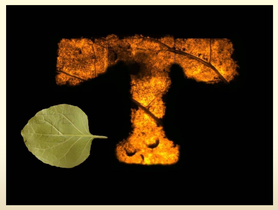

Functional analysis of a putative membrane-bound endo-β-1,4-glucanase from *Panicum virgatum*

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Introduction

Cellulose, the main component of plant cell walls, is composed of a simple polymer of unbranched β-1,4 linked glucan chains[1]. Mutational studies of glucan chains in model species have allowed for the determination of genes involved in cellulose synthase[1]. The cellulose produced by plants needs to be enzymatically modified for integration into plant tissues[2]; to accomplish this, plants produce enzymes which degrade cellulose[2]. One of these enzymes is an endo-β-1,4-glucanase (EGase), which hydrolyses β-1,4 linkages in the cellulose structure[3].

EGases, which digest cellulose and release glucose, exist in plant, fungi, metazoa, and bacteria. EGases are grouped into families with plant EGases predominately belonging to glycoside hydrolase family 9 (GHF9)[4]. The confirmation of a functional EGase from *Panicum virgatum* (switchgrass) may aid in the development of switchgrass transformants with an amorphous cellulose structure, thereby reducing the amount of resources required during biofuel refinery. The reduction of inputs required to process switchgrass into fuel will decrease the cost of producing ethanol.

Objectives

The intent of this research project is the functional characterization of the putative gene. This project involves the following experiments:

- Determine functional homolog through an *Arabidopsis* gene rescue experiment
- Determine anatomical differences between wild-type (WT) and an overexpression (OE) via microscopy

Arabidopsis Overexpression/Rescue

Cultures of transformant *Agrobacterium tumefaciens* were transformed to contain the gene of interest. Cultures were grown overnight and then used to transform three Arabidopsis lines known to be deficient in a single EGase to conduct the gene rescue experiment. In addition to the three EGase deficient lines, their respective backgrounds were also transformed to test for phenotypic backgrounds.

The *Arabidopsis thaliana* plants were transformed via floral dip. *Agrobacterium tumefaciens* cultures containing the gene of interest were grown overnight to an OD of 0.8, spun down, and placed in a sugar solution containing an inducing agent. Plants were then transformed by dipping the flowers into the solution. After one week, the Arabidopsis plants were given a second dipping to ensure adequate transformation.

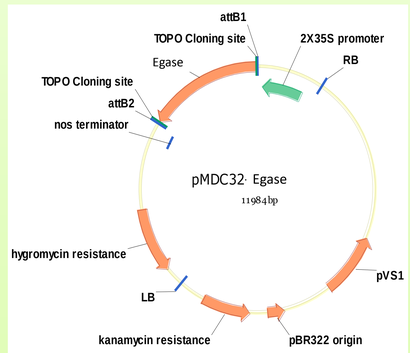

Figure 1. Map of the Curtis vector.

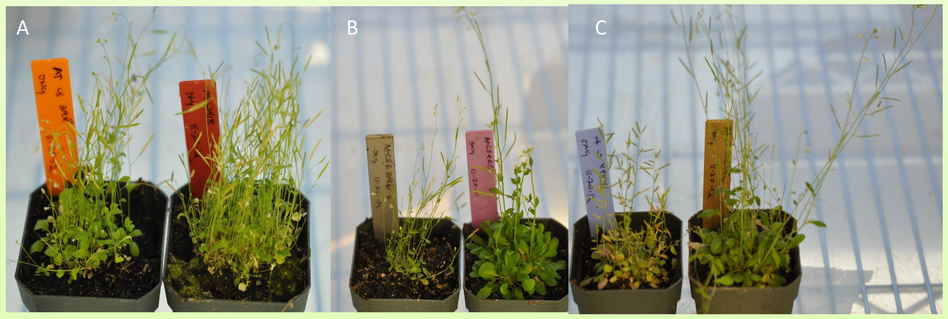

Figure 2. Comparison of Mutant *Arabidopsis* and their respective backgrounds. A: mutant and its Wassilewskija ecotype. **B:** mutant and its C24 ecotype. **C:** mutant and its Columbia-0 ecotype.

Microscopy

Samples were collected from switchgrass transformed with an overexpression of the Egase. Seven parts from each plant were collected in triplicate including the first three nodes, the leaf tip, the middle of the leaf, the leaf base, and the stem below the first node. These samples were then placed in Formalin-Acetic Acid-Alcohol. The samples were then dehydrated with 95% ethanol for two days. After dehydration, the samples were infiltrated with JB-4 (catalyzed Monomer A) . The first step in this infiltration was to use a solution containing one quarter JB-4 in three-quarters 95% ethanol for five days. Next, a 1:1 ratio of JB-4 to 95% ethanol was infiltrated for two days. Then, three-quarters JB-4 and one-quarter 95% ethanol was infiltrated for two days. Finally, the samples were placed in 100% JB-4 for two days. The samples were then removed and placed in a plastic molding tray. Monomer A was combined with Monomer B to initiate the hardening reaction. The molds were then placed under nitrogen gas and hardened. The samples were then cut using a glass blade on a microtome. The plastic samples were then sectioned in 5 micron sections and placed on slides. The samples were stained with Pontamine Fast Scarlet 4B, then viewed using the 40x objective under identical epifluorescent parameters.

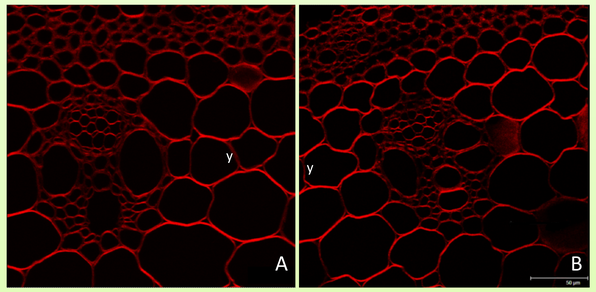

Figure 3. Vascular bundle comparison of WT (A) and Egase OE (B). These pictures were taken with identical settings using a laser confocal microscope. Notice lines indicated the increase in malleability in cell structure (y).

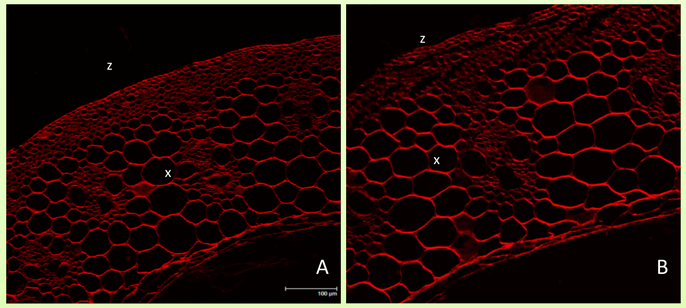

Figure 4. Stem comparison of WT (A) and Egase OE (B) stems. Notice the decreased order in cells near the epidermis (z) and the increase in overall cell wall size in the overexpression (x).

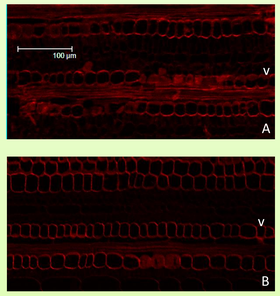

Figure 5. Comparison of WT (A) and Egase OE (B) leaf tissue. Notice the increased definition of cells (v) in B. Also note the decrease of cellulose found in the vascular bundles.

Conclusions

Switchgrass contains a functional EGase, which closely resembles other EGases from GHF9. The *Arabidopsis* overexpression and rescue experiments will need to be repeated, as no positive transformants were recovered. Microscopy work indicated anatomical differences between WT and Egase OE, but more work is needed to quantify these differences.

Future Research

Further research to enhance utility of this EGase:

- Enzymatic characterizations
- Determine relative gene expression level through qRT-PCR
- Carry the Rescue/Overexpression *Arabidopsis* to the T3 generation
- Spectral analysis of the microscopy work

Acknowledgements

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