Unlocking the Future of Bioenergy in Nigeria Using Genetic Modification Framework (GMF) of Switchgrass

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Abstract. The use of biofuel is gradually becoming more attractive universally as convenient substitute for fossil fuels due to the current increase in the demand for clean and renewable energy. This is because of its contribution towards lowering the dependence on crude oil, in view of the current global decline of fuel reserves. Switchgrass (Panicum virgatum L.) has been developed into a herbaceous bioenergy crop. The processing of non-edible switchgrass biomass for fuel production will impact on rural and national development without negatively affecting food security. One of the main challenges of the production of biofuel from plant biomass is the technological impediment of breaking down plant biomass (lignin and cellulose in plant cell walls) that can be converted into biofuel. This research work will employ the use of genomic and transgenic aspects of biotechnology, such as callus induction and culture media optimization of native switchgrass, genes and constructs, cloning and sequencing, transformation and characterization analyses to invent a genetic modification framework (GMF) technology that will result in genetically modified switchgrass capable of coproducing cellulase which will culminate to drastic reduction in the cost of production of biofuel and thereby encourage its commercialization.

Keywords: Bioenergy, Genetic Modification Framework (GMF), cell wall, switchgrass, Panicum virgatum L.

1 Introduction

Biofuel is globally becoming more attractive as suitable substitute for fossil fuels due to increasing demand for clean energy, declining fuel reserves and its contribution towards reducing dependence on crude oil. The processing of non-edible switchgrass biomass for fuel production will impact on rural and national economic development, without compromising food security.

Lignocellulosic biomass is an abundant, domestic, renewable feedstock source that can be converted to liquid transportation fuels and other chemicals by fermentation. Cellulosic ethanol is a promising near-term technological option to reduce transportation sector greenhouse gas emissions [1]. Because lignocellulosic biomass is made up of the complex structures of cellulose, hemicellulose, and lignin, such feedstock is highly recalcitrant to bioconversion of its carbohydrates into ethanol compared with starch [2–3]. Current biomass fermentation processes for fuels and chemicals have a relatively high cost primarily because of this recalcitrance, which in turn has limited commercialization of biomass ethanol [4].

Switchgrass (Panicum virgatum) is an important biofuel crop candidate due to high production yields, low energy and nutrient inputs, short growing time and wide adaptability. It is one of the grasses (family Poaceae) which have been selected as preferred feedstock for bioenergy [5–10].

The main challenge of cellulosic biofuels, therefore, is the comparatively low rate of cellulosic conversion. In addition, switchgrass is naturally outcrossing; therefore, maintenance of genotype is difficult through sexual propagation. Efforts have been made, especially by researchers in North...
America to improve the biofuel efficiency of the existing switchgrass cultivars using conventional plant breeding techniques [11]. These have not yielded the expected results. To achieve sustainable energy production, it is necessary to overcome the chemical and structural properties of biomass that inhibit its deconstruction in dedicated bioenergy crops [12].

Inspite of the interest in switchgrass as a bioenergy crop, few studies have been undertaken to elucidate cell wall structure and genes involved in cell wall biogenesis of this species, in recent years [13 – 15]. There is need to use transgenic and genomic aspects of biotechnology for the modification of cell wall of switchgrass to improve cellulase expression, which could enhance the bioconversion of biomass into biofuel. This is yet to be reported.

The main objective of this research therefore is to develop high cellulosic ethanol yielding switchgrass cultivars with increased cellulosic capabilities for biofuel production using genetic engineering. The research will employ the use of genomic and transgenic aspects of biotechnology to modify the cell wall composition of switchgrass for improved cellulose expression. The cellulase enzyme if successfully engineered into switchgrass would potentiate the use of switchgrass as an economically viable biofuel generating plant. Genetic information would be available to scientists and researchers for further exploitation/exploration.

The genetic modification framework (GMF) technology of switchgrass will coproduce cellulase that will result in drastic reduction in the cost of production of biofuel, and thereby encourage the commercialization of non-edible switchgrass biofuel. As Nigeria moves forward with expanding the renewable bioeconomy, the characteristics and research history of switchgrass make it well suited to large-scale production of feedstock in many agroecoregions of the country. This work if accomplished will result in genetically modified switchgrass; then that potential would be explored among farmers to grow/produce switchgrass.

2 Materials and methods

2.1 Framework for Genetic Modification of Switchgrass for Enhanced Bioconversion into Biofuel

The framework of the genetic modification of switchgrass involves three main phases (Figure 1). A small part of the leaf of a healthy wild native switchgrass is cut and surface sterilized using an established protocol [16]. The sterilized explant is inoculated into appropriate growth media for callus generation (phase 1).

The process of switchgrass transformation (ST) begins with access to DNA templates of gene of interest through primer design, resulting in designed DNA editing cassette (gene construct) carrying the gene of interest for use in cloning. Cloning and sequencing prepare the cloned gene of interest for use in transformation (phase 2).

Figure 1. The genetic modification framework (GMF) for switchgrass transformation and determination of biofuel efficiency.
The transformed switchgrass with modified cell wall for expression of cellulose enzyme is then subjected to molecular and biochemical characterization (phase 3) which reveals indices of determination of biofuel conversion efficiency. Figure 2 shows the overview of concepts relationship that will result in certain derivable which include: a genetic modification framework (GMF) production of economically viable biofuel and provision of important genetic information.

2.2 Generating Switchgrass Cultivars from Native Switchgrass as a Pre-requisite for Genetic Transformation (Phase 2)
Switchgrass cultivars would be generated from calluses induced from mature seeds of native switchgrass using tissue culture technology [17–18]. This will require the optimization of culture media for callus induction and regeneration, as the regeneration capacity of switchgrass is highly genotype-dependent. The young inflorescence leaves that would be regenerated from the callus, serve as explants for generation of fresh callus to be used for Agrobacterium mediated transformation of the gene of interest. Specific tasks for this phase include:

2.2.1 Callus Induction and Culture from Native Switchgrass
Mature seeds of native switchgrass are harvested and surface-sterilized with full strength disinfectant and then rinsed with distilled water. The seeds would be kept overnight in the dark at a specified temperature. They are further sterilized, followed by another round of rinsing with distilled water. The sterilized seeds will be placed on callus induction medium fortified with growth hormones (type and concentrations as would be optimized). These would be cultured for six to eight weeks, after which the embryogenic calli would be picked for sub-culturing on MP medium.

2.2.2 Optimization of Culture Medium for the Native Switchgrass
It has been reported that current switchgrass tissue culture and transformation systems are not very efficient and limited to derivatives of a single variety [18]. There is the need to optimize the culture medium for generation of callus from the native switchgrass. This would be done by varying the composition of the growth medium supplements. MS medium (being the most commonly used and available commercially) would be the basic medium, with agar as the solidifying agent, while the
other parameters would be varied. This would involve varying the concentrations of growth regulators (auxins and cytokinins)[18].

2.2.3 Regeneration of Subcultured Callus
Embryogenic calli, would be placed in regeneration medium for regeneration. Regenerated plants would be hardened and cultivars of about 35–40 cm transplanted to pots filled with the same soil mix. Plants would be adequately watered daily.

2.2.4 Induction of Callus Using Leaf Explants from the Laboratory Cultivated Switchgrass
Inflorescences from the regenerated calli are utilized as leaf explant for fresh callus induction. The resultant callus would then be used for the agrobacterium mediated genetic transformation.

2.3 Genetically Engineering Switchgrass Callus for Heterologous Expression of Cellulase Enzyme (Phase 2)
In this phase, having made switchgrass available for use in genetic engineering, designed DNA editing cassette (gene construct) carrying the gene of interest is used for cloning and sequencing prior to transformation. Cell-wall hydrolysis enzymes can potentially be produced in all feedstock crops that are to be used for cellulosic ethanol production. The plant-based production of these enzymes has a crucial advantage, in that growing transgenic plants in the field requires a much lower energy input than microbial production of these enzymes. The following tasks would be undertaken in this phase:

2.3.1 Genes and Constructs
The gene for manipulation is chosen based on the best gene sequence information available. The identified gene of interest had previously been expressed successfully in both rice and maize [19]. Sequences of the gene of interest would be accessed from GenBank (NIH), while the DNA template would be obtained from partners who had earlier worked on the genome, identified from the literature and GenBank. cDNA would be used to design the gene construct for cloning the gene of interest for over expression.

2.3.2 Expression of Target Gene
Overexpression would be controlled using a maize promoter ubiquitin 1 (ZmUb). This would be carried out using a selected pANIC plant vector. pANIC series of vectors are gateway compatible for overexpression via RNA, specifically created by the BioEnergy Science Center, USA, as an enablement for switchgrass transformations. pANIC is a versatile set of 16 Gateway compatible destination vectors, primarily targeted for switchgrass, from which it owes its name Panicumvirgatum L.[19].

2.3.3 Cloning and sequencing
Cleaned PCR products will be ligated into apANIC vector, transformed into E. coli chemically competent cells and this would be plated on antibiotic containing LB agar supplemented with IPTG and X-gal. This will be incubated overnight. White colonies would be picked and propagated in LB broth containing ampicillin. Plasmid DNA would be isolated using a Plasmid Midi Kit (available commercially) and the resulting DNA would be digested with EcoRI to confirm the presence of the gene of interest through sequence analysis. Nucleotide sequence translation, nucleotide alignments, and the deduced amino acid sequences would be performed online using the bioinformatics tools available online.

Alignment of the obtained sequences would also be carried out using basic local alignment search tool (BLAST) also available online through National Center for Biotechnology information (NCBI: http:// www.ncbi.nlm.nih.gov). Confirmed cloned sequences would be digested with restriction enzymes and this would be ligated into a pANIC vector and transformed into agrobacterium for onward transformation into switchgrass calli.
2.3.4 Switchgrass Transformation
Among the various vectors used in plant transformation, the Ti-plasmid of Agrobacterium tumefaciens has been widely used. This bacterium is known as “natural genetic engineer” of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall [20]. Ti-plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti-plasmid in place of unwanted sequences.

To transform switchgrass plants, embryogenic callus (in case of monocots) will be collected and infected with Agrobacterium carrying recombinant disarmed Ti-plasmid vector. The infected tissue will be cultured (co-cultivation) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes would take place. After this, the transformed calli are transferred onto selection cum plant regeneration medium supplemented, usually with lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots will be transferred to root-inducing medium, and after another 3-4 weeks, complete plants will be transferred to soil following the hardening (acclimatization) of regenerated plants [21].

2.4 Switchgrass Characterization and Analyses (Phase 3)
The segregation and stability of the transgene integration and expression in the subsequent generation will be investigated by genetic and molecular analyses using Polymerase Chain Reaction (PCR). This will be used for verification of insertion of RNAi fragments (foreign genes) in the transformed switchgrass. All the transgenic plants that would be produced will be treated as regulated materials for environmental concern.

2.4.1 Gene Expression Analysis
Reverse transcription-quantitative PCR (RT-qPCR) would be carried out to determine the expression profile of the targeted gene in the transgenic switchgrass using standard procedures [22], using a superscript kit (available commercially).

2.4.2 Enzyme Assay and Determination of Total Soluble Proteins
This involves the assay of extractable cellulase activity and determination of total soluble proteins (tsp). This will be carried out according to NREL Laboratory Analytical Procedures for Standard [23].

2.4.3 Saccharification Analysis
This would be carried out on the tillers at the developmental stage, according to standard techniques [24 – 26].

2.5 Industrial Testing of Bioconversion Efficiency
The transformed switchgrass will be used to produce biofuel and the efficiency of the process will be determined. This will be compared with the control.

3 Progress report, expected results and potential impact
3.1 Field Work
Domestication of switchgrass from wild native switchgrass was done in three locations; Research and Botanical Garden of the Department of Plant Science and Biotechnology, Nasarawa State University, Research Gardens of Contec Global Agro Ltd and African University of Science and Technology, Abuja, Nigeria.

3.2 Callus Induction and Culture Media Optimization of Switchgrass
Invitro culture of switchgrass is currently being undertaken to generate optimized conditions/protocol of callus from domesticated switchgrass which will be made available for use in genetic engineering.
3.3 Expected Results

3.3.1 Callus Induction and Culture Media Optimization of Native Switchgrass
Determination of optimized conditions/protocol for generation of local switchgrass cultivars using tissue culture. Switchgrass callus available for use in genetic engineering.

3.3.2 Genes and Constructs
Designed DNA editing cassette (gene construct) carrying the gene of interest for use in cloning.

3.3.3 Cloning and Sequencing
Cloned gene of interest, ready for use in transformation.

3.3.4 Transformation and Characterization
Transformed switchgrass with modified cell wall for expression of cellulose enzyme.

3.3.5 Gene Expression Analysis
Expression profile of targeted gene in transformed switchgrass is determined/documentated.

3.3.6 Enzyme Assay and Determination of Total Soluble Proteins (tsp)
The information will be made available for use in protein/proteome analysis of the transgenic switchgrass.

3.3.7 Saccharification Analysis
Susceptibility of transformed switchgrass to enzymatic hydrolysis in comparison to the control is determined/documentated.

3.4 Potential Impact
Large scale production of biofuels in the country will encourage international collaboration and partnerships with some of the few developed and developing countries that have biofuel and biodiesel programmes as benchmarks for alternative and renewable fuel sources.

The global demand and desire for this type of technology are apparent, because the current biomass fermentation processes for fuels and chemicals have relatively high cost primarily due to recalcitrance of the lignocellulosic biomass, which in turn has limited commercialization of biomass ethanol.

In the long run, it is expected to provide large scale production of clean renewable energy source with the potential to solve series of problems related to climate and sustainability. Beyond the advantages of positive impacts on climate and sustainability, there will be creation of thousands of new jobs and a drastic reduction in Nigeria’s dependence on fossil fuels.

4 Conclusions
The genetic modification framework (GMF) technology of switchgrass will coproduce cellulase that will result in drastic reduction in the cost of production of biofuel, and thereby encourage the commercialization of non-edible switchgrass biofuel.

This work if accomplished will result in genetically modified switchgrass; then that potential would be explored among farmers to grow/produce switchgrass. This will be a big step forward for Nigeria’s energy security and make the nation a major player in the global bio economy.

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References

[1] Somerville C, Youngs H, Taylor C, Davis S C and Long S P 2010 Feedstocks for lignocellulosic biofuels. Science.329 pp 790–792. DOI: 10.1126/science.1189268
[2] Abramson M, Shoseyov O and Shani Z 2010 Plant cell wall reconstruction toward improved lignocellulosic production and processability. Plant Sci178 pp 61– 72
[3] Aden A, Foust T 2009 Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose16 pp 535–545
[4] Himmel M E 2007 Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science315 pp 804–807
[5] Parrish D, Casler M D and Monties A. 2012 The evolution of switchgrass as an energy crop. In: Monties A, editor. Switchgrass: a valuable biomass crop for energy. London: Springer; pp 1–28.
[6] Bouton J H, 2007 Molecular breeding of switchgrass for use as a biofuel crop. CurrOpin Genet Dev.17(6)pp 553–8
[7] McLaughlin S B, Kszos L A 2005. Development of switchgrass (Panicumvirgatum) as a bioenergy feedstock in the United States. Biomass Bioenergy. 28 pp 515–535. Doi: 10.1016/j.biombioe.2004.05.006
[8] Daniel B and Paulo S 2007 “Brazil Institute Special Report: The global dynamics of biofuels” Brazil Institute of Woodrow Wilson Centre. Retrieved 2015-11-22
[9] Onowo J C, Dashe E S and Onwualu A P 2019 Switchgrass – an important non-edible biocrop for future biofuel production in Nigeria. J. Nat. and Appl. Sci. 7(2)pp18-21
[10] Dashe E S, Onowo J C, Omojola M O, Onwualu A P 2019 A comparative study of the effect of different pre-treatments on lipase catalyzed transesterification of used cooking oil. J. Nat. and Appl. Sci. 7(2)pp42-56
[11] Vogel K P and Mitzhell R B 2008 Heterosis in switchgrass: biomass yield in switchgrass. Crop Science48pp 2159-2164
[12] McLaughlin S B, Adams L 2005 Development of switchgrass (Panicumvirgatum) as a bioenergy feedstock in the United States. Biomass Bioenergy28 pp 515-535
[13] Rao X. Lu. N. Li G, Nakashima J, Tang Y, Dixon R A 2016 Comparative cell-specific transcriptomics reveals differentiation of C4 photosynthesis pathways in switchgrass and other C4 lineages. J ExpBot.67(6) pp 1649–62
[14] Chen X, Ma Q, Rao X, Tang Y, Wang Y and Li G 2015Genome-scale identification of cell-wall-related genes in switchgrass through comparative genomics and computational analyses of transcriptomic data. BioEnergyRes.9(1)pp172–80
[15] Willis J, Smith J, Mazarei M, Zhang J, Turner G, and Decker S 2016 downregulation of the UDP-arabinomutase gene in switchgrass(Panicumvirgatum L.) results in increased cell wall lignin while reducing arabino-glycans. Front Plant Sci. 7 p 1580
[16] Garbel G, Denchv P and Conger B V 1996 Micropropagation of switchgrass by node culture. Crop Science36(6) pp 1709-11
[17] Burris J N, Mann D G F, Joyce B L and Steward Jr C N 2009 An improved tissue culture system for embryogenic callus production and plant regeneration in switchgrass (Panicumvirgatum L.). Bioenerg. Res. 2 pp 267 – 274
[18] Gimeno J, Eattock N, van Deynze A and Blumwald E 2014 Selection and validation of reference genes for gene expression analysis in switchgrass (Panicum virgatum L.) Using Quantitative Real-Time RT-PCR. Journal.pone. 0091474
[19] Nelson RS, Steward Jr C N and Davison B H 2017 Development and use of a switchgrass (PanicumvirgatumL.) transformation pipeline by the bioenergy science center to evaluate plants for reduced cell wall recalcitrance. Biotechnology for Biofuels. 10 p 309
[20] Hwang H H, Yu M and Lai E M 2017 "Agrobacterium-Mediated plant transformation: biology and applications," The Arabidopsis Book (15). https://doi.org/10.1199/tab.0186

[21] Hardin C F, Fu C, Hisano H, Xiao X, Shen H, Stewart 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 pp755–62

[22] Fu C, Xiao X, Xi Y, Ge Y, Chen F, Bouton J, Dixon R A and Wang Z Y 2011 downregulation of cinnamyl alcohol dehydrogenase (Cad) leads to improved saccharification efficiency in switchgrass. Bio Energy Res. 4 pp153-164

[23] Decker S R, Carlile M, Selig M J, Doeppke C, Davis M, Sykes R, Turner G and Ziebell A 2012 Reducing the effect of variable starch levels in biomass recalcitrance screening. In: Himmel ME, editor. Biomass conversion: methods and protocols. Totowa: Humana Press; p. 181–95.

[24] Selig M J, Tucker M P, Sykes R W, Reichel K L, Brunecky R, Himmel M E, Davis M F and Decker S R 2010 Lignocellulose recalcitrance screening by integrated highthroughput hydrothermal pretreatment and enzymatic saccharification. IndBiotechnol. 6 pp104–11

[25] Steel G D and J H. Torrie 1980 Principles and procedures of statistics: abiotmetrical approach. 3rd edition. Hill Book Company Inc. New York. 633pp.

[26] Obi I U 1986 Statistical methods of detecting differences between treatment means. Department of Crop Sc., UNN, Nigeria, pp 1-45