GENETIC ENGINEERING OF SORGHUM AND SWITCHGRASS FOR IMPROVED BIOFUEL PRODUCTION

A Dissertation

Presented to

The Faculty of the Graduate School

At the University of Missouri

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

By

PHAT TIEN DO

Dr. Zhanyuan J. Zhang, Dissertation Supervisor

JULY, 2016

The undersigned, appointed by the dean of the Graduate School,
have examined the dissertation entitled

GENETIC ENGINEERING OF SORGHUM AND SWITCHGRASS FOR IMPROVED BIOFUEL PRODUCTION

Presented by Phat Tien Do,

A candidate for the degree of

Doctor of philosophy,

And hereby certify that, in their opinion, it is worthy of acceptance.

_____

Dr. Zhanyuan Zhang

_____

Dr. David Mendoza

_____

Dr. Xi Xiong

_____

Dr. David Braun

_____

Dr. William Folk

ACKNOWLEDGMENTS

I would first like to thank my dissertation supervisor, Dr. Zhanyuan J. Zhang, for all the advices, time, knowledge, and sacrifices he has given in supervising me to complete this degree of study. I could not have had the opportunity to attend and study at the University of Missouri, Columbia without his helps and supports. Dr. Zhang, thank you very much for your instructions and suggestions for my research. You let me work independently while I could get your timely advices.

I would also like to express my great appreciation to my dissertation committee, Drs. David Mendoza, David Braun, Xi Xiong and William Folk. Thank you very much for taking your time to participate in my dissertation committee, to give me helpful suggestions and discussions about my study plan and my research directions. Dr. Folk, thanks for all the comments, instructions and proofreading my publications.

I want to thank Vietnamese Government, The Minister of Education and Training of Vietnam for the scholarship ward to pursue my Ph.D. program study in the United States.

I would like to acknowledgment Drs. Alexander Jurkevich and Frank Baker at the Molecular Cytology Core at University of Missouri for microscopy analysis, and Dr. Sophie Alvarez and the Proteomics Facility at The Donald Danforth Plant Science Center, St. Louis, MO, for assistance with the mass spectrometry.

In addition, I extend my special thanks to all current and former members in Dr. Zhang's Lab, Neng Wan who provided me the useful help with greenhouse work, Joann Rose De Tar, Hyeyoung Lee, Muruganantham Mookkan and Michelle Folta who participated in

my research and shared their knowledge, Kaixuan Duan, Hanbing Li, Hua Liu, Chris Willig and Chu Wu who gave me their discussions and suggestions for research. Thanks are also extended to former postdoc SoYon Park for her supports as I began my experiments.

Most importantly, I would like to thank to my family and friends for all their love and support. To my parents, parents-in-law, brothers and sisters, thank you very much for your encouragement and support for my study, and also for taking care of my children. To my amazing wife Ngoc Thi Hong Le, I could not have done this work without your love and emotional support. I could focus on my research and study because I knew that you could do great jobs for our daughters and family as I was not at home.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENETIC ENGINEERING OF SORGHUM AND SWITCHGRASS FOR IMPROVED BIOFUEL PRODUCTION

Phat T. Do

Dr. Zhanyuan J. Zhang, Dissertation Supervisor

Abstract

Biofuels, energy sources generated from biomass, have been seen as a potential route to meet energy demand and avoid political instability and environmental issues worldwide. Switchgrass has been considered as an excellent feedstock for biofuels due to the high cellulosic content, wide adaptation as well as the lower input energy for production. Sorghum is the fifth most important crop in the world for human staple food and also a versatile feedstock for grain, sugar, and biomass production. In current study, we demonstrated that expression of the *Zea mays gibberellin 20-oxidase* (ZmGA20ox) cDNA in switchgrass improved biomass production. Under greenhouse conditions, selected transgenic plants exhibited longer leaves, internodes and tillers, which resulted in 2-fold increased biomass. This is the first parallel report on the switchgrass biomass increase through genetic engineering approach. Our results suggest that the employment of ectopic ZmGA20ox, or selection for natural variants with high level expression of endogenous GA20ox are appropriate approaches to increase biomass production of switchgrass and possible other monocot biofuel crops. Additional contribution of this study is to optimize sorghum regeneration and transformation processes using standard binary vectors and *bar* gene as a plant selectable marker. The optimized transformation process enables reproducibly to achieve over 14% transformation frequency, the highest

transformation efficiency through *Agrobacterium*-mediated transformation among the public laboratories. Of randomly analyzed independent transgenic events, 40-50% events carried a single copy of integrated T-DNA. The system developed here should be beneficial to sorghum biology study and genome exploration including genome editing.

Chapter 1
Literature Review

Biofuels

Global warming has become a major concern in recent years and it has caused short and long term effects around the world. This has caused more frequent abnormal weather conditions in recent years. Human activities such as using fossil fuels and delivering greenhouse gas emissions are thought to be main reasons for global warming (Ragauskas et al., 2006). Moreover, energy consumption by human has increased drastically. As a result, finding and developing alternative, renewable energy resources that can replace fossil energy are the focal research.

Biofuels, energy sources generated from biomass, have been seen as a potential route to avoid the global political instability, environmental issues and energy demand (Chum and Overend, 2003; Ragauskas et al., 2006). Based on characteristics of materials and manufacturing processes, biofuels are usually classified into three main generations: (1) First-generation biofuels include ethanol and biodiesel, the majority of which is used around the world today. These biofuels are directly related to the biomass that is generally edible. (2) Second-generation biofuels are produced from a wide array of different feedstock, ranging from lignocellulosic feedstock to municipal solid wastes. (3) Third-generation biofuels would be produced from algal biomass (Lee and Lavoie, 2013). The first generation biofuels are very advantageous for producers and widely used today. However, they may be hindered by the fuel-versus-food debate and the restrictions on energy consumption and land utilization. The third generation biofuels have technical and

geographical challenges in production of algal biomass. The second generation biofuels use bioenergy crops that are able to grow on area not suited for food crops such as marginal and low-cost land. In addition, plant residues could be used as materials for the second generation biofuels. Therefore, by using less expensive biomass, the second generation biofuels are considered to have more dominance than others and gaining increasing attentions from scientists in recent years (Ruth, 2008; Sticklen, 2008; Lee and Lavoie, 2013).

Switchgrass - tissue culture and genetic improvement

Switchgrass

Switchgrass (*Panicumvirgatum* L) is a perennial warm-season (C4) grass that is native to most of North America except for areas west of the Rocky Mountains and north of 55°N latitude. This grass can grow 3 to 10 feet tall, typically as a bunchgrass, but the short rhizomes can form a sod over time. This feedstock can grow in a wide range of habitats and climates and also has fewer major insect or disease pests (Vogel, 2004). Root depth of established switchgrass may reach 10 feet, but most of the root mass is in the top 12 inches of the soil profile. Based on the distribution, switchgrass has two genetically and phenotypically distinct forms or ecotypes. Upland ecotypes occur in upland areas that are not subject to flooding, whereas lowland ecotypes are found on floodplains and other areas that receive run-on water. Generally, lowland plants have a later heading date and are taller with larger and thicker stems. Upland ecotypes are either octaploids or tetraploids, whereas lowland ecotypes are tetraploids (Casler et al., 2004).

Switchgrass has been considered as an excellent potential feedstock for biofuels due to the high cellulosic content as well as the lower input energy for production (Schmer et al., 2008). In addition, this feedstock has wide adaptation, excellent conservation attributes and ease of harvesting and storage. Switchgrass biomass could be used for biofuel production as wet and dry feedstock (Nageswara-Rao et al., 2013). Establishment by seeds is another advantage of switchgrass as compared to other potential feedstock such as *Miscanthus* (Gonzalez-Hernandez et al., 2009). Therefore, switchgrass was selected as the herbaceous model species for biomass energy in the Bioenergy Feedstock Development Program of the U.S. Department of Energy (DOE). In this program, the cellulosic biofuel was predicted to supply 20% of national transportation fuels and about one-third of the biomass comes from perennial crops like switchgrass (Sanderson et al., 2006). Achieving greater biomass and getting more efficient conversion of lignocellulose to biofuels are two constraints remaining in term of using switchgrass for biofuel production. Genetic improvements with various target genes can be used as a promising area of research to overcome these constraints.

Switchgrass tissue culture and transformation systems

A long-term improvement of switchgrass was initiated by the U.S. Bioenergy Feedstock Development Program in the 1990s. Then, studies on tissue culture and regeneration of switchgrass were carried out and optimized to develop essential systems for genetic improvements (Nageswara-Rao et al., 2013). Somatic embryos and regenerated plants were obtained from different explants of switchgrass cultivar Alamo such as mature caryopses, leaves and young seedling explants (Denchev and Conger, 1994, 1995). These studies indicated that mature caryopses were valuable explants for

switchgrass regeneration due to the ease of handling and high callus induction frequency. Cell suspension systems were established to produce embryogenic callus and regenerated plants from young inflorescences (Gupta and Conger, 1999). Then, several parameters such as osmotic treatments and inoculum were optimized to increase embryogenic respond and regeneration frequency of switchgrass Alamo (Odjakova and Conger, 1999). The suspension systems are known to be advantageous due to the rapid propagation, the ease for protoplast isolation and mutant selection. However, higher cost and genotype-dependence are disadvantages as compared to solidified systems using mature caryopses.

The first report of genetic transformation in switchgrass was reported by using bombardment method (Richards et al., 2001). In this study, a construct containing reporter gene (Green fluorescent protein - *sgfp*) and selectable gene (Basta tolerance - *bar*) was transferred into immature inflorescence-derived embryogenic callus of switchgrass. The presence and expression of transgenes were confirmed by selection medium (amended with bialaphos), Southern blot and GFP expression. The inheritance of *bar* was exhibited in T1 switchgrass. *Agrobacterium tumefaciens*–mediated transformation has been seen as the most common method for switchgrass transformation since it showed high transformation frequency and low copy number of transgenes (Nageswara-Rao et al., 2013). Somleva et al., (2002) firstly used *Agrobacterium* – mediated method to transfer *bar* and *gus* genes to variety of explants including somatic embryos, embryogenic calli, plantlet segments and mature caryopses. The transformation frequency was indicated to vary from 0% to nearly 100% affected by genotypes and explants. One or two copies of T-DNA were exhibited in most of tested transgenic switchgrass. The inheritance and segregation of both transgenes were observed in T1

progeny. An improved tissue culture system was utilized for switchgrass through somatic embryogenesis by using a novel LP9 medium (Burris et al., 2009). This led to a longer periods of callus maintenance but good callus quality. Moreover, the genetic transformation capacity of this system was confirmed by 4.4% using *pporRFP* gene. The inheritance and silencing of transgenes related to different copy number were shown in the study of Xi et al., (2009). The transgene silencing was found not only in the progeny with multiple inserts but also with single copy number. As mentioned, the transformation frequency of switchgrass was highly depended on genotypes. For example, Performer, an elite variety, has been the most efficient for genetic engineering due to the high capacity in tissue cultre and transformation (Li and Qu, 2011). Its transformation frequency reached over 90% by *Agrobacterium*-mediated method after modifications of medium, infection and co-cultivation conditions. To reduce the time for transgenic production, Song et al., (2012) used basal parts of seedling as explants for *Agrobacterium*-mediated transformation in the presence of selectable marker. The transformation process was shortened by 4-5 weeks. As an alternative, a transient gene expression system has been employed as a rapid tool to test gene constructs. Agroinfiltration was successfully applied to *Agrobacterium*-mediated transient expression system of switchgrass (VanderGheynst et al., 2008; Chen et al., 2010). The transient GUS expression was observed in seedlings or harvested switchgrass leaves within 2-3 days after inoculation. Furthermore, GUS expression enabled to quantify transgene expression level by 4-Methylumbelliferyl beta-D-galactopyranoside (MUG) assays. Mazarei et al., (2008) utilized a protoplast system for transient expression of switchgrass. In this system, protoplasts were isolated from leaves and roots of two switchgrass genotypes (Alamo and Alamo2), and the expression

of *gus* gene controlling by 35S and ubiquitin promoters was observed in isolated protoplasts after using PEG-mediated DNA uptake method. In addition, other attempts have been made to improve switchgrass transformation such as promoter discovery and testing (Mann et al., 2011) and vector construction (Mann et al., 2012). Therefore, the efficient regeneration and transformation systems have been developed to provide wide applicability for switchgrass genetic engineering with agronomic and economic candidate genes.

Biomass quality improvements

Since switchgrass is considered as a model herbaceous energy cop, improving biofuel conversion from this crop biomass is an important target that is attracting more attention from scientists. The presence of lignin has been considered the most significant constraint for second generation biofuel (Simmons et al., 2010; Hisano et al., 2009; Sticklen, 2008). For these reasons, modifications of the chemical structure of lignin components and reducing lignin content of cell wall are being performed to improve switchgrass biomass quality.

Results in regulating lignin biosynthesis by RNA interference in switchgrass have been demonstrated in several recent reports. The activity of 4-Coumarate:coenzyme A ligase (*4-CL*), at a upstream in lignin biosynthesis pathway, was dramatically reduced by RNAi, leading to lignin content reduction with less guaiacyl component (Xu et al., 2011). Down-regulation of the switchgrass caffeic acid O-methyltransferase gene reduced lignin contents and increased ethanol production by 38% (Fu et al., 2011a). The decrease in the syringyl:guaiacyl mononignol ratio and the improvement of biomass quality were also

obtained. Consequently, the cost of biofuel production from transgenic switchgrass biomass was reduced due to the lower chemical and energy inputs. Furthermore, the reduction of overall lignin content and the altering of lignin composition were indicated in most of the cinnamyl alcohol dehydrogenase (CAD)-suppressed transgenic switchgrass lines. Significantly, saccharification efficiency transgenic biomass was also increased (Fu et al., 2011b; Saathoff et al., 2011).

Other efforts have also been utilized to improve switchgrass biomass quality for bioenergy application. A transcription factor (PvMYB4) was identified, characterized and overexpressed in switchgrass (Shen et al., 2012). The PvMYB4-expression significantly reduced expression levels of lignin biosynthesis genes and resulted in the reduction in lignin content and ster-linked *p*-CA : FA ratio. Transgenic switchgrass exhibited the decreased plant height and the increased tillering. In addition, the higher sugar release efficiency from cell wall was also indicated. More recently, Wuddineh et al., (2015) indicated that overexpression of gibberellin 2-oxidases not only changed switchgrass plant architecture but also reduced lignin content and syringyl/guaiacyl lignin monomer ratio.

Biomass yield improvements

Achieving greater biomass is another strategy in switchgrass utilization for biofuel production. Chuck et al., (2011) reported that overexpression of the maize *Corngrass1* microRNA in switchgrass resulted in the increase in the starch content by up to 250%. Biomass digestibility was improved with the higher release of glucose from saccharification assays. Moreover, the concern about transgene flow was reduced because

switchgrass flowering was completely inhibited under both greenhouse and field conditions. The overexpression of miRNA156 in switchgrass was shown to have morphological alterations, non-flowering phenotypes and increased biomass production (Fu et al., 2012). Of these, transgenic switchgrass exhibited 58% to 101% higher biomass yield than control wild-type. Furthermore, the increase in tiller number, sugar release and forage digestibility was also exhibited. In the research for overexpression of gibberellin 2-oxidases, transgenic switchgrass showed modified plant phenotypes and increased glucose release (Wuddineh et al., 2015). The semi-dwarf transgenic lines exhibited 35% and 24% increase in fresh and dry biomass, respectively, as compared to wild-type plants. A 41% increase in fresh-to-dry weight ratios was also recorded in these transgenic lines. Thus, the successes in using genetic engineering to improve switchgrass biomass was limited to few reports.

Increasing gibberellins biosynthesis pathway was reported to improve biomass production in various plant species such as poplar, citrus, tobacco (Eriksson et al., 2000; Biemelt et al., 2004; Fagoaga et al., 2007). In addition, the function of gibberellins in plant architecture and biomass production of switchgrass was illustrated in the research by Wuddineh et al., (2015). Therefore, modifying plant regulators by genetic engineering could be seen as a viable venue to improve biomass production.

Sorghum Transformation: Achievements, Challenges and Perspectives*

* The information in this section was published in Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants (Springer): 291-312

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a drought tolerant crop which can grow in marginal land areas where the growth of other cereals is limited. It is the fifth most important cereal after wheat, rice, maize and barley (Food and Agricultural Organization of the United Nations 2013). Sorghum can be used as a source of food for humans and animals, as well as raw materials for the production of alcoholic beverages and bioenergy (Dahlberg et al., 2011). The gluten-free flour of sorghum makes it suitable for celiac patients. In addition, sorghum consumption can improve human health due to its high antioxidant phenolics and low cholesterol content (Taylor et al., 2006; Dahlberg et al., 2011). Sorghum is a dietary staple for about 500 million people in more than 30 countries of the semi-arid tropics, especially in Africa and Asia (Dahlberg et al., 2011). In 2011, in excess of 55 million tons of sorghum was harvested from about 35 million ha grown worldwide, with an average yield of 1.5 tons per hectare. Of these, the US dedicated about 1.6 million ha and produced over 5.4 million tons with an average yield of 3.4 tons per hectare (Food and Agricultural Organization of the United Nations. 2013). Recently, ethanol production has become one of the fastest growing segments in the US sorghum industry and has led to the single largest value-added market for grain sorghum producers in America. Currently, about 15-20% of the US domestic sorghum production is used for manufacturing of ethanol and its co-products (Dahlberg et al., 2011).

Both natural and man-made interventions affect sorghum production. Natural factors include fungal diseases (Little et al., 2012; Tesso et al., 2012) insects (Guo et al., 2011), abiotic stress (Tari et al., 2012) and the parasitic weed-like *Striga* (Khan et al., 2000). Biofuel conversion not only cuts into food-based yields, but also presents new problems on how to gain the most efficiency from sorghum plants for the ethanol process. Therefore, efforts have been made to improve sorghum varieties to reduce the impacts of these limiting factors on sorghum agronomical performance. To date, most sorghum varietal improvements have been achieved through conventional breeding (Grootboom et al., 2010). However, traditional breeding for crop improvement has several limitations, including its inability to sustain yield and productivity indefinitely (Vasil, 1994). In recent years, plant biotechnology, including molecular genetics and genomics as well as plant transformation, has provided a powerful means to supplement traditional breeding approaches. Plant transformation has a unique role in varietal improvement and offers a much faster approach to accomplish genetic gains for various traits (Gurel et al., 2009; Grootboom et al., 2010). These gains will contribute to both food and biofuel industries as they relate to sorghum production.

Despite the difficulties in sorghum tissue culture and transformation progresses have been made (Zhu et al., 1998; O'Kennedy et al., 2006), twenty years after the first transgenic sorghum was developed (Casas et al., 1993), several successes in sorghum transformation have been reported which employ different transformation methods such as *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and pollen-mediated transformation. More recently, transformation studies have focused primarily on using marker genes to establish, develop and improve transformation and

regeneration processes (Nguyen et al., 2007). The production of transgenic sorghum with agronomic traits such as nutrient improvement, pest resistance, disease and stress tolerance have been reported (Zhao and Tomes, 2003; Gao et al., 2005a; Arulselvi et al., 2010; Maheswari et al., 2010). Low transformation frequency and transgene silencing are limiting factors for sorghum varietal improvement by genetic engineering. As a result, more attempts have been made to overcome these obstacles in order to meet the requirements of sorghum consumption and biofuel production.

This review discusses the contributions of genetic transformation to sorghum improvements with emphasis on transformation methods, sources of explant tissues, promoters and various candidate genes. In addition, challenges and possible strategic solutions to sorghum transformation are also discussed.

Transformation methods employing different types of explants

Although a tissue culture system for sorghum was reported about four decades ago (Gamborg et al., 1977), less progress has been made in sorghum transformation than in other cereals (Nguyen et al., 2007). Microprojectile- and *Agrobacterium*- mediated transformation methods are two main approaches that have been developed and applied for sorghum transformation. Other methods such as electroporation - and pollen-mediated transformation have also been reported.

Microprojectile transformation

Due to the host limitations by *Agrobacterium tumefaciens*, early studies on sorghum transformation focused on direct DNA delivery methods. The first two reports on sorghum transformation described the use of protoplasts and cell suspension cultures

combined with electroporation, but without success in obtaining stable transgenic sorghum plants (Battraw and Hall, 1991; Hagio et al., 1991). Fertile transgenic sorghum plants were first obtained by microprojectile bombardment of immature embryos of sorghum genotype P898012 (Casas et al., 1993). This method was later applied to transformation of immature inflorescences and other explants, such as leaf tissues and calli, with constructs carrying reporter, selectable marker and target genes (Hasegawa et al., 1995; Casas et al., 1997; Zhu et al., 1998). The transformation efficiency of the above bombardment method was very low, around 0.08 to 1%, despite some modifications (Casas et al., 1997; Able et al., 2001; Emani et al., 2002). The transformation efficiency was improved to 1.3% by the optimization of transformation conditions, including bombardment parameters such as acceleration pressure, target distance and gap width, as well as experimentation with different types of explants (Tadesse et al., 2003). Although immature, mature embryos, shoot tips and embryogenic calli were used in this study, transgenic sorghum plants were obtained only from immature embryos and shoot tips. Using shoot apices as explants for bombardment reduced the time for transgenic sorghum regeneration, but could cause transgene instability in transgenic plants (Girijashankar et al., 2005). Consequently, immature embryos were used thereafter as favored explants for microprojectile bombardment. Recently, many studies aiming at introducing different genes of interest have employed alternative explant tissues, which included inflorescences, shoot tips, or calli derived from immature embryos for sorghum transformation (Grootboom et al., 2010; Maheswari et al., 2010; Raghuwanshi and Birch, 2010; Kosambo-Ayoo et al., 2011; Brandão et al., 2012). However, these studies showed low transformation efficiencies from 0.3 to 1.3%.

Most recently, Liu and Godwin, (2012) reported a substantial improvement in particle bombardment-mediated sorghum transformation with a frequency of 20.7%; furthermore, more than 90% of transgenic plants exhibited normal growth and fertility under glasshouse condition. High frequencies of callus induction and shoot regeneration were achieved by using genotype Tx430 and an increase or addition of $CuSO_4$, KH_2PO_4, L-proline, and L-asparagine in the culture medium. DNA delivery conditions were also optimized with 0.6 µm gold particles, 18.5cm flying distance, and 1000 psi helium pressure.

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation has been used in many sorghum transformation studies. However, as with other cereal plants, this method is still subject to certain limitations that hinder sorghum transformation progress and reduce transformation efficiency. In 2000, Zhao and his colleagues first reported the production of stable transgenic plants obtained using *Agrobacteium*-mediated transformation. In this study, immature embryos were used as explants and the transformation frequency ranged from 0.95 % to 2.34%, greater than the frequency of the bombardment method used at that time. Later studies showed further improvement of *Agrobacterium*-mediated transformation. (Carvalho et al., 2004) increased the transformation to 3.5% by optimization of the infection, co-cultivation and selection conditions. By using mannose and kanamycin instead of herbicidal agents, the transformation rate was achieved at 3.3 to 4.5% (Gao et al., 2005b; Howe et al., 2006). Transgenic plant recovery further reached 5% as some factors related to callus induction, inducible treatments (e.g., cold-pretreatment of immature seeds, reduction of phenolic compounds, and tissue culture

microenvironment) were considered and optimized (Nguyen et al., 2007). Gurel et al., (2009) reported an 8.3% transformation frequency by utilizing the heat treatment of immature embryos before inoculation. Other attempts have been made to optimize parameters related to co-cultivation and regeneration media, but further improvements have not been reported (Shridhar et al., 2010; Kimatu et al., 2011). Recently, the frequency of sorghum transformation via *Agrobacterium*-mediated delivery was improved dramatically by 33% (Wu et al., 2014). This was achieved by modifications of media and the utilizing of supper binary vectors. In general, all previous results demonstrated that immature embryos were the most efficient explants for sorghum transformation by *Agrobacterium*-mediated method.

Other transformation methods

Electroporation was first utilized by combining with protoplast culture for sorghum transformation (Ou-Lee et al., 1986; Battraw and Hall, 1991). Nevertheless, this method could not be further developed and applied widely because of the lack of a protoplast-to-plant regeneration system. The electroporation of protoplasts for transformation utilizes high-voltage electric pulses applied either directly or indirectly to a solution containing plasmid DNA and protoplasts (Ou-Lee et al., 1986). To date, as is the case with most plant species, electroporation of sorghum protoplasts has been reported only for transient transgene expression and no transgenic plant has ever been obtained using this method.

Pollen-mediated transformation was another approach in sorghum transformation, inspired by previous success in several plant species including maize (Wang et al., 2001).

Pollen was subjected to ultra-sonication in a sucrose solution containing plasmid, and then the treated pollen was used to pollinate stigmas of the male sterile plants. In the case of sorghum transformation, the integration and inheritance of the introduced gene was confirmed in T0 plants using Southern-blot hybridization and antibiotic resistance in the T1 generation (Wang et al., 2007). The disadvantages of this method include low transformation frequency and difficulties in seed production due to damage of pollen after ultra-sonication. Furthermore, as is the case with other direct transformation methods, a large number of transgene copies inserted into the sorghum genome were observed as the target for gene silencing. Table 9.1 summarizes key studies in sorghum transformation.

Promoters

Promoters have drastic effects on the success of plant transformation. Using suitable promoters is essential to improve the transgenic frequency and transgene expression and, therefore, it gains considerable attention from many laboratories. It is desirable to identify strong promoters that not only provide a high expression level of the introduced genes, but also avoids transgene-induced gene silencing in the target cells.

In most early studies of sorghum transformation, the cauliflower mosaic virus (CaMV35S) promoter was used in both bombardment and *Agrobacterium*-mediated delivery methods. Despite the lower efficiency in monocotyledon than in dicotyledonous cells, this promoter has been used extensively for transformation of sorghum and other monocotyledons. The strength of the CaMV35S promoter was determined by the expression levels of transgenes in T0 and T1 plants (Casas et al., 1993, 1997; Carvalho et

al., 2004). To improve the expression of transgenes in sorghum and other cereals, an intron sequence (i.e., *il* sequence of maize) was inserted in the 5' untranslated region (5' UTR) behind the 35S promoter (Gallie and Young, 1994; Vain et al., 1996; Tadesse et al., 2003).

Monocotyledonous promoters were utilized as a potential way to enhance sorghum transformation. The *uidA* and *hpt* genes controlled by the maize alcohol dehydrogenase promoter (*adh1*) were transferred into sorghum via bombardment in the earliest study (Hagio et al., 1991). Although stable transformation was reported using sorghum cell suspension cultures, the efficiency was very low. The maize ubiquitin 1 promoter (*ubi1*) was first used for transgenic sorghum through *Agrobacterium*-mediated transformation (Zhao et al., 2000). Mendelian segregation in the T1 generation was confirmed by screening for herbicide resistance. Furthermore, by using the *ubi1* promoter and a good source of embryos, a higher frequency of stable transformation was reported than in previous studies. Able et al., (2001) evaluated the influence of three promoters involving *actin1*, CaMV35S and *ubi1* on sorghum transformation by expressing two reporter genes, *uidA* and *gfp*. This study indicated that the transient expression of *uidA* gene controlled by *ubi1* was significantly higher than with the other promoters.

In separate efforts to improve transformation efficiency, various promoters including *actin1*, *adh1*, CaMV35S, HBT (a chimeric promoter with the 35S enhancer fragment) or *ubi1*, were fused with a reporter gene and transferred into sorghum (Jeoung et al., 2002). The strength of these promoters was explained by the order *ubi1*>CaMV 35S>*HBT* for GFP expression in calli of Tx430 genotype and *ubi1*>CaMV35S>*act1*>*adh1* for GUS constructs. The activities of these heterologous

promoters *adh1*, *act1*, CaMV35S and *ubi1* were compared by using the *uiA* gene in an effort to optimize transformation conditions (Tadesse et al., 2003). The histochemical staining and enzymatic activity assay of the *gusA* gene in samples demonstrated that *ubi1* was the strongest promoter followed by *actin1*, *Adh1* and *CaMV35S*. The *ubi1* promoter was also used with different target genes, such as *manA* and *tlp*, for sorghum transformation (Gao et al., 2005b; Gurel et al., 2009). To date, *ubi1* is still considered to be the most efficient promoter for transgene expression in sorghum and is used predominantly in sorghum studies (Grootboom et al., 2010; Kosambo-Ayoo et al., 2011; Raghuwanshi and Birch, 2010; Liu and Godwin, 2012; Shridhar et al., 2010)

Several promoters of plant genes were also exploited successfully in sorghum genetic engineering in some individual studies. In a maize study (applicable to sorghum), the protease inhibitor gene *mpiC1* was induced in response to mechanical wounding and insect feeding. In an attempt to increase insect resistance, Girijashankar et al., (2005) used the maize *mpiC1* promoter to drive *CryIAc* and introduce the transgene into sorghum via shoot apices-based transformation. These authors observed a stronger expression of the *Cry*IAc gene under the control of the *mpiC1* promoter than the maize *polyubiquitin1* promoter. Recently, the kafirin promoter (*α or β kaf*) was used in sorghum transformation (Ahmad et al., 2012; Wu et al., 2014). This promoter contained endosperm specificity-determining motifs, a prolamin-box, the O2-box 1, CATC, and TATA boxes required for *α-kafirin* gene expression. This report showed that *ubi1*-GFP expression was detected throughout the plant, while the *α-kafirin*-GFP was expressed only in seeds. This success suggested a new venue for studying sorghum grain quality by using the *α-kaf* seed-specific promoter through genetic transformation.

Selectable marker and reporter genes

Selectable marker genes

An efficient selection system can be seen as the key for successful transformation. Monocotyledons are known to have a more narrow range of available marker genes than dicotyledons due to a natural endogenous resistance to some selective agents (Tadesse et al., 2003). However, various selectable marker genes have been utilized in sorghum transformation. These maker genes could be divided into three main groups, including antibiotic resistance (*hpt, npt*II), herbicide resistance (*bar)* and nutrient assimilation (*man A*).

The stable integration of neomycin phosphotransferase II (*npt*II) *gene* in transgenic sorghum was first reported by Tadesse et al., (2003). In this study, geneticin selection was used to avoid the release of phenolic substances. Mendelian inheritance of *npt*II in T1 generation was confirmed by using geneticin resistance analysis of T1 seedlings. Later studies also verified that *npt*II was an efficient antibiotic marker for transgenic selection (Howe et al., 2006; Mall et al., 2011; Liu and Godwin, 2012). Likewise, the hygromycin phosphotransferase gene (*hpt)* conferring hygromycin resistance was also used as a good selectable marker for sorghum transformation (Hagio et al., 1991; Carvalho et al., 2004; Nguyen et al., 2007; Raghuwanshi and Birch, 2010). However, as is the case with other plants, the disadvantage of using antibiotic-resistance selectable markers for sorghum is the possible migration of these genes to infectious bacteria (Balter, 1997).

The bialaphos resistance gene, *bar*, encodes *p*hosphinothricin *a*cetyl *t*ransferase (PAT) conferring herbicide resistance and is one of the most efficient selectable markers for sorghum transformation. Some glufosinate ammonium-based herbicides, such as phosphinothricin (PPT), Basta, and Bialaphos, could be used as selection agents in experiments that utilize the *bar* gene. Different concentrations of these herbicides have been used to select transgenic plants based on the types of explants and different stages during the regeneration process. For example, a 0.6% aqueous solution of Ignite/Basta (glufosinate 200 mg/ml) was used for leaf painting (Casas et al., 1993); up to 10 mg/l PPT was supplemented to callus induction medium, while lower concentrations of PPT from 1mg/l to 5 mg/l were applied in different stages of callus development and shoot regeneration (Zhao et al., 2000; Emani et al., 2002; Tadesse et al., 2003; Lu et al., 2009). Basta was used for the selection of embryogenic calli and somatic embryos at concentrations from 1 mg/l to 2.5 mg/l (Girijashankar et al., 2005; Arulselvi et al., 2010; Grootboom et al., 2010). The advantage of using the *bar* gene is to produce herbicide resistant plants. Nevertheless, *bar* selection seems to be a leaky system resulting in many escapes in sorghum. In addition, there was concern about transmission of the *bar* gene via pollen to wild relatives of sorghum (Gao et al., 2005a).

The phosphomannose isomerase (*pmi*) gene, isolated form *Escherichia coli*, has been used as a positive selectable marker gene to eliminate the risk of herbicide and antibiotic resistance genes in other monocotyledons such as maize, rice and wheat (Wright et al., 2001; Lucca et al., 2001). The *pmi* enzyme converts mannose-6-phosphate into fructose-6-phosphate, which can be used as a carbon source for plant cells. The mannose selection system was used for sorghum transformation initially by Gao et al.,

(2005a). In this study, medium containing 1% to 2% mannose was applied for embryogenic callus selection; the integration and expression of the *pmi* gene in progeny was confirmed by Southern and Western blots, respectively. The high transformation efficiency was indicated to be 2.88% for Pioneer 8505 and 3.30% for C401 genotypes. Afterwards, other independent reports again indicated the efficiency of mannose selection in sorghum transformation (Gurel et al., 2009; Grootboom et al., 2010). Until now, the highest frequency of *Agrobacteium*-mediated sorghum transformation was obtained by using the *mpi* selection system (Gurel et al., 2009; Wu et al., 2014).

Reporter genes

Among the various reporter genes, *uidA* and *gfp* are used extensively for transformation of most plant species. The *uidA* gene coding for β-glucuronidase (GUS) has been utilized in many sorghum transformation studies employing all transfer methods (Casas et al., 1993, 1997; Lu et al., 2009; Arulselvi et al., 2010; Grootboom et al., 2010; Brandão et al., 2012). The chief advantage of *uidA* is its simple detection system when compared to other reporter genes because the transient and stable expression of GUS in tissue is easily visualized without specific equipment. However, the *uidA* detection system is limited by the loss of tissue samples to the destructive assay, X-Gluc staining.

The green fluorescent protein (GFP) gene, isolated from jellyfish (*Aequorea victoria*), can be used as a reporter gene to monitor stable expression and avoid destructive assays. GFP has been found to be superior to other markers in many cases because of some favorable properties such as no need for exogenous substrates and easy visualization (Able et al., 2001; Hravska et al., 2006). In many previous studies, the

marker gene, *gfp*, was transferred into sorghum alone or together with other target genes by different methods (Jeoung et al., 2002; Gao et al., 2005a; Gurel et al., 2009; Ahmad et al., 2012; Liu and Godwin, 2012; Shridhar et al., 2010). Using the *gfp* gene to detect transgenic materials for plant transformation has two advantages because it is highly sensitive and non-destructive. Conversely, *gfp* detection requires expensive equipment, which is a disadvantage of *gfp* as a reporter gene. Another disadvantage is that high concentrations of *gfp* could adversely affect organogenesis, which in turn can cause sterility (Jeoung et al., 2002). The reduced regeneration efficiency by *gfp* accumulation in the cell organelles was also reported in some plant species (Haseloff and Amos, 1995; Able et al., 2001).

In some studies, other reporter genes have been introduced into sorghum. Casas et al., (1993) reported that the stable expression of R and C1 maize anthocyanin regulatory elements was obtained in transgenic sorghum plants under control of the CaMV35S promoter. In this study, anthocyanin accumulation could be seen in order to initially evaluate the efficiency of the sorghum transformation system. In addition, the *luc+* gene coding for firefly luciferase was transferred into both grain sorghum (Hasegawa et al., 1995) and sweet sorghum (Raghuwanshi and Birch, 2010). The integration and expression of this gene in transformed sorghum plants was confirmed by genomic Southern blot analysis and the luciferase assay. Recently, DsRed-encoded 28-kDa red fluorescent protein was overexpressed in sorghum genotype Tx430 and the expression of this protein was observed in different organs such as roots, leaves, shoots, and seeds (Wu et al., 2014).

Stress tolerance genes

Pest tolerance

In order to reduce the damage on sorghum development and yields caused by many insect species, *Bacillus thuringiensis* (*Bt*) toxin genes have been transferred into this crop. Girijashankar et al., (2005) introduced different constructs involving *ubi-cry1Ab*, *ubi-cry1Ac* and *mpiC1-cry1Ac* into sorghum by particle bombardment. The expression and inheritance of the *Bt* genes were confirmed in T1 plants by partial tolerance against first instar larvae of the spotted stem borer (*Chilopartellus Swinhoe*). However, *Bt* protein accumulated at very low contents of 1-8 ng per gram of fresh tissue of mechanically wounded leaves. In a recent report, Zhang et al., (2009) utilized *Agrobacterium*-mediated transformation to transfer the *CryIAb* gene into three sorghum cultivars, 115, ICS21B and 5-27, with an average transformation efficiency of 1.9%. Different expression levels of *Bt* protein in transgenic plants was detected by Western blotting and ELISA assays, respectively. Furthermore, transgenic plants with a high content of *Bt* protein displayed a tolerance to pink rice borer (*Sesamina inferens*). The barrier for utilization of *Cry* family genes is the very low content of *Bt* protein obtained in transgenic sorghum plants. These contents are far below the lethal dose required to give complete protection against some major insect species (Girijashankar et al., 2005).

Fungi tolerance

The rice chitinase gene (*Chi11*), which may have a protective role against fungal pathogens, is known as the first potentially agronomically useful gene introduced into sorghum. The presence of *Chi11* in transgenic sorghum was confirmed by Southern

blotting, and the expression was indicated by the improvement of resistance to disease incited by fungus (Zhu et al., 1998; Krishnaveni et al., 2001; Arulselvi et al., 2010). Both chitinase (*harchit*) and chitosanase (*harcho*) genes, isolated from *Trichoderma harzianum*, were introduced into sorghum in attempts to improve resistance to fungal diseases such as anthracnose caused by *Colletotrichum sublineolum* (Kosambo-Ayoo et al., 2011). The transgenic plants displayed greater tolerance to anthracnose as compare to the parent wild-types in both *in planta* and *ex planta* infection assays with *C. sublineolum*. Similarly, the *tlp* gene, i.e., encoding thaumatin-like protein (TLP), enhanced resistance to fungal diseases and drought and was transferred into sorghum with the *gfp* gene (Gao et al., 2005b). The result showed a 100% correlation between *gfp* expression and the presence of the *tlp* gene in transgenic plants. In addition, the strong expression of TLP was indicated by Western blot analysis.

Abiotic stress tolerance

Although the *tlp* gene, which has a function of enhancing drought tolerance, was introduced into sorghum, and the presence of this transgene was verified in T0 and T1 generations. However, the response of transgenic plants to fungus or drought was not shown (Gao et al., 2005b). To enhance the tolerance to water deficit and NaCl stress, the *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase from *E. coli* was used for sorghum transformation (Maheswari et al., 2010). The improved drought tolerance of transgenic sorghum was illustrated by the increased retention of leaf water. Moreover, there was a significantly improved maintenance in root and shoot growth of transformed plants under NaCl stress (200 mM).

Calcium-dependent protein kinases (CDPKs) are known as key players in the responses of plants to environmental attacks. Therefore, the CDPK-7 gene isolated from rice (genotype Nipponbare) was transferred into sorghum to enhance abiotic stress tolerance (Mall et al., 2011). The presence and expression of this gene was confirmed in transformed sorghum by molecular analysis. However, improvement in the tolerance to cold and salt stress was not observed under tested conditions. Instead, the result showed a lesion mimic phenotype and up-regulation of a number of pathogen related proteins along with transcripts linked to photosynthesis.

Nutrient modifications

Despite the use of sorghum as a human and animal food source, it has a low nutritional quality, e.g., being relatively poor in protein and lipid. Overproduction of the essential, but limiting amino acid, lysine, is known as a good strategy to improve sorghum grain quality. The first study on genetic engineering to improve sorghum grain quality was accomplished by Yohannes et al., (1999). In this investigation, a mutated *dhdps-rl* gene, encoding a feedback-insensitive dihydro-picolinate synthetase enzyme leading to increased lysine accumulation, was introduced into sorghum by bombardment. Later, Zhao and Tomes, (2003) used the high-lysine protein gene (HT12) for sorghum transformation via *Agrobacterium*-mediated transformation. The reported transformation rate was 2.1% and expression of HT12 in transgenic plants led to a 50% increase in total grain lysine. Sorghum lys1 tRNA synthase elements (TC2 or SKRS), together with the *bar* gene in a 2 T-DNA system, were introduced into sorghum (Lu et al., 2009). The average transformation frequency was 0.7%; the presence of the target gene was confirmed in T1 generation plants, and marker-free transgenic sorghum plants were

obtained. However, the expression of this gene and the change in lysine content were not described. Recently, Wu et al., (2013) used a super binary vector, PHP166, for sorghum transformation with the aim to improve the concentration of pro-vitamin A, mineral bioavailability, protein quality, and protein digestibility in seeds. The multiple and single-copy intact integrations of the T-DNA were verified in transgenic plants, but transgene expression was not reported.

Challenges in sorghum transformation

Clearly, transformation plays a unique role in sorghum genetic improvement and biological studies and has gained significant attention from scientists around the world. However, the transformation efficiency, even two decades after the first production of fertile transgenic sorghum, remains too low to satisfy the requirements of sorghum genetic engineering. This is in sharp contrast with some other cereal crops, whose transformation protocols have been improved considerably. Progress in sorghum transformation has been hampered by many difficulties associated with tissue culture, the transformation process itself and transgene silencing.

Tissue culture barrier

Reproducible generation of transgenic plants depends on an efficient tissue culture system. However, sorghum is considered to be the most recalcitrant crop among the cereals for its *in vitro* response (Gao et al., 2005b; Pola and Sarada, 2006; Girijashankar et al., 2007; Arulselvi and Krishnaveni, 2009; Sadia et al., 2010). Accumulation of phenolic compounds and a high degree of genotype dependence are known as the major barriers for sorghum tissue culture.

The release of phenolics into the medium was a well-known problem for tissue culture due to strong negative effects on cell differentiation, somatic development and plant regeneration (Zhao et al., 2000; Tadesse et al., 2003; Gao et al., 2005a; Howe et al., 2006). These compounds not only decreased the frequency of sorghum regeneration, but also were toxic to *Agrobacterium* cells in transformation experiments (Nguyen et al., 2007). More phenolic substances were observed in red sorghum, hybrid sorghum and some public varieties, hinder the use of these genotypes for regeneration and transformation (Gao et al., 2005a; Nguyen et al., 2007). A number of culture manipulations have been developed to alleviate the effects of phenolic compounds in tissue culture such as reducing the sub-culturing intervals, the addition of polyvinylpolypyrrolidone (PVPP) to the medium (Zhao et al., 2000; Gao et al., 2005a; Lu et al., 2009), and the use of activated charcoal and cold pretreatment (Nguyen et al., 2007). However, short subculture intervals require more labor and materials, which raise the cost of the culture process. PVPP and activated charcoal reduce the effective concentration of certain growth regulators and therefore, affect the *in vitro* response of the tissue (Howe et al., 2006).

To date, the successful recovery of transgenic plants through *Agrobacterium*-mediated or particle bombardment was achieved mainly using immature embryos, in spite of various explants utilized, which include immature embryos, inflorescences or shoot tips. Nevertheless, the frequency of callus induction and plant regeneration from immature embryos varies widely and depends especially on plant genotype. Consequently, different genotypes have different transformation efficiencies even though the same culture and transformation conditions are employed (Casse et al., 1993, 1997;

Zhao et al., 2000; Able et al., 2001; Gao et al., 2005a; Howe et al., 2006; Raghuwanshi and Birch, 2010; Kosambo-Ayoo et al., 2011). Casse et al., (1993) reported that after DNA delivery, only three of eight genotypes produced embryogenic calli on selection medium, and only genotype P898012 regenerated plants under bialaphos selection. Genotype dependence was again demonstrated as the drawback for tissue culture in recent reports on sorghum regeneration (Maheswari et al. 2006; Jogeswar et al. 2007; Arulselvi and Krishnaveni 2009). Sorghum genotypes such as Tx430 and P898012 have been considered to be appropriate materials for regeneration and transformation, regardless of the fact that many sorghum genotypes have been screened and used in studies. Therefore, it is imperative to compare these genotypes alongside experiments to identify highly regenerable genotypes (Kumar et al., 2011; Gurel et al., 2009; Howe et al., 2006), and to establish further an optimal protocol for tissue culture and transformation.

Transformation conditions

Agrobacterium-mediated sorghum transformation is known to have advantages over other methods, especially for generating a high proportion plants with single copy of transgenes and reduced chances of gene silencing and instability (Zhao et al., 2000; Gao et al., 2005a, b; Howe et al., 2006; Nguyen et al., 2007; Lu et al., 2009). However, similar to some other cereals, sorghum has been recalcitrant to *Agrobacterium*-mediated transformation. The interaction between bacterial cells and sorghum tissue could be improved by pre-induction of *Agrobacterium* with acetosyringone, using tissues that have actively dividing cells, and heat-cold pretreatment of explants (Verma et al., 2008; Gurel et al., 2009). Other ways to increase transformation include the use of greater

concentrations of *Agrobacterium* or longer co-cultivation time (Zhao et al., 2000). Nevertheless, the above treatment conditions could be plant species- or genotype-dependent and, therefore, may not necessarily promote high transformation efficiency and could even cause negative effects on transgenic plant recovery. Zhao et al., (2000) reported that too high concentration of bacteria caused serious damage of explant tissues during the *Agrobacterium* inoculation period, and the overgrowth of bacteria interfered with callus growth on the medium. This observed when high concentrations of bacteria were used, contributing to the failure in transgenic regeneration (Gao et al., 2005b). Moreover, *Agrobacterium* is a plant pathogen which is capable of inducing plant necrosis; it also reduces regeneration and transformation efficiency (Hansen, 2000). In fact, this problem has been reported in several sorghum transformation studies (Gao et al., 2005b; Nguyen et al., 2007). Additionally, immature embryos proved to be sensitive to *Agrobacterium* infection and embryo death after co-cultivation was the limiting factor in improving transformation efficiency (Carvalho et al., 2004).

Likewise, the low frequency of sorghum transformation via microparticle bombardment was known to be associated with the difficulty of DNA delivery and tissue damage (Able et al., 2001). Increasing particle flow by using a higher acceleration pressure could improve DNA delivery, but at the same time, it could cause more extensive tissue damage which is detrimental to callus induction, cell differentiation and plant recovery. For example, at a high pressure of particle flow (1800 psi), more than 90% of bombarded tissues became necrotic; regenerable calli and somatic embryos did not develop (Tadesse et al., 2003). Similarly, in a separate study, 10% of the shoot apices were killed when high helium gas pressure was employed for bombardment

(Girijashankar et al., 2005). Although several parameters such as the microprojectile size, DNA coating of the microprojectiles, distance to the target tissue and the velocity of gas flow were evaluated and optimized, the efficiency of sorghum transformation via bombardment was still less than those of other crops (Able et al., 2001; Tadesse et al., 2003; Liu and Godwin, 2012).

Finally, selection pressures influence cell differentiation and reproduction of transgenic tissue. Negative selective agents, such as antibiotics or herbicides, have been known to cause detrimental effects on plant tissue culture and hinder the regeneration process (Zhao et al., 2000; Gao et al., 2005b). Untransformed cells subjected to stress by selection substrates release phenolic compounds that are toxic for transformed cells. For example, the release of phenolic substances from herbicide-treated explants during the regeneration process was a key reason for failure in the production of transgenic sorghum plants via phosphinothricin-selection (Tadesseet al., 2003; Lu et al., 2009). In some cases, the selection pressure on sorghum tissue could be reduced by using a low concentration of selection agents in combination with rapid selection to regenerate plants (Lu et al., 2009), or by using visual maker genes such as *gfp* without using antibiotics or herbicides as the selection agents (Gao et al., 2005b). However, these approaches would allow generating more "escapes" (i.e., non-transgenic events), decrease the efficiency of selection process, and increase the time and resources necessary for the analysis of transformed plants.

Transgene silencing

Transgene silencing has been observed in both dicotyledons (Matzke and Matzke, 1995) and monocotyledons (Iyer et al., 2000). Methylation of the introduced DNA and homology-dependent ectopic pairing were known as the major pathways leading to transgene inactivation (Demeke et al., 1999; Iyer et al., 2000; Fagard and Vaucheret, 2000). In sorghum transformation, transgene silencing appears to be a problem because it is not attributed to variation in copy number, or the method of transformation. For example, the GUS gene has been widely used in sorghum transformation. However, the silencing of this gene was indicated in many reports. Early studies showed that GUS-transformed cells did not display blue staining upon incubation with the histochemical substrate X-Gluc, or they showed a very low level of ß-glucuronidase activity (Hagio et al., 1991; Battraw and Hall, 1991). Casas et al., (1993) observed that the GUS gene was not expressed after sustained periods of culture although the presence of this gene was confirmed by Southern analysis. They suggested that the expression of transgenes was inactivated by DNA methylation in the transformed sorghum cells. In 1997, Casas and his colleagues also observed that GUS activity could not be detected in T1 plants containing the GUS gene. Zhu et al., (1998) also found that both *bar* and rice chitinase genes were present, but silenced at certain developmental stages in a few primary transgenic plants (T0) as confirmed by Southern and Western blots, respectively. Emani et al., (2002) confirmed that multiple copies of the *bar* as well as the *gus* genes had integrated into the sorghum genome. The expression of the *bar* gene was observed in T0, T1 and T2 generations. However, GUS expression was not found in all tissues tested from regenerated T0 plants. Moreover, by using reactivation agents and different

promoters, these workers demonstrated that methylation-based transgene silencing was the reason for the suppression and inactivation of transgenes.

Future perspectives

Over the past two decades since the production of the first transgenic sorghum plants, many sorghum transformation studies with various DNA delivery methods have been reported. Not only various marker genes have been used to establish, confirm and optimize sorghum transformation protocols, but also some agronomical important genes such as genes for pest, disease and abiotic tolerance have been transferred into sorghum. Future sorghum transformation research efforts will continue to focus on enhancing the value of sorghum for food consumption and biofuel production.

Improvement of grain quality

Grain sorghum is a major staple for millions of people in Africa and Asia, and a major livestock feed in developing countries. Nevertheless, the low nutritional content is limiting its value as food and feed. Attempts to improve the lysine content of sorghum grain using transformation was reported in early studies (Yohannes et al., 1999; Zhao and Tomes, 2003), and the need for such an improvement has gained more attention recently from scientists around the world. As discussed earlier, Ahmad et al., (2012) studied the endosperm-specific expression of the α-kafirin promoter that was isolated from sorghum using the *gfp* gene as a reporter. This result implied that the identification of a sorghum grain-specific promoter could open up the opportunity to express ectopically candidate genes in endosperm for grain quality improvement.

Sorghum grains are known to have relatively poor digestibility in comparison to those of other cereal grains. Kafirins, the main sorghum proteins resistant to digestion, account for more than 80% of the protein in the endosperm of the sorghum grain (Hamaker et al., 1995). These proteins are co-translationally translocated to the endoplasmic reticulum (ER) and assembled into discrete protein bodies which tend to be poorly digestible in food and feed applications (Kumar et al., 2011). Therefore, using genetic engineering techniques to reduce the expression of different kafirin subclasses is a promising approach to improve sorghum grain quality (Da Silva et al., 2011; Kumar et al., 2011).

In the attempt to improve the staple food for about 300 million people in Africa, the Africa Biofortified Sorghum (ABS) project was established by the collaboration of 13 organizations with two main phases. It was initiated by 2005 and scheduled for completion in 2015. Achieving increased beta carotene concentration and stabilization, increasing iron and zinc bioavailability, and improvement in protein digestibility, are targeted traits that have been the main focus in this project. The progress of ABS updated on September 2012 showed that hundreds of transgenic events have been produced and analyzed for enhanced beta carotene. The next steps of the ABS is to determine and optimize the final transgenic constructs for the β-carotene gene and Fe and Zn bioavailability gene. Moreover, transgenic sorghum should be evaluated by using animal model systems (The Africa Biofortified Sorghum 2012).

Increase biofuel conversion

Due to the multiple uses of sorghum, there are now several research programs being developed that emphasize the development of grain, particularly sweet and cellulosic sorghums, for biofuel production (Rooney et al., 2007). Sorghum starch and sugar are now being used for biofuel production. Modifications in starch deposition, digestibility and sugar content would strongly influence ethanol production from sorghum grain (Rooney et al., 2007). Thus, the improvement of starch and sugar contents of sorghum grain using genetic engineering is predicted to gain more effort from researchers globally. In addition, a large and sustainable supply of biomass must be made for profitable biofuel production from lignocellulose. This will require the development of specialty crops for bioenergy production (Rooney et al., 2007). However, high biomass but low saccharification potential would waste energy and labor for harvesting, storing, transporting, and biofuel production. Hence, increasing biomass as well as saccharification yield will maximize biofuel yield. As a consequence, this could be another area in which sorghum transformation could play a role to accelerate energy production. Wang et al., (2011) identified two markers on sorghum chromosomes which are associated with saccharification yield. They found that these markers are physically close to genes which encode plant cell wall synthesis enzymes. They further proposed to evaluate the impact of these candidate genes on saccharification in sorghum through genetic transformation.

For the second generation biofuel (cellulose ethanol), lignin is known to impede conversion of lignocellulose into ethanol. Cellulosic biomass is always more difficult than starch to be broken down into sugars due to the presence of lignin and the complex structure of cell walls. Modifying the chemical structures of lignin components and/or

reducing plant lignin could decrease pretreatment costs in bioethanol production from cellulosic biomass (Ragauskas et al., 2006). Using genetic engineering to reduce lignin content has been attempted for some plant species such as hybrid poplar (Hu et al., 1999) and switchgrass (Fu et al., 2011a; Xu et al., 2011). Recently, Dien et al., (2009) indicated that some *brown midrib (bmr)* mutations in forage sorghum not only reduced lignin content significantly, but also improved glucose yields of sorghum biomass. Therefore, changing lignin components and content by genetic engineering would be important strategies to increase the potential of sorghum as a biofuel feedstock.

Exploitation of sorghum genomes

The sorghum genome has been sequenced by the whole-genome shotgun (WGS) method and approximately 98% of the total predicted genes (34,496) have been placed in their chromosomal context (Paterson et al., 2009). These genomics resources offer great potential to improve sorghum genetically. Using genetic transformation to introduce, express, and modulate genes in transgenic plants represents a very powerful tool to examine directly gene functions, and also provides a means to broaden the sorghum germplasm for genetic improvement. Verma et al.,(2011) induced and generated stable *Ds*-tagged mutants in sorghum via *Agrobacterium*-mediated transformation. The *Ds*-tagged mutants are used commonly for mutagenesis and functional genomics. Thus, this result could be seen as a good example for the utilization of sorghum transformation to study genome functions. Most recently, precise genome editing technologies have emerged and advanced rapidly. These technologies, particularly CRISPR/Cas9 [Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated (Cas) 9] as a simple and powerful approach (Gaj et al., 2013; Li et al., 2013; Shan et al.,

2013), deems to enhance sorghum genome exploitation, benefiting sorghum genetic studies and transgene-free variety development.

Conclusion

Sorghum is one of the most important crops in the world due to its food value and potential for bioenergy production. Genetic engineering is capable of supplementing traditional methods of improving sorghum as a food and feedstock. Among the DNA-delivery methods that have been utilized for sorghum transformation, the bombardment and *Agrobacterium*-mediated methods are the most efficient. Some agronomical traits such as nutrient improvement, pest resistance, disease tolerance and stress tolerance have been achieved through sorghum genetic engineering. Several factors are known to play an important role in sorghum genetic engineering. Promoters have great impact on the success of sorghum genetic engineering because they directly influence the expressions of transgenes in sorghum. *Ubi1*, a maize ubiquitin 1 promoter, was indicated as the strongest promoter for sorghum transformation and was used in recent studies with both marker genes and genes of interest. Furthermore, the use of *mpiC1* and *α-kafirin* promoters through transgenic approaches has excellent potential for sorghum genetic improvement. Herbicide and antibiotic selection systems have been used widely in sorghum transformation. However, the high pressure of these negative selective agents on cell differentiation and development reduces regeneration and transformation efficiency. Moreover, there is a concern about possible migration of *bar* and antibiotic genes to wild relatives of sorghum, or to infectious bacteria. Using mannose selection as a positive selection system has overcome the side effect of the negative selective agents and has indeed increased sorghum transformation efficiency. Sorghum has been known to be the

most recalcitrant crop for genetic engineering. Nevertheless, to date, sorghum engineering frequency has increased significantly due to improvements in tissue culture and transformation conditions. Finally, genome sequencing, together with discovery of candidate genes and promoters, will continue to be very useful for sorghum genetic engineering. These new genetic resources provide opportunities to develop sorghum varieties with important traits required for food consumption and bioenergy production. New emerging transgene technologies especially precise genome editing technology including CRISPR/Cas9 should revolutionize sorghum genetic improvements and biology studies.

References

Able, J.A., Rathus, C., and Godwin, I.D. (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *Vitro Cell. Dev. Biol.-Plant* **37**: 341–348.

Ahmad, N., Sant, R., Bokan, M., Steadman, K.J., and Godwin, I.D. (2012). Expression Pattern of the Alpha-Kafirin Promoter Coupled with a Signal Peptide from Sorghum bicolor L. Moench. *J. Biomed. Biotechnol*., doi:10.1155/2012/752391

Arulselvi, I. and Krishnaveni, S. (2009). Effect of hormones, explants and genotypes in in vitro culturing of sorghum. *J. Biochem. Technol*. **1**: 96–103.

Arulselvi, P.I., Michael, P., Umamaheswari, S., Krishnaveni, S., and others (2010). Agrobacterium mediated transformation of Sorghum bicolor for disease resistance. Int. *J. Pharma Bio Sci*. **1**: 272–281.

Balter, M. (1997). Transgenic corn ban sparks a furor. *Science* **275**: 1063–1063.

Battraw, M. and Hall, T.C. (1991). Stable transformation of Sorghum bicolor protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes. *Theor. Appl. Genet.* **82**: 161–168.

Biemelt, S., Tschiersch, H., and Sonnewald, U. (2004). Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiol*. **135**: 254–265.

Brandão, R.L., Carneiro, N.P., de Oliveira, A.C., Coelho, G.T., and Carneiro, A.A. (2012). Genetic Transformation of Immature Sorghum Inflorescence via Microprojectile Bombardment. *Transgenic Plants - Adv Limit*:133-148

Burris, J.N., Mann, D.G., Joyce, B.L., and Stewart Jr, C.N. (2009). An improved tissue culture system for embryogenic callus production and plant regeneration in switchgrass (Panicum virgatum L.). *BioEnergy Res*. **2**: 267–274.

Carvalho, C.H.S., Zehr, U.B., Gunaratna, N., Anderson, J., Kononowicz, H.H., Hodges, T.K., and Axtell, J.D. (2004). Agrobacterium-mediated transformation of sorghum: factors that affect transformation efficiency. *Genet Mol Biol* **27**: 259–269.

Casas, A.M., Kononowicz, A.K., Haan, T.G., Zhang, L., Tomes, D.T., Bressan, R.A., and Hasegawa, P.M. (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *Vitro Cell. Dev. Biol.-Plant* **33**: 92–100.

Casas, A.M., Kononowicz, A.K., Zehr, U.B., Tomes, D.T., Axtell, J.D., Butler, L.G., Bressan, R.A., and Hasegawa, P.M. (1993). Transgenic sorghum plants via microprojectile bombardment. *Proc. Natl. Acad. Sci*. **90**: 11212–11216.

Casler, M.D., Vogel, K.P., Taliaferro, C.M., and Wynia, R.L. (2004). Latitudinal adaptation of switchgrass populations. *Crop Sci*. **44**: 293–303.

Chen, X., Equi, R., Baxter, H., Berk, K., Han, J., Agarwal, S., and Zale, J. (2010). Research A high-throughput transient gene expression system for switchgrass (Panicum virgatum L.) seedlings. *Biotechnology for Biofuels*., doi: 10.1186/1754-6834-3-9

Chuck, G.S., Tobias, C., Sun, L., Kraemer, F., Li, C., Dibble, D., Arora, R., Bragg, J.N., Vogel, J.P., Singh, S., and others (2011). Overexpression of the maize Corngrass1 microRNA prevents flowering, improves digestibility, and increases starch content of switchgrass. *Proc. Natl. Acad. Sci*. **108**: 17550–17555.

Chum, H.L. and Overend, R.P. (2003). Biomass and bioenergy in the United States. *Adv. Sol. Energy* **15**: 83–148.

Dahlberg, J., Berenji, J., Sikora, V., and Latkovic, D. (2011). Assessing sorghum [Sorghum bicolor (L) Moench] germplasm for new traits: food, fuels & unique uses. *Maydica* **56**: 85–92.

Demeke, T., Hucl, P., B\aaga, M., Caswell, K., Leung, N., and Chibbar, R.N. (1999). Transgene inheritance and silencing in hexaploid spring wheat. *Theor. Appl. Genet*. **99**: 947–953.

Denchev, P.D. and Conger, B.V. (1995). In vitro culture of switchgrass: influence of 2, 4-D and picloram in combination with benzyladenine on callus initiation and regeneration. *Plant Cell Tissue Organ Cult.* **40**: 43–48.

Denchev, P.D. and Conger, B.V. (1994). Plant Regeneraton from Callus Cultures of Switchgrass. *Crop Sci*. **34**: 1623–1627.

Dien, B.S., Sarath, G., Pedersen, J.F., Sattler, S.E., Chen, H., Funnell-Harris, D.L., Nichols, N.N., and Cotta, M.A. (2009). Improved sugar conversion and ethanol yield for forage sorghum (Sorghum bicolor L. Moench) lines with reduced lignin contents. *BioEnergy Res*. **2**: 153–164.

Emani, C., Sunilkumar, G., and Rathore, K.S. (2002). Transgene silencing and reactivation in sorghum. *Plant Sci*. **162**: 181–192.

Eriksson, M.E., Israelsson, M., Olsson, O., and Moritz, T. (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol*. **18**: 784–788.

Fagard, M. and Vaucheret, H. (2000). (Trans) gene silencing in plants: how many mechanisms? *Annu. Rev. Plant Biol*. **51**: 167–194.

Fagoaga, C., Tadeo, F.R., Iglesias, D.J., Huerta, L., Lliso, I., Vidal, A.M., Talon, M., Navarro, L., García-Martínez, J.L., and Peña, L. (2007). Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture. *J. Exp. Bot*. **58**: 1407–1420.

Food and Agricultural Organization of the United Nations, 2013 FAOSTAT ProdSTAT, Production Crops http://faostatfaoorg/site/567/defaultaspx#ancor (accessed 16 January 2013)

Fu, C., Mielenz, J.R., Xiao, X., Ge, Y., Hamilton, C.Y., Rodriguez, M., Chen, F., Foston, M., Ragauskas, A., and Bouton, J. (2011a). Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proc. Natl. Acad. Sci*. **108**: 3803–3808.

Fu, C., Sunkar, R., Zhou, C., Shen, H., Zhang, J.-Y., Matts, J., Wolf, J., Mann, D.G., Stewart, C.N., and Tang, Y. (2012). Overexpression of miR156 in switchgrass (Panicum virgatum L.) results in various morphological alterations and leads to improved biomass production. *Plant Biotechnol. J*. **10**: 443–452.

Fu, C., Xiao, X., Xi, Y., Ge, Y., Chen, F., Bouton, J., Dixon, R.A., and Wang, Z.-Y. (2011b). Downregulation of cinnamyl alcohol dehydrogenase (CAD) leads to improved saccharification efficiency in switchgrass. *BioEnergy Res*. **4**: 153–164.

Gaj, T., Gersbach, C.A., and Barbas III, C.F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. **31**: 397–405.

Gallie, D.R. and Young, T.E. (1994). The Regulation of Gene Expression in Transformed Maize Aleurone and Endosperm Protoplasts (Analysis of Promoter Activity, Intron Enhancement, and mRNA Untranslated Regions on Expression). *Plant Physiol*. **106**: 929–939.

Gamborg, O.L., Shyluk, J.P., Brar, D.S., and Constabel, F. (1977). Morphogenesis and plant regeneration from callus of immature embryos of sorghum. *Plant Sci. Lett*. **10**: 67–74.

Gao, Z., Jayaraj, J., Muthukrishnan, S., Claflin, L., and Liang, G.H. (2005a). Efficient genetic transformation of sorghum using a visual screening marker. *Genome* **48**: 321–333.

Gao, Z., Xie, X., Ling, Y., Muthukrishnan, S., and Liang, G.H. (2005b). Agrobacterium tumefaciens-mediated sorghum transformation using a mannose selection system. *Plant Biotechnol. J*. **3**: 591–599.

Girijashankar, V., Sharma, H.C., Sharma, K.K., Swathisree, V., Prasad, L.S., Bhat, B.V., Royer, M., San Secundo, B., Narasu, M.L., and Altosaar, I. (2005). Development of transgenic sorghum for insect resistance against the spotted stem borer (Chilo partellus). *Plant Cell Rep*. **24**: 513–522.

Girijashankar, V., Sharma, K.K., Balakrishna, P., and Seetharama, N. (2007). Direct somatic embryogenesis and organogenesis pathway of plant regeneration can seldom occur simultaneously within the same explant of sorghum. *J. SAT Agric. Res*. **3**: 1–3.

Gonzalez-Hernandez, J.L., Sarath, G., Stein, J.M., Owens, V., Gedye, K., and Boe, A. (2009). A multiple species approach to biomass production from native herbaceous perennial feedstocks. *Vitro Cell. Dev. Biol.-Plant* **45**: 267–281.

Grootboom, A.W., Mkhonza, N.L., O'Kennedy, M.M., Chakauya, E., Kunert, K.J., and Chikwamba, R.K. (2010). Biolistic mediated sorghum (Sorghum bicolor L. Moench) transformation via mannose and bialaphos based selection systems. *Intl J Bot* **2**: 89-94.

Guo, C., Cui, W., Feng, X., Zhao, J., and Lu, G. (2011). Sorghum Insect Problems and ManagementF. *J. Integr. Plant Biol*. **53**: 178–192.

Gupta, S.D. and Conger, B.V. (1999). Somatic embryogenesis and plant regeneration from suspension cultures of switchgrass. *Crop Sci*. **39**: 243–247.

Gurel, S., Gurel, E., Kaur, R., Wong, J., Meng, L., Tan, H.-Q., and Lemaux, P.G. (2009). Efficient, reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. *Plant Cell Rep*. **28**: 429–444.

Hagio, T., Blowers, A.D., and Earle, E.D. (1991). Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Rep*. **10**: 260–264.

Hamaker, B.R., Mohamed, A.A., Habben, J.E., Huang, C.P., and Larkins, B.A. (1995). Efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. *Cereal Chem*. **72**: 583–588.

Hansen, G. (2000). Evidence for Agrobacterium-induced apoptosis in maize cells. *Mol. Plant. Microbe Interact*. **13**: 649–657.

Hasegawa, P.M., Kononowicz, A.K., Casas, A.M., Tomes, D.T., and Bressan, R.A. (1995). New vistas are opened for sorghum improvement by genetic transformation. *Afr. Crop Sci. J.* **3**: 171–180.

Haseloff, J. and Amos, B. (1995). GFP in plants. *Trends Genet*. **11**: 328–329.

Hisano, H., Nandakumar, R., and Wang, Z.-Y. (2009). Genetic modification of lignin biosynthesis for improved biofuel production. *Vitro Cell. Dev. Biol.-Plant* **45**: 306–313.

Howe, A., Sato, S., Dweikat, I., Fromm, M., and Clemente, T. (2006). Rapid and reproducible Agrobacterium-mediated transformation of sorghum. *Plant Cell Rep*. **25**: 784–791.

Hra\vska, M., Rakouskỳ, S., and \vCurn, V. (2006). Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants. *Plant Cell Tissue Organ Cult*. **86**: 303–318.

Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J., and Chiang, V.L. (1999). Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nat. Biotechnol*. **17**: 808–812.

Iyer, L.M., Kumpatla, S.P., Chandrasekharan, M.B., and Hall, T.C. (2000). Transgene silencing in monocots. *Plant Mol. Biol*. **43**: 323–346.

Jeoung, J.M., Krishnaveni, S., Muthukrishnan, S., Trick, H.N., and Liang, G.H. (2002). Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. *Hereditas* **137**: 20–28.

Khan, Z.R., Pickett, J.A., Berg, J. van den, Wadhams, L.J., and Woodcock, C.M. (2000). Exploiting chemical ecology and species diversity: stem borer and striga control for maize and sorghum in Africa. *Pest Manag. Sci*. **56**: 957–962.

Kimatu, J.N., Diarso, M., Song, C.D., Agboola, R.S., Pang, J.S., Qi, X., and Liu, B. (2011). DNA cytosine methylation alterations associated with aluminium toxicity and low pH in Sorghum bicolor. *Afr J Agr Res* **6**: 4579–4593.

Kosambo-Ayoo, L.M., Bader, M., Loerz, H., and Becker, D. (2011). Transgenie sorghum (Sorghum bicolor L. Moench) developed by transformation with chitinase and chitosanase genes from Trichoderma harzianum expresses tolerance to anthracnose. *Afr. J. Biotechnol*. **10**: 3659–3670.

Krishnaveni, S., Joeung, J.M., Muthukrishnan, S., and Liang, G.H. (2001). Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. *J. Genet. Breed*. **55**: 151–158.

Kumar, V., Campbell, L.M., and Rathore, K.S. (2011). Rapid recovery-and characterization of transformants following Agrobacterium-mediated T-DNA transfer to sorghum. *Plant Cell Tissue Organ Cult.* **104**: 137–146.

Lee, R.A. and Lavoie, J.-M. (2013). From first-to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. *Anim. Front*. **3**: 6–11.

Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., and Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol*. **31**: 688–691.

Li, R. and Qu, R. (2011). High throughput Agrobacterium-mediated switchgrass transformation. *Biomass Bioenergy* **35**: 1046–1054.

Little, C., Perumal, R., Tesso, T., Prom, L.K., Odvody, G.N., and Magill, C.W. (2012). Sorghum pathology and biotechnology-A fungal disease perspective: part I. Grain mold, head smut, and ergot. *Eur J Plant Sci Biotech* **6**: 10–30.

Liu, G. and Godwin, I.D. (2012). Highly efficient sorghum transformation. *Plant Cell Rep*. **31**: 999–1007.

Lucca, P., Ye, X., and Potrykus, I. (2001). Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Mol. Breed*. **7**: 43–49.

Lu, L., Wu, X., Yin, X., Morrand, J., Chen, X., Folk, W.R., and Zhang, Z.J. (2009). Development of marker-free transgenic sorghum [Sorghum bicolor (L.) Moench] using standard binary vectors with bar as a selectable marker. *Plant Cell Tissue Organ Cult*. **99**: 97–108.

Maheswari, M., Varalaxmi, Y., Vijayalakshmi, A., Yadav, S.K., Sharmila, P., Venkateswarlu, B., Vanaja, M., and Saradhi, P.P. (2010). Metabolic engineering using mtlD gene enhances tolerance to water deficit and salinity in sorghum. *Biol. Plant.* **54**: 647–652.

Mall, T.K., Dweikat, I., Sato, S.J., Neresian, N., Xu, K., Ge, Z., Wang, D., Elthon, T., and Clemente, T. (2011). Expression of the rice CDPK-7 in sorghum: molecular and phenotypic analyses. *Plant Mol. Biol*. **75**: 467–479.

Mann, D.G., King, Z.R., Liu, W., Joyce, B.L., Percifield, R.J., Hawkins, J.S., LaFayette, P.R., Artelt, B.J., Burris, J.N., Mazarei, M., and others (2011). Switchgrass (Panicum virgatum L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. *BMC Biotechnol*., doi:10.1186/1472-6750-11-7**4**

Mann, D.G., LaFayette, P.R., Abercrombie, L.L., King, Z.R., Mazarei, M., Halter, M.C., Poovaiah, C.R., Baxter, H., Shen, H., Dixon, R.A., and others (2012). Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (Panicum virgatum L.) and other monocot species. *Plant Biotechnol. J*. **10**: 226–236.

Matzke, M.A. and Matzke, A.J. (1995). How and why do plants inactivate homologous (trans) genes? *Plant Physiol*. **107**: 679.

Mazarei, M., Al-Ahmad, H., Rudis, M.R., and Stewart, C.N. (2008). Protoplast isolation and transient gene expression in switchgrass, Panicum virgatum L. *Biotechnol. J.* **3**: 354–359.

Nageswara-Rao, M., Soneji, J.R., Kwit, C., Stewart Jr, C.N., and others (2013). Advances in biotechnology and genomics of switchgrass. *Biotechnol Biofuels* **6**: 77.

Nguyen, T.-V., Thu, T.T., Claeys, M., and Angenon, G. (2007). Agrobacterium-mediated transformation of sorghum (Sorghum bicolor (L.) Moench) using an improved in vitro regeneration system. *Plant Cell Tissue Organ Cult.* **91**: 155–164.

Odjakova, M.K. and Conger, B.V. (1999). The influence of osmotic pretreatment and inoculum age on the initiation and regenerability of switchgrass suspension cultures. *Vitro Cell. Dev. Biol.-Plant* **35**: 442–444.

O'Kennedy, M.M., Grootboom, A., and Shewry, P.R. (2006). Harnessing sorghum and millet biotechnology for food and health. *J. Cereal Sci.* **44**: 224–235.

Ou-Lee, T.-M., Turgeon, R., and Wu, R. (1986). Expression of a foreign gene linked to either a plant-virus or a Drosophila promoter, after electroporation of protoplasts of rice, wheat, and sorghum. *Proc. Natl. Acad. Sci.* **83**: 6815–6819.

Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., and Poliakov, A. (2009). The Sorghum bicolor genome and the diversification of grasses. *Nature* **457**: 551–556.

Pola, S.R. and Sarada, M.N. (2006). Somatic embryogenesis and plantlet regeneration in Sorghum bicolor (L.) Moench, from leaf segments. J Cell Mol Biol 5: 99–107.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., and Liotta, C.L. (2006). The path forward for biofuels and biomaterials. *science* **311**: 484–489.

Raghuwanshi, A. and Birch, R.G. (2010). Genetic transformation of sweet sorghum. *Plant Cell Rep.* **29**: 997–1005.

Richards, H.A., Rudas, V.A., Sun, H., McDaniel, J.K., Tomaszewski, Z., and Conger, B.V. (2001). Construction of a GFP-BAR plasmid and its use for switchgrass transformation. *Plant Cell Rep.* **20**: 48–54.

Rooney, W.L., Blumenthal, J., Bean, B., and Mullet, J.E. (2007). Designing sorghum as a dedicated bioenergy feedstock. *Biofuels Bioprod. Biorefining* **1**: 147–157.

Ruth, L. (2008). Bio or bust? *EMBO Rep.* **9**: 130–133.

Saathoff, A.J., Sarath, G., Chow, E.K., Dien, B.S., and Tobias, C.M. (2011). Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PloS One* **6**: e16416.

Sadia, B., Josekutty, P.C., Potlakayala, S.D., Patel, P., Goldman, S., and Rudrabhatla, S.V. (2010). An efficient protocol for culturing meristems of sorghum hybrids. *Phyton-Rev. Int. Bot. Exp*. **79**: 177.

Sanderson, M.A., Adler, P.R., Boateng, A.A., Casler, M.D., and Sarath, G. (2006). Switchgrass as a biofuels feedstock in the USA. *Can. J. Plant Sci*. **86**: 1315–1325.

Schmer, M.R., Vogel, K.P., Mitchell, R.B., and Perrin, R.K. (2008). Net energy of cellulosic ethanol from switchgrass. *Proc. Natl. Acad. Sci*. **105**: 464–469.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., and Qiu, J.-L. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol*. **31**: 686–688.

Shen, H., He, X., Poovaiah, C.R., Wuddineh, W.A., Ma, J., Mann, D.G., Wang, H., Jackson, L., Tang, Y., Neal Stewart, C., and others (2012). Functional characterization of the switchgrass (Panicum virgatum) R2R3-MYB transcription factor PvMYB4 for improvement of lignocellulosic feedstocks. *New Phytol.* **193**: 121–136.

Shridhar, J., Bhat, R.S., Bhat, S., and Kuruvinashetti, M.S. (2010). Agrobacterium-mediated transformation studies in sorghum using an improved gfp reporter gene. *J SAT Agri Res* **8**:1-5.

Da Silva, L.S., Jung, R., Zhao, Z., Glassman, K., Taylor, J., and Taylor, J. (2011). Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein body structure and protein nutritional quality in improved sorghum lines. *J. Cereal Sci*. **54**: 160–167.

Simmons, B.A., Loqué, D., and Ralph, J. (2010). Advances in modifying lignin for enhanced biofuel production. *Curr. Opin. Plant Biol*. **13**: 312–319.

Somleva, M.N., Tomaszewski, Z., and Conger, B.V. (2002). -Mediated Genetic Transformation of Switchgrass. *Crop Sci*. **42**: 2080–2087.

Song, G., Walworth, A., and Hancock, J.F. (2012). Factors influencing Agrobacterium-mediated transformation of switchgrass cultivars. *Plant Cell Tissue Organ Cult*. **108**: 445–453.

Sticklen, M.B. (2008). Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nat. Rev. Genet*. **9**: 433–443.

Tadesse, Y., Sagi, L., Swennen, R., and Jacobs, M. (2003). Optimisation of transformation conditions and production of transgenic sorghum (Sorghum bicolor) via microparticle bombardment. *Plant Cell Tissue Organ Cult*. **75**: 1–18.

Tari, I., Laskay, G., Takács, Z., and Poór, P. (2012). Response of Sorghum to Abiotic Stresses: A Review. *J. Agron. Crop Sci*. **199**(4):264-274

Taylor, J., Schober, T.J., and Bean, S.R. (2006). Novel food and non-food uses for sorghum and millets. *J. Cereal Sci*. **44**: 252–271.

Tesso, T., Perumal, R., Little, C., Adeyanju, A., Radwan, G., Prom, L., and Magill, C. (2012). Sorghum pathology and biotechnology-A fungal disease perspective: Part II. Anthracnose, stalk rot, and downy mildew. Biol. Biotechnol. Health Nutr. Sorghum Eur. *J. Plant Sci. Biotechnol*. **6**: 31–44.

The Africa Biofortified Sorghum (ABS) project (2012) Project update http://ksiconnecticrisatorg/wp-content/uploads/2012/11/8-Florence-Wambugu-ABS-for-Florencepdf

Vain, P., Finer, K.R., Engler, D.E., Pratt, R.C., and Finer, J.J. (1996). Intron-mediated enhancement of gene expression in maize (Zea mays L.) and bluegrass (Poa pratensis L.). *Plant Cell Rep*. **15**: 489–494.

VanderGheynst, J.S., Guo, H.-Y., and Simmons, C.W. (2008). Response surface studies that elucidate the role of infiltration conditions on Agrobacterium tumefaciens-mediated transient transgene expression in harvested switchgrass (Panicum virgatum). *Biomass Bioenergy* **32**: 372–379.

Vasil, I.K. (1994). Molecular improvement of cereals. Plant Mol. Biol. 25: 925–937.

Verma, A.K., Patil, V.U., and Bhat, R.S. (2011). A transiently expressed transposase system to generate Ds-tagged mutants for functional genomics in sorghum. *Plant Cell Tissue Organ Cult.* **107**: 181–185.

Verma, A., Nain, V., Kumari, C., Singh, S.K., Narasu, M.L., and Kumar, P.A. (2008). Tissue specific response of Agrobacterium tumefaciens attachment to Sorghum bicolor (L) Moench. *Physiol. Mol. Biol. Plants* **14**: 307–313.

Vogel, K.P. (2004). Switchgrass. p. 561-588 In: L.E. Moser et al., eds. Warm-season (C4) Grasses. ASA-CSSA-SSSA, Madison, WI

Wang, J.X., Sun, Y., Cui, G.M., and Hu, J.J. (2001). Transgenic maize plants obtained by pollen-mediated transformation. *Acta Bot. Sin*. **43**: 275–279.

Wang, W., Wang, J., Yang, C., Li, Y., Liu, L., and Xu, J. (2007). Pollen-mediated transformation of Sorghum bicolor plants. *Biotechnol. Appl. Biochem*. **48**: 79–83.

Wang, Y.-H., Poudel, D.D., Hasenstein, K.H., and Van Deynze, A. (2011). Identification of SSR markers associated with saccharification yield using pool-based genome-wide association mapping in sorghum. *Genome* **54**: 883–889.

Wright, M., Dawson, J., Dunder, E., Suttie, J., Reed, J., Kramer, C., Chang, Y., Novitzky, R., Wang, H., and Artim-Moore, L. (2001). Efficient biolistic transformation of maize (Zea mays L.) and wheat (Triticum aestivum L.) using the phosphomannose isomerase gene, pmi, as the selectable marker. *Plant Cell Rep*. **20**: 429–436.

Wuddineh, W.A., Mazarei, M., Zhang, J., Poovaiah, C.R., Mann, D.G., Ziebell, A., Sykes, R.W., Davis, M.F., Udvardi, M.K., and Stewart, C.N. (2015). Identification and overexpression of gibberellin 2-oxidase (GA2ox) in switchgrass (Panicum virgatum L.) for improved plant architecture and reduced biomass recalcitrance. *Plant Biotechnol. J*. **13**: 636–647.

Wu, E., Lenderts, B., Glassman, K., Berezowska-Kaniewska, M., Christensen, H., Asmus, T., Zhen, S., Chu, U., Cho, M.-J., and Zhao, Z.-Y. (2014). Optimized Agrobacterium-mediated sorghum transformation protocol and molecular data of transgenic sorghum plants. *Vitro Cell. Dev. Biol.-Plant* **50**: 9–18.

Xi, Y., Fu, C., Ge, Y., Nandakumar, R., Hisano, H., Bouton, J., and Wang, Z.-Y. (2009). Agrobacterium-mediated transformation of switchgrass and inheritance of the transgenes. *BioEnergy Res*. **2**: 275–283.

Xu, B., Escamilla-Treviño, L.L., Sathitsuksanoh, N., Shen, Z., Shen, H., Percival Zhang, Y.-H., Dixon, R.A., and Zhao, B. (2011). Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New Phytol*. **192**: 611–625.

Yohannes T, Frankard V, Sagi L, Swenen R, Jacobs M (1999) Nutritional quality improvement of sorghum through genetic transformation. In A Altman, M Ziv, and S Izhar (Eds.), Plant biotechnology and in vitro biology in the 21st century (pp. 617-620). Netherlands: Springer.

Zhang, M., Tang, Q., Chen, Z., Liu, J., Cui, H., Shu, Q., Xia, Y., and Altosaar, I. (2009). Genetic transformation of Bt gene into sorghum (Sorghum bicolor L.) mediated by Agrobacterium tumefaciens]. *Sheng Wu Gong Cheng Xue Bao Chin. J. Biotechnol*. **25**(3):418-423

Zhao, Z., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., and Seltzer, J. (2000). Agrobacterium-mediated sorghum transformation. *Plant Mol. Biol*. **44**: 789–798.

Zhao Z, Tomes D (2003) Sorghum transformation. In JF Jackson, HF Linskens, and RB Inman (Eds.), Genetic Transformation of Plants (pp.91-102). Verlag Berlin Heidelberg: Springer.

Zhu, H., Jeoung, J.M., Liang, G.H., Muthukrishnan, S., Krishnaveni, S., and Wilde, G. (1998). Biolistic transformation of sorghum using a rice chitinase gene [Sorghum bicolor (L.) Moench-Oryza sativa L.]. *J. Genet. Breed*. **52**(3):243-252.

Tables

Table 1.1 Information about transgenes, promoters and DNA delivery methods in sorghum transformation

Features	Transgenes	Promoters	DNA-delivery methods
Reporter	*gus* (*uidA*)	*CaMV35S; adh1; act1; ubi1*	Bombardment; *Agrobacterium*-mediated; Electroporation; pollen-mediated transformation
	gfp, Sgfp65T (improved *gfp*)	*CaMV35S; act1; ubi1; α-kaf*	Bombardment; *Agrobacterium*-mediated
	luc^+ (luciferase)	*ubi1*	Bombardment
	R and Cl maize anthocyanin regulatory elements	*CaMV35S*	Bombardment
Selectable	*bar*	*CaMV35S; act1; ubi1*	Bombardment; *Agrobacterium*-mediated
	pmi	*ubi1*	*Agrobacterium*-mediated
	htp	*CaMV35S; ubi1*	Bombardment, *Agrobacterium*-mediated
	nptII	*act1; CaMV35S; ubi1*	Bombardment; *Agrobacterium*-mediated; PEG-mediated transformation
	CAT gene (chloramphenicol acetyltransferase)	*CaMV35S*	Electroporation
Stress tolerance	*CryIAb*	*ubi1*	Bombardment; *Agrobacterium*-mediated
	CryIAc	*mpiC1; ubi1*	Bombardment
	harchi (chitinase) and *harcho* (chitosanase)	*ubi1*	Bombardment
	Chi11(rice chitinase)	*ubi1*	*Agrobacterium*-mediated
	mtlD gene encoding for mannitol-1-phosphate dehydrogenase	*CaMV35S*	Bombardment
	tlp (encoding thaumatin-like protein-TLP)	*ubi1*	*Agrobacterium*-mediated
	OsCDPK-7 (Calcium dependent	*ubi1*	*Agrobacterium*-mediated

	protein kinases (CDPKs))		
Nutrient improvement	*dhdps-rl*	-	Bombardment
	lysine-rich HT12	-	*Agrobacterium*-mediated
	sorghum lys1 tRNA synthase elements (TC2 or SKRS)	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum gamma-kafirin-1	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum gamma-kafirin-2	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum delta-kafirin-2	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	lysine alpha-ketogluterate reductase	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	CrtI	*sorghum beta-kafirin promoter*	*Agrobacterium*-mediated

Chapter 2

Expression of *ZmGA20ox* cDNA alters plant morphology and increases biomass production of switchgrass (*Panicum virgatum* L.) *

*The information in this chapter was published in Plant Biotechnol. J., doi: 10.1111/pbi.12514

Summary

Switchgrass (*Panicum virgatum* L.) is considered a model herbaceous energy crop for the USA, for its adaptation to marginal land, low rainfall and nutrient deficient soils; however, its low biomass yield is one of several constraints, and this might be rectified by modulating plant growth regulator levels. In this study we have determined whether expression of the *Zea mays gibberellin 20-oxidase* (*ZmGA20ox)* cDNA in switchgrass will improve biomass production. The *ZmGA20ox* gene was placed under the control of constitutive CaMV35S promoter with a strong TMV omega enhancer, and introduced into switchgrass via *Agrobacterium*-mediated transformation. The transgene integration and expression levels of *ZmGA20ox* in T0 plants were analyzed using Southern blot and qRT-PCR. Under greenhouse conditions, selected transgenic plants exhibited longer leaves, internodes and tillers, which resulted in 2-fold increased biomass. These phenotypic alterations correlated with the levels of transgene expression and the particular gibberellin content. Expression of *ZmGA20ox* also affected the expression of genes coding for key enzymes in lignin biosynthesis. Our results suggest that the employment of ectopic *ZmGA20ox*, or selection for natural variants with high level

expression of endogenous *GA20ox* are appropriate approaches to increase biomass production of switchgrass and other monocot biofuel crops.

Keywords: *gibberellin, gibberellin 20-oxidase,* biofuel, biomass, switchgrass.

Introduction

Biofuels are an important component of the energy sources of the planet, and there is great need for developing biofuel feedstock crops (Sticklen, 2008; Carroll and Somerville, 2009). Switchgrass (*Panicum virgatum* L.) was the first plant selected for bioenergy by U.S. Department of Energy in the 1990s (McLaughlin and Kszos, 2005). This perennial C4 grass has high productivity across different environments, and is adapted to marginal land, low rainfall regions and nutrient deficient soil (Fike *et al.*, 2006). Switchgrass produces net positive renewable energy and has positive environmental benefits (Schmer *et al*., 2008). U.S. Department of Energy and U.S. Department of Agriculture have projected a national goal for biofuel to supply 20% transportation fuels by 2030. About 1 billion dry tons of biomass will be annually required for the goal, of which one-third of the biomass will come from perennial feedstock such as switchgrass. Therefore, increasing biofuel crop yields is a major goal of U.S. biomass energy research program (Sanderson *et al*., 2006). Numerous factors affecting plant biomass production have been studied and applied in attempts to gain higher crop vegetative yields (Demura and Ye, 2010). Of these, manipulation of endogenous plant hormone contents is one of the most effective in improving plant growth, development and biomass.

Gibberellins (GAs) comprise a large family of diterpenoid carboxylic acids of more than one hundred compounds currently known in higher plants, fungi and bacteria (Hedden and Phillips, 2000; Olszewski *et al*., 2002; Yamaguchi, 2008). In higher plants, GA_1, GA_3, GA_4 and GA_7 are the most common active GAs that control diverse processes of plant growth and development. The complex pathways of bioactive GA biosynthesis in higher plant require three different classes of enzymes and the participation of different cell components (Olszewski *et al*., 2002; Yamaguchi, 2008). In the cytoplasm, the last steps of GA biosynthesis are catalyzed by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) to form various GA intermediates and mature bioactive GAs (Hedden and Phillips, 2000; Olszewski *et al*., 2002). GA20-oxidase, a multifunctional enzyme, catalyzes several sequential reactions in the formation of inactive gibberellins (GA_9, GA_{20}), then GA3ox introduces a 3β-hydroxyl group to form the mature products (Yamaguchi, 2008).

GA20ox is encoded by genes that have been cloned from various dicots (Phillips *et al*., 1995; Kang *et al*., 1999; Carrera *et al*., 2000; Eriksson *et al*., 2000) and monocots (Toyomasu *et al*., 1997; Du *et al*., 2009). The ectopic expression of genes coding for GA20ox has been shown to increase the levels of bioactive GAs and to affect plant growth and morphology. For example, overexpression of *GA20ox* caused a higher level of GA_4 in *Arabidopsis thaliana* and consequently to accelerate elongated hypocotyls of seedlings, increasing shoot growth and early flowering (Croker *et al*., 1999). Overexpression of *GA20ox* in potato resulted in taller plants and longer leaf petioles (Carrera *et al*., 2000). Ectopic expression of *Arabidopsis GA20ox* increased bioactive GAs in transgenic tobacco, leading to increased plant growth and biomass production

(Biemelt *et al*., 2004). In citrus, overexpression of *GA20ox* modified plant architecture. Transgenic citrus plants had much longer thorns and typical organs at juvenile stages. In addition, higher levels of active GA_1 were also observed in these plants (Fagoaga *et al*., 2007). In hybrid aspen (Eriksson *et al*., 2000), ectopic expression of *GA20ox* gene increased growth rate and biomass, and caused more and longer fibers compared to wild-type plants.

In rice, Ayano *et al* (2014) showed that expression of *GA20ox* correlated with GA_1 and GA_4 content and has a role in internode elongation, but most studies in monocots have focused on down-regulation of *GA20ox* gene to reduce plant height and increase reproductive yields (Sasaki *et al*., 2002). Overall, these results indicate that altering expression of *GA20ox* changes GA levels, and consequently also plant growth, development and biomass production.

Here, we report that the expression of *ZmGA20ox* cDNA in switchgrass results in elevation of bioactive GA levels and altered plant architecture with longer internodes, leaves and increased fresh and dry biomass. Moreover, the expression of *ZmGA20ox* was found to affect expression of genes in lignin biosynthesis. Our results suggested that ectopic *ZmGA20ox* is a viable approach for increased biomass for biofuel production by switchgrass and possibly other energy monocots.

Results

Generation of transgenic switchgrass plants with *ZmGA20ox*

Using binary construct for overexpression of *ZmGA20ox* and *Agrobacterium*-mediated transformation, more than 20 transgenic switchgrass events were produced and

confirmed based on the leaf painting and genomic PCR using specific primers for *ZmGA20ox* and *hptII* genes (Figure 2.1, Figure 2.2, and Table 2.1). After two months growth under greenhouse conditions, phenotypic differences became obvious among the transgenic events compared to WT control plants. All transgenic events exhibited longer tillers (Figures 2.3, 2.4, and 2.5), and could be divided into four groups: group 1 - more tillers but the same growth as WT; groups 2 - more tillers and much faster growth than WT; group 3 –fewer tillers and much faster growth than WT; group 4 – very thin leaves and more tillers and much faster growth than WT. Groups 1 to 4 exhibited 12.7%, 39.8%, 47.1%, and 80% increase in tiller height, respectively, as compared to WT (Figure 2.4). The longer tillers of transgenic plants were the result of longer internodes and leaves, typical of morphological changes caused by GA_3. Group 1 plants displayed less change in internode and leaf elongation than remaining groups. Of all groups, group 4 had the largest increase in leaf (42.7%) and internode (approximately 120%) length (Figure 2.4 and 2.5).

All transgenic switchgrass plants exhibited a reduction in internode diameter and leaf width. The internode diameter of transgenic plants was reduced to between 2.90 mm and 3.84 mm compared to 4.23 mm of WT plants (Figure 2.4 and 2.5). The average leaf width of transgenic plants was between 10 mm (group 4) and 12.5 mm (group 2) whereas that of WT plants was 15.2 mm. There was a correlation between the increase in internode and leaf length and the reduction in internode diameter and leaf width of transgenic plants except for group 1. Transgenic plants of this group showed a significant reduction in internode diameter as compared to group 2. Interestingly, a substantial increase in the number of tillers appears to be compensated by the decreased internode

diameter and leaf width. Other groups displayed similar growth phenotypes, that is, the longer leaves and internodes were compensated by the narrow leaves and small internode diameters. In addition, group 4 exhibited a weak tiller phenotype that could not stand up well. Curling leaves also occurred in plants of this group.

Effects of ectopic *ZmGA20ox* on growth rate, biomass and flowering time

ZmGA20ox transgenic switchgrass plants exhibited increased growth rate, especially during vegetative development stage, and faster tiller emergence and elongation were observed in all transgenic lines. For tiller number, groups 2, 3, 4 transgenic and WT plants had no significant difference in the number of tillers of each plant. By contrast, group 1 transgenic plants had a remarkable increase in the number of tillers (by approximately 130%) compared to WT plants (Table 2.2). Interestingly, all transgenic groups showed increases in both fresh and dry biomass weight, but a 19.7% to 34.8% reduction in fresh to dry weight ratios, respectively. Specifically, groups 1, 2 and 3 displayed a 1.8 to 2 fold increase in the whole dry biomass as compared to WT plants whereas group 4 had insignificant increase in dry biomass. To reconfirm the faster growth of transgenic plants, the tillers were cut and the growth rates measured again. One month after cutting back, tillers of ectopic *ZmGA20ox* plants were 50.5% to 86.9% higher than WT plants (Figure 2.6).

Switchgrass flowering was observed at the R1 stage of individual tiller (Hardin *et al*., 2013), but flowering times were not correlated with faster growth rate. At 12 weeks under greenhouse condition, wild-type plants exhibited more than 17 % flowering tillers, but for transgenic plants, that varied from 0% to 10.6% (Figure 2.7). Flowering tillers of

wild-type switchgrass reached a peak (96%-100%) at 17 weeks. However, the rate of flowering tillers of transgenic switchgrass gradually increased and ranged from 48.1% to 70.2%.

Cell size changed in *ZmGA20ox* transgenic plants

We examined the effect of *ZmGA20ox* overexpression on internode cell size of transgenic plants from group 4 using fluorescence microscopy (Figure 2.8). Both longitudinal and cross sections of transgenic plant (G4-1) showed smaller pith and xylem cells, and transgenic xylem cells displayed 22.8% reduction in average cell size while the reduction in pith cell size was 36.6% as compared to wild-type plants (Table 2.3). This data was consistent with decreased tiller thickness and smaller internode diameter. There was no difference in the vascular bundle distribution in stems and the pith cell length between transgenic plants and wild-type control (Figure 2.8 and Table 2.3). Both wild-type and transgenic plants exhibited three circles of vascular bundles in the cross section of fully elongated internodes at the same developmental stage. Therefore, the longer internodes and leaves of *ZmGA20ox* transgenic plants could be caused by the increase in cell division at the position of leaf divisional zones and intercalary meristems.

Transgenic phenotypes correspond to *GA20ox* transcript and GAs levels

Transgene integration patterns of various phenotypic groups were analyzed. Several events in each group were randomly selected for Southern blot using *ZmGA20ox* and *hptII* partial open-reading frames as probes, respectively. Genomic DNA was digested with restriction enzyme *Bam*HI, which cuts once within the T-DNA region, allowing identification of different events and estimation of the number of transgene

copies (Figure 2.1 and Figure 2.9). When the *hptII* probe was used, varying banding patterns were shown in transgenic samples, whereas no hybridizing band was detected in wild-type plants (Figure 2.9a). However, when the *ZmGA20ox* probe was used, besides varying molecular weight bands corresponding to the transgene, a 4 kb hybridizing band was shown in all samples including wild-type plant. This band is believed to be the endogenous *GA20ox* gene which shares a high degree of sequence homology with *ZmGA20ox* (Figure 2.9b).

We analyzed transgene expression and gibberellin levels using a representative event from each group. RNA samples isolated from whole tillers at elongation stage E1 of these events were used for cDNA synthesis, and then for both RT-PCR and quantitative real-time PCR (qRT-PCR) with *GA20ox* primers (Table 2.1). The RT-PCR and qRT-PCR results were consistent, showing increases in the transcript abundance of transgenic events from the four main groups (Figure 2.10). Transgenic event G1-3 showed 4.4 fold increase in the transcription level of *GA20ox* compared to WT control, while that for other events ranged from 16.3 to 17.7 fold. These results agreed with the phenotypic changes of these transgenic groups as described above. No significant difference in the transcript abundances was found between transgenic lines among groups 2, 3 and 4.

We then analyzed gibberellin in *ZmGA20ox* transgenic events, and focused on GA_1 and GA_4, two bioactive GAs in higher plants (Table 2.4). The levels of endogenous gibberellins were correlated to the degrees of altered transgenic phenotypes. Wild-type and G1-3 plants had very low levels of GA_1 and GA_4 that could not be detected by our current GAs detection system. A high concentration of GA_4 (7.4 ng/g) was recorded

whereas GA_1 was not detectable in transgenic event G2-2. Both GA_1 and GA_4 were detected in transgenic events G3-1 and G4-1 at high levels. Importantly, the highest content of bioactive GAs occurred in G4-1 transgenic plant that had the most significant alteration of phenotype. Furthermore, a higher concentration of GA_4 than GA_1 was found in all GA-detectable transgenic plants.

Effects of ectopic *ZmGA20ox* on lignin gene expression

To explore whether changes in *GA20ox* expression affected lignin biosynthesis, transcripts of three genes coding for enzymes in lignin biosynthesis pathway were analyzed by qRT-PCR. Of these, 4CL (4-coumarate: CoA ligase) is an enzyme in the early steps of this pathway, while CAD (cinnamyl alcohol dehygrogenase) and COMT (caffeic acid 3-O-methyltransferase) catalyze the final steps of monolignol biosynthesis. In group 4 transgenic plants (G4-1), *GA20ox* expression level correlated with transcript levels of lignin genes, showing a significant increase in the transcript abundance of all three lignin genes (Figure 2.11). However, remaining groups had only a minor change in the expression levels of these lignin genes. For example, in group 2 plants (G2-2), the expression of *4CL* gene was clearly increased but *CAD* and *COMT* expressions had no significant change. The phloroglucinol-HCl staining for lignin was associated with an altered expression of pathway-specific genes (Figure 2.12). Much higher lignin accumulation was observed in internode cross sections of transgenic group 4, where lignin staining was exhibited not only in sclerenchyma cell walls but also clearly in parenchyma cell walls. Compared to wild-type, transgenic group 1 did not show a clear change in lignin accumulation. In addition, groups 2 and 3 had a slight increase in lignin content.

Discussion

GA20 oxidase is a key enzyme in the pathways of bioactive GA biosynthesis in higher plants, and its overexpression has been shown to alter plant phenotypes and to increase relative growth rates in many plant species. Biomass improvement by modulation of *GA20ox* genes has been achieved in some dicot plants such as tobacco and hybrid aspen (Eriksson *et al*., 2000; Biemelt *et al*., 2004). In this study, the observed alteration of plant architecture of transgenic switchgrass with the *ZmGA20ox* gene under the control of the CaMV35S promoter resembled that in dicots (Croker *et al*., 1999; Carrera *et al*., 2000; Biemelt *et al*., 2004; García-Hurtado *et al*., 2012), maize (Voorend *et al*., 2015) as well as that of exogenous applications of GA_3 to other monocots (Tsai and Arteca, 1985). These architecture changes included longer leaves, internodes and tillers but smaller leaves and internode diameters compared to wild-type control plants. These morphological changes were highly corresponded with expression of *GA20ox* and bioactive gibberellin levels, which were higher than the wild-type control. Furthermore, varied phenotypes of these groups were correlated to the different contents and forms of bioactive gibberellins, respectively. Finally, higher biomass and reduced dry-fresh weight ratios were obtained in all transgenic plants. The present work is the first study to report the ectopic expression of *GA20ox* in switchgrass for improving biomass.

Bioactive GA_1 and GA_4 are produced at the final stage of GA biosynthesis catalyzed through two parallel pathways, involving 13-hydroxylation and the non-13-hydroxylation (Vidal *et al*., 2001). Higher contents of GA_1 than GA_4 were shown in the *GA20ox* overexpressing transgenic citrus (*Citrus sinensis*) (Fagoaga *et al*., 2007) and

Populus (Eriksson *et al*., 2000). In contrast, ectopic *GA20ox* in potato (*Solanum tuberosum*) displayed much higher contents of GA_4 in apical shoots (Carrera *et al*., 2000). The same result was obtained in both shoots and fruits of transgenic tomato (*Solanum lycopersicum*). The phenotypes of *GA20ox* transgenic tomato plants were due to the increase of bioactive GA_4 content (García-Hurtado *et al*., 2012), whereas both GA_4 and GA_1 had function in internode elongation of deepwater rice (Ayano *et al*., 2014). In the current study, the same altered phenotypes were exhibited by G2-2 and G3-1 lines, even though the lower content of GA_4 but higher level of GA_1 occurred in the G3-1 line. In addition, the largest phenotypic alteration of transgenic line G4-1 was consistent with the highest levels of GA_1 and GA_4 (Table 2.4). These data indicate that the elongated phenotypes of *ZmGA20ox* transgenic switchgrass resulted from the activation of both bioactive GAs.

Longer internodes in *CcGA20ox1* overexpression citrus could be correlated to cell divisions (Fagoaga *et al*., 2007). Similar results were observed in the transgenic *AtGA20ox* Populus trees (Eriksson *et al*., 2000). By contrast, transgenic *AtGA20ox* tobacco plants showed longer shoots that resulted from both cell divisions and elongation (Biemelt *et al*., 2004). The occurrence of cell division and elongation events corresponded to the regions of active GA biosynthesis and signaling (Kaneko *et al*., 2003). Moreover, in the study of gibberellin biosynthesis and signal transduction in rice, Ayano *et al* (2014) found that internode elongation was induced by the accumulation of GA during submergence. The activation of intercalary meristem located in the nodes was proposed as a driving force in internode elongation. Gibberellin levels were suggested to regulate the growth of maize leaves by spatial control of cell division (Nelissen *et al*.,

2012). In our study, no difference in the length of pith cells between transgenic lines and wild-type control plant was found. Therefore, the elongated internodes and leaves of *ZmGA20ox* transgenic plants were possibly consequences of the increased cell divisions in the leaf divisional zones, leaf primordia and intercalary meristems under higher levels of GAs (Figure 2.8 and Table 2.3).

GA deficiency by ectopic expression of *GA2ox* promoted earlier tiller formations and higher tiller number was indicated in rice (Lo *et al*., 2008) and switchgrass (Wuddineh *et al*., 2015). However, the number of switchgrass tillers was not correlated with the GA levels. The semi-dwarf switchgrass lines showed an increase in tiller number whereas dwarf lines displayed a reduction in number of tillers per plant relative to wild-type controls (Wuddineh *et al*., 2015). On the other hand, GAs were shown to increase tiller numbers of Welsh onion by initiating and promoting axillary bud development (Yamazaki *et al*., 2015). In addition, Ni *et al* (2015) reported that GAs induced the formation of secondary buds as well as promoted shoot branching of some perennial woody plants by synergistically acts with cytokinin. In our study, there was no significant difference in tiller number between transgenic groups showing high levels of GAs and non-transgenic wild-type plants. However, a remarkable increase in the number of tillers was observed in transgenic group 1, which showed a slight increase in *GA20ox* transcript abundance. To reconcile the effects upon switchgrass tillering by different levels of GA deficiency (semi-dwarf and dwarf switchgrass) (Wuddineh *et al*., 2015), it is possible that switchgrass tiller formation and development may be impacted by different levels and components of bioactive gibberellins.

The overexpression of *GA20ox* in *Arabidopsis* promoted flowering (Blázquez and Weigel, 2000; Rieu *et al*., 2008). By contrast, GAs were indicated to inhibit flowering in grapevine (Boss and Thomas, 2002) and a similar observation was made in tomato (García-Hurtado *et al*., 2012). Recently, Yamaguchi et al (2014) reported that GA inhibits switch flower formation in *Arabidopsis* by interactions with genes promoting floral fate such as the EUI-LIKE P450 A1 gene (ELA1), LEAFY transcription factor (LFY) and also DELLA proteins. The up-regulation of ELA1 and LFY reduces the levels of GAs such as GA_4. In our study, *ZmGA20ox* transgenic switchgrass exhibited a slight delay of flowering time compared to wild-type control plants (Figure 2.7). Therefore, high levels of GAs, especially GA_4, may negatively affect switchgrass flower formation. This speculation could be supported by the correlation between the slow flowering and the high GA_4 contents in transgenic lines G2-2 and G4-1 (Figure 2.7 and Table 2.4). Moreover, no effect on flowering was observed in some plant species as a result of either GA abundance (Gallego-Giraldo *et al*., 2007) or GA deficiency (Dijkstra *et al*., 2008). Therefore, the role of gibberellins on flowering varies and depends on the species. More research needs to be conducted to understand this complex mechanism.

Biomass yield and quality traits are two important criteria in selecting switchgrass for biofuel production. In our study, we found the association between the altered lignin gene expression, the lignin staining results and the levels of bioactive gibberellins in ectopic *ZmGA20ox* transgenic switchgrass. In transgenic group 4 (line G4-1) the stronger lignin gene expression and histological staining correlated with the higher contents of GA_1 and GA_4. This observation is consistent with the results of lignin deposition study in tobacco under the *GA20ox* overexpression and different GA_3 concentration treatments

(Biemelt *et al*., 2004). Interestingly, in our study some transgenic groups showed increased biomass production while displaying no significant change in lignin genes expression.

In summary, this is the first study on the effects of ectopic *GA20ox* expression on morphology and biomass of switchgrass. *ZmGA20ox* transgenic plants exhibited drastic alterations in plant phenotypes resulting in longer leaves and internodes. The increased growth rate caused increased fresh and dry biomass, and demonstrates a means to improve the biomass production of this feedstock and possible other cellulosic crops. Furthermore, the insignificant increase of lignin gene expression and lignin contents in those good phenotype groups should be desirable as bioenergy feedstock. Thus, the expression of ectopic *GA20 oxidase* could be a good experimental approach to benefit biomass production of monocot plants. Results from this study also implies that the use of variation in the natural *GA20ox* gene expression would be a viable means to select for improved varieties with higher biomass, avoiding the outcrossing risk of transgenic switchgrass pollen.

Experimental procedures

Vector construction and plant transformation

The open reading frame (1116 bp) of *Zea mays GA20 oxidase* (*ZmGA20ox*) from Genbank (NM_001112453.1) coding for 311 amino acids was synthesized by GenScript (GenScript, USA).The *EcoRI/Hind*III fragment encompassing the *ZmGA20ox* gene and 35S promoter plus TMV Omega enhancer sequence was inserted into binary vector pCAMBIA1300 to generate transgenic T-DNA construct (Figure 2.1), which was

mobilized into *Agrobacterium tumefaciens* strain AGL1 for switchgrass transformation by the protocol of (Li and Qu, 2011) with modifications. Embryogenic calli were induced from mature seeds of switchgrass cultivar Alamo. Hygromycin B (Invitrogen^{TM} Life Technologies, USA) was added to selected medium at 50 mg/l.

Growth condition, leaf painting, sample collection and measurement

Transgenic switchgrass events were grown in greenhouses with day/night temperatures of 28/21^{o}C, a photoperiod of 16 h light/8 h dark, in 3-gal pots containing Promix soil supplemented by Osmocote (14-14-14) (Hummert International, Earth City, MO). Leaf painting was carried out by swiping 1g/l hygromycin B solution onto the upper suffice of a leaf and results were recorded one week later (Figure 2.2). The phenotypic data including the morphology of leaves, internodes and tillers were collected at R1 stage (Hardin *et al*., 2013). Internodes (I3 and I4) and their leaves were subjected to phenotypic observations. The above ground tissues were harvested when 50% of tillers reached R2 stage for biomass measurements. Dried weight was calculated after switchgrass samples were dried in an oven at 45^{o}C for 48h.

PCR and Southern blot

Gene-specific primers (Table 2.1) were used for PCR reactions to confirm the presence of transgenes and Southern blots were used to confirm their integration into the genome. Genomic DNA was extracted from switchgrass leaf tissue using a CTAB procedure modified from (Dellaporta *et al*., 1983). For Southern blot analysis, 30 μg purified DNA was digested by a restriction enzyme that cut once within the T-DNA region. Digested DNA fragments were fractionated on a 2.0% agarose gel prior to transfer to Zeta-Probe®

GT nylon membrane (Bio-Rad, USA). DNA was fixed to nylon membrane by UV cross-link. Hybridization and membrane washing were conducted based on the Zeta-Probe® GT manufacturer's instructions at 65^{o}C. Prime-It® RmT Random Primer Labeling Kit (Stratagene, USA) was used to generate 32P-labeled probes of *ZmGA20ox* (from synthetic transgene template) or *hptII* (from pCAMBIA1300 vector).

Quantitative real-time PCR

Total RNA was extracted from a whole tiller of wild-type and transgenic switchgrass plants at elongation E1 stage (Hardin *et al*., 2013) using TRIZOL® Reagent according to the manufacture's protocol (InvitrogenTM Life Technologies, USA). The isolated RNA was treated with DNase-I (InvitrogenTM Life Technologies, USA) to remove genomic DNA contamination. The first-strand cDNA was synthesized from the DNase-treated RNA using M-MLV Reverse Transcriptase and Oligo-dT primer (Promega, USA). RT-PCR was carried out using specific primers for *ZmGA20ox* gene (Table 2.1). qRT-PCR was conducted using iQ™ SYBR® Green Supermix (BIO-RAD, USA). The data were normalized using the levels of swithcgrass ubiquitin (UBQ) transcripts (Xu *et al*., 2011). The primers used for qRT-PCR were the same as described above for RT-PCR (Table 2.1). Transcript abundance was quantified using three independent biological replicates.

Microscopy and cell size measurement

Images of cross and longitudinal sections of fully elongated internodes were captured under Olympus IX70 Inverted Microscope with ORCA-ER Digital Camera fluorescence optics at 10x. Images were analyzed by MetaMorph Microscopy Automation and Image Analysis software to identify cell size, length and number.

Lignin staining

For lignin staining, internode samples were collected at reproduction developmental stage (R1). Internode were cut by Vibratome series 3000 to generate 60 µm cross sections and cleared by ethanol overnight. Cleared sections were immersed in 1% chloroglucinol staining solution (in 2:1 ethanol/HCl) for 2 min (Baum, 2008; Bart *et al*., 2010; Wuddineh *et al*., 2015). The cross sections were placed on microscopy slides and covered by coverslip. The edges of the slides were sealed with commercial sealant and examined under Leica DM 5500B Compound Microscope with Leica DFC290 Color Digital Camera at 10x.

Gibberellin quantification

Gibberellins were extracted in cold methanol:isopropanol:acetic acid (20:79:1, v/v/v) from samples spiked with deuterium-labelled internal standards of GA_1 (D2-GA1, Olkemim ltd, Czech Republic). After centrifugation at 16,000 g, the supernatants were collected and pellet extraction repeated. The pooled supernatants were evaporated and the resulting pellet re-dissolved in 200 µL of 30% methanol. Chromatographic separation of metabolites was accomplished using a 3C18-EP-120 column (0.5 mm × 100 mm, Eksigent) using a mobile gradient of 85% solvent A (0.1% acetic acid in HPLC-grade water, v/v) to 95% solvent B (0.1% acetic acid in 90% acetonitrile, v/v) in 6 min at a flow rate of 15 µL min-1. A 6500-QTRAP (AB Sciex, Foster city, USA) was used to acquire MS spectra. Parameters for analysis were set as follows: ESI in the negative mode (TurboIonSpray), capillary voltage -4500, nebulizer gas 25 arbitrary units (a.u.), heater gas 25 a.u., curtain gas 10 a.u., collision activation dissociation -2, temperature 250 °C.

Gibberellins GA_1 and GA_4 were detected using multiple reaction monitoring (MRM) transitions that were optimized using the standards (GA_1 and GA_4, Olkemim ltd, Czech Republic) and the deuterium-labeled standard. Concentrations were determined from standard curves of known gibberellin concentrations.

Data analysis

Comparisons between transgenic and wild-type control plants were made by Turkey's least significant difference procedure using one-way ANOVA and T-Test in SPSS software (ver.20, Chicago, IL, USA). Standard errors are provided for statistical diagrams as appropriate. The asterisks on the bars in the figures and the tables indicate a significant difference from the wild-type controls at $P<0.05$ or 0.01 levels.

Acknowledgements

The authors thank Dr. William Folk for proofreading of the manuscript and helpful suggestions, and Drs. David Braun and David Mendoza for helpful discussions. Thanks are also extended to Neng Wan (Dr. Zhang's lab) for his help with greenhouse work, Dr. Sophie Alvarez and the Proteomics Facility at The Donald Danforth Plant Science Center, St. Louis, MO, for assistance with the mass spectrometry (supported by NSF Grant No. DBI-1427621 for acquisition of the QTRAP LC-MS/MS), and the Molecular Cytology Core at University of Missouri for microscopy analysis. P.T. Do was supported by the Vietnam Educational Foundation. The authors have no conflict of interest to declare.

References

Ayano, M., Kani, T., Kojima, M., Sakakibara, H., Kitaoka, T., Kuroha, T., ANGELES-SHIM, R.B., Kitano, H., Nagai, K., and Ashikari, M. (2014). Gibberellin biosynthesis and signal transduction is essential for internode elongation in deepwater rice. *Plant Cell Environ*. **37**, 2313–2324.

Bart, R.S., Chern, M., Vega-Sánchez, M.E., Canlas, P., Ronald, P.C., and others (2010). Rice Snl6, a cinnamoyl-CoA reductase-like gene family member, is required for NH1-mediated immunity to *Xanthomonas oryzae pv. oryzae*. *PLoS Genet* **6**: e1001123.

Baum, S. (2008). Preparation of Arabidopsis tissue sections of fixed material. *Cold Spring Harb Protoc* **10**, 1101.

Biemelt, S., Tschiersch, H., and Sonnewald, U. (2004). Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiol*. **135**, 254–265.

Blázquez, M.A. and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. *Nature* **404**, 889–892.

Boss, P.K. and Thomas, M.R. (2002). Association of dwarfism and floral induction with a grape "green revolution" mutation. *Nature* **416**, 847–850.

Carrera, E., Bou, J., García-Martínez, J.L., and Prat, S. (2000). Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *Plant J*. **22**, 247–256.

Carroll, A. and Somerville, C. (2009). Cellulosic biofuels. *Annu. Rev. Plant Biol*. **60**, 165–182.

Croker, S.J., García-Lepe, R., Lewis, M.J., Hedden, P., and others (1999). Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. *Plant J*. **17**, 547–556.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Report*. **1**, 19–21.

Demura, T. and Ye, Z.-H. (2010). Regulation of plant biomass production. *Curr. Opin. Plant Biol*. **13**, 298–303.

Dijkstra, C., Adams, E., Bhattacharya, A., Page, A.F., Anthony, P., Kourmpetli, S., Power, J.B., Lowe, K.C., Thomas, S.G., Hedden, P., and others (2008). Over-expression of a gibberellin 2-oxidase gene from *Phaseolus coccineus* L. enhances gibberellin inactivation and induces dwarfism in *Solanum* species. *Plant Cell Rep*. **27**, 463–470.

Du, J., Yao, Y., Ni, Z., and Sun, Q. (2009). Cloning and characterization of an up-regulated GA 20-oxidase gene in hybrid maize. *Prog. Nat. Sci*. **19**, 161–166.

Eriksson, M.E., Israelsson, M., Olsson, O., and Moritz, T. (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol*. **18**, 784–788.

Fagoaga, C., Tadeo, F.R., Iglesias, D.J., Huerta, L., Lliso, I., Vidal, A.M., Talon, M., Navarro, L., García-Martínez, J.L., and Peña, L. (2007). Engineering of gibberellin levels in citrus by sense and antisense overexpression of a *GA 20-oxidase* gene modifies plant architecture. *J. Exp. Bot*. **58**, 1407–1420.

Fike, J.H., Parrish, D.J., Wolf, D.D., Balasko, J.A., Green, J.T., Rasnake, M., and Reynolds, J.H. (2006). Long-term yield potential of switchgrass-for-biofuel systems. *Biomass Bioenergy* **30**, 198–206.

Gallego-Giraldo, L., García-Martínez, J.L., Moritz, T., and López-Díaz, I. (2007). Flowering in tobacco needs gibberellins but is not promoted by the levels of active GA_1 and GA_4 in the apical shoot. *Plant Cell Physiol*. **48**, 615–625.

García-Hurtado, N., Carrera, E., Ruiz-Rivero, O., López-Gresa, M.P., Hedden, P., Gong, F., and García-Martínez, J.L. (2012). The characterization of transgenic tomato overexpressing gibberellin 20-oxidase reveals induction of parthenocarpic fruit growth, higher yield, and alteration of the gibberellin biosynthetic pathway. *J. Exp. Bot*. **63**, 5803–5813.

Hardin, C.F., Fu, C., Hisano, H., Xiao, X., Shen, H., Stewart Jr, C.N., Parrott, W., Dixon, R.A., and Wang, Z.-Y. (2013). Standardization of switchgrass sample collection for cell wall and biomass trait analysis. *BioEnergy Res*. **6**, 755–762.

Hedden, P. and Phillips, A.L. (2000). Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci*. **5**, 523–530.

Kaneko, M., Itoh, H., Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Ashikari, M., and Matsuoka, M. (2003). Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? *Plant J*. **35**, 104–115.

Kang, H.-G., Jun, S.-H., Kim, J., Kawaide, H., Kamiya, Y., and An, G. (1999). Cloning and molecular analyses of a gibberellin 20-oxidase gene expressed specifically in developing seeds of watermelon. *Plant Physiol*. **121**, 373–382.

Li, R. and Qu, R. (2011). High throughput *Agrobacterium-mediated* switchgrass transformation. *Biomass Bioenergy* **35**, 1046–1054.

Lo, S.-F., Yang, S.-Y., Chen, K.-T., Hsing, Y.-I., Zeevaart, J.A., Chen, L.-J., and Yu, S.-M. (2008). A novel class of *gibberellin 2-oxidases* control semidwarfism, tillering, and root development in rice. *Plant Cell* **20**, 2603–2618.

McLaughlin, S.B. and Kszos, L.A. (2005). Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass Bioenergy* **28**, 515–535.

Nelissen, H., Rymen, B., Jikumaru, Y., Demuynck, K., Van Lijsebettens, M., Kamiya, Y., Inzé, D., and Beemster, G.T. (2012). A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. *Curr. Biol.* **22**, 1183–1187.

Ni, J., Gao, C., Chen, M.-S., Pan, B.-Z., Ye, K., and Xu, Z.-F. (2015). Gibberellin promotes shoot branching in the perennial woody plant Jatropha curcas. *Plant Cell Physiol*. **56**, 1655–1666.

Olszewski, N., Sun, T., and Gubler, F. (2002). Gibberellin signaling biosynthesis, catabolism, and response pathways. *Plant Cell Online* **14**, S61–S80.

Phillips, A.L., Ward, D.A., Uknes, S., Appleford, N.E., Lange, T., Huttly, A.K., Gaskin, P., Graebe, J.E., and Hedden, P. (1995). Isolation and expression of three *gibberellin 20-oxidase* cDNA clones from Arabidopsis. *Plant Physiol*. **108**, 1049–1057.

Rieu, I., Ruiz-Rivero, O., Fernandez-Garcia, N., Griffiths, J., Powers, S.J., Gong, F., Linhartova, T., Eriksson, S., Nilsson, O., Thomas, S.G., and others (2008). The gibberellin biosynthetic genes *AtGA20ox1* and *AtGA20ox2* act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant J.* **53**, 488–504.

Sanderson, M.A., Adler, P.R., Boateng, A.A., Casler, M.D., and Sarath, G. (2006). Switchgrass as a biofuels feedstock in the USA. *Can. J. Plant Sci*. **86**, 1315–1325.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., Khush, G.S., and others (2002). Green revolution: a mutant gibberellin-synthesis gene in rice. *Nature* **416**, 701–702.

Schmer, M.R., Vogel, K.P., Mitchell, R.B., and Perrin, R.K. (2008). Net energy of cellulosic ethanol from switchgrass. *Proc. Natl. Acad. Sci*. **105**, 464–469.

Shen, H., He, X.Z., Poovaiah, C.R., Wuddineh, W.A., Ma, J., Mann, D.G.J., Wang, H.Z., Jackon, L., Tang, Y., C.N. Stewart, Jr., Chen, F., and Dixon, R.A. (2012). Functional characterization of the switchgrass (Panicum virgatum) R2R3-MYB transcription factor PvMYB4 for improvement of lignocellulosic feedstocks. *New Phytol.* **193**, 121–136

Sticklen, M.B. (2008). Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nat. Rev. Genet*. **9**, 433–443.

Toyomasu, T., Kawaide, H., Sekimoto, H., Numers, C. von, Phillips, A.L., Hedden, P., and Kamiya, Y. (1997). Cloning and characterization of a cDNA encoding *gibberellin 20-oxidase* from rice (*Oryza sativa*) seedlings. *Physiol. Plant*. **99**, 111–118.

Tsai, D.-S. and Arteca, R.N. (1985). Effects of root applications of gibberellic acid on photosynthesis and growth in C3 and C4 plants. *Photosynth. Res*. **6**, 147–157.

Vidal, A.M., Gisbert, C., Talon, M., Primo-Millo, E., Lopez-Diaz, I., and Garcia-Martinez, J.L. (2001). The ectopic overexpression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. *Physiol. Plant*. **112**, 251–260.

Voorend W., Nelissen H., Vanholme R., De Vliegher A., Van Breusegem F., Boerjan W., Roldán-Ruiz I., Muylle H. and Inzé D. (2015) Overexpression of *GA20-OXIDASE1* impacts plant height, biomass allocation and saccharification efficiency in maize. *Plant Biotechnol. J.,* doi: 10.1111/pbi.12458

Xu, B., Escamilla-Treviño, L.L., Sathitsuksanoh, N., Shen, Z., Shen, H., Zhang, Y.H., Dixon, R.A., and Zhao, B. (2011). Silencing of 4-coumarate:coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New Phytol*. **192**, 611–625

Yamaguchi, N., Winter, C.M., Wu, M.-F., Kanno, Y., Yamaguchi, A., Seo, M., and Wagner, D. (2014). Gibberellin acts positively then negatively to control onset of flower formation in Arabidopsis. *Science* **344**, 638–641.

Yamazaki, H., Shiraiwa, N., Itai, A., and Honda, I. (2015). Involvement of Gibberellins in the Regulation of Tillering in Welsh Onion (Allium fistulosum L.). *Hortic. J.* http://doi.org/10.2503/hortj.MI-050

Tables

Table 2.1 Primers used in this study

Primer name	Sequences	References
	Primers for PCR	
hptII-F	CAGGACATTGTTGGAG	Li and Qu. 2010
hptII-R	TCTGTCGAGAAGTTTC	Li and Qu. 2010
ZmGA20-F	GCTCTGAGATGAGCCGTCTG	
ZmGA20(T)-R	ATTTGGAGAGGACACGCTCG	
	Primers for qRT-PCR	
GA20-F	GAGATGGACAAGGTGGTCAG	
GA20-R	GTAGTGCCTCATGGTGAAGT	
Pv4CL1-F	CGAGCAGATCATGAAAGGTTACC	Shen *et al*., 2012
Pv4CL1-R	CAGCCAGCCGTCCTTGTC	Shen *et al*., 2012
PvCAD-F	TCACATCAAGCATCCACCATCT	Shen *et al*., 2012
PvCAD-R	GTTCTCGTGTCCGAGGTGTGT	Shen *et al*., 2012
PvCOMT-F	CAACCGCGTGTTCAACGA	Shen *et al*., 2012
PvCOMT-R	CGGTGTAGAACTCGAGCAGCTT	Shen *et al*., 2012
PvUbi-F	CAGCGAGGGCTCAATAATTCCA	Xu *et al*., 2011
pvUbi-R	TCTGGCGGACTACAATATCCA	Xu *et al*., 2011

Table 2.2 Tiller number and biomass

Groups	Tiller number	Dry weight (g)	Fresh/Dry ratio
WT	23.7	50.7	4.51
G1	54.5*	108.9*	3.01*
G2	31.5	103.1*	3.31*
G3	17.3	94.2*	2.94*
G4	28.8	80.6	3.62*

* Significance relative to wild type at $p \leq 0.05$

Table 2.3 Cell measurements

Plants	# pith cells/mm2	Pith cell length (μm)	Xylem cell size (μm^2)
WT	440.31±10.20	247.47±6.3	3773.78±73.24
G4-1	694.62±35.44*	243.43±6.6	2914.67±77.05*

* Significance relative to wild type at $p \leq 0.01$

Table 2.4 Concentration of bioactive GAs in whole tiller at E1 stage *

GAs	WT	G1-3	G2-1	G3-1	G4-1
GA_1	n.d.	n.d.	n.d.	1.2 ± 0.12	1.6 ± 0.12
GA_4	n.d.	n.d.	7.4 ± 0.94	4.3 ± 0.40	10.5 ± 0.92

*Concentration in nanograms per gram fresh weight, as means of three independent measurements. nd – not detectable.

Figures

Figure 2.1 Schematic of the T-DNA region of the binary construct for switchgrass transformation. 35S Poly A: CaMV35 poly A terminator; *hptII:* hygromycin phosphotransferase II gene; CaMV35S, CaMV35S promoter; *ZmGA20ox*, *Z. mays* Gibberellin (GA) 20-oxidase; OCS poly A, octopine synthase terminator.

Figure 2.2 Switchgrass leaf painting using hygromycin B. Circles point leaf painting areas

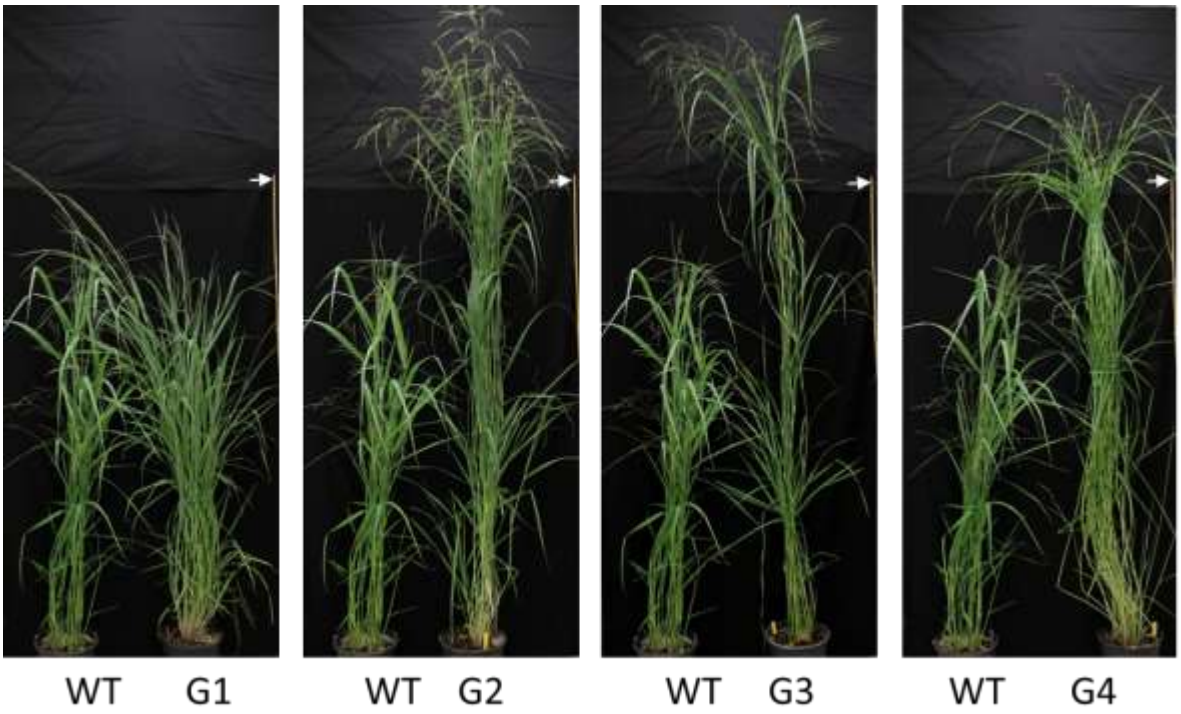


Figure 2.3 Switchgrass phenotypes. G1-G4, transgenic groups 1-4, respectively; WT, wild-type control * Arrow indicates height measurement (bar = 180 cm)

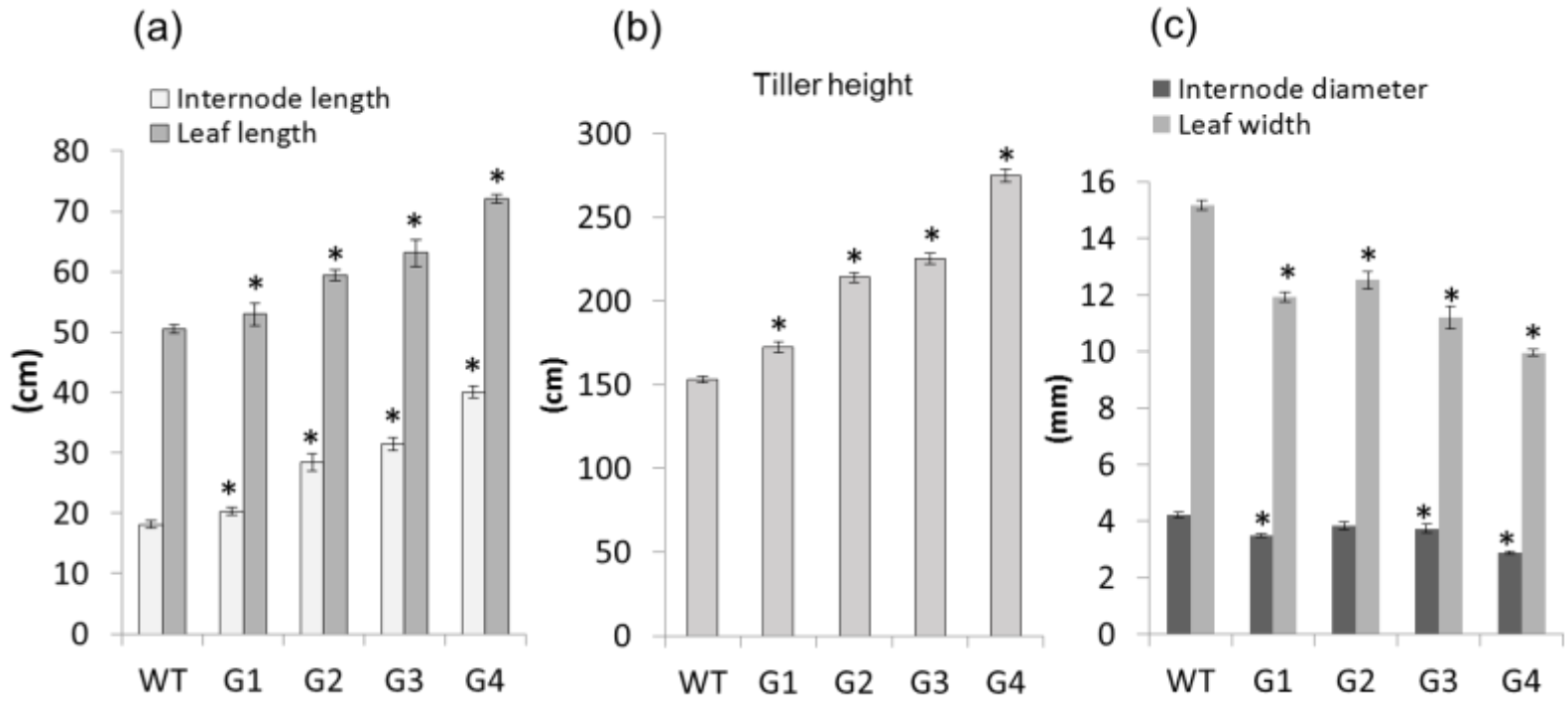


Figure 2.4 Morphology of T0 transgenic plants. G1-G4, transgenic groups 1-4, respectively; WT, wild-type control. * Indicates significant difference at $p<0.05$

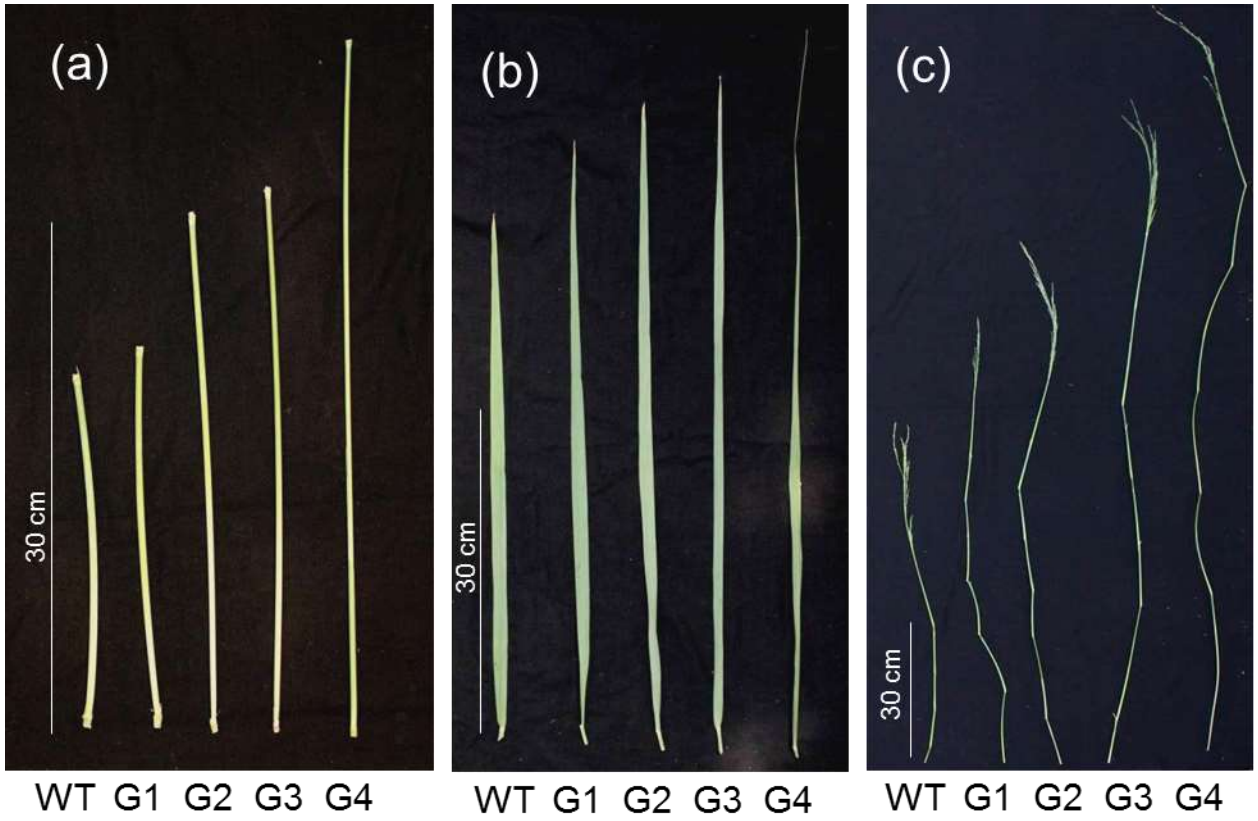


Figure 2.5 T0 plant morphology. (a) Internodes; (b) Leaves; (c) Tillers. G1-G4, transgenic groups 1-4; WT, wild-type.

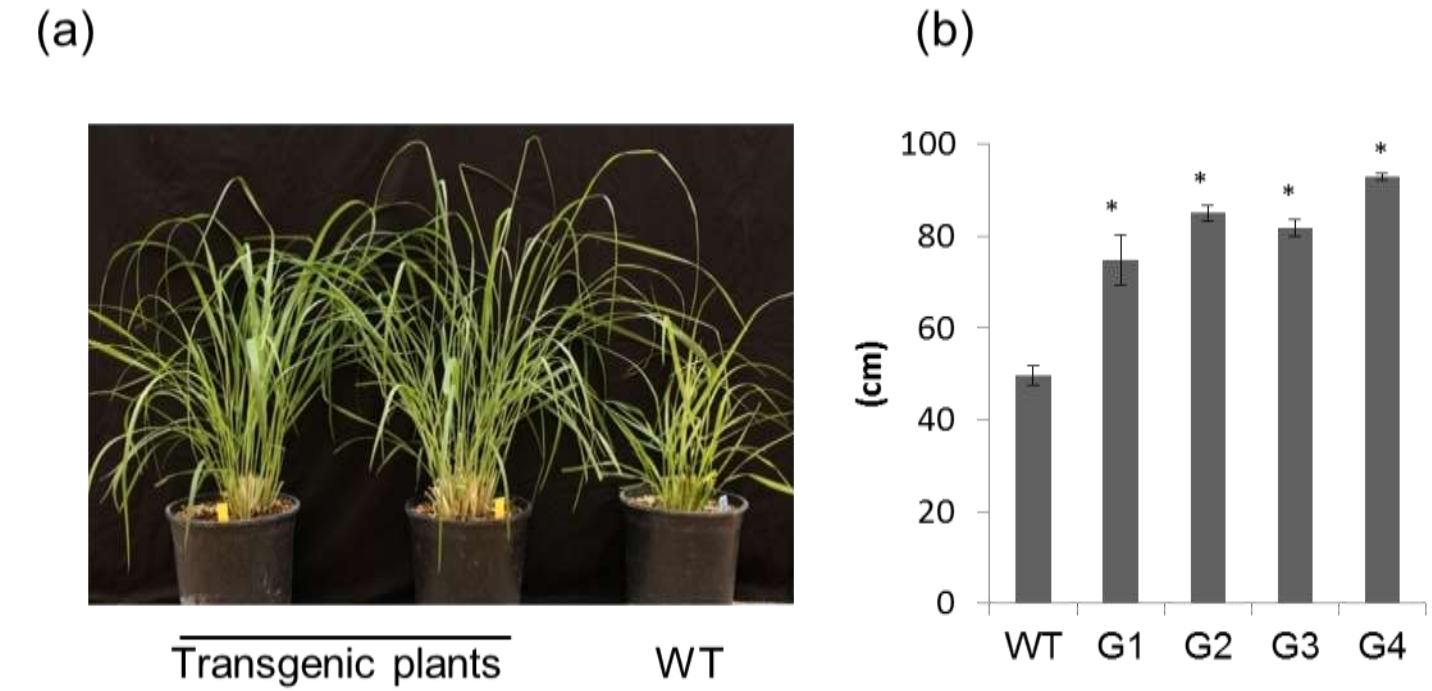


Figure 2.6 Effects of ZmGA20ox overexpression on plant growth rate

(a) Switchgrass plants grown in greenhouse (b) Tiller height at one month after cutting back

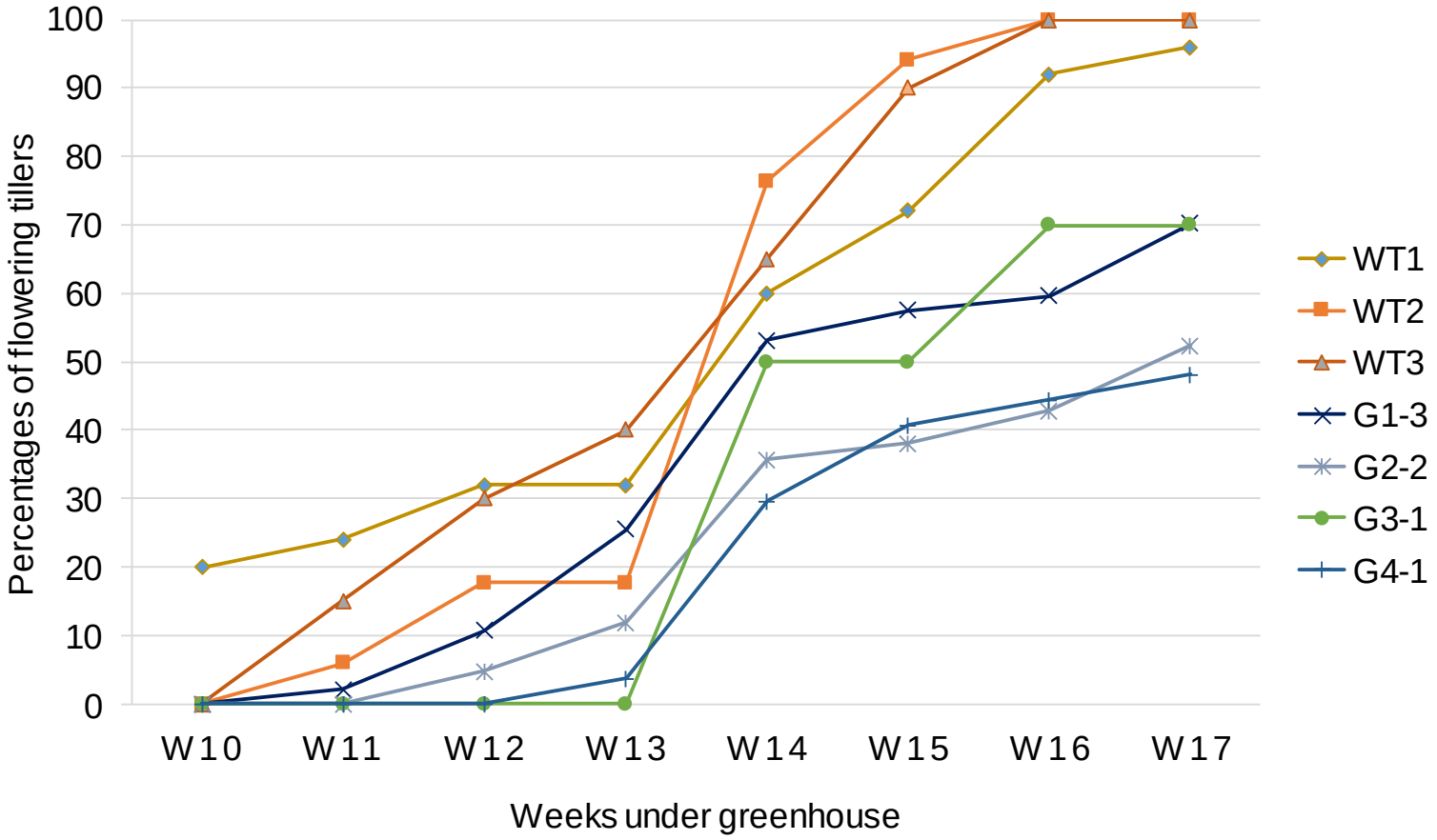


Figure 2.7 Flowering time of ZmGA20ox overexpression plants. Switchgrass plants under greenhouse condition. Transgenic lines of different groups (G1-3; G2-2; G3-1; G4-1) and wild-type control plants (WT1-WT3)

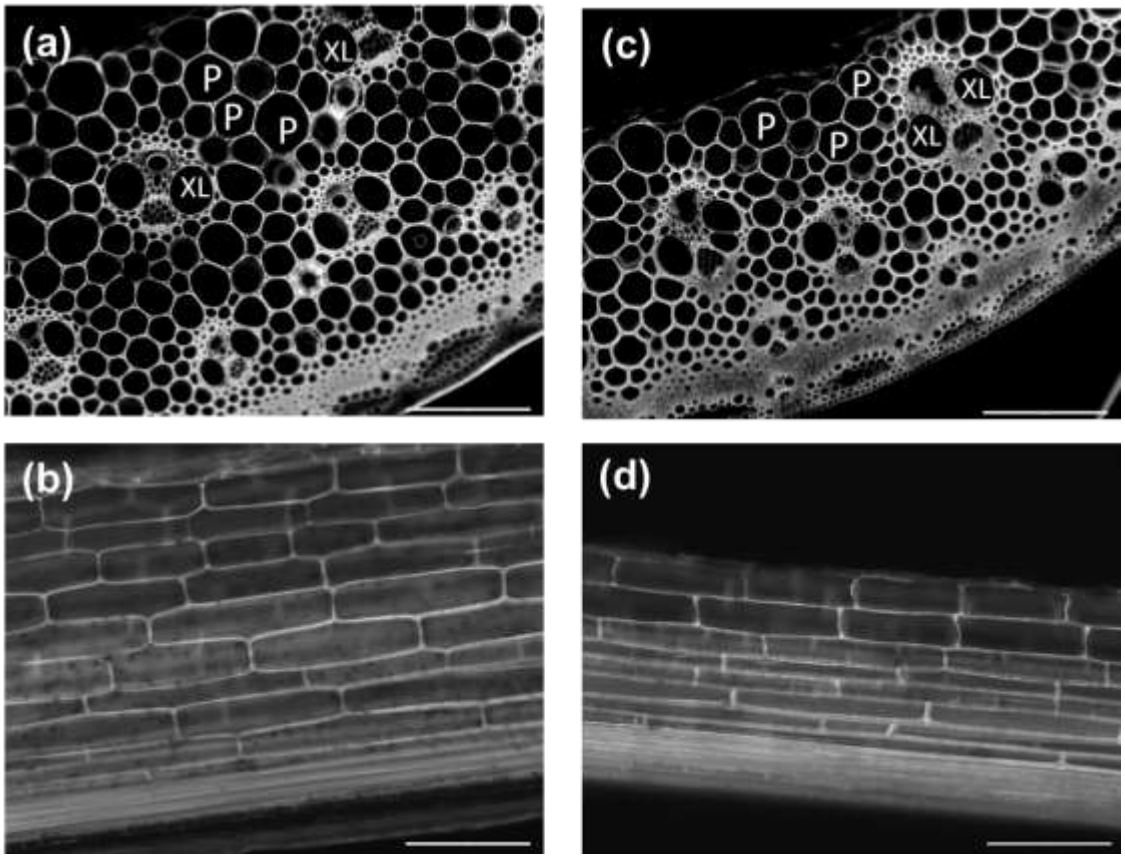


Figure 2.8 Fluorescent microscopy of plant tissues. P, pith cells; XL, xylem cells

a, b. Cross and longitudinal sections of wild-type internodes, respectively.

c, d. Cross and longitudinal sections of transgenic internodes, respectively

Bar: 200 µm at 10X magnification

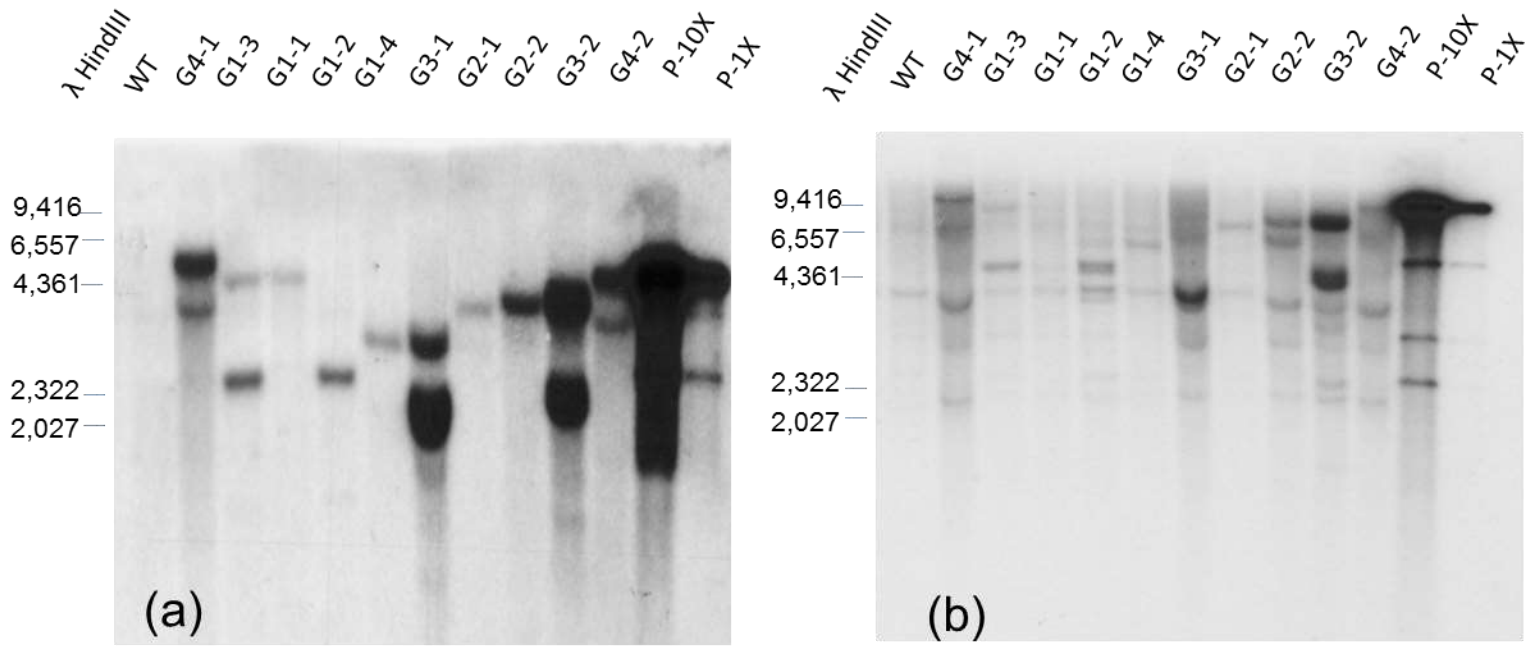


Figure 2.9 Southern blot analysis of T0 events using *hptII* probe (a) and *GA20ox* probe (b), WT, Wild-type control; G1-G4, random samples from various groups; P-10X and P-1X, plasmid digestion representing 10x and 1x genome equivalents.

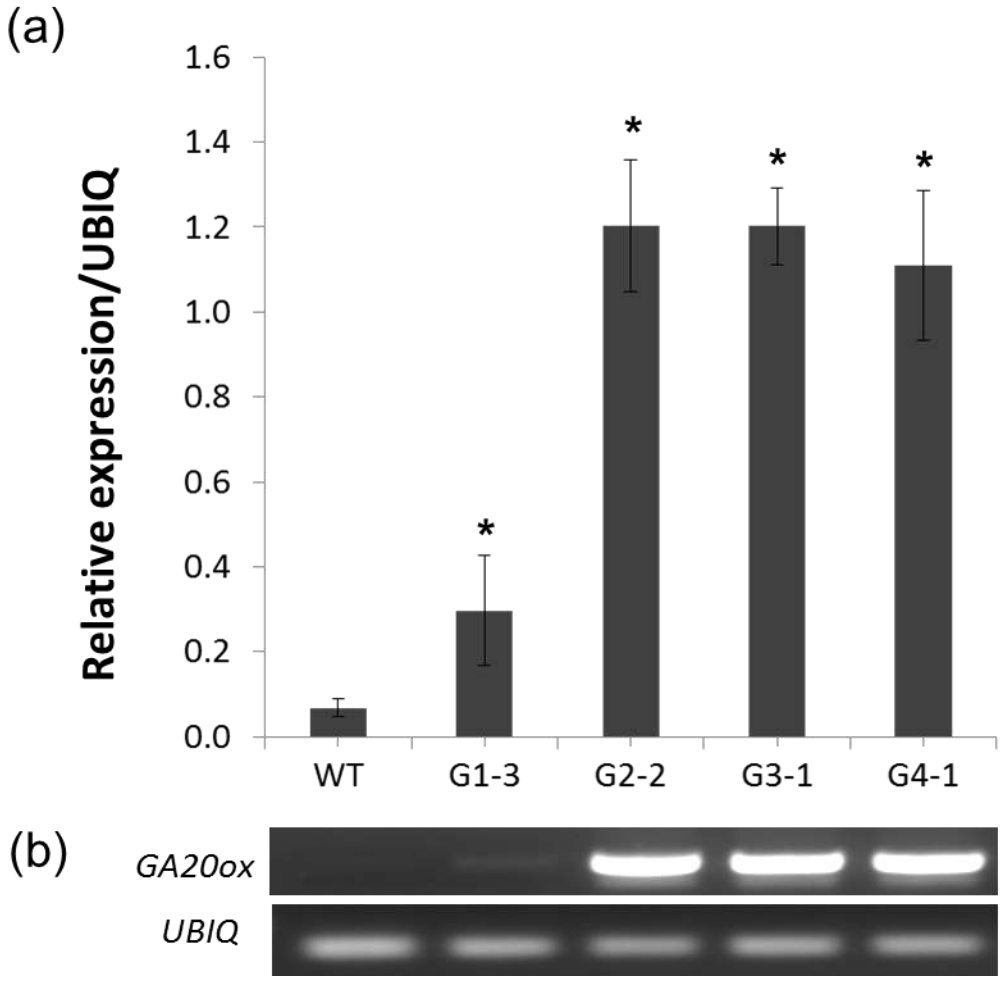


Figure 2.10 Transcript abundance of GA20ox in representative event of each group (G1-3, G2-2, G3-1, and G4-1) compared to wild-type (WT). Quantitative real-time PCR analysis of GA20ox transcript levels (a) and RT-PCR gel analysis of GA20ox and UBIQ transcripts (b).* significant relative to WT ($p<0.05$)

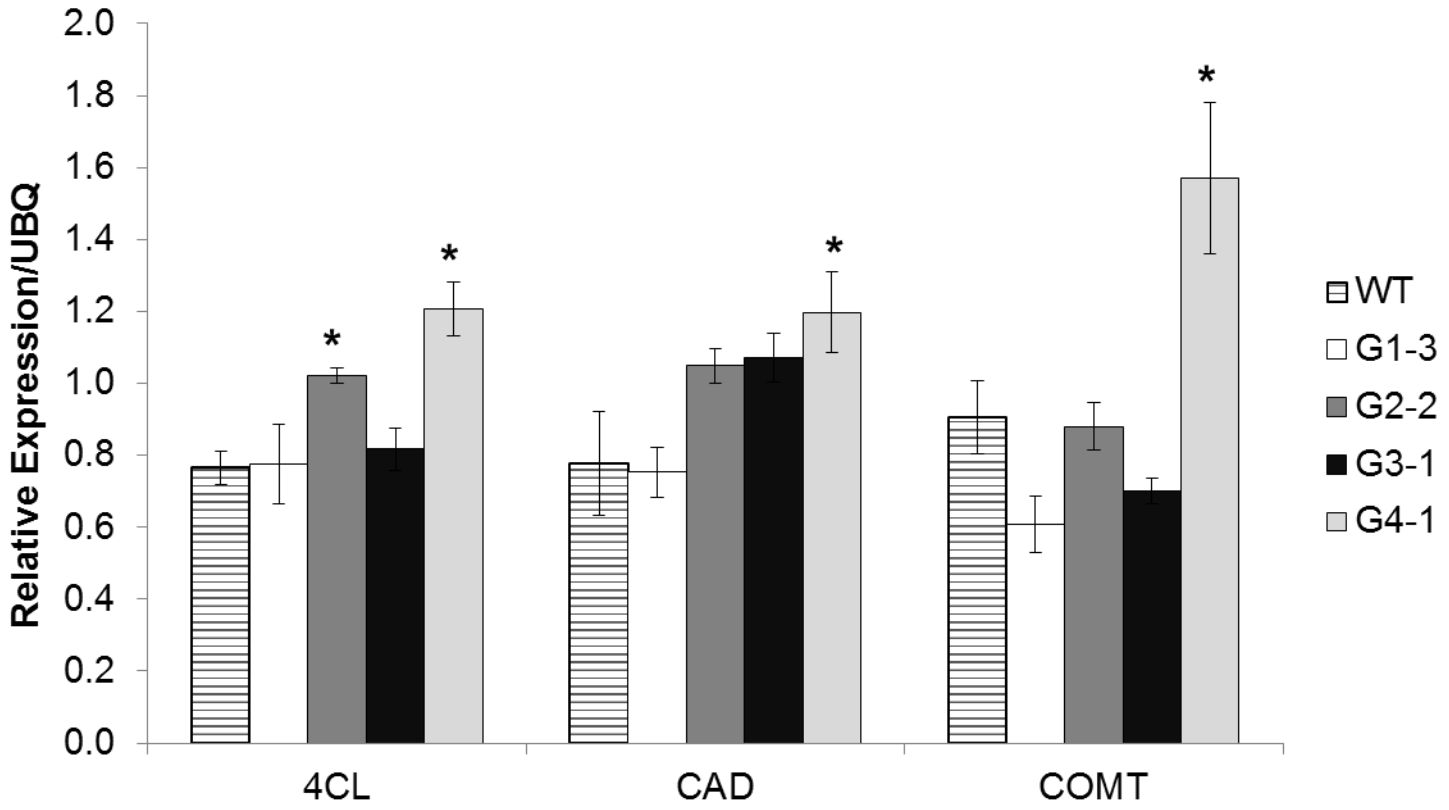


Figure 2.11 Relative expressions of lignin genes in transgenic switchgrass and wild-type by qRT-PCR. 4CL, 4-coumarate:CoA ligase; CAD, cinnamyl alcohol dehygrogenase; COMT, caffeic acid 3-O-methyltransferase. * Significant relative to wild-type ($p<0.05$)

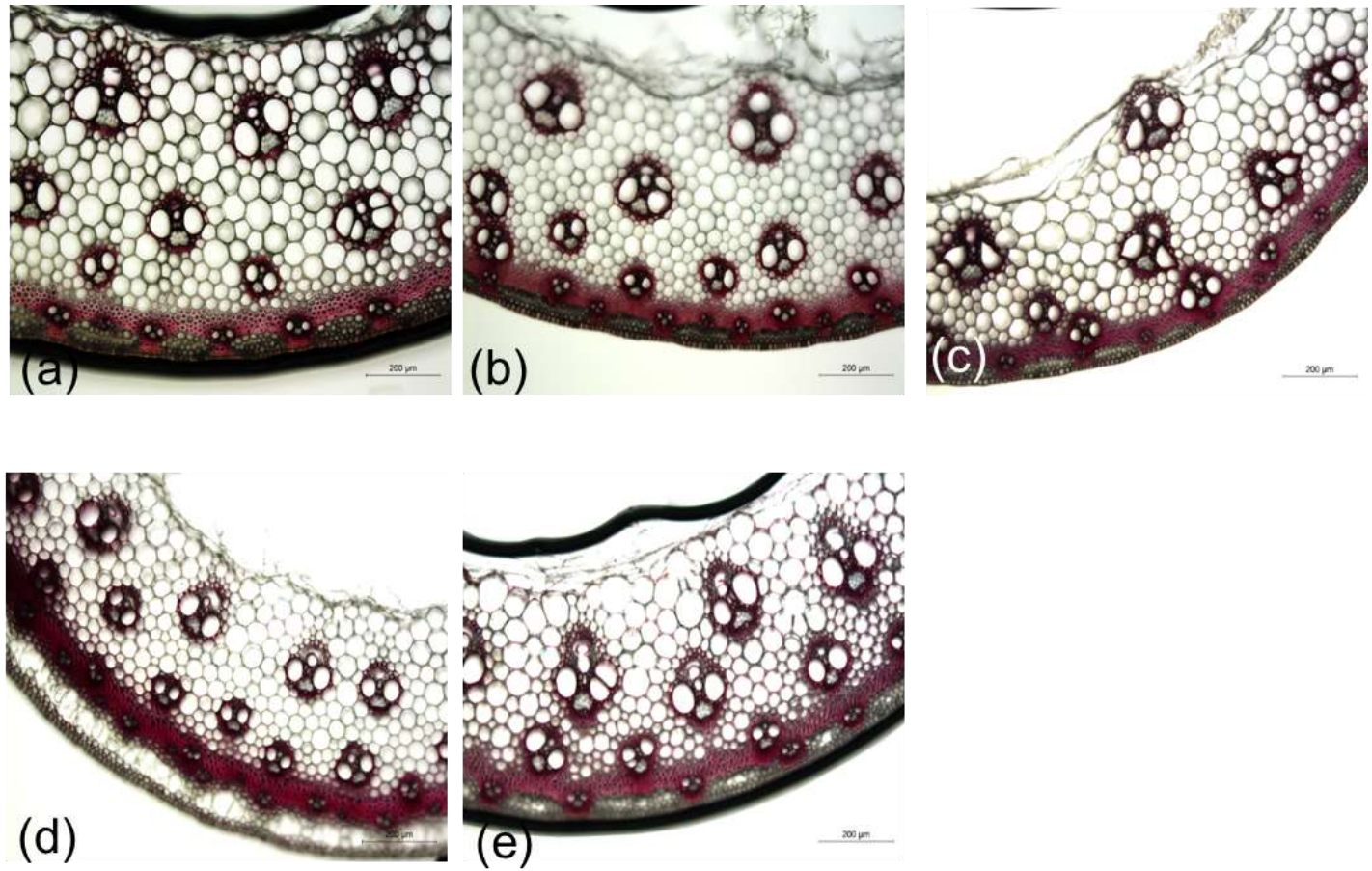


Figure 2.12 Lignin staining of internode cross sections. (a) Wild-type; (b) G1-3; (c) G2-2; (d) G3-1; (e) G4-1

Chapter 3

Rapid and efficient *Agrobacterium*-mediated transformation of sorghum (*Sorghum bicolor*) employing standard binary vectors and *bar* gene as a plant selectable marker*

*****The information in this section was submitted in Plant Cell Report

Summary

Sorghum (*Sorghum bicolor*) is an important food and biofuel crop worldwide, for which improvements in genetic transformation are needed to study its biology and facilitate agronomic and commercial improvement. Here we report optimization of regeneration and transformation of public sorghum genotype P898012 using standard binary vectors and *bar* gene as a selectable marker. The tissue culture timeframe has been reduced by 7-12 weeks with a regeneration capacity of over 18 plants per callus, and the optimized transformation procedure employing *Agrobacterium* strain AGL1 and the *MAS* promoter-containing construct driving *bar* reproducibly achieved over 14% transformation frequency. Of randomly analyzed independent transgenic events, 40-50% may carry a single copy of integrated T-DNA. Some independent transgenic events were derived from the same embryogenic callus lines, the 3:1 Mendelian segregation ratio was found in all transgenic events with one copy of integrated T-DNA, as estimated by Southern blots. The system described here should facilitate better studies of sorghum biology and agronomic improvement.

Keywords: *Agrobacterium*, sorghum, plant transformation, standard binary vectors, *bar*

Introduction

Sorghum [*Sorghum bicolor* (L) Moench] is one of the most important cereal crops in the world, especially because of its drought tolerance and cultivation on marginal soils. In 2014, over 60 million tons of sorghum grain were harvested from 38 million hectares with an average yield of 1.6 tons per hectare. Sorghum is a dietary staple for a half billion people in more than 30 countries, especially in Africa and Asia (Dahlberg et al., 2011). Sorghum also may become an important crop for biofuel production, and can be used in a variety of industrial materials. Furthermore, sorghum is the first C4 crop with a full genome sequence and is a model monocot using C4 metabolism (Chibani et al., 2009; Wang et al., 2009). However, sorghum production is affected by many biotic factors such as fungal diseases, insects, and abiotic stresses, and more attention must be given to improve nutrient values and biofuel conversion.

Significant progress has been made in *Agrobacterium*-mediated transformation of sorghum (in review, Do and Zhang, 2015). However, as compared with many other cereal crops, sorghum remains to be more recalcitrant to *Agrobacterium*-mediated transformation. Since the first report of sorghum transformation using *Agrobacterium* (Zhao et al., 2000), various sorghum genotypes and explant tissues have been explored with a goal of improving transformation efficiency (Zhao et al., 2000; Able et al., 2001; Jeoung et al., 2002; Carvalho et al., 2004; Gao et al., 2005b, 2005a; Howe et al., 2006; Nguyen et al., 2007; Gurel et al., 2009; Lu et al., 2009; Shridhar et al., 2010). The utilization of super binary vectors has dramatically improved transformation frequency of low or non-tannin sorghum genotypes (Wu et al., 2014), but this usage is subject to proprietary restrictions. By contrast, the use of standard binary vectors for sorghum

transformation can overcome this limitation but has not been significantly improved (Nguyen et al., 2007; Lu et al., 2009).

The earliest successes in transformation of sorghum were achieved in genotype P898012 through both particle bombardment or *Agrobacterium*, but yielded low frequencies (Casas et al., 1993; Zhao et al., 2000). These methods were further improved to introduce target genes of research interest (Able et al., 2001; Carvalho et al., 2004; Lu et al., 2009). Gurel et al., (2009) discovered that heat treatment of sorghum immature embryos of genotype P898012 significantly improved transformation, achieving up to 8.3% transformation frequency.

Here, we report another milestone in the improvement of *Agrobacterium*-mediated sorghum transformation system employing standard binary vectors. The improved transformation was achieved by systematic study of several regeneration and transformation conditions. The improved regeneration process enables a high rate of embryogenic callus induction and 90.6% shoot regeneration with 18.6 plants per callus, in addition to a shortened tissue culture period (only 7 to 12 weeks). Moreover, optimization of transformation conditions led to a transformation frequency over 14%. Transgene integration and inheritance were verified using histochemical GUS staining, herbicide screening, PCR and Southern blot analysis.

Results

Sorghum regeneration improvement

Immature zygotic embryos (IEs) of five sorghum genotypes were initially cultured on callus induction medium (Liu and Godwin, 2012) for 4 weeks in dark. Of

these, TBx623 represents sequenced sorghum genome; Tx430 was shown a high frequency regeneration; Tx2737 and Wheatland showed some regeneration ability in our preliminary experiments (data not shown) and P898012 has been successfully transformed before. Our screening results indicated that TBx623 and Wheatland displayed low frequencies of callus induction (less than 20%) and callus was small, white and compact, with pronounced phenolic release (Figure 3.1). By contrast, over 80% of IEs of genotypes P898012, Tx2737 and Tx430 produced highly embryogenic calli that were light yellow, friable and white in appearance (Figure 3.1), so these three genotypes were selected for further regeneration experiments.

We chose three sorghum genotypes, Tx2737, Tx430 and public genotype P898012, to evaluated impact of varying 2,4-D concentrations (CIM1-1.0 mg l^{-1}; CIM2-1.5 mg l^{-1}; CIM3- 2.0 mg l^{-1}) on callus induction for the three genotypes: All displayed high frequencies of callus induction ranging from 85.3% to 100% at three weeks on callus induction media, of which CIM1 induced a slightly lower induction efficiency and smaller size of calli than CIM2 and CIM3. Importantly, somatic embryos were obtained from all calli of genotype P898012 at three weeks (on all callus induction media) and regenerated shoots emerged at two weeks on regeneration medium. Significant increases in the frequency of regenerated plants were obtained with CIM2 and CIM3 for both P898012 and Tx430 as compared to medium CIM1 (Table 3.2). However, no difference in regeneration frequency of three tested media was observed for genotype Tx2737, thus CIM2 was used for further experiments. The regeneration capacity varied from 2.8 to 18.9 regenerants per explant depending on genotypes. The highest number of regenerated plants per callus were 3.4 and 4.8 for genotype Tx430 and Tx2737 whereas it reached

18.9 regenerated plants per callus for genotype P898012. Due to the high regeneration capacity, genotype P898012 was selected for further optimization of regeneration and transformation.

Rooting is an important step for regeneration and transformation, so we compared different plant growth regulators (Table 3.3) for induction of root formation. The addition of growth regulator to rooting medium increased the frequency of root induction from 92.3% to 100% compared to 75% of control medium without growth regulator. No significant difference in root induction frequency was found between four tested media, i.e., R1, R2, R3 and R4. However, significant differences in the number of roots, root quality and root induction time were observed. All sorghum shoots produced roots within 8 days on media R1 and R2, whereas root induction took 12 days on medium R3. Of three plant growth regulators, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) induced a high root quality (Figure 3.2 and Table 3.3). However, 1-Naphthaleneacetic acid (NAA) induced low root quality, and on medium R2 (1 mg l^{-1} NAA), multiple roots were formed but were swollen. In addition, callus was formed around these roots and inhibited shoot and root elongation (Figure 3.2). The same root morphology also was observed on R4 medium with the combination of NAA, IAA and IBA. This suggested that NAA has negative effect on sorghum root induction and development. Based on the root induction frequency, timeframe, and quality, medium R1 (1 mg l^{-1} IBA) was selected for sorghum root induction as our standard sorghum regeneration protocol and for subsequent transformation experiments. Regenerated sorghum plants showed normal growth, pollen development and seed production. As presentative, the rapid and efficient sorghum regeneration process is showed in Figure 3.3 and Table 3.1.

Agrobacterium tumefaciens strain AGL1 improved transformation efficiency

We compared three *Agrobacterium* strains: AGL1, EHA101 and GV3101, on infections of sorghum immature embryos of genotype P898012 (Table 3.4), after introduction of standard binary vector pZY102 into these strains. We observed no difference in callus induction between *Agrobacterium* strains AGL1, EHA101, and control (uninfected), which varied from 96.4% to 98.2% ($p > 0.05$); however, a significant decrease in callus induction (78.7%, $p \leq 0.05$) was observed by using *Agrobacterium* strain GV3101. Moreover, higher phenolic release was observed on culture medium surrounding infected immature embryos of this treatment. At 7 days after inoculation, calli were collected from different treatments for GUS transient assays. The GUS staining rate was 41.3% for AGL1 and 48.9% for EHA101, while that for GV3101 was only 6.7%. Finally, the stable transformation efficiency of treatment using AGL1 was 9.6 %, much higher than that of EHA101 (6.4%) (Table 3.4). The lower rate of callus induction and GUS transient assay correlated with the reduced transformation using GV3101, i.e., 1.1%, while all tested plants displayed GUS staining and herbicide resistance. Selected T0 AGL1- or EHA101-transformed events and their T1 generation were used for further molecular and segregation analysis.

Impact of promoters on sorghum transformation

Three different vectors pZY102, pFGC5941 and pFGC161 with the *bar* gene under the control of *CaMV35S*, *MAS* and *Zm-Ubi* promoters, respectively, (Figure 3.4) were mobilized into *Agrobacterium* strain AGL1 and used for sorghum transformation. These binary constructs contain the same backbone, with identical replicons for plasmid

replication. Callus induction frequency was measured at 4 weeks after inoculation, and glufosinate - ammonium (2.5 mg l^{-1}) was added to callus induction and regeneration medium for transgenic selection. The regeneration frequency using pFGC5941 was 8.8%, but reduced to 6.0% for pFGC161 and 4.1% for pZY102, respectively. The stable transgenic events were calculated from herbicide resistant sorghum plants after leaf painting and PCR using *bar* gene primers. The stable transformation frequency was consistent with the regeneration rate, except the treatment using pFGC161 for which higher rate of "escaped" (non-transgenic) plants dramatically reduced the stable transformation frequency to 3.1%, even though a high rate of regenerated plants (6.0%) was observed from this treatment. Evidently, use of vector pFGC5941 carrying *bar* gene controlled by *MAS* promoter exhibited an over two-fold increase in sorghum transformation frequency as compared to pZY102 and pFGC161 (Table 3.5). This indicated a higher transformation efficiency could obtain by the *MAS* promoter than CaMV35S or Zm-Ubi promoters.

Co-cultivation with filter papers negatively impacts sorghum stable transformation

Filter paper wicks as a culture support or co-cultivation with filter papers during the co-cultivation stage has been employed in some sorghum transformation efforts to improve transformation efficiency. To examine filter paper's impact, explants infected with *Agrobacterium* strain EHA101 harboring binary vector pZY102 were placed directly on agar co-cultivation medium (Method I) or the medium was overlaid with a piece of sterilized filter paper (Method II), before explants were transferred to the same media for all next steps. Although higher phenolic release was observed at three days of co-cultivation time (Figure 3.5), method II displayed positive effects on the survival rate of

immature embryos as well as callus induction and transient expression rate (Figure 3.6). The callus induction frequency was 85.9% when immature embryos were directly placed on agar co-cultivation medium, and increased to 98.5% by using filter-papers. Moreover, over 60% calli from method II showed *gus* transient expression, while that was 44% on method I. In addition, there was a slight increase in the number of blue dots (GUS staining) per explant in method II, respectively. However, using filter paper for co-cultivation reduced the stable transformation efficiency: While 11.4% of callus placed directly on agar co-cultivation produced transgenic plants, only 5.7% of callus cultured on filter papers gave rise to transgenic plants (Figure 3.6). Selected transgenic plants from two treatments were transferred to the soil, and the presence of *gus* and *bar* genes of these events were confirmed by GUS staining and leaf painting.

Optimized procedure increases sorghum transformation efficiency

To measure the overall transformation efficiency using the above improved regeneration and transformation conditions, we conducted stable sorghum transformation employing *Agrobacterium* strain AGL1 containing the binary vector pFGC5941 in which *MAS* promoter drives *bar*. A total of 656 sorghum immature embryos of genotype P88012 were deployed in three independent experiments. The callus induction frequency varied from 87.1% to 92.3% (Table 3.6). The lower callus induction frequency may have resulted from low quality sorghum immature embryos harvested from late fall season, which is known to be unfavorable for sorghum growth. Despite the lower quality of immature embryos, sorghum transformation efficiency over the three independent experiments was 14% with a total of 93 independent herbicide resistant plants.

GUS staining and herbicide selection

The efficiency of various treatment comparisons was first evaluated by the transient expression of *gus* gene displayed in sorghum callus at 10 days after inoculation. The insertion and expression of *gus* gene was indicated by blue color on leaves, roots and florets of T0 transgenic plants (Figure 3.7). In addition, *gus* gene inheritance and expression in progeny were exhibited by GUS staining of T1 immature embryos and seedlings (Figure 3.7). No GUS staining observed on various tissues of non-transgenic wild-type plants, such as leaves, roots, florets, immature embryos and seedlings, indicating the *gus* gene was transferred, integrated and inherited in transgenic sorghum. On selection medium amended with 2.5 mg l^{-1} glufosinate, non-transgenic calli turned brown and died quickly as they were transferred to the light condition (Figure 3.8a), while the transgenic calli remained growth and regenerated shoots. After leaf painting, transgenic plant leaves showed no necrotic symptoms while wild type plant leaves displayed severe damage (Figure 3.8b). For herbicide spraying, leaves of susceptible seedlings became yellow and then brown 5 days after herbicide spray. The whole seedling turned dry and died at 10 days of spraying. However, resistant plants showed normal growth without necrotic damage (Figure 3.8c).

Molecular analysis of T0 transgenic sorghum

Randomly selected, herbicide resistant sorghum plants were subjected to further molecular analysis. The presence of *bar* and *gus* genes were confirmed by PCR using *bar* and *gus* specific primers, perspectively. PCR-positive plants were randomly selected for Southern blot to determine transgene integration into the sorghum genome (Table 3.7 and

Figure 3.9). Restriction enzyme *BamH*I, which cuts only once within the T-DNA of plasmids pFGC5941 and pFGC161, was used for DNA digestion of transgenic plant transformed with those plasmids. Furthermore, restriction enzyme *Xho*I was used for transgenic plants of pZY102. Four of ten transgenic events derived from *Agrobacterium* strain AGL1 displayed single T-DNA insertions, with average 2.1 ± 0.3 copy numbers of integrated T-DNA (Table 3.7 and Figure 3.9a). Transgenic events derived from strain EHA101 showed a slight reduction in single copy insertion (27.3%) as compared to those from AGL1. Moreover, the average copy numbers of T-DNA insertion increased to 2.4 ± 0.4 (Table 3.7 and Figure 3.9b). This result indicated that *Agrobacterium* strain AGL1 shall be preferred for sorghum transformation. Interestingly, Southern blot results exhibited different insertion patterns of transgenic lines regenerated from the same callus. For example, transgenic lines A1-1, A1-2 and A1-3 were regenerated from one callus of the experiment using strain AGL1. While A1-1 and A1-3 lines presented the same banding pattern, A1-2 showed different bands (Figure 3.9a, b) suggesting a different different event. Different banding patterns were also obtained from transgenic lines E2-1 and E2-2 regenerated from one callus of EHA101 treatment. Together, the results here show the average copy numbers of integrated T-DNA varied from 2.0 to 2.5 and the frequency of single insertion ranged from 27.3% to 50%, depending on the choice of *Agrobacterium* strain and promoter driving the *bar* gene.

Progeny segregation analysis

Seeds of different T0 transgenic events randomly-selected from above AGL1-transformation experiments were used for T1 segregation analysis. Herbicide screening was used to confirm the inheritance and expression of the *bar* gene and GUS assay was

utilized for *gus* gene confirmation. Transgenic events derived from binary vector pZY102 were subjected to analysis of both *bar* and *gus* genes. Chi-square test confirmed that all transgenic events with one insertion (as estimated from Southern blot) showed the Mendelian inheritance (3:1) (Table 3.8). These transgenic events were from different binary vectors: pZY102 (A6, Z1 and Z8); pFGC5941 (F8 and F9); pFGC161 (G4) (Table 3.8 and Figure 3.9). Of these, transgenic lines A6, Z1 and Z8 displayed 3:1 segregation ratio for both *bar* and *gus* genes. The consistency between T1 segregation ratio and insertion pattern of Southern blot indicated that these transgenic events contained single copy T-DNA. On other hand, transgenic events with multiple copies of integrated T-DNA exhibited complex segregation patterns. Most of them showed a significant difference from 3:1 segregation ratio (Table 3.8). However, the 3:1 segregation of *bar* and *gus* genes were observed from events A1-3 and Z4, which showed 3 insertions of T-DNA. Moreover, transgenic events E4 and A1-2 displayed 3:1 segregation for *gus* gene but different patterns were obtained with the *bar* gene. By contrast, the 3:1 segregation occurred in events A4 and Z5 for *bar* gene but not for the *gus* gene. The 3:1 ratio also was displayed in transgenic events F3 and G7 that have more than one copy of *bar* gene. In addition, the expression of *gus* gene was not observed from T1 generation of transgenic event Z2 that contained 6 insertions of T-DNA. Furthermore, this event showed the distorted segregation frequency of *bar* gene: Only 16 out of 108 seedlings were resistant to herbicide, while no seedling had GUS staining. These results indicated the unpredictable inheritance and segregation patterns of transgenic plants with multiple copies of transgenes.

Discussion

The higher capacity of super-binary vectors in plant transformation was exhibited in some previous studies. However, the utilization of super-binary vectors is subjected to the challenges in vector construction, cloning and transformation. Moreover, their capacity is most evident as combined with only certain *Agrobacterium* strains (Komari et al., 2006). Here, we have developed a rapid and efficient *Agrobacterium*-mediated transformation process for sorghum using standard binary vector system and *bar* gene as a plant selectable marker. The use of optimal concentration of 2,4-D (1.5mg/L**)** and effective rooting medium as well as *Agrobacterium tumefaciens* strain AGL1 and a desirable promoter driving selectable marker gene *bar* have been critical to achieve this. Because the immature embryos we deployed in our stable sorghum transformation experiments were from late fall and winter season, we anticipate a higher transformation frequency to be achieved using higher quality immature embryos.

High phenolic release requires more frequent culture transfer and also is thought to be toxic to *Agrobacterium* cells (Zhao et al., 2000; Nguyen et al., 2007), consequently limiting the transformation efficiency. Therefore, reduction of tissue culture timeframe and increase in regenerated plant potential are important strategies to improve transformation efficiency. Grootboom et al., (2008) reported an efficiency of 6.13 regenerants per callus explant using genotype P898012 and the tissue culture timeframe ranged from 10 to 14 weeks. In this present study, by the optimization of callus induction and rooting media, the shortest tissue culture timeframe was reduced to 7 weeks and plant regeneration capacity reached 18.9 regenerated plants per callus. Moreover, the high frequency of root formation and recovered plants contribute to a greater potential of

transgenic event recovery. An additional important change in our protocol is the elimination of maturation medium. The maturation medium was used to develop somatic embryos before shoot development (Zhao et al., 2000; Grootboom et al., 2008). Through our morphological observations we found that somatic embryos already have developed to more advanced developmental (maturation) stage towards to end of callus induction, allowing us to eliminate the maturation stage without compromising shoot development.

Different *Agrobacterium tumefaciens* strains exhibit different effects on plant transformation frequency and transgenic event quality with various plant species (Chetty et al., 2013; Cao et al., 2014; Cho et al., 2014). By using supper binary vectors for sorghum transformation of genotype Tx430, Wu et al., (2014) reported a higher transformation frequency but a lower transgenic event quality of strain AGL1 as compared to strain LB4404. In our study with standard binary vectors and genotype P898012, we found that *Agrobacterium* strain AGL1 displayed a higher transformation frequency and good transgenic event quality compared to strains EHA101 and GV301. Southern blot using *bar* or *gus* partial open reading frame detected that 40% of transgenic events displayed single insertion of transgenes. Moreover, transgenic events with single insertion displayed the Mendelian inheritance. Therefore, *Agrobacterium tumefaciens* strain AGL1 is recommended for sorghum transformation using standard binary vectors. Co-cultivation with filter papers or filter paper wicks have been shown to increase Co-cultivation with filter papers or filter paper wicks have been utilized to eliminate the over-growth of bacteria, increase transient gene expression and regeneration potential as well as transformation frequency of various plant species including both monocot and dicots (Cheng et al. 2003; Howe et al. 2006; Lu et al. 2009; Ozawa 2009; Nanasato et al.

2011; Nanasato et al. 2013; Yang et al. 2013; Jia et al. 2015; Nanasato et al. 2015). We evaluated the effect of filter papers on the callus induction frequency, the transient expression of transgenes and the stable sorghum transformation frequency. In current study, the over-growth of *Agrobacterium* was not observed at three days on co-cultivation medium. However, higher phenolic pigment accumulation on filter papers indicated the capacity of filter papers to absorb phenolic substances. Similar to the utilization of PVPP and activated charcoal (Zhao et al. 2000; Nguyen et al. 2007), higher callus induction frequency and GUS transient expression were observed with filter paper treatment as compared to the solid agar system. However, utilizing these materials may reduce the effective concentration of certain medium components, growth regulators, phenols and therefore, affect the efficiency of transformation progress. Consequently, the stable transformation was much lower in the presence of filter papers.

The choice of promoters is important for transformation and transgene expression. Promoters that have been utilized for sorghum transformation *CaMV35S*, maize alcohol dehydrogenase promoter (*adh1*), *Zm-Ubi*, rice actin promoter (*actin1*) and a chimeric promoter with the 35S enhancer fragment (HBT) (Casas et al., 1993; Gallie and Young, 1994; Vain et al., 1996; Casas et al., 1997; Zhao et al., 2000; Jeoung et al., 2002; Tadesse et al., 2003; Carvalho et al., 2004; Gao et al., 2005b; Nguyen et al., 2007; Gurel et al., 2009; Lu et al., 2009). The activities of different promoters have been compared by using reporter genes (*uidA* and *gfp*) for both bombardment and *Agrobacterium*-mediated methods (Able et al., 2001; Jeoung et al., 2002; Tadesse et al., 2003). The *Zm-Ubi* has been considered to be the most efficient for transgene expression and has been utilized in most recent sorghum transformation studies (Gurel et al., 2009; Liu and Godwin, 2012;

Wu et al., 2014). However, Zhao et al., (2000) found that the level of transient expression of reporter genes was not directly correlated with the frequency of stable transformation. In our experiments, the activities of the modified CaMV35S, *Zm-Ubi* and *MAS* promoters were evaluated, with the highest stable transformation frequency being achieved by the utilization of the *MAS* promoter. In addition, there was no significant difference in stable transformation between the enhanced CaMV35S and *Zm-Ubi* promoters ($p > 0.05$). This is the first comparison of different promoters on sorghum stable transformation.

Materials and methods

Plant materials

Five sorghum genotypes, P898012 (public genotype), TBx603 (sequencing genotype), Tx2737, Tx430 (inbred lines) and Wheatland (short growing variety) (generous gifts of Dr. Yinghua Huang at USDA-ARS, OK) were used for the regeneration tests and genotype P898012 was used for optimization of transformation conditions. Sorghum plants were grown in glasshouse at the University of Missouri, Columbia, MO, with day/night temperatures of 28/21^{o}C, a photoperiod of 16 h light/8 h dark, in 3-gallon pots containing Promix soil supplemented by Osmocote (14-14-14) (Hummert International, Earth City, MO). Sorghum plants were watered and fertilized as needed. Each sorghum head was covered by a tassel bag before pollination. Immature seeds were collected from sorghum panicles 11–14 days after pollination and used for regeneration and transformation experiments.

Regeneration

Immature seeds of different sorghum genotypes were sterilized with 50% bleach for 15 min with gentle agitation and rinsed 3-4 times with sterile water. For genotype screening, immature embryos, 1.0-1.5 mm in length, were isolated and placed on callus induction medium with scutellum face up; callus morphology, quality and induction frequency were evaluated after three weeks. Medium compositions modified from previous work (Liu and Godwin, 2012) are listed in Table 3.1.

***Agrobacterium* strains and binary vectors**

Agrobacterium tumefaciens strains AGL1, EHA101 and GV3101 were used with three different binary vectors pZY102, pFGC5941 and pFGC161 (Figure 3.4) containing the *bar* gene under the control of the cauliflower mosaic virus (*CaMV35S*), mannopine synthase (*MAS),* or maize ubiquitin (*Zm-Ubi*) promoters. These binary constructs contain the same backbone, with identical replicons for plasmid replication. Moreover, the β-glucuronidase gene (*gus)* harbored by pZY102 was employed for histochemical GUS staining for both transient and stable transformation event evaluation.

Transformation

Briefly, *Agrobacterium tumefaciens* harboring binary vectors were clonally isolated from a -80°C glycerol stock onto a YEP medium plate containing appropriate antibiotics and incubated at 28ºC in the dark for 2-3 days. A single colony was streaked out onto new YEP plate with the same antibiotics and kept at the same condition for 2-3 days until bacterial colonies developed. One loop of *Agrobacterium tumefaciens* taken from YEP plate was suspended in infection medium (IM) to reach cell density of 0.4 at OD_{550}

(optical density). Bacteria were incubated at 100 rpm for 4 hours at room temperature before inoculation. About 50-70 sorghum immature embryos were subjected to heat treatment for 3 min at 43 ^{o}C, followed by 2 min at 25^{o}C before being inoculated with 1 ml bacterial suspension for 10 min. The embryos were placed on co-cultivation medium (TM) with scutellum face up. The plates were kept at 25^{o}C in dark for 3 days, then embryos were transferred to resting medium (RM) for 10 days with a subculture after each 5-days. Calli were transferred to callus induction medium (CM) for 10 days before being transferred to shoot regeneration medium (SM). Embryogenic calli on SM medium were exposed to light of 100-150 μmol $m^{-2}s^{-1}$ and 18:6 h photoperiod at 26^{o}C for shoot induction (for 6-10 weeks), sub-cultured to fresh medium every two weeks). Elongated shoots with 3-4 leaves were transferred to rooting medium (RT) for 2-3 weeks, and regenerated shoots with healthy roots were transferred to Promix soil in a growth chamber before moving to greenhouse.

Herbicide resistance screen

Herbicide resistance screening of primary (T0) sorghum events was performed three times onto three different leaves by swiping 100 mg l^{-1} glufosinate-ammounium solution onto the upper leaf surface. Results were recorded after one week. For transgenic T_1 plants, seeds germinated in Promix soil and seedlings with 3-4 young leaves were subjected to Liberty® spray (100 mg l^{-1} glufosinate-ammonium) three times. Results were recorded after one week.

Histochemical GUS staining

Transgenic plant tissues (calli, leaves, roots, florets, immature embryos and seedlings) were assayed with X-Gluc staining solution at 37°C for 24 h (Jefferson et al., 1987). Chlorophyll of plant tissue was removed by soaking in 70% ethanol or by optical clearing (Warner et al., 2014). Sixty calli of each treatment were used for GUS staining, the number of blue spots were observed under microscopes and recorded.

PCR and genomic Southern blot

DNA used for PCR and Southern blots were isolated from leaf tissue using a CTAB procedure modified from (Dellaporta et al., 1983). The primer pairs for PCR included forward-bar (5'-AAACCCACGTCATGCCAGTT-3'), reverse-bar (5'-CATCGAGACAAGCACGGTCA-3'), forward-gus (5'- GCTAACGTATCCACGCCGTA-3'), reverse-gus (5'-CATGAAGATGCGGACTTGCG-3'). For Southern blot analysis, 30 µg purified DNA was digested by restriction enzymes that cut once within the T-DNA region and DNA fragments were separated on a 1.0% agarose gel prior to transfer to Zeta-Probe® GT nylon membrane (Bio-Rad, USA). DNA was fixed to nylon membranes by UV cross-link. Hybridization and membrane washing were conducted at 65^{o}C by the manufacturer's instructions. Prime-It® RmT Random Primer Labeling Kit (Stratagene, USA) was used to generate ^{32}P-labeled probes of *bar* gene (from pZY102, pFGC5941, pFGC161) or *gus* gene (from pZY102).

Progeny segregation analysis

Chi-square test was used to analyze the segregation ratios of *bar* gene and *gus* gene of T1 sorghum plants based on herbicide resistance and GUS staining, respectively. For each

independent event (T0), about one hundred T1 plants were screened. Chi-square values greater than 3.84 indicated that the observed ratio was different from the expected Mendelian ratio of 3:1 for independent segregation of a single locus (at $P \leq 0.05$).

Experimental design and data analysis

Regeneration and transformation experiments were arranged as Randomized Complete Block Design, or Complete Random Design (when block effect was not significant). Each experiment was repeated three times and the data was collected for statistical analysis. Comparisons between different treatments were made by Turkey's least significant difference procedure using one-way ANOVA and *t*-Test in SPSS software (ver.20, Chicago, IL, USA). Standard errors are provided as appropriate. Callus induction anh transformation frequency was calculated by the number of callus or independent herbicide resistant sorghum plants over the total number of immature embryos to start with. All randomly sampled herbicide resistant plants were later confirmed to carry transgenes by GUS-staining, PCR, and Southern blot as well as displayed transgene inheritance by progeny segregation analysis.

Acknowledgements

The authors thank Dr. Yinghua Huang (USDA-ARS, OK) for providing sorghum genotypes, Dr. Lu Lu for informative suggestions and Drs. David Braun, David Mendoza and Xi Xiong for helpful discussions. Thanks are also extended to Neng Wan for his help with greenhouse work and Cuong X. Nguyen for help with GUS assay. This research was supported by Vietnam Educational Foundation (VIED) (awarded to P.T. Do). The authors have no conflict of interest to declare.

References

Able, J.A., Rathus, C., and Godwin, I.D. (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. In Vitro Cell. Dev. Biol.-Plant **37**: 341–348.

Cao, S., Masilamany, P., Li, W., and Pauls, K.P. (2014). Agrobacterium tumefaciens-mediated transformation of corn (Zea mays L.) multiple shoots. Biotechnol. Biotec. Eq. **28**: 208–216.

Carvalho, C.H.S., Zehr, U.B., Gunaratna, N., Anderson, J., Kononowicz, H.H., Hodges, T.K., and Axtell, J.D. (2004). Agrobacterium-mediated transformation of sorghum: factors that affect transformation efficiency. Genet Mol Biol **27**: 259–269.

Casas, A.M., Kononowicz, A.K., Haan, T.G., Zhang, L., Tomes, D.T., Bressan, R.A., and Hasegawa, P.M. (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. In Vitro Cell. Dev. Biol.-Plant **33**: 92–100.

Casas, A.M., Kononowicz, A.K., Zehr, U.B., Tomes, D.T., Axtell, J.D., Butler, L.G., Bressan, R.A., and Hasegawa, P.M. (1993). Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. **90**: 11212–11216.

Cheng, M., Hu, T., Layton, J., Liu, C.-N., and Fry, J.E. (2003). Desiccation of plant tissues post-Agrobacterium infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In Vitro Cell. Dev. Biol.-Plant **39**: 595–604.

Chetty, V.J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., and Orozco-Cardenas, M.L. (2013). Evaluation of four Agrobacterium tumefaciens strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom. Plant Cell Rep. **32**: 239–247.

Chibani, K., Wingsle, G., Jacquot, J.-P., Gelhaye, E., and Rouhier, N. (2009). Comparative genomic study of the thioredoxin family in photosynthetic organisms with emphasis on Populus trichocarpa. Mol. Plant **2**: 308–322.

Cho, M.-J., Wu, E., Kwan, J., Yu, M., Banh, J., Linn, W., Anand, A., Li, Z., TeRonde, S., Register III, J.C., and others (2014). Agrobacterium-mediated high-frequency transformation of an elite commercial maize (Zea mays L.) inbred line. Plant Cell Rep. **33**: 1767–1777.

Dahlberg, J., Berenji, J., Sikora, V., and Latkovic, D. (2011). Assessing sorghum [Sorghum bicolor (L) Moench] germplasm for new traits: food, fuels & unique uses. Maydica **56**: 85–92.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Report. **1**: 19–21.

Do, P.T. and Zhang, Z.J. (2015). Sorghum Transformation: Achievements, Challenges, and Perspectives. In Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants (Springer), 291-312.

Gallie, D.R. and Young, T.E. (1994). The Regulation of Gene Expression in Transformed Maize Aleurone and Endosperm Protoplasts (Analysis of Promoter Activity, Intron Enhancement, and mRNA Untranslated Regions on Expression). Plant Physiol. **106**: 929–939.

Gao, Z., Jayaraj, J., Muthukrishnan, S., Claflin, L., and Liang, G.H. (2005a). Efficient genetic transformation of sorghum using a visual screening marker. Genome **48**: 321–333.

Gao, Z., Xie, X., Ling, Y., Muthukrishnan, S., and Liang, G.H. (2005b). Agrobacterium tumefaciens-mediated sorghum transformation using a mannose selection system. Plant Biotechnol. J. **3**: 591–599.

Grootboom AW, Mkhonza NL, O'Kennedy MM, Chakauya E, Kunert KJ, Chikwamba RK (2010) Biolistic mediated sorghum (*Sorghum bicolor* L. Moench) transformation via mannose and bialaphos based selection systems. Intl J Bot **6:** 89-94

Grootboom, A.W., OKennedy, M.M., Mkhonza, N.L., Kunert, K., Chakauya, E., and Chikwamba, R.K. (2008). In vitro culture and plant regeneration of sorghum genotypes using immature zygotic embryos as explant source. Int. J. Bot. **4**, 450-455

Gurel, S., Gurel, E., Kaur, R., Wong, J., Meng, L., Tan, H.-Q., and Lemaux, P.G. (2009). Efficient, reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. Plant Cell Rep. **28**: 429–444.

Howe, A., Sato, S., Dweikat, I., Fromm, M., and Clemente, T. (2006). Rapid and reproducible Agrobacterium-mediated transformation of sorghum. Plant Cell Rep. **25**: 784–791.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**: 3901.

Jeoung, J.M., Krishnaveni, S., Muthukrishnan, S., Trick, H.N., and Liang, G.H. (2002). Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. Hereditas **137**: 20–28.

Jia, Y., Yao, X., Zhao, M., Zhao, Q., Du, Y., Yu, C., and Xie, F. (2015). Comparison of Soybean Transformation Efficiency and Plant Factors Affecting Transformation during the Agrobacterium Infection Process. Int. J. Mol. Sci. **16**: 18522–18543.

Komari T, Takakura Y, Ueki J, Kato N, Ishida Y, Hiei Y (2006) Binary vectors and super-binary vectors. Methods Mol Biol **343**:15–41.

Liu, G. and Godwin, I.D. (2012). Highly efficient sorghum transformation. Plant Cell Rep. **31**: 999–1007.

Lu, L., Wu, X., Yin, X., Morrand, J., Chen, X., Folk, W.R., and Zhang, Z.J. (2009). Development of marker-free transgenic sorghum [Sorghum bicolor (L.) Moench] using standard binary vectors with bar as a selectable marker. Plant Cell Tissue Organ Cult. PCTOC **99**: 97–108.

Nanasato, Y., Kido, M., Kato, A., Ueda, T., Suharsono, S., Widyastuti, U., Tsujimoto, H., and Akashi, K. (2015). Efficient genetic transformation of Jatropha curcas L. by means of vacuum infiltration combined with filter-paper wicks. In Vitro Cell. Dev. Biol.-Plant **51**: 399–406.

Nanasato, Y., Konagaya, K., Okuzaki, A., Tsuda, M., and Tabei, Y. (2011). Agrobacterium-mediated transformation of kabocha squash (Cucurbita moschata Duch) induced by wounding with aluminum borate whiskers. Plant Cell Rep. **30**: 1455–1464.

Nanasato, Y., Konagaya, K., Okuzaki, A., Tsuda, M., and Tabei, Y. (2013). Improvement of Agrobacterium-mediated transformation of cucumber (Cucumis sativus L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. Plant Biotechnol. Rep. **7**: 267–276.

Nguyen, T.-V., Thu, T.T., Claeys, M., and Angenon, G. (2007). Agrobacterium-mediated transformation of sorghum (Sorghum bicolor (L.) Moench) using an improved in vitro regeneration system. Plant Cell Tissue Organ Cult. **91**: 155–164.

Ozawa, K. (2009). Establishment of a high efficiency Agrobacterium-mediated transformation system of rice (Oryza sativa L.). Plant Sci. **176**: 522–527.

Shridhar, J., Bhat, R.S., Bhat, S., and Kuruvinashetti, M.S. (2010). Agrobacterium-mediated transformation studies in orghum using an improved gfp reporter gene. Journal of SAT Agricultural Research **8**, 1-5

Tadesse, Y., Sagi, L., Swennen, R., and Jacobs, M. (2003). Optimisation of transformation conditions and production of transgenic sorghum (Sorghum bicolor) via microparticle bombardment. Plant Cell Tissue Organ Cult. **75**: 1–18.

Vain, P., Finer, K.R., Engler, D.E., Pratt, R.C., and Finer, J.J. (1996). Intron-mediated enhancement of gene expression in maize (Zea mays L.) and bluegrass (Poa pratensis L.). Plant Cell Rep. **15**: 489–494.

Wang, X., Gowik, U., Tang, H., Bowers, J.E., Westhoff, P., Paterson, A.H., and others (2009). Comparative genomic analysis of C4 photosynthetic pathway evolution in grasses. Genome Biol 10. doi:10.1186/gb-2009-10-6-r68.

Warner, C.A., Biedrzycki, M.L., Jacobs, S.S., Wisser, R.J., Caplan, J.L., and Sherrier, D.J. (2014). An optical clearing technique for plant tissues allowing deep imaging and compatible with fluorescence microscopy. Plant Physiol. **166**: 1684–1687.

Wu, E., Lenderts, B., Glassman, K., Berezowska-Kaniewska, M., Christensen, H., Asmus, T., Zhen, S., Chu, U., Cho, M.-J., and Zhao, Z.-Y. (2014). Optimized Agrobacterium-mediated sorghum transformation protocol and molecular data of transgenic sorghum plants. In Vitro Cell. Dev. Biol.-Plant **50**: 9–18.

Yang, J., Yi, J., Yang, C., and Li, C. (2013). Agrobacterium tumefaciens-mediated genetic transformation of Salix matsudana Koidz. using mature seeds. Tree Physiol. **33**: 628–639.

Zhao, Z., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., and Seltzer, J. (2000). Agrobacterium-mediated sorghum transformation. Plant Mol. Biol. **44**: 789–798.

Tables

Table 3.1 Sorghum transformation medium

Medium components	Unit per liter	Concentrations					
		Inoculation (IM)	Co-cultivation (TM)	Resting (R)	Callus induction (CIM)	Regeneration (SM)	Rooting (RM-IBA)
MS salts	g	4.3	4.3	4.3	4.3	4.3	4.3
MES	g	0.5 [a]	0.5 [a]	0.5 [a]	0.5 [a]	0.5 [a]	0.5 [a]
Proline	g	-	0.7	1	1	-	-
2,4-D	mg	1.5 [a]	1.5 [a]	1.5 [a]	1.5 [a]	-	-
Sucrose	g	68.5	20	30	30	30	30
Glucose	g	36	10	-	-	-	-
Vitamin B5 (100X)	ml	10	10	10	10	10	10
Ascorbic Acid	mg	-	10	-	-	-	-
BAP	mg	-	-	-	-	1	-
IAA	mg	-	-	-	-	1	-
IBA	mg	-	-		-	-	1
Agar	g	-	8	8	8	8	8
PVPP	g	-	10	10	10	10	10
AS	µM	100	100	-	-	-	-
$CuSO_4$	mg	-	-	0.16	0.16	0.16	0.16
Asparagine	g	-	-	1	1	-	-
KH_2PO_4	g	-	-	1	1	-	-
Cefotaxime [a]	mg	-	-	400	300	300	300
Glufosinate [a]	mg	-	-	-	2.5	2.5	-
pH		5.2	5.8	5.8	5.8	5.8	5.8
Period [a]		10 min	3 days	10 days	10-15 days	4-6 weeks	2-3 weeks

MS salts: (Murashige and Skoog 1962). Vitamin B5: (Gamborg et al., 1968) MES, 2-(4-morpholino) ethane sulfonic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; acetosyringone

,3',5'-dimethoxy-4'-hydroxyacetophenone; BAP, 6-Benzylaminopurine; IAA, indole-3-acetic acid; IBA, Indole-3-butyric acid; PVPP, Polyvinylpolypyrrolidone. Most of reagents were supplied by Sigma-Aldrich Inc., USA, except MES (Fisher Scientific, USA), Cefotaxime (Caisson Laboratories, USA), Glufosinate (Plantmedia, USA). [a] The optimized conditions were made by this current study.

Table 3.2 Sorghum regeneration of different genotypes in different concentrations of 2,4-D

Genotypes	**Medium**	**IEs**	**Callus induction (%)**	**Regeneration (%)**	**# Plants/callus (5 weeks on RM)**
Tx430	CIM1 (1mg/l 2,4-D)	200	92.6	37.7 **	2.8 ± 1.3 ns
	CIM2 (1.5mg/l 2,4-D)	200	99.5	68.4 ns	3.2 ± 1.2 ns
	CIM3 (2mg/l 2,4-D)	188	99.4	62.5 ns	3.4 ± 1.5 ns
Tx2737	CIM1 (1mg/l 2,4-D)	182	85.3	63.6 ns	3.4 ± 2.1 ns
	CIM2 (1.5mg/l 2,4-D)	181	95.3	73.4 ns	4.7 ± 2.3 ns
	CIM3 (2mg/l 2,4-D)	181	92.9	76.1 ns	4.8 ± 2.5 ns
P898012	CIM1 (1mg/l 2,4-D)	205	94.5	71.3 **	10.8 ± 4.6 **
	CIM2 (1.5mg/l 2,4-D)	214	100	90.3 ns	18.9 ± 6.2 ns
	CIM3 (2mg/l 2,4-D)	202	100	90.6 ns	17.6 ± 4.5 ns

** Significant at $P<0.01$

ns Non significant

[a] Total number of immature embryos (IEs) used for three replicates, 60-70 IEs for each replicate.

Table 3.3 Sorghum root formation in different rooting media

Rooting medium	Explants*	Root induction (%)	Number of roots	Root quality
RM: 4.3 g/l MS salts; 10ml/l VTMB5; 0.5 g/l MES; 0.16 mg/l $CuSO_4$; 30 g/l Sucrose; 8 g/l Agar; 10 g/l PVPP; pH 5.7	72	75.0	3.1	+++
R1: RM + 1 mg/l IBA	73	100	5.1	+++
R2: RM+ 1 mg/l NAA	72	100	>10	+
R3: RM+ 1 mg/l IAA	72	100	4.4	+++
R4: RM + 1mg/l NAA + 1mg/l IBA + 1mg/l IAA	73	92.3	>10	+

* Total sorghum shoots in rooting medium for three replicates, 24-25 shoots per each replicate.

Table 3.4 The effects of Agrobacteria strains on sorghum transformation

Agro strains	IEs*	Callus induction (%)	Gus transient (%)	# Gus spots	Transformation (%)	Leaf painting resistance (%)
EHA101	170	96.4 [a]	48.9[a]	1.9 ± 0.24	6.4 [bc]	100
AGL1	167	98.2 [a]	41.3 [a]	1.8 ± 0.19	9.6 [a]	100
GV3101	164	78.7 [b]	6.7 [b]	1.5 ± 0.50	1.1 [c]	100
Control	93	96.8 [a]	0.0	-	0.0	-

Values presented by different letters are significantly different at $p \leq 0.05$
* Total number of IEs used for three replicates, 50-60 IEs for each replicate of infected treatments, around 30 EIs for control treatments

Table 3.5 Sorghum transformation using different binary vectors

Vectors	IEs*	Callus induction (%)	Regeneration (%)	Transformation (%)
pZY102	317	84.1 [ab]	4.1 [bc]	4.1 [b]
pFGC616	320	69.3 [bc]	6.0 [ab]	3.1 [b]
pFGC5941	305	84.6 [ab]	8.8 [a]	8.5 [a]
No infection	161	87.6 [a]	-	-

Values presented by different letters are significantly different at $p \leq 0.05$
* Total number of IEs used for three replicates, around 100 IEs for each replicate of infected treatments, around 50 EIs for control treatments

Table 3.6. Sorghum transformation used the optimized protocol

Replication	IEs	Callus	Callus induction (%)	Transgenic events	Transformation (%)
R1	220	203	92.3	36	16.4
R2	211	186	88.2	33	15.6
R3*	225	196	87.1	24	10.7

* Immature embryos were collected from sorghum plants grown in the winter season.

Table 3.7 Southern blot results of transgenic sorghum

Agrobacterium strains and vectors	**Probes**	**Independent events**	**Number copies of integrated T-DNA**	**% single integration**
AGL1-pZY102	*gus* gene	10	2.1 ± 0.3	40
AGL1-pZY102	*bar* gene	10	2.5 ± 0.5	40
AGL1-pFGC5941	*bar* gene	10	2.1 ± 0.4	40
AGL1-pFGC161	*bar* gene	10	2.0 ± 0.5	50
EHA101-pZY102	*gus* gene	11	2.4 ± 0.4	27.3

Table 3.8 The segregation of T1 transgenic plants

Events	Genes	Positive	Negative	Total seeds	X^2	Number of insertion
E1	*gus*	49	53	102	39.54	2
	bar	227	125	352	20.74	
E2-1	*gus*	61	1	62	18.08	5
	bar	47	48	95	33.01	
E2-2	*gus*	146	1	147	46.36	2
	bar	290	43	333	25.94	
E4	*gus*	74	34	108	2.419*a	2
	bar	103	5	108	23.90	
A1-2	*gus*	80	33	113	1.064*a	2
	bar	75	38	113	4.486	
A1-3	*gus*	79	32	111	0.867*a	3
	bar	83	28	111	0.003*a	
A4	*gus*	71	37	108	4.938	3
	bar	85	23	108	0.790*a	
A6	*gus*	73	29	102	0.640*	1
	bar	82	20	102	1.581*	
A11	*gus*	95	18	113	4.958	2
	bar	110	3	113	30.09	
Z1	*gus*	77	36	113	2.834*	1
	bar	87	26	113	0.238*	
Z2	*gus*	0	108	108	324.0	6
	bar	16	92	108	208.6	
Z4	*gus*	81	28	109	0.028*a	3

	bar	78	27	105	0.029*a	
Z5	*gus*	70	44	114	11.23	2
	bar	83	31	114	0.292*a	
Z8	*gus*	81	33	114	0.947*	1
	bar	89	25	114	0.573*	
F3	*bar*	83	30	113	0.145*a	2
F4	*bar*	62	50	112	23.04	2
F5	*bar*	100	11	111	13.48	2
F7	*bar*	106	2	108	30.86	3
F8	*bar*	82	31	113	0.356*	1
F9	*bar*	83	31	114	0.292*	1
G10	*bar*	88	25	113	0.498	5
G3	*bar*	75	40	115	5.869	2
G4	*bar*	67	18	85	0.663*	1
G7	*bar*	91	21	112	2.333*a	4

[a]Not consistent to locus number

*Non-significant difference from 3:1 segregation ratio at $p \leq 0.05$

Figures

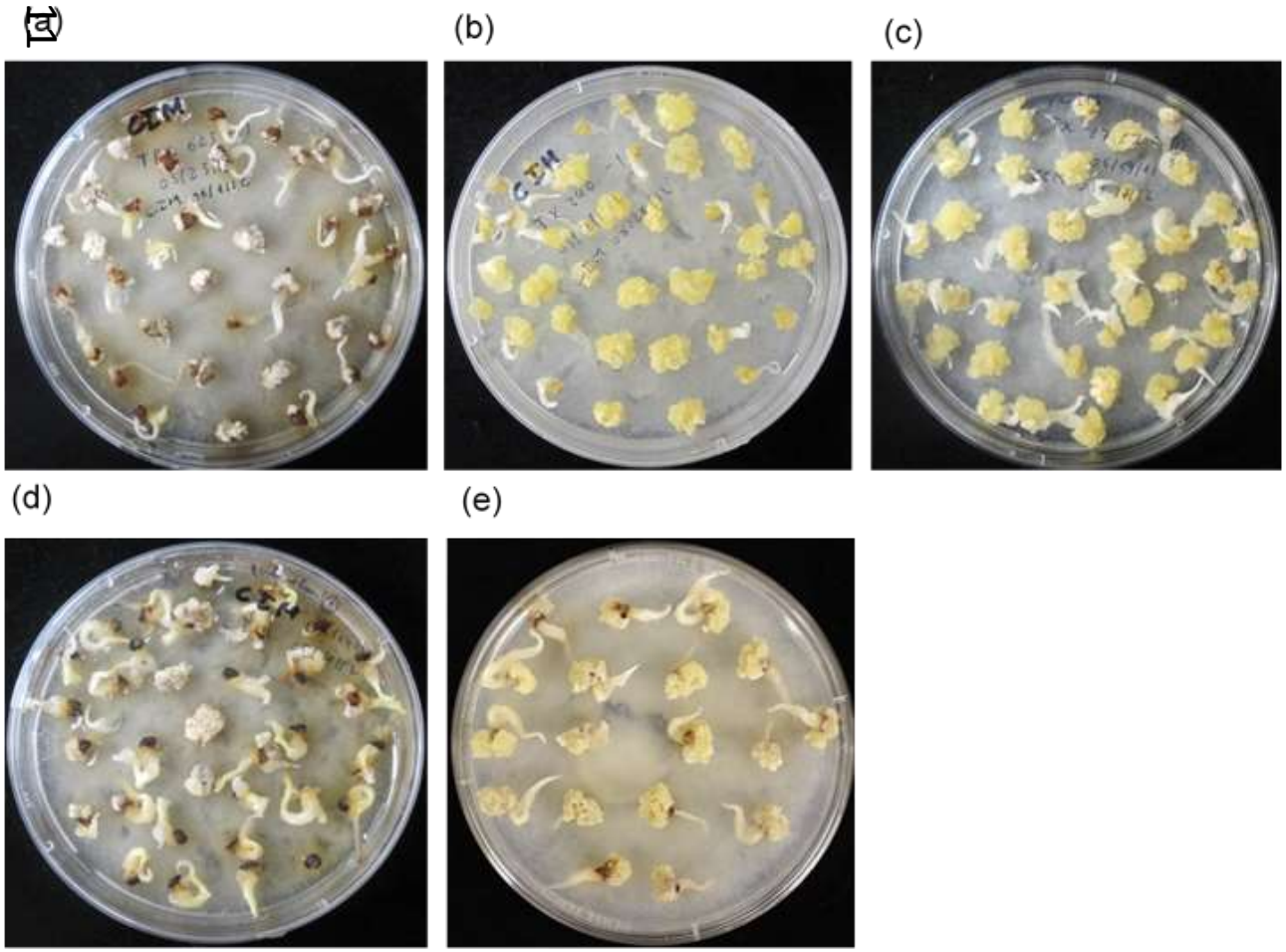


Figure 3.1 Callus phenotypes from different germplasm. a TBx623. b Tx430. c Tx2737. d Wheatland. e P898012.

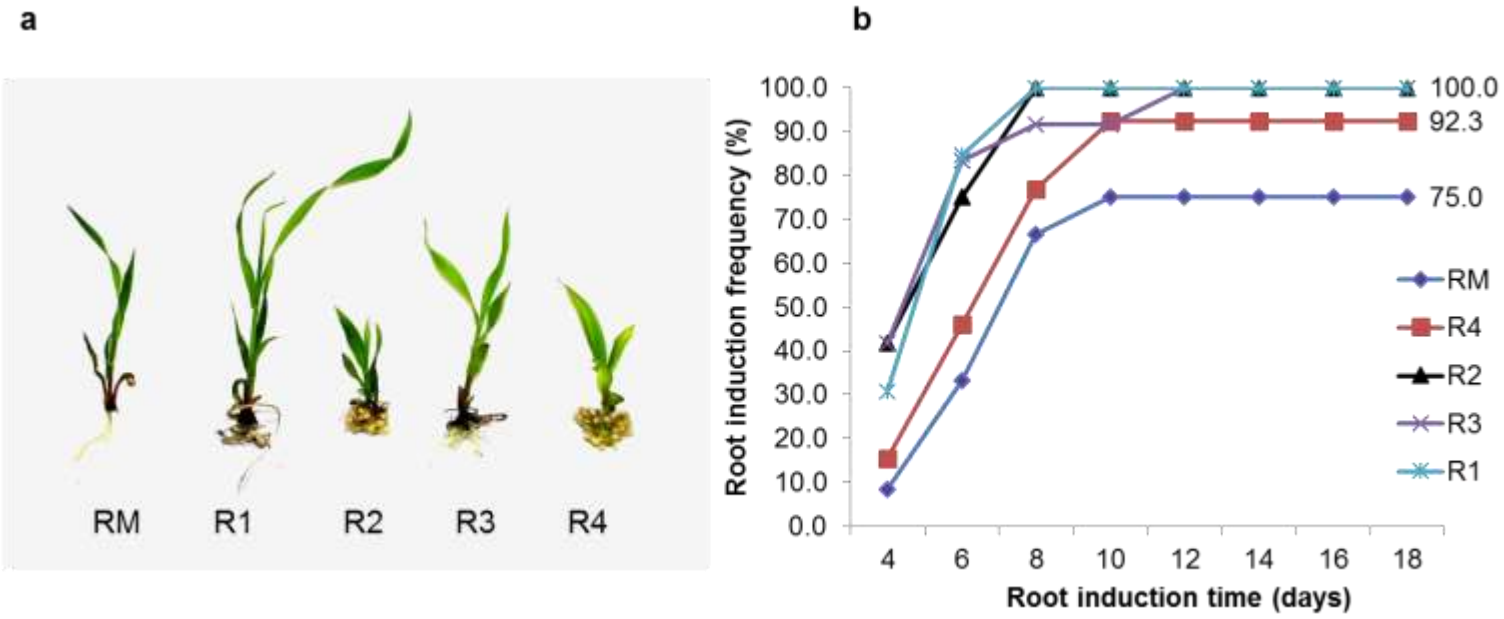


Figure 3.2 Sorghum root formation. a sorghum root quality with different rooting media. b root induction frequency for given timelines

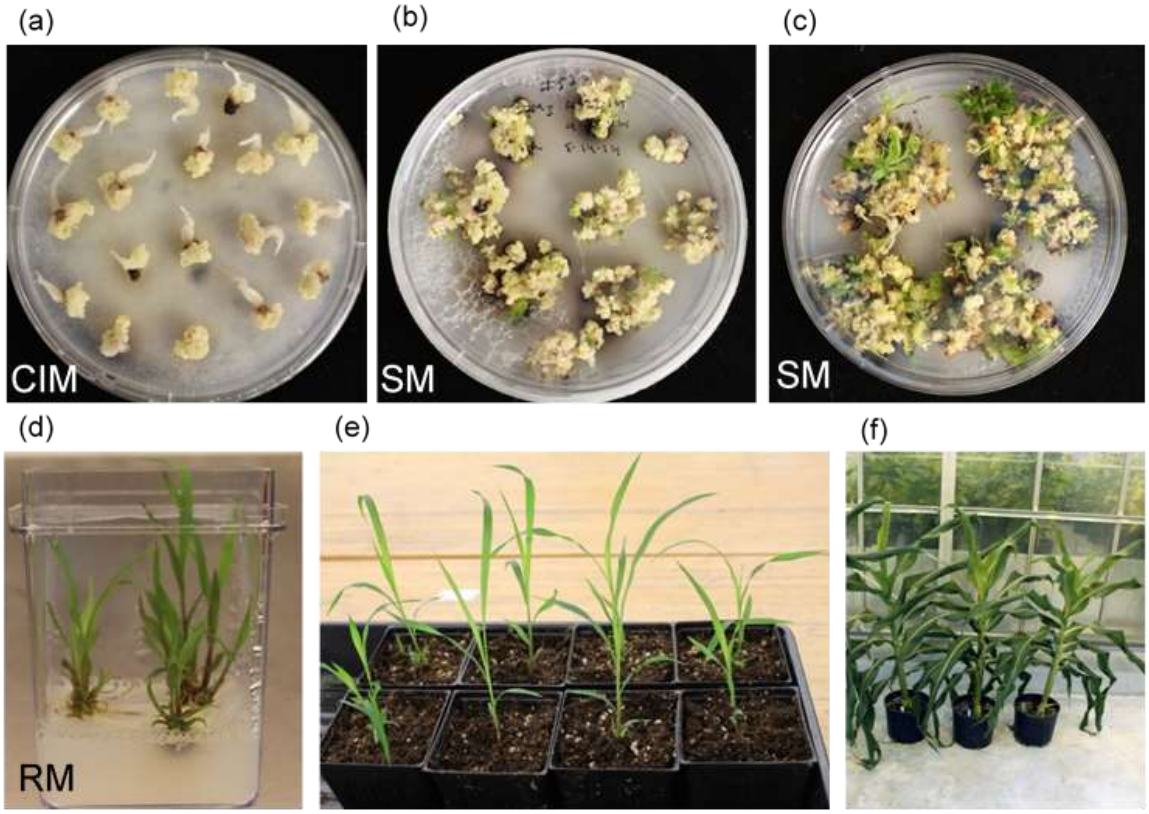


Figure 3.3 Tissue culture procedure of genotype P898012 from zygotic immature embryos. a Callus induction. b Somatic embryos. c Regenerated shoots. d Root induction. e Hardened plants in soil. f plants growing in the glasshouse. CIM, SM and RM: Medium components from table 3.1 without antibiotic and glufosinate. Tissue culture periods: CIM (3-4 weeks); SM (2-5 weeks); RM (2-3 weeks)

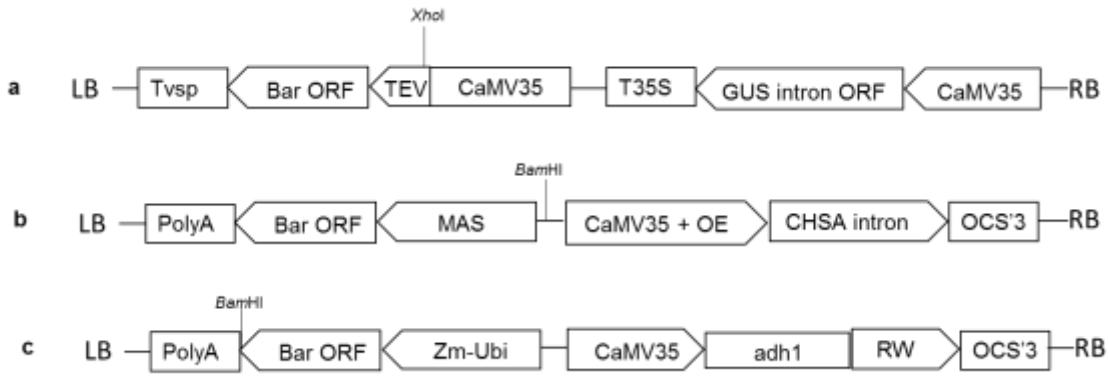


Figure 3.4 Diagram of the binary transformation vectors used in the study.

Shown are T-DNA regions of standard binary vectors pZY102 (**a**), pFGC5941 (**b**), and pFGC16 (**c**). LB, T-DNA left border; RB, T-DNA right border; Tvsp, terminator from soybean vegetative storage protein gene; Bar ORF, an open reading frame of bialaphos resistance gene; TEV, tobacco etch virus 5′ untranslated region; CaMV35, promoter from cauliflower mosaic virus; PolyA, terminators (poly A signals); T35S, T35S terminator; MAS, mannopine synthase promoter; Zm-Ubi, maize ubiquitin promoters; GUS intron ORF, an open reading frame of the GUS reporter gene containing a functional intron; OE, omega enhancer; CHSA intron, chalcone synthase A gene intron; adh1, alcohol dehydrogenase gene intron; RW, rice waxy-a gene intron; OCS3□, octopine synthase terminator; *Bam*HI and *Xho*I, restriction enzyme sites used to digest DNA for Southern Blot.

(a) (b)

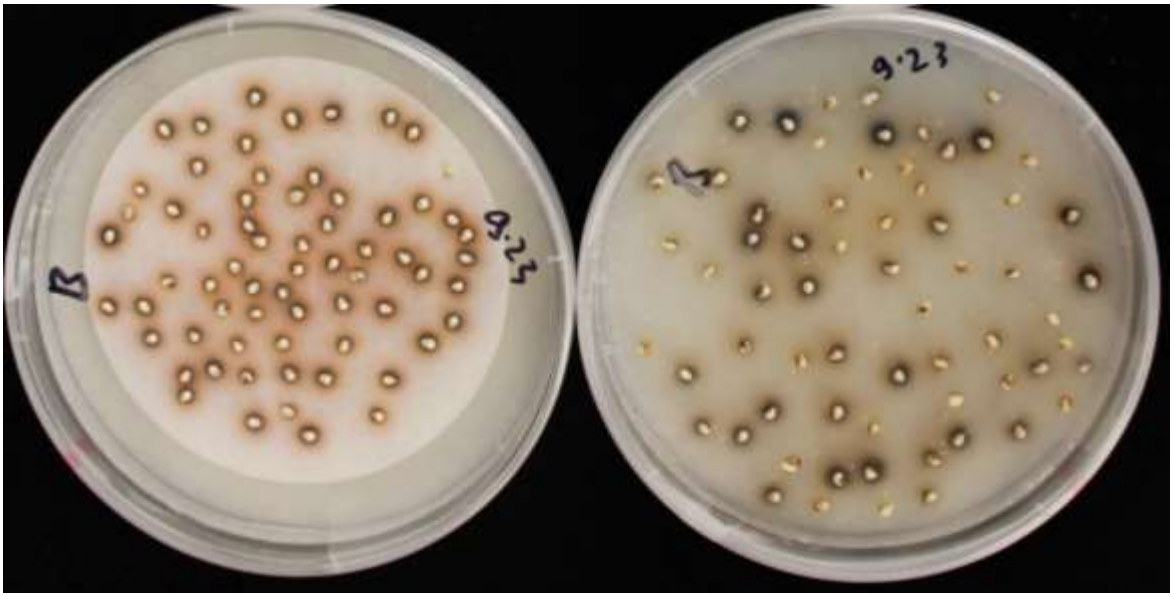
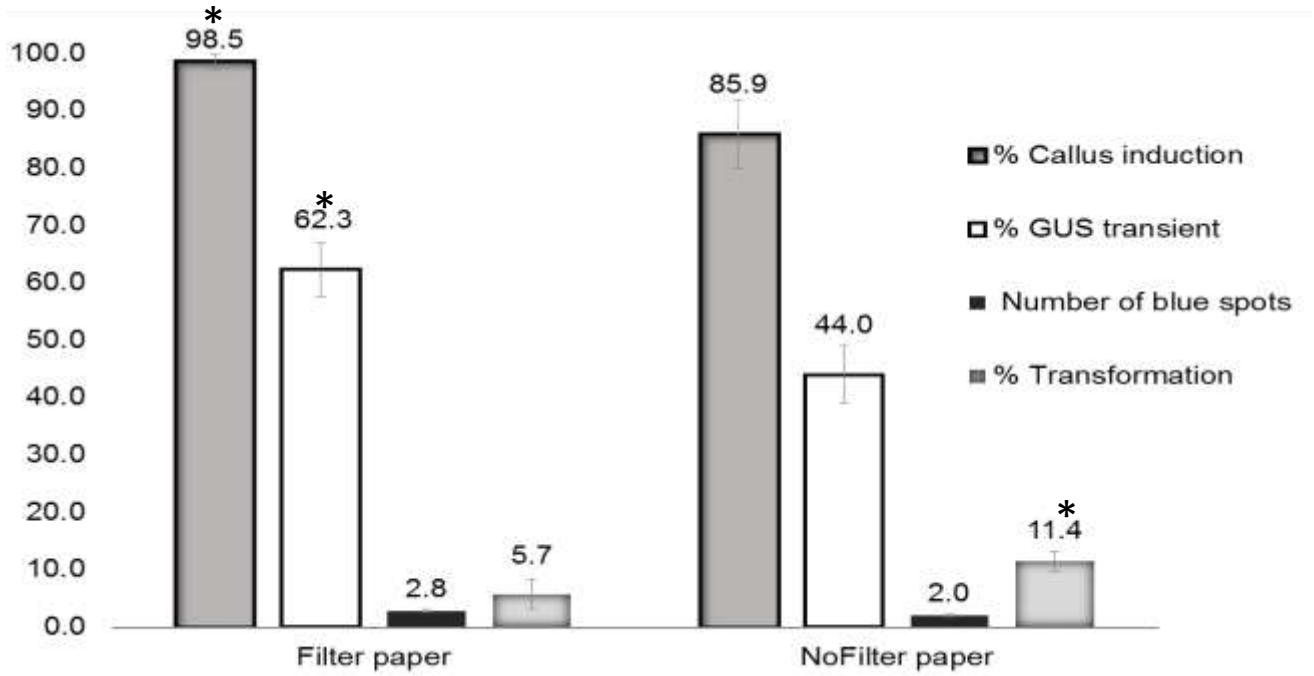


Figure 3.5 Phenolic release of infected immature embryos on co-cultivation medium. a Filter paper treatment. b No filter paper treatments.



3.6 Effects of co-cultivation with filter papers on sorghum transformation. *indicate a significant difference between different treatments at P<0.05

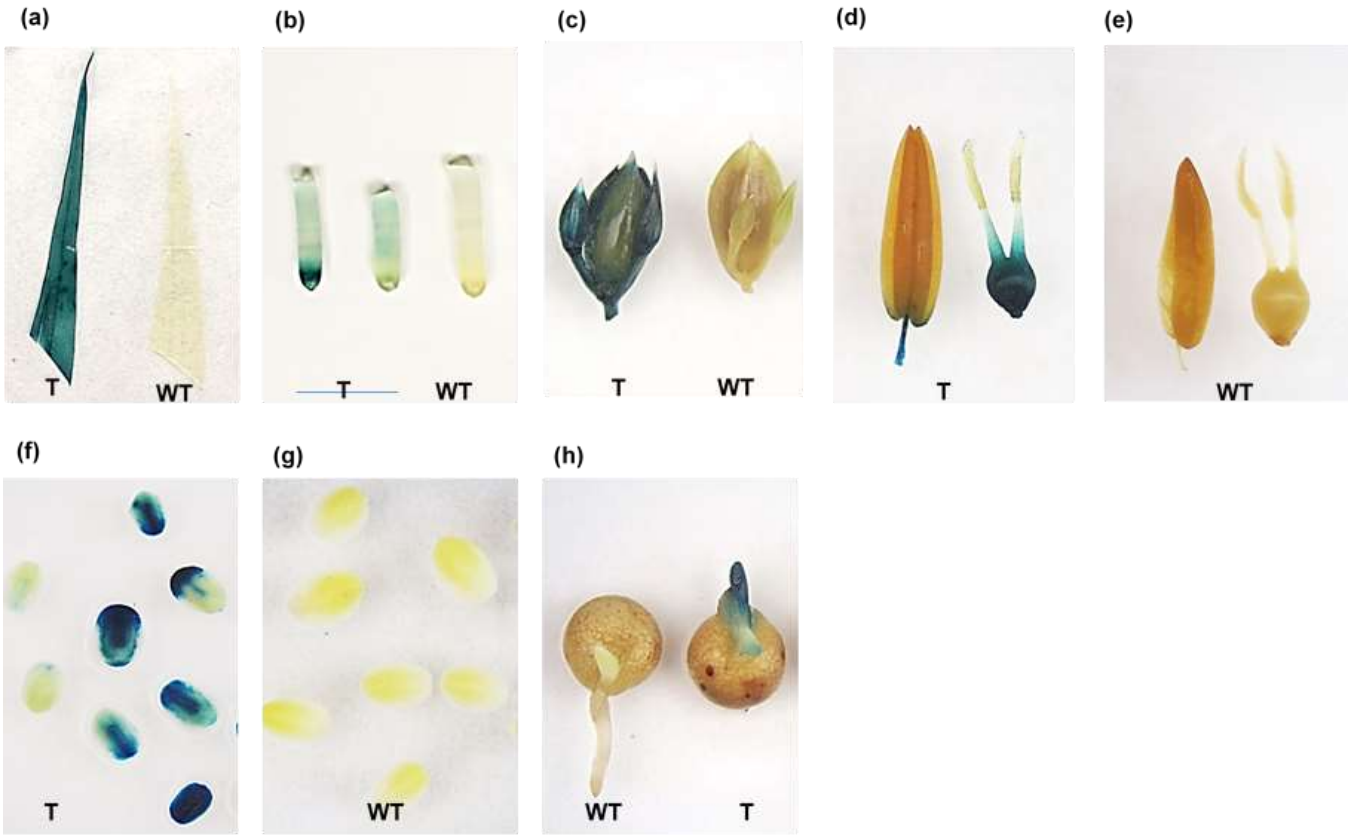


Figure 3.7 GUS assay of transgenic sorghum. a-c Leaves, roots, florets, stamen and ovary of sorghum. f-g T1 immature embryos. h T1 seedling. T: Transgenic plant. WT: Wild-type control

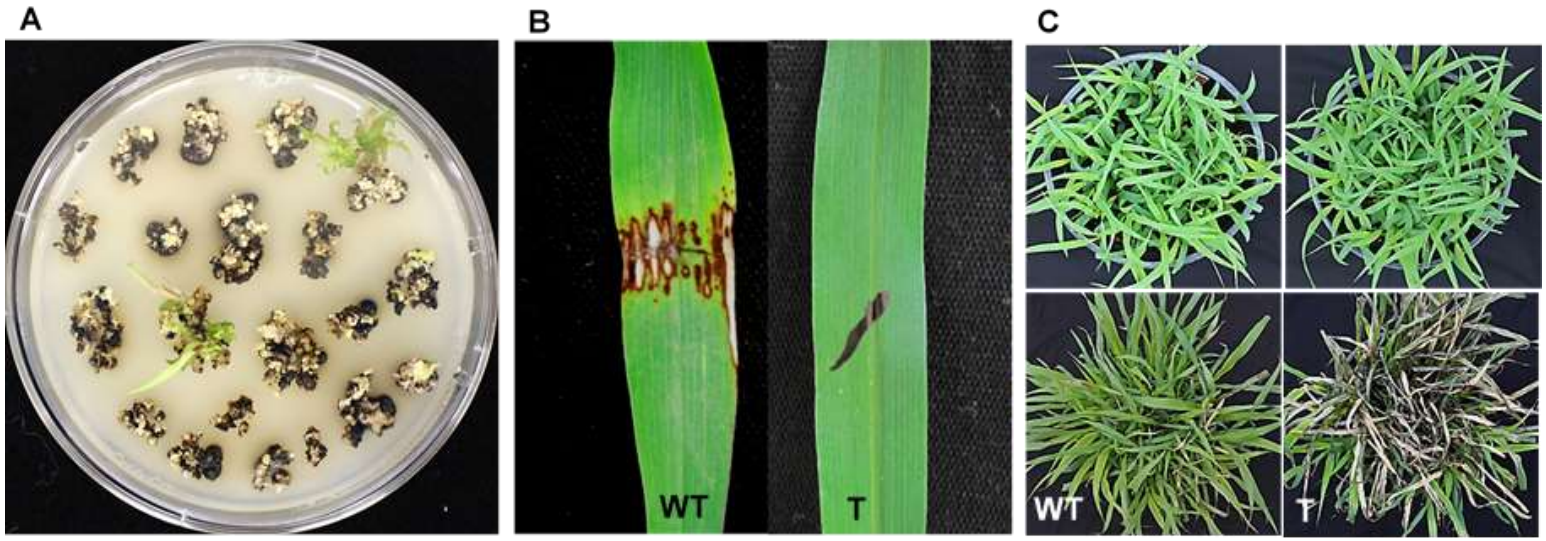


Figure 3.8 Transgenic sorghum selection using herbicide. a Regenerated sorghum on selection medium. b Leaf painting. c Herbicide screening of T1 (above: before spraying; bellow: 12 days after spraying). T. transgenic plants, WT. Wild-type plants.

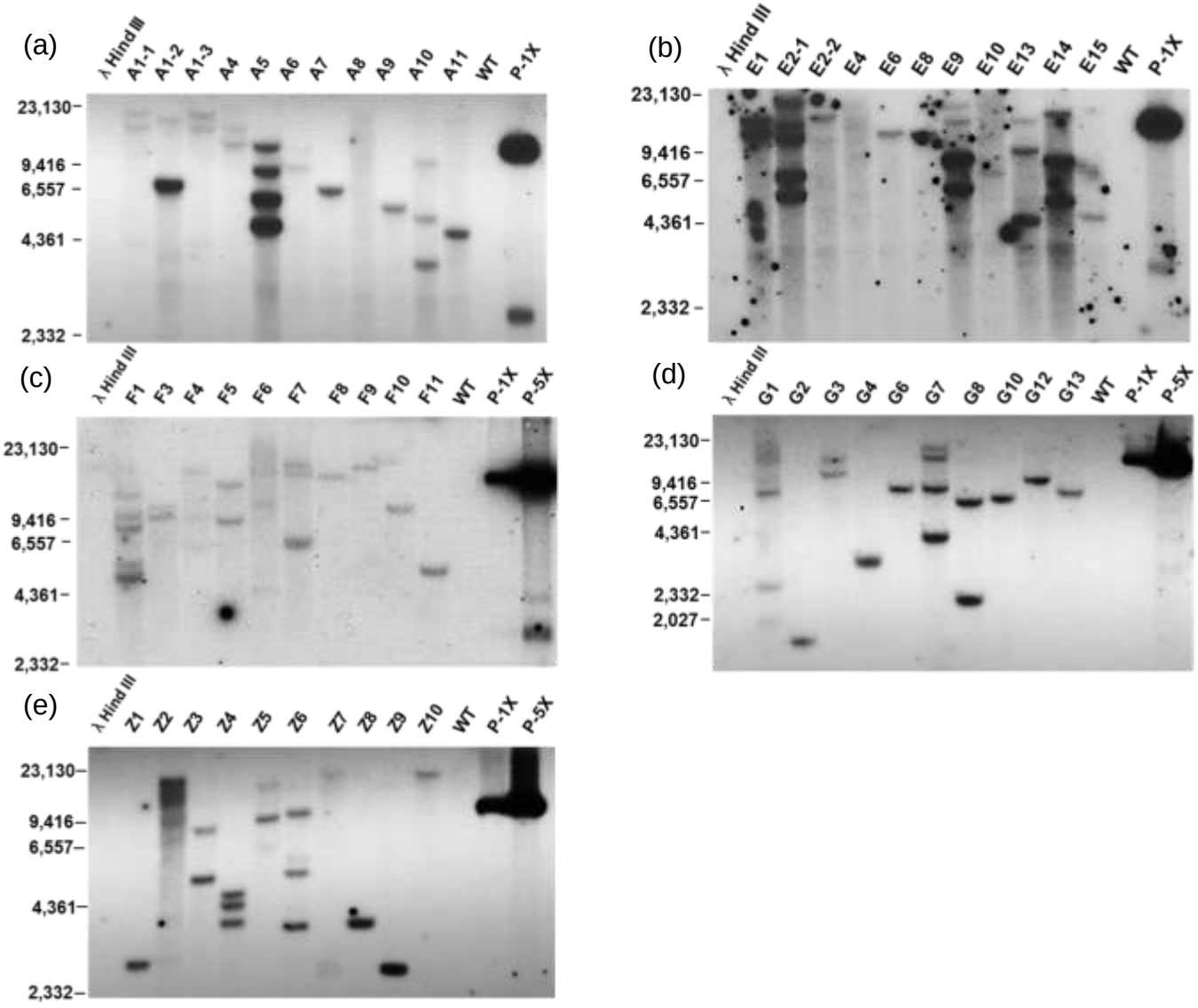


Figure 3.9 Southern blot analysis of transgenic sorghum.

T0 transgenic events derived from AGL1/pZY102 (a**)** and EHA101/pZY102 (b) in the experiments comparing different *Agrobacterium* strains; from AGL1/pFGC5941 (c), AGL1/pFGC161 (d) and AGL1/ pZY102 (e) in the experiments comparing different standard binary vectors. λ Hind III, DNA ladder; P-1X and P-5X, 1X and 5X genome

equivalent copy number controls, respectively, using plasmid DNA; WT, wild-type control plant. Note that *gus* was used as probe for membranes **a** and **b**. whereas *bar* probe was used for membranes **c, d** and **e.**

Conclusion and future perspectives

Biofuels have been considered as alternative, renewable energy resources that have potential to avoid environmental issue and compete with the demand of energy consumption by human. Switcghrass and sorghum have been seen as two potential crops for biofuel production. Of which, switchgrass is known as a material for the second generation of biofuel, while sorghum could be used for both the first and second generations. Therefore, the increased research has been focused to utilize genetic engineering for genomic studies and variety improvements of these two important crops, recently.

Switchgrass was first selected as a potential feedstock for biofuel production by the U.S department of energy. It has been predicted to contribute the main part of biomass required for the national goal of biofuel production. Improving total biomass production and getting higher efficiency of biofuel conversion from biomass are two important targets in the utilization of switchgrass for biofuel application. Gibberellins are important plant hormones that play critical roles in plant growth, development and biomass production. The regulation of genes encoded enzymes in gibberellin biosynthesis pathway exhibited critical effects in gibberellin levels, and substantial changes in plant morphology, architecture and biomass of both monocot and dicot. In current study, an open reading frame of GA20 *oxidase* gene from *Zea mays* derived by CaMV35S promoter plus omega-enhancer sequence was transferred into switchgrass with the aim of improving total biomass production. The insertion, expression of transgenes was confirmed in the correlation with bioactive gibberellin levels using molecular and

biochemical analysis. Transgenic switchgrass exhibited the alteration in morphology and phenotype etc. longer leaves, longer internodes and increased tiller height. Further more, the representative plant architecture of the overexpression of GA20ox was also observed such as smaller leaves and internode diameters. The effects of ectopic ZmGA20ox on lignin gene expression was exhibited in some transgenic events. In addition, all transgenic events displayed the delay in flowering as compared to wild-type non-transgenic plants. More importantly, faster growth and higher biomass production was achieved in all ZmGA20ox transgenic switchgrass, respectively. The current research is the first report utilizing GA20 *oxidase* for switchgrass biomass improvement. Therefore, it provides the great strategy to overcome the constraints in using switchgrass for biofuel production and opens potential applications for other monocot biofuel crops. In addition, research questions raised from our results open new directions for future studies. For example, a further research should be established to identify potential events for biofuel application based on biomass productivity in the field condition as well as biofuel conversion efficiency from transgenic switchgrass biomass. Another research direction is to study effects of bioactive gibberellin levels on switchgrass tiller formation and flowering in the correlation with other plant hormones and comparison to other plant species.

Sorghum is one of the most important cereal crop providing the staple food for more than half billion people in the world. Recently, sorghum has been considered as potential materials for biofuel production. Plant transformation has provided powerful means for sorghum genomic studies and cultivar improvements. However, as the other recalcitrant crops for tissue culture, low transformation efficiency has restricted the wide

application of this approach. Many attempts have been carried out in the aim of sorghum transformation improvement such as the using various explants and selectable marker genes, the modification medium components, the utilization of cold and heat treatments etc. Standard binary vectors have been widely used for plant transformation including sorghum due to the critical advantages in construction, cloning, mobilization and transformation. However, the efficiency of sorghum transformation using standard binary vectors is being lower as compared to many other crops. Therefore, the main goal of current research is to establish rapid and efficient *Agrobacterium*-mediated transformation of sorghum (*Sorghum bicolor*) employing standard binary vectors. The systematic optimization of regeneration and transformation conditions improved sorghum transformation efficiency to over 14%. Of which, 40-50% tested transgenic events exhibited single insertions of integrated T-DNA estimated by Southern blots. The key improvements of this system included the utilization of potential genotypes, the modifications in callus induction and rooting medium as well as the employment of *Agrobacterium* strain AGL1 harbored standard binary vector with MAS promoter driving *bar* gene. The current system should be potential for studies of sorghum genetic engineering and variety improvements. As a biofuel crop, the new procedure should be employed for studies in modifications of starch deposition, sugar content and biomass digestibility. In addition, the exploitation of sorghum genomes as well as the precise genome editing technologies such as TALLEN, CRISPR/Cas9 have advanced studies in sorghum genome functions. Candidate genes in starch metabolism and lignin biosynthesis pathway should be considered in future research employing the new transformation system. As a staple food for human, the new procedure has potential for

studies in improvements of sorghum grain quality with candidate genes in beta carotene and kafirins biosynthesis.

In conclusion, the results of this study contributed the great applications for improved biofuel production from two potential energy crops, sorghum and switchgrass. This also open new directions for future studies in genetic engineering and functional genomics of these two crops and other plant species.

VITA

Phat Tien Do was born on January 28, 1981 in Hanoi, Vietnam. He is son of Trinh Doan Do and Tho Thi Cao. Do graduated from Ngoc Tao High School in 1999. He earned Bachelor of Agronomy from Vietnam National University of Agriculture in 2003. Then, he chose to become a senior researcher at the Plant Cell Biotechnology Laboratory, Institute of Biotechnology, which is a part of Vietnam Academy of Science and Technology. He received Master of Science degree in Genetics and Plant Breeding at the same university in 2009. Phat Do was awarded a Vietnamese Government Scholarship to pursue his Ph.D. program study in the United States. At the University of Missouri-Columbia, he joined and began his research with biofuel crops in Dr. Zhanyuan J. Zhang‘s Lab in August, 2011. He finished his doctorate degree in Plant Biology and Genetics in July 2016. He is the first author of one peer-reviewed research article, one book chapter and one peer-reviewed manuscript. Phat Do plan to go back to Vietnam and develop his own independent research programs on plant science.