron microscopy image of a six week old culture showing a cluster of somatic embryos at different stages of development. (E) Somatic embryo with well developed scutellum and scutellar notch (arrowhead) from which coleoptiles emerge. (F) Plantlets regenerated from somatic embryos.

Fig.2 Plants developed from *in vito* generated plantlets

2. A number of wounding treatments and additives have been shown to increase *Agrobacterium*-mediated transient gene expression and stable genetic transformation. Thiol compounds [2-3], sonication assisted *Agrobacterium* transformation (SAAT) [4], a combination of SAAT and vacuum infiltration [5], heat and centrifugation [6], and surfactants [7], have been applied successfully to a variety of explants of different species.

Calli were subjected to differential centrifugation treatments after sonication and the application of thiol compounds in an effort to increase transient GUS expression. Explants of immature inflorescences were induced to form calli using the protocol described in 'Section 1'. *Agrobacteria* harboring pCambia1305.1 which carries the GUSPlus^{TM} reporter were grown overnight to OD_{600}=1.165, the cells were spun down and re-suspended in infiltration media (0.1X MS, 1X B5 vitamin, 3% sucrose,1.2 gl^{-1} MES, pH 5.4), and diluted to OD_{600} = 1.0. A 1M stock solution of acetosyringone dissolved in DMSO was added to a final concentration of 100 µM for a three hour induction period. The calli were sonicated for 1 minute followed by shaking at 150 rpm for 30 minutes, and then differential centrifugation treatments were applied for 1 minute at various speeds (5,000, 7,000, 9,000, 11,000, and 13,000 rpm). After centrifugation, the calli were transferred to sterilized filter paper wetted by 200 µM acetosyringone, DTT and cysteine for 5 days of co-cultivation in the dark at room temperature (70^{o}F). GUS assays were performed, and the highest centrifugation speed at 13,000 rpm produced 72% GUS positive foci (Table 3; Fig 3) compared to the slower centrifugation speeds which produced fewer foci (Table 3; Fig 3). Plants regenerated from these calli are being grown to maturity to test for the presence of the GUS transgene in Southern analyses.

Table 3. The Percentage of GUS Positive Foci Due to Different Centrifugation Treatments		
Centrifugation Speed[1]	**GUS Positive Calli**	**Percentage (%)†**
5,000 rpm	17/27	63% NS
7,000 rpm	12/34	35% NS
9,000 rpm	11/25	44% NS
11,000 rpm	10/22	45% NS
13,000 rpm	18/25	72% **

[1] All centrifugation treatments were for one minute.
†NS= not significant; **=significant at the 5% level.

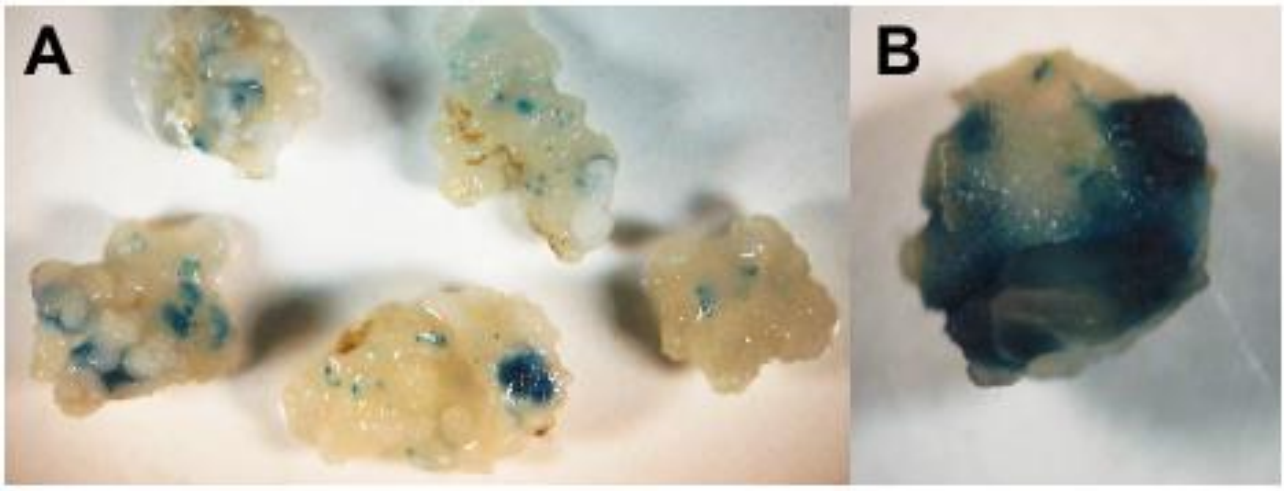

Fig 3 – Transient GUSPlus™ expression in foxtail millet calli after centrifugation for one minute at (A) 7,000 rpm and (B) 13,000 rpm. The higher centrifugation speed increased the number of GUS foci.

Sonication, centrifugation and thiol compounds have been applied to all calli that have been co-cultivated with *Agrobacterium* harboring *tb*1 and *moc1* genetic constructs (see section 3), and plantlets are currently being regenerated and analyzed for stable genetic transformation.

During the year 2009-2010 we did not test lethal dosage of hygromycin for foxtail millet callus. In the year 2011-2012, calli induced from explants described in "section 1" were tested on different hygromycin concentrations for four weeks(Table.4). After four weeks,

approximately 50% calli survived on medium containing 1.5mg l^{-1} hygromycin. Therefore, this concentration was employed for stable transformation.

Table 4. Effect of different hygromycin concentrations on callus growing

Hygromycin	Calli number	Survival Percentage (%)
0.5mgl^{-1}	100	100 % NS†
1.0mgl^{-1}	100	80 % NS†
1.5mgl^{-1}	100	50 % *
2.0 mgl^{-1}	100	20 % *
5.0 mgl^{-1}	100	0% *

†NS= not significant; *=significant at the 5% level.

Explants were transferred onto callus induction medium after three days co-cultivation. They were subcultured every two weeks, did not produce any transformants and were dead after five month culture and selection. The results indicated that hygromycin plus Agrobacterium inhibits cell to further develop.

During the year2009-2010, we used calli induced from immature inflorescence and seeds, and hygromycin as seelcdtion marker, we did not succeed in producing transformants. From the year 2011-2012, we continued to use calli from immature inflorescence and seeds on different section markers, and tried to use immature embryos for transformation.

Immature embryos as explants for transformation

Immature embryos are widely employed for genetic transformation in monocots [8-10]. Immature embryos were isolated from inflorescence pollinated in 10 days, inoculated with AGL1 harbored with pCAMBIA1305.1 for 10 minutes. Inoculation procedure was using the protocol described above. Four experiments were carried out for this purpose. The average of GUS positive foci was 43.7% (Table 5, Fig.4), which indicated that embryos can be used for stable transformation.

Table 5. The Percentage of GUS Positive Foci in immature embryos

Experiments	GUS Positive Calli	Percentage (%)
Experiment#1	41/100	41%
Experiment#2	36/100	36%

Experiment#3	47/100	47%
Experiment#4	51/100	51%
Average		43.7%

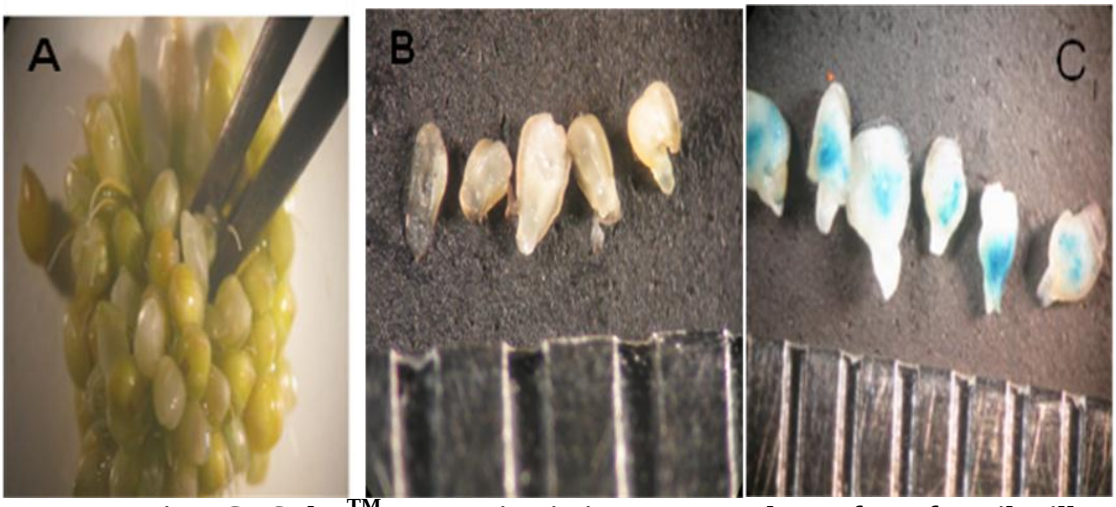

Fig.4 **Transient GUSPlus™ expression in immature embryos from foxtail millet. A. Immature seeds; B. isolated embryos; C. GUS expressed in immature embryos**

The year 2011-2012, we tried to generate transsformants using germinating seedlings from foxtail millet.

Germinating seedlings of foxtail millets were used for transformation. The junction areas between the root and shoot of seedlings were pierced two or three times with a sterile needle that had been dipped in *Agrobacterium* AGL1 harboring pCAMBIA1305.1 under a dissecting microscope. After wounding, the entire seedlings were placed in *Agrobacterium* resuspension solution for 30 minutes, and placed onto sterile filter paper in a 100 × 15 mm Petri dish wetted with 1.6 ml of a solution comprising DTT (154 mg/l; [12]), L-cysteine (400 mg/l; [11,12]) and Acetosyringone (100 μM), incubated at room temperature in the dark for 3 days of cocultivation. After three days, half of them were stained with X-Gluc, and were planted into soil respectively. After three days, 10% seedling showed GUS foci on roots or shoots or both (Fig.5). Leaf samples from these seedlings were screened with PCR screened with PCR or southern blot. No PCR or southern blot positive plants were identified.

Fig5. Transient GUS expression in seedlings after three days co-cultivation

Effect of different selection makers on transformation of foxtail millet

Due to failure in transformation in hygromycin, Kanamycin and mannose were selected for transformation. All medium and inoculation procedure were followed described above. AGL1 harbored with pCAMBIA2201 which carries GUS as s reporter gene and NPTII as selection marker were used for kanamycin test. The results showed that AGL1 harbored with pCAMBIA2201 was less infected than pCAMBIA1305.1, and calli were dead after 2 month culture and did not get transformed calli (Table 6.)

Table 6 Effect of Kanamycin on transformation of foxtail millet

Kanamycin	Calli Plated	GUS Positive	Callus cultured in medium with Kanamycin in two months
10mgl-1	500	15%	0
35mgl-1	800	19%	0
45mgl-1	780	21%	0

Kanamycin did not produce promising results. Mannose was used as selection for foxtail millet transformation. Many plants cannot grow in medium containing mannose, however, plants transformed with the phosphomaanose isomerase(PMI) gene are able to grow by utilize mannose as a carbohydrate [11]. AGL1 harbored with pPZP201 which contains PMI as a selection markers and sGFP as a reporter gene. Inoculation protocol was followed as described above with modification. After three days co-cultivation, calli were transferred onto callus induction medium containing $25gl^{-1}$ sucrose and 5 gl^{-1} for two weeks, then subculture into medium containing different concentrations of

maanose(Table 7). Although the carbohydrate mannose as a selective agent, calli did not survive in medium containing mannose, which indicated that mannose may be used for foxtail millet transformation.

Table 7 Effect of mannose on transformation of foxtail millet

Sucrose/ Mannose concentration	Calli Plated	GFP positive	Callus cultured in medium with mannose in four months
10/20	200	30%	0
10/25	200	21%	0
5/25	200	35%	0
0/30	200	27%	0

3. Two candidate genes to be examined in this work include those identified from other cereals that affect meristem initiation and elongation. One gene that alters axillary meristems is *MONOCULM1* (*moc1*) from rice, a GRAS transcription factor family member [12]. Of the many genes that affect axillary meristem outgrowth, the best known is *teosinte branched1* (*tb1*) from maize [13]. cDNAs for *moc1* and *tb1* were sub-cloned into *Agrobacterium* and biolistics over-expression vectors (Fig 3). The pANIC series of vectors (Neal Stewart, unpublished) are monocot friendly vectors designed for either over-expression or knockdown of Gateway compatible cDNAs, originally intended for the genetic transformation of switchgrass. Each gene of interest is driven by a promoter: intron for better expression in monocots. It should be noted that we chose not use foxtail millet cDNAs in genetic transformation due to the potential confounding problems of detecting homologs in Southern analyses.

The over-expression vectors and the genetic constructs used for *Agrobacterium*-mediated genetic transformation are illustrated in Figs 6a-6c, whereas the biolistics vectors and genetic constructs are illustrated in Figs 6d-6f:

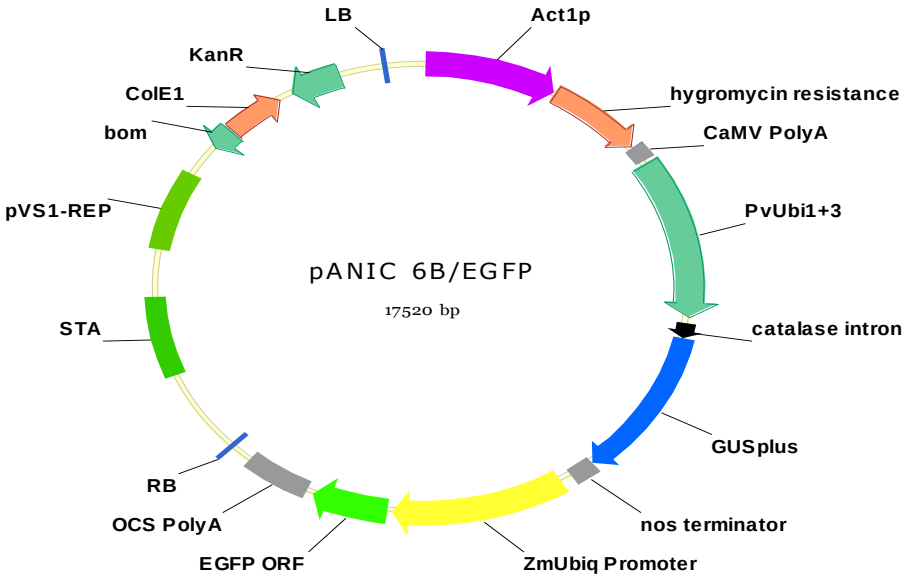

Fig 6a - The pANIC 6B *Agrobacterium* vector with the hygromycin selectable marker and the GFP, GUS reporters.

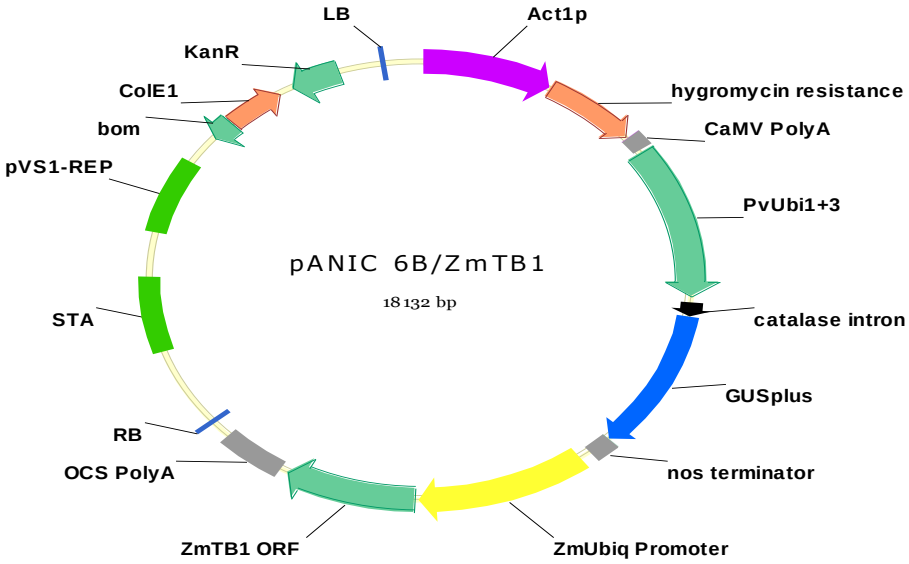

Fig 6b - The pANIC 6B *Agrobacterium* vector with the hygromycin selectable marker, the GUSPlus reporter, and the *Zea mays tb1* sequence cloned behind the *Zea mays* ubiquitin promoter.

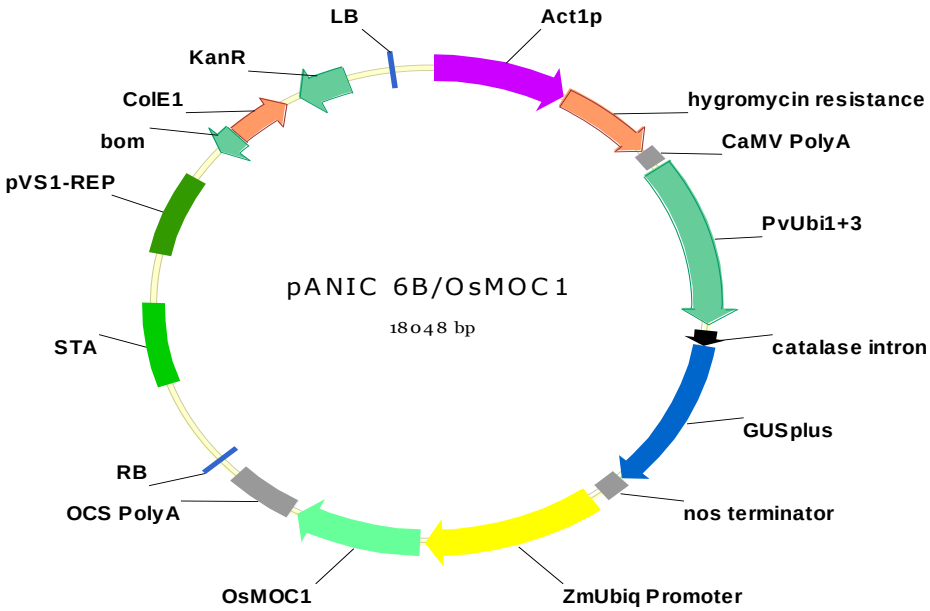

Fig 6c - The pANIC 6B *Agrobacterium* vector with the hygromycin selectable marker, the GUSPlus reporter, and the *Orysa sativa moc1* sequence cloned behind the *Zea mays* ubiquitin promoter.

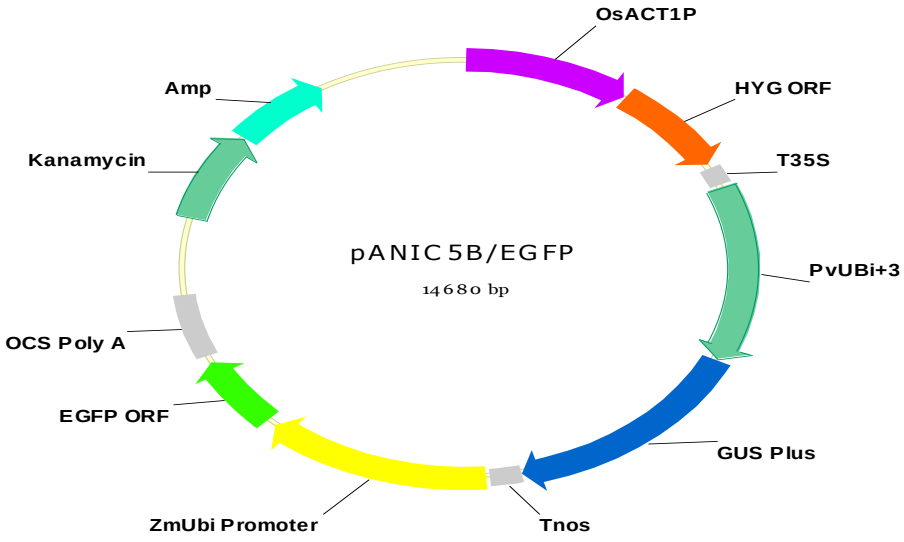

Fig 6d - The pANIC 5B biolistics vector with the hygromycin selectable marker and the GUSPlus, GFP reporters.

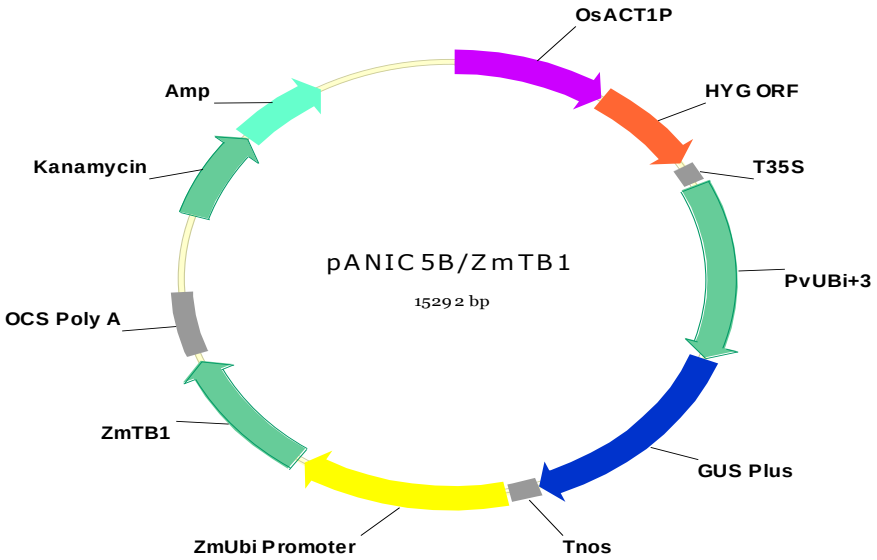

Fig 6e - The pANIC 5B biolistics vector with the hygromycin selectable marker, GUSPlus reporter, and the *Zea mays tb1* sequence cloned behind the *Zea mays* ubiquitin promoter.

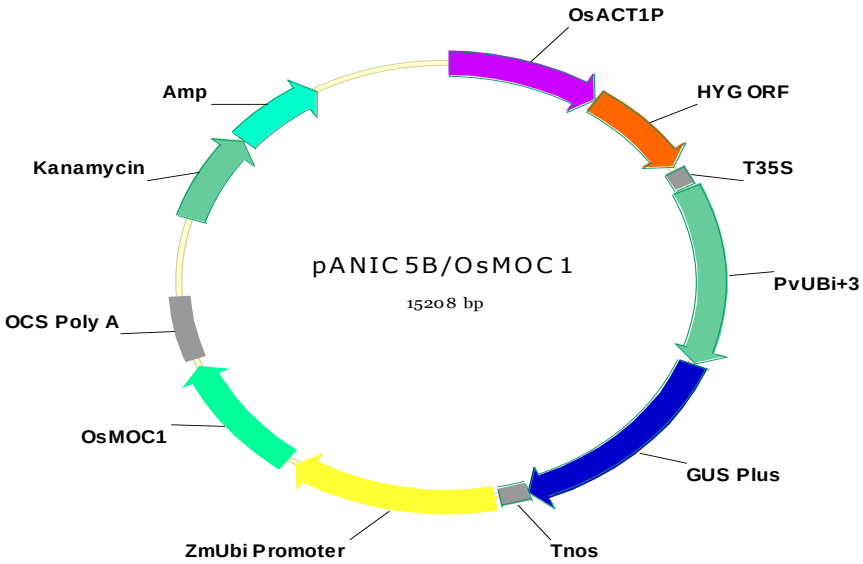

Fig 6f - The pANIC 5B biolistics vector with the hygromycin selectable marker, GUSPlus reporter, and the *Orysa sativa moc1* sequence cloned behind the *Zea mays* ubiquitin promoter.

4. In the 2009 annual report, we presented data showing the change in seed color after floral dip with *Agrobacterium* strains EHA105 and AGL1 harboring a vector that carried the maize anthocyanin *B-Peru* reporter using the floral dip. Unfortunately, only some of those seeds germinated and of those that germinated, all died in the seedling stage, possibly due to toxicity because of over-expression of the anthocyanin reporters.

In the Year 2010 we have produced putative transformants after floral dip with Agro harboring a vector carrying the hygromycin selectable marker and the red fluorescence protein (*rfp*) (Fig 7a). These seedlings grew on hygromycin selection media (20-25 gl^{-1}). There are 56/6000 and 108/4000 plants resistant to hygromycin(Table 5). Some were PCR positive for the hygromycin gene (Fig 7b) and the ubiquitin promoter: intron primer sequences (Fig 7c). The midrib of some mature plants tested positive for expression of the red fluorescence protein (Fig 7d). Unlike the anthocyanin reporters, the red fluorescence protein does not appear to negatively affect plant growth. F1 Seed were collected and tested in Southern analysis for the presence of the *rfp* sequences. Southern bolt showed there were no positive (Fig8).

Table 5 In planta transformation results

	AGL1-p1305.2	**AGL1-pANIC-2B**
Seeds screened	**6000**	**4000**
Hyg(20-25mg/l)	**56**	**108**
PCR/GUS	**0**	**19**
Sounthern blot	**n/a**	**0**

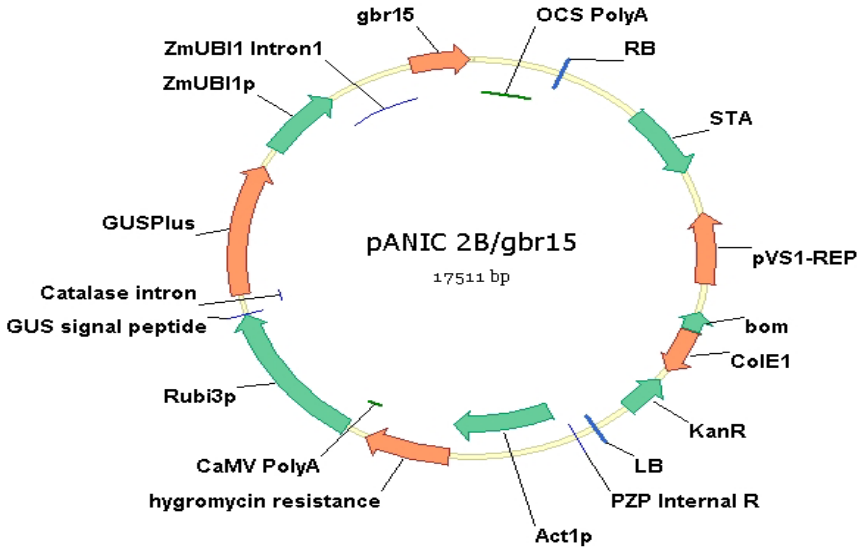

Fig 7a - The pANIC 2B over-expression vector for *Agrobacterium*-mediated transformation. This vector carries the hygromycin resistance selectable marker, GUSPlus and red fluorescence protein (*rfp*) reporter.

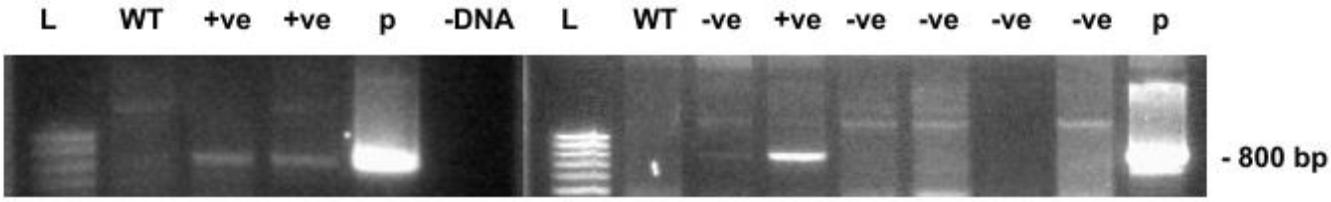

Fig 7b - Putative transformants screened for the presence of the hygromycin gene. L=ladder; WT=Wild Type; -DNA= no DNA control; +ve= positive; -ve= negative; p=pANIC vector 2B.

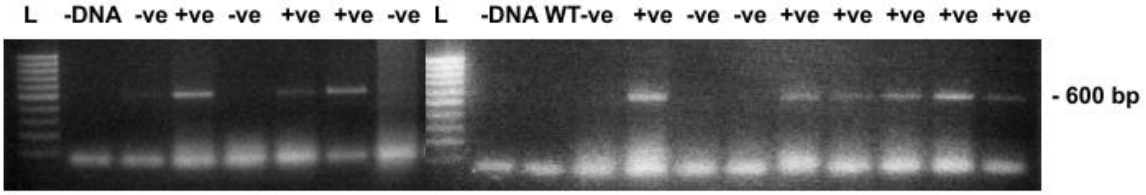

Fig 7c - Putative transformants screened for the presence of the ubiqutin promoter:intron. L=ladder; WT=Wild Type; -DNA= no DNA control; ve= positive; -ve= negative; p=pANIC vector 2B.

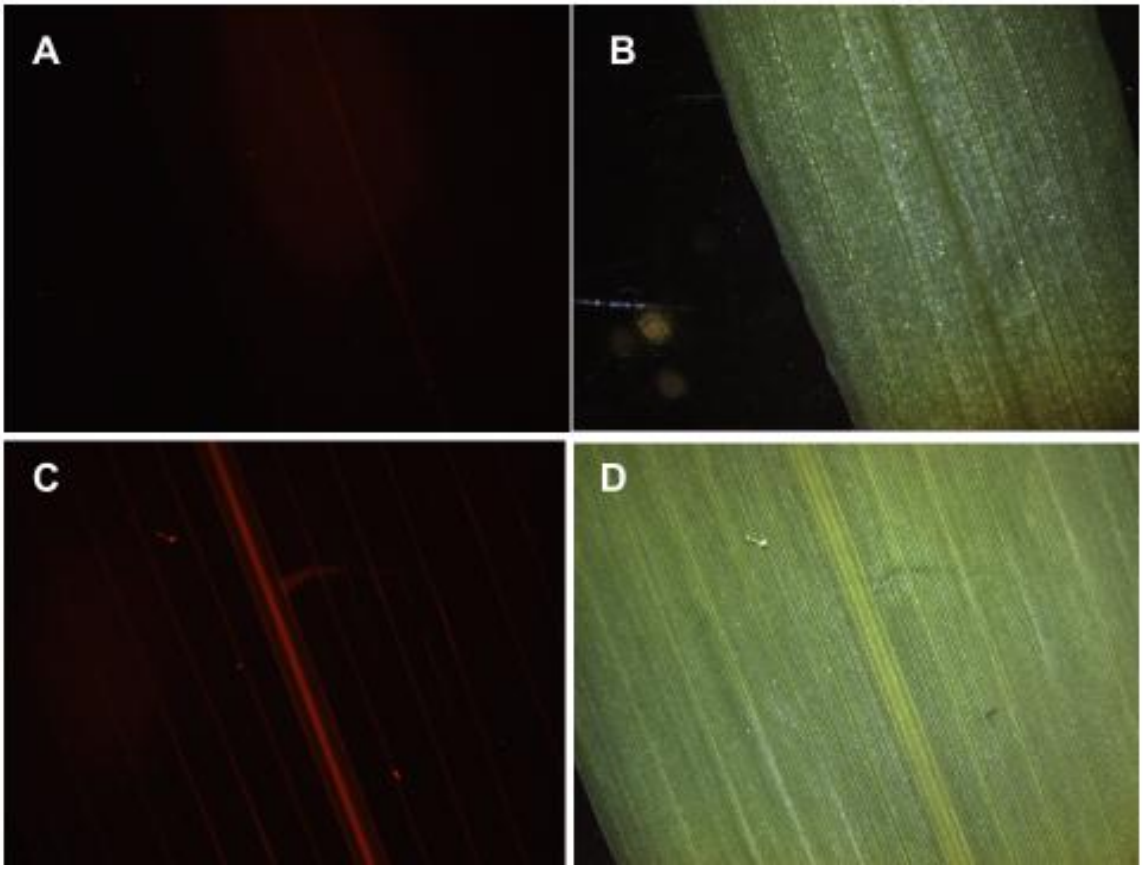

Fig 7d – Red fluorescence protein expression in the midrib of putatively transformed millet leaves vs. the control. (A) Control leaf fluorescence at 600 nm. (B) Control leaf under visible light. (C) Putatively transformed leaf fluorescence at 600 nm. (D) Putatively transformed leaf under visible light.

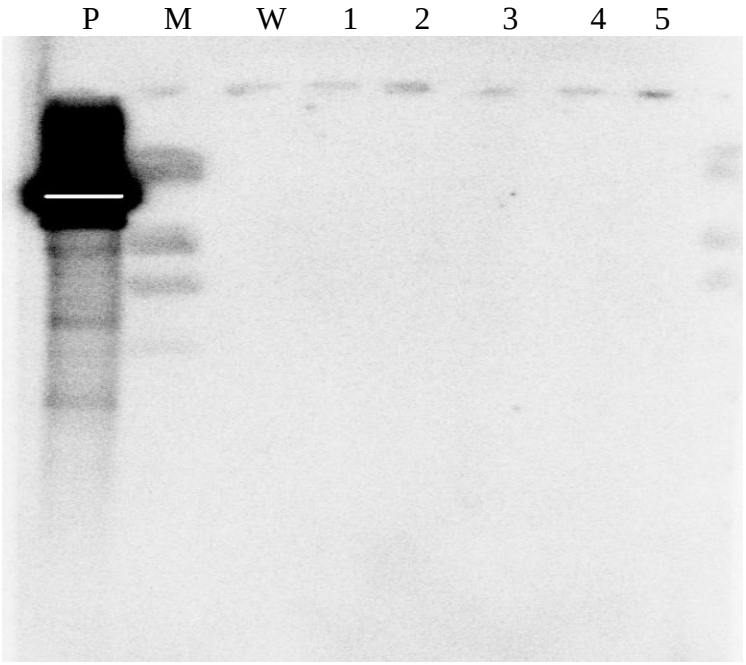

Fig8. Putative transformants tested with Southern bot.
P, rfp plasmid as positive control; M, marker; W, wild type; 1-5 putative transformants.

Prospective

Through our efforts, different DNA delivery methods, selection markers and explants were employed for foxtail millet tissue culture and transformation during the funding period. We optimized tissue culture and regeneration of foxtail millet, inoculation protocols and selection markers, which provide valuable information for future research in foxtail millet and other plants. Based on our research, future research for foxtail millet should be considered in several aspects. There are thousands of genotypes of foxtail millet around the world, certain genotypes may be more suitable for genetic transformation. In addition, new selection makers and/or DNA delivery methods can be considered for recalcitrant plants such as foxtai millet cultivar Yugu1.

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