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Potential and possibilities of *genetically modified switchgrass* (*Panicum virgatum* L.) for high biofuel production- A review

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Abstract
Ligno-cellulosic biomass is one of the most promising renewable and clean energy resources to reduce greenhouse gas emissions and dependence on fossil fuels. However, the resistance to accessibility of sugars embedded in plant cell walls (so-called recalcitrance) is a major barrier to economically viable cellulosic ethanol production. A recent report from the US National Academy of Sciences indicated that, "absent technological breakthroughs", it was unlikely that the US would meet the congressionally mandated renewable fuel standard of 35 billion gallons of ethanol-equivalent biofuels with 1 billion gallons of biodiesel by 2022. In this context, genetically engineered switchgrass may provides a novel system for understanding cell wall recalcitrance as well as provides new germplasm for developing switchgrass cultivars as biomass feed stocks for biofuel production. By keeping these things in mind and to promote the research in this area, properties of genetically engineered switchgrass (*Panicum virgatum*) biomass for cellulosic ethanol production have been reviewed.

Keywords: Switchgrass, Bioenergy, Cellulosic ethanol, PvMYB4, Cell wall, Recalcitrance, Lignin, Hemi-cellulose.

1. Introduction
The widespread use of fossil fuels has led to increased greenhouse gas emissions (Leal *et al.,* 2013) [1] and exacerbated climate change. If left unchecked, the average global temperature could increase by 6°C by 2050, while an increase of only 2°C is considered the maximum that we can reasonably endure (Leal *et al.,* 2013) [1]. The use of biofuels has been proposed to reduce fossil fuel use in transportation (Fulton *et al. 2005*) [2]. First generation biofuels are produced from sugar and starch, i.e. traditional sources of food (Edgerton *et al.,* 2009) [3]. Their use has led to unforeseen problems, such as food shortages, and is often seen negatively for being associated with a loss of diversity and environmentally unfavorable changes in land use (Nageswara-Rao *et al.,* 2013) [4] which could increase greenhouse gas emissions (Searchinger *et al.,* 2008) [5]. Additionally, the demand for grain worldwide is expected to increase by about 15% over the next decade, due in part to the growing demand for meat in developing countries.

Switchgrass has been used as a forage grass in pastures and rangelands on the Great Plains of the United States for over 60 years and within the past 20 years it has become increasingly important on pastures in the central and eastern United States (Vogel *et al., 2004*) [4]. Switchgrass has attractive features as a dedicated lignocellulosic feedstock for bioenergy production in the United States (Keshwani DR, & Cheng *et al*., 2009, McLaughlin SB, & Adams Kszos L *et al*., 2005, Schmer MR, Vogel KP *et al*., 2008) [7, 8, 9], and recent studies report partial success in overcoming recalcitrance.

Genetic transformation of switchgrass is an important tool that can be used to elucidate the function of genes and to develop novel germplasm with increased biomass yield, improved biomass quality, and tolerance to biotic and abiotic stresses. Establishing a robust and efficient genetic transformation protocol is therefore critically important. In this review, discussion has been made on recent progress in switchgrass transformation and its applications.

Table 1.1: Major biofuels and their sources.

Type of Biofuel	Feedstock	State where produced
Conventional Bioethanol	Corn Sugarcane Sugar beets Cereals (e.g. milo, wheat, barley) Cassava	United States, Canada South America (primarily Brazil), Central America, Asia, Africa Europe Europe Canada Asia, South and Central America
Advanced Bioethanol	Cellulosic biomass • Grass (e.g. switchgrass, miscanthus, mixed species) • Short-rotation woody crops (e.g. poplar) • Plant waste (e.g. corn stover, wood waste)	In development
Conventional biodiesel	Rapeseed (canola) Soybean Sunflower Palm Jatropha Castor	Europe, Canada, Asia Europe, Canada, South and Central America, Africa, Asia, United States Europe, Canada, Africa, Asia South and Central America, Africa, Asia South and Central America, Africa, Asia South and Central America
Advanced biodiesel, bioethanol, biobutanol, aviation fuels	Algae	In development

2. Plant Regeneration

The first report on regeneration of a switchgrass plant used two sources as explants, mature caryopses and young leaf pieces from the cultivar 'Alamo'. The young leaf pieces were produced by cutting 1 to 1.5 meter tillers back to either the first node or the crown, then excising the newly formed shoots (secondary tillers) that had grown out of the cuts and removing the whorled leaf pieces from within the secondary tillers (Denchev and Conger *et al*., 1994) [10]. Previous work had successfully used mature caryopses in different species of grasses to produce embryogenic calli (Conger and Gray *et al*., 1984; Morrish *et al*., 1987; Vasil *et al*., 1987) [11, 12]

The regeneration of plants from embryogenic suspension cultures in switchgrass has been accomplished (Mazarei *et al*. 2011). [13]

2.1 Culture Medium

In the initial study to regenerate switchgrass from callus, sucrose was used as the carbon 110 source in the MS based medium (Denchev and Conger *et al*., 1994) [10]. In some studies (Denchev and Conger *et al*., 1995; Alexandrova *et al*., 1996a, b; Gupta and Conger *et al*., 1998, 1999; Odjakova and Conger *et al*., 1999) [10, 15, 16, 17] as well as others, maltose was substituted for sucrose, however this is not a universal adaptation as others have continued to use sucrose (Mann *et al*., 2011) [18], or saw no improvement in callus induction after replacing sucrose with maltose (Xi *et al*., 2009) [10].

Regeneration of switchgrass plants of lowland ecotypes is routinely achieved by means of embryogenic callus induction from cultures of mature caryopses in the dark. Plant regeneration of upland cultivars remains challenging. The most commonly used basal medium for embryogenic callus induction is MS supplemented with 3% (w/v) sucrose or maltose and 22.5 µM 2, 4-D with or without BAP. Addition of 17mmol (2g L-1) L-Proline is beneficial for both the quality and quantity of embryogenic callus produced from mature caryopsis culture.

2.2 Genetic Transformation

The first genetic transformation for switchgrass was via particle bombardment which obtained 4% transformation efficiency (Richards *et al*., 2001) [20]. The plasmid *psGFP-BAR* was constructed which contained the *gfp* (*green fluorescent protein*) reporter gene and the *phosphinothricin acetyltransferase* (*bar*) gene that confers bialaphos resistance. The plasmid was coated onto tungsten particles and propelled into inflorescence derived embryogenic callus (Richards *et al*., 2001) [20]. Plants resistant to Basta® (herbicide containing bialaphos) were regenerated and resistance was inherited by offspring of the crosses between transgenic and non-transgenic controls (Richards *et al*., 2001) [20]. The first use of agrobacterium mediated transformation in switchgrass came in 2002 when *Agrobacterium*, carrying the binary vector pDM805 harboring the *bar* and the reporter *gus* (β-*glucuronidase*) genes, was used to infect various genotypes of Alamo (Somleva *et al*., 2002) [29]. Reciprocal crosses between non-transgenic control and transformed plants were made and Basta® tolerant T1 progeny was obtained (Somleva *et al*., 2002) [29]. Basta® resistant switchgrass created a concern that transgenic switchgrass would be difficult to control if grown in areas adjacent to other Basta® resistant food crops. The switchgrass could become invasive in the crop field and hard to control. To promote the feasibility of using transgenically improved switchgrass an alternate selection system was developed (Xi *et al*., 2009) [10]. Transgenic 'Alamo' was created using *Agrobacterium* with a chimeric *hpt* gene (also denoted as *hph, hygromycin phosphotransferase B* confers hygromycin, hyg B, resistance) and *gus* gene (Xi *et al*., 2009) [10].

PCR screening revealed that 80% of putative transgenic plants selected on 60 mg L-1 or less of hyg B were escapes. One of the escapes, plant TB3-6-2, was in fact a transgenic plant, 201 however it contained multiple copies of the transgene and after further testing showed susceptibility to hyg B. RT-PCR of it and some of its progeny revealed little transcript

accumulation and its progeny also showed susceptibility to hyg B. However RT-PCR of the progeny which received only one copy of the transgene revealed transcript accumulation and hyg B resistance was seen (Xi *et al*., 2009) [10].

3. Applications of Genetic Transformation in Switchgrass Germplasm Improvement

3.1 Improving Biomass Quality

Switchgrass contains large amounts of cellulose and hemicellulose, sugars, which can undergo hydrolysis and then be fermented for the production of ethanol. The difficulty in producing this second generation biofuel is due to the association of cellulose and hemicellulose with lignin (Fu *et al*., 2011b) [22]. Lignin is a complex phenolic polymer and the lignin content in switchgrass is negatively correlated with the amount of glucose recovered, and thus the amount of ethanol able to be produced (Fu *et al*., 2011b) [22].

CAD (*Cinnamyl alcohol dehydrogenase*) gene produces the enzyme which catalyzes the final step in the synthesis of the monomer precursors to lignin. *Agrobacterium*-mediated transgenic switchgrass plants were produced by RNAi in which the *CAD* gene was downregulated (Fu *et al*., 2011b) [22]. The plants showed reduced *CAD* enzyme activity, and the lignin content was reduced by 14-22%. This had the effect of increasing saccharification of the cellulose and hemicellulose, increasing the glucose available for fermentation by 15-35% (Fu *et al*., 2011b). A separate study also used RNAi and achieved similar results in the down regulation of *CAD* (Saathoff *et al*., 2011). RNAi was also used to down regulate *COMT* (*caffeic acid O-methyltransferase* gene) by *Agrobacterium* - mediated transformation in switchgrass with the intent of lowering lignin content (Fu *et al*., 2011a) [23]. It has been achieved a 38% increase in ethanol yield while using 300-400% less cellulase (enzymes that catalyze cellulolysis) in the production of ethanol (Fu *et al*., 2011a) [23]. However the COMT deficient transgenic plants produced less ethanol than the wild types when milder pretreatment conditions were employed (Tschaplinski *et al*., 2012) [24].

Another enzyme involved in the synthesis of lignin precursors is 4CL (4-coumarate: coenzyme A ligase) (Xu *et al*., 2011a) [25]. Two homologous genes were identified, *Pv4CL1* and *Pv4CL2*, which putatively coded for 4CL. Using *Agrobacterium*- mediated RNAi of *Pv4CL1* they reduced the activity of 4CL by 80% (Xu *et al*., 2011a) [25]. This resulted in 17-32% less lignin content in the T0 plants, and 22% less lignin in the T1 transgenic plants when compared to the control. The T1 transgenic plants also yielded 57.2% more fermentable sugar than the control. RNAi of *PvCL2* did not show the same affect, however (Xu *et al*., 2011a) [25].

Shen *et al*., (2012) [26] found that a R2R3-MYB transcription factor, *PvMYB4*, when over expressed in transgenic switchgrass, reduced the lignin content which resulted in a three-fold increase in the efficiency of the release of sugar from cell wall residues. In a follow up study the *PvMYB4-OX* (over expression) lines produced 2.6 times the amount of ethanol as the control without pre-treatment (Shen *et al*., 2013) [27].

3.2 Improve Biomass Yield

Over expression of the micro-RNA miR156b via *Agrobacterium*-mediated transformation yielded several different results based on the level of over expression. This miRNA controls apical dominance and floral transition in switchgrass, an important consideration to make when concerned about increasing biomass yield (Fu *et al*., 2012) [28]. When switchgrass transitions to the reproductive mode, its vegetative growth ceases; conversely failing to halt vegetative growth before winter comes could kill the plant. Correct timing is needed to maximize biomass production. Low levels of miR156 overexpression led to increased biomass with an otherwise normal phenotype (Fu *et al*., 2012) [28]. Moderate overexpression also improved biomass production but the transgenic plants were non-flowering. High levels of overexpression led to stunted growth (Fu *et al*., 2012) [28].

Switchgrass may also be exploited to produce specialty products. PHAs (polyhydroxyalkanoates) are naturally occurring biodegradable plastics which are found in certain microbes, when faced with nutrient limitations, as a storage reserve (Somleva *et al*., 2008) [29]. The genes controlling the synthesis of PHB (polyhydroxybutyrate), a member of the PHA family, were introduced into switchgrass via *Agrobacterium* (Somleva *et al*., 2008) [29]. They were able to produce this plastic at a maximum rate of 3.72% dry weight of the leaf tissue. This is below the estimated 7.5% dry weight threshold needed for commercialization, but represents a significant step in the right direction (Somleva *et al*., 2008) [29].

4. Conclusions

It has been demonstrated that over expression of PvMYB4, a general transcriptional repressor of the phenylpropanoid /lignin biosynthesis pathway, can lead to very high yield ethanol production through dramatic reduction of recalcitrance. MYB4-OX switchgrass is an excellent model system for understanding recalcitrance, and provides new germplasm for developing switchgrass cultivars as biomass feed stocks for biofuel production. However, a better strategy for reducing recalcitrance is required for the development of improved lignocellulosic bioenergy feedstocks.

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