EXPEDITING CELLULOSIC BIOFUELS AGENDA: PRODUCTION OF HIGH VALUE-LOW VOLUME CO-PRODUCTS AND LIGNIN DOWN-REGULATION OF BIOENERGY CROPS

By

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ABSTRACT

EXPEDITING CELLULOSIC BIOFUELS AGENDA: PRODUCTION OF HIGH VALUE-LOW VOLUME CO-PRODUCTS AND LIGNIN DOWN-REGULATION OF BIOENERGY CROPS

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One barrel of oil contains 81% fuels (46% gasoline, 9% jet fuel, 26% diesel) and 19% petrochemical co-products (lubricants, paints, solvents, adhesives, wax, greases, tar, asphalt, paraffin wax and more). For cellulosic biofuels (ethanol and butanol) to become commercially profitable, the efforts should not only be to improve the efficiency and economics of biofuels production, but also to mimic the petro-industry model by selfproducing of high value co-products including microbial heterologous cellulases and recombinant bio-pharmaceuticals in the non-food portions of cellulosic bioenergy crops.

Cellulases are needed to convert plant cellulose into fermentable sugars for biofuels. At present microbial cellulase mix are produced at cost of \$1.0 per gallon of ethanol. This dissertation will present co-production of biologically active *Acidothermus cellulolyticus* (E1) endo-cellulase, *Trichoderma reesei* 1,4- β -cellobiohydrolases I (CBH I) exo-cellulase and cow rumen *Butyrivibrio fibrisolvens* microbial cellobiase in corn (corn) plant endoplasmic reticulum, apoplast (cell wall area) and vacuole respectively. It is estimated that in this research the heterologous E1 and cellobiase are respectively produced at 0.8 and 1.2 kilograms per ton of corn silage.

Another high-value co-product is human saliva secretory leukocyte protease inhibitor (hsSLPI) which is well known as an anti-HIV protein and as the reason that HIV is not transmitted via human saliva. Biologically active human SLPI can not be produced in *E. coli*

due to SLPI's anti-microbial activity. This dissertation will present production of biologically active human SLPI in plant apoplast for preclinical testing.

Pretreatment of lignocellulosic biomass is required to breakdown lignin in order to expose crop cellulose to cellulases. Depending of the method used, pretreatment processes cost \$1.0 to \$2.15 per gallon of ethanol. This dissertation will also present down-regulation of corn stover (leaf and stem) lignin content via RNA interference (RNA1) of corn Cinnamoyl-CoA reductase (*CCR*). Such lignin down-regulation concomitantly increased corn crystalline cellulose. In addition, enzymatic hydrolysis of ammonia fiber expansion (AFEX)-pretreated corn stover of low lignin transgenic corn lines had a higher percent glucan conversion rate.

Following the petro-industry model, co-production of high value recombinant coproducts, and lignin down-regulation of bioenergy crops are expected to expedite commercialization of cellulosic biofuels.

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KEY TO SYMBOLS AND ABBREVIATIONS

SLPI: secretory Leukocyte Protease Inhibitor

WAP: whey acidic protein

WFDC: WAP four-disulfide core domain

Trappin: TRansglutaminase substrate and WAP motif-containing ProteIN

LPS: lipopolysaccharides

IL-1: interleukin-1

HIV-1: human immunodeficiency virus type 1

HSV: herpes simplex virus

ARDS: adult respiratory distress syndrome

COPD: chronic obstructive pulmonary diseases

P-RbcS1: Ribulose bisphosphate carboxylase promoter from *Asteraceous chrysanthemum*.

T-RbcS1: Ribulose bisphosphate carboxylase small unit terminator from Asteraceous

chrysanthemum.PAT : phosphinothricin acetyltransferase

Act1: the rice actin promoter

PPT: phosphinothricin

ER: endoplasmic reticulum

ERDS: ER-associated degradation system

E1: endo-cellulases (β -1,4-glucanases)

CBH I: exo-cellulases (1,4- β -cellobiohydrolases I)

AFEX: Ammonia Fiber Explosion

MU: fluorophore 4-methylumbelliferone

*p*NPβG: *p*-nitro-phenyl-β-D-glucopyranoside

CMC: carboxymethyl cellulose

4CL: 4-coumarate CoA ligase

CCR: cinnamoyl-CoA reductase

CAD: cinnamyl alcohol dehydrogenase

bm: brown-midrib

PAL: phenyl ammonia lyase

TBO: toluidine blue O

SEM: scanning electron microscope

GC: gas chromatography

CHAPTER I

In the quest for an alternative to microbial cellulase mix production: Corn stoverproduced heterologous multi-cellulases readily deconstruct lignocellulosic biomass into fermentable sugars for biofuels

- 1. The author is authorized to reuse the article (Park et al., 2011) as a Chapter I by the permission of Journal of Chemical Technology and Biotechnology.
- 2. This research is also contributed by Dr. Chuangsheng Mei (CBH I) and Dr. Callista Ransom (cellobiase).

I. INTRODUCTION

With the 2003 awakening report that the United States held 3% of the world's petroleum reserves, and consumed 25% of the world's petroleum consumption (http://www1.eere.energy.gov/vehiclesandfuels/facts/2004/fcvt_fotw336.html), the U.S. government urged the agricultural and petrochemical industries to find and implement biofuels as alternatives to fossil fuels to reduce the nation's dependence on foreign oil. A report resulted in the 2005 publication of the USDA-DOE documents the availability of U.S. lands for annual production of one billion tons of lignocellulosic matter in order to replace 30% of the foreign oil import to the U.S. by 2030 (http://www1.eere.energy.gov/biomass/pdfs/final billionton vision report2.pdf).

Plant lignocellulosic biofuels are considered as excellent alternative to petroleum fuel, gasoline. Plants annually produce 180 billion tons of cellulose at the global level (Emons and Mulder, 2000) and, as the most abundant biopolymer on earth, cellulose is indeed the most promising renewable energy source for biofuels production.

Despite the great potential of lignocellulosic biofuels, their production costs heavily depend on how cheap cellulase enzymes are produced and how efficiently lignocellulosic materials are broken down. At present, cellulase enzymes are produced in microbial bioreactors at approximate costs of \$1.00 per gallon of ethanol (Mei et al., 2009) which impedes the commercialization of cellulosic bioethanol. Therefore, the production costs of the microbially-produced commercial cellulases need to be further reduced in order to make the cellulosic biofuel technology competitive with corn grain ethanol.

At least three different cellulase enzymes are required to break down plant cell wall cellulose for cellulosic biofuel production. The plant secondary cell walls are mainly composed of crystalline cellulose, varying mixtures of hemicellulose and lignin. Pretreatment of the lignocellulosic biomass is necessary prior to enzymatic hydrolysis because the access of enzymes to cellulose is restricted by lignin-hemicellulose interference. Pretreatments (e.g., Ammonia Fiber Explosion; AFEX) break the lignin seal, disrupt the crystalline structure of macro- and microfibrils and increase the pore volume and available surface area. These physicochemical changes allow the enzymes to penetrate into the lignocellulosic fibers which render them amenable to enzymatic hydrolysis (Sticklen, 2007; Chundawat et al., 2010).

The three cellulases include endo- and exo-cellulases and cellobiases. The endocellulases such as β -1,4-glucanases (e.g., Cel5a; E1; EC 3.2.1.4, Accession no. U33212) randomly cleave β -1,4-glucan along the polysaccharide chain and produce a new reducing and non-reducing end of the cellulose strand. After the reaction of an endo-cellulase, the smaller glucan chains are further hydrolyzed by exo-cellulases such as 1,4- β cellobiohydrolases I or CBH I (Cel7a; EC 3.2.1.91. Accession no. E00389) which cleaves from the reducing ends, or the CBH II (Cel6a; EC 3.2.1.21, Accession no. M55080) which cleaves from the non-reducing ends of cellulose chains (Miettinen-Oinonen et al., 2005).

The hydrolysis of cellulose due to synergistic action of endo- and exo- cellulases results in dimer glucose chains or cellobiose. The cellobiose can be further converted into the monomer glucose by cellobiases such as β -1,4-glucosidase 1 (EC 3.2.1.21, Accession no. M31120). The β -1,4-glucosidase 1 has been grouped into two glycosyl hydrolase subfamilies, sub-family A and sub-family B. Sub-family A includes plant and non-rumen prokaryotic cellobiases. Sub-family B includes fungal cellobiases such as the one produced in *T. reesei*, *Aspergillus niger*, and *A. aculeatus* (Rojas and Romeu, 1996; Murray et al., 2004), and rumen bacteria such as the anaerobic bovine symbiotic *Butyrivibrio fibrisolvens* used in our studies. Cellobiases also act as cellulase inducers and transcriptional regulators (Fowler and Brown, 1992). Cellobiase is only needed at about 100-1000 times lower amounts than endo- and exo-cellulases for hydrolysis of cellulose (Hood et al., 2007).

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To reduce the costs of cellulases, we produced biologically active *Acidothermus cellulolyticus* E1, *Trichoderma reesei* CBH I, and bovine rumen *Butyrivibrio fibrisolvens* cellobiase in three different sub-cellular compartments of three different sets of transgenic corn plants. Then, we extracted plant-produced crude proteins containing each heterologous cellulase, mixed them together and added the mixture in certain ratios to Ammonia Fiber Explosion (AFEX) pretreated corn stover (Vega-Sachez and Ronald, 2010). We found that under our conditions, a certain ratio of the heterologous multicellulase mix was the most effective for cellulose conversion into glucose. In this research, we accomplish production of all three heterologous cellulases in corn plants in a cost-effective manner and suggest the feasible application of the plant-produced heterologous multicellulase mix in biofuel industries.

Previously, we found the composition of corn stover to include 34.4% glucan and 22.8% xylan (Chundawat et al., 2008). Theoretically, production of a few heterologous cellulases should have no effect on corn stover composition. Furthermore, the composition of corn stover is nearly identical in AFEX-pretreated and untreated corn stover (Chundawat et al., 2007; Chundawat et al., 2010).

II. MATERIALS AND METHODS

1. Co-transformation vectors



Figure 1. Schematic drawing of the plasmid vectors E1, CBH I and cellobiase (*bg1A*). Plasmids include pE1ER containing the *A. cellulolyticus* E1 targeted into ER; pDM302: plasmid containing the *bar* gene; pCBH-IApo: Plasmid containing the *T. reesei* CBH I targeted into apoplast; pBG1Vac: Plasmid containing the *Butyrivibrio fibrisolvens* cellobiase targeted into vacuole; and pGreen: plasmid containing the *bar* and the FLOWERING LOCUS C (FLC) genes.

There are five transformation vectors included in our experiments (Fig. 1). The pE1ER contains the *A. cellulolyticus* E1 gene (Tucker et al., 1989) included in ImpactVectorTM. This vector has been designed based on the green-specific Rubisco promoter and the signal peptide sequences to target E1 into the ER as described (Mei et al., 2008). The pDM302 (Accession no. X17220) contains the *bar* gene encoding phosphinothricin acetyltransferase (PAT) as a selectable marker. The gene regulated by the rice actin 1 (*Act1*) promoter and *nos* terminator (McElroy et al., 1990b).

The pApo is a binary vector targeting the CBH I gene (Klarskov et al., 1997a) into apoplast. This vector was constructed using the *T. reesei* CBH I gene. The gene was obtained from digestion of the pMZ766-CBH I with *XbaI* enzyme and the released CBH I gene cassette was then ligated into pCAMBIA3303. This vector contains the CaMV 35S promoter, the tobacco mosaic virus translational enhancer (Ω), the tobacco pathogenesis-related protein 1a (Pr1a) signal peptide for apoplast targeting, the six histidines, enterokinase recognition site (EK) and the polyadenylation signal from nopaline synthase gene (3' *nos*).

The pBGVac, or pUC1813 (Yao, 2004) contains the *bglA* gene (Lin et al., 1990) encoding *B. fibrisolvens* H17c β -glucosidase, the ER leading sequence, the vacuole-targeting signal peptide (VT), and the CaMV 35S promoter and terminator.

The pGreen (Hellens et al., 2000) is a binary vector containing the *bar* selectable marker gene regulated by the CaMV 35S promoter and nos terminator, and the FLOWERING LOCUS C (*FLC*) gene regulated by the CaMV 35S promoter and nos terminator. This vector also contains T-DNA left and right borders and carries the *nptII* gene for bacterial resistance to kanamycin.

2. Corn genetic transformation and production of transgenic progenies

Highly proliferating, immature-embryo-derived Hi II embryogenic corn calli were co-bombarded via the BiolisticTM gun with a 1:1 ratio of the pE1ER, pCBH-IApo or pBGVac, and either the pDM302 (McElroy et al., 1990b) or pGreen constructs (Hellens et al., 2000) containing the *bar* herbicide resistance selectable marker gene. In vitro culture phosphinothricin (PPT) resistant callus was selected based on our standard procedures (Biswas et al., 2006). The herbicide resistant plants were acclimated in a growth chamber, and then transferred to a greenhouse until maturity. Fertile first generation transgenic plants were self-pollinated and dried seeds were harvested 35-45 days after pollination.

3. Transgene integration and transcription analyses

The PCR analyses were performed on both first (T0) and second (T1) generation transgenic plants to confirm the presence of transgenes. Northern blotting was performed to confirm transcription of transgenes. Total RNA was isolated from putatively transgenic and wild-type control untransformed plants using Trizol reagent following the manufacturer instructions (Invitrogen, Carlsbad). RNA gel blot analysis was carried out following modifications of our previous procedure (Biswas et al., 2006).

4. Preparation of crude plant protein extracts and western blotting

Proteins were extracted from wild-type control untransformed and T0 E1 transgenic leaf tissues as described before (Mei et al., 2008). For crude protein extraction from T0 CBH I transgenic corn, 100 mg of leaf disks was ground in 4 volumes of ice-cold extraction buffer. The extract buffer contained 80 mM MES, pH 5.5, 10 mM 2-mercaptoethanol, 10 mM EDTA 0.1% sodium *N*-lauroylsarcosinate, 0.1% Triton X-100, 1 mM PMSF, 10 M leupeptin, and 1 g/ml each of aprotinin, pepstatin A, and chymostatin. The supernatant from the crude extract which was centrifuged at 15,000 g and 4 °C for 10 min was quantified using Bradford method (Bradford, 1976).

The Invitrogen NuPAGE[®] Bis-Tris Discontinuous Buffer System with a 10% $NuPAGE^{®}$ Novex Bis-Tris Pre-Cast Gel was used for Western blotting of T0 transgenic plants according to the manufacturer's instruction (Invitrogen, Carlsbad).

5. Biological activities of heterologous E1, CBH I and cellobiase

The biological activities of heterologous E1 and CBH I were measured in T0 transgenic plants following our previous research (Mei et al., 2009). Briefly, 10 μ l of a set of diluted crude protein containing each heterologous cellulase extract was mixed with 100 μ l

reaction buffer (50 mM sodium acetate pH 5.0 containing 1.0 mM of substrate MUC, 4methylumbelliferone β -D-cellobioside) in 96-well plates. Plates were covered and incubated at 65 °C in the dark for 30 min. Then, 100 µl of stop buffer (100 mM glycine, pH 10.3) was added and the fluorophore 4-methylumbelliferone (MU; the product of E1 or CBH I hydrolysis of the substrate MUC) was measured by reading the fluorescence at 465 nm using SPECTRAmax M2 device (Molecular Devices Inc., Sunnyvale) at of 360 nm excitation wavelength. After subtracting the background, the activity of each sample was calculated using a MU standard curve which contributed to deactivated enzyme extract.

The biological activity of heterologous cellobiase of T0 plants was measured via the modification of our standard procedure (Mei et al., 2008) measuring the hydrolysis of *p*-nitrophenyl- β -D-glucopyranoside (*p*NP β G), The incubation mixture included 2 mM *p*NP β G, 50 mM sodium phosphate buffer (pH 6.5) and 30 µl crude protein in a total volume of 100 µl. The reaction was conducted at 40 °C for 15 min and stopped by the addition of 300 µl 1.0 M Na₂CO₃. The amount of *p*-nitrophenol (*p*NP) released was determined using a spectrophotometer via measuring the absorbance of the solution at 415 nm. Standard solutions between 0~100 nmol *p*NP were also included.

6. Percent heterologous E1 and cellobiase in plant crude protein extracts

The percentage of heterologous E1 in crude protein extract was measured in T0 transgenic plants based on densitometry analysis of Western blot X-ray film. The percentages of the heterologous cellobiase in crude protein extract was measured via the standard curve representing the biological activities of different dilutions of the purified *A. niger* cellobiase (Gao et al. 2010) (80 % pure; isolated from NovozymeTM 188).

7. Estimation of heterologous cellulases per ton of dry mature corn stover versus corn silage

Based on their plant crude protein extracts, two reports were used to estimate the amount of heterologous cellulases per ton of dry mature corn stover versus corn silage. The first report is from the Department of Animal Science at North Carolina State University (http://www.agr.state.nc.us/drought/documents/InterpretingForageAnalysisReportsforcornstal ks.pdf). We calculated the amount of heterologous cellulases based on this report showing that 5 % of dry mature corn stover is protein, and approximately 40 % of these proteins are water soluble (total soluble proteins). The second report is from Manitoba Agriculture, Food and Rural Initiatives (http://www.gov.mb.ca/agriculture/crops/specialcrops/bii01s02.html) which indicates that about 9.4 % of corn silage is protein.

8. Optimization of ratio of E1 to CBH I for maximizing CMC conversion

Different ratios of E1 to CBH I in T0 transgenic plants were used in order to find an ideal ratio for carboxymethyl cellulose (CMC) conversion. The enzymatic hydrolysis experiment took place in a vial containing 1% CMC (Sigma-Aldrich, St Louis, MO) substrate in a 15 ml reaction buffer (7.5 ml of 100 mM sodium citrate buffer, pH 4.8). In addition, 60 µl (600 µg) tetracycline and 45 µl (450 µg) cycloheximide were added to each vial to prevent the growth of microorganisms during incubation and hydrolysis reaction. The reaction was supplemented with *A. niger* cellobiase (NovozymeTM 188) to convert the cellobiose to glucose. Distilled water was added to bring the total volume in each vial to 15 ml. All reactions were performed in duplicate to test reproducibility. The hydrolysis reaction was carried out at 50 °C with a shaker speed of 90 rpm. About 1 ml of each sample was taken out from the hydrolysis reaction after 72 hr of hydrolysis, and filtered using a 0.2 µm syringe filter and kept frozen. The amount of glucose produced in the enzyme blank and substrate blank were subtracted from the respective hydrolyzed glucose levels. The equivalent glucose

concentration was quantified using Glucose Analyzer (YSI 2700 SELECTTM Biochemistry Analyzer, Yellow Springs, OH) using glucose as the standard.

9. Optimization of ratio of E1 to CBH I to cellobiase for maximizing AFEX pretreated corn stover conversion

The DNS assay was employed to quantify the reducing sugar produced as the result of enzymatic hydrolysis, determining the optimum ratio of all three heterologous enzymes produced in T0 transgenic plants on conversion of AFEX pretreated corn stover into fermentable sugars (Cai et al., 1999). DNS is a colorimetric reagent used in standard assays to detect reducing sugars. For conversion, 1 % glucan loading equivalent AFEX pretreated corn stover was hydrolyzed using the microplate hydrolysis conditions as described elsewhere (Chundawat et al., 2008). Also, different ratios of E1:CBH I:Cellobiase were produced by diluting of crude proteins of different transgenic plants. Each of the different crude cellulase mix ratios were added to 1% glucan loading equivalent AFEX pretreated corn stover in microplates. After hydrolysis, 50 µl sample supernatant from each vial was taken and placed in each well of a 96 well plate, 100 µl DNS was added to each well, and the color was developed at 100 °C for 30 min (Gao et al., 2010; Chundawat et al., 2008). Heat resistant sticky film lid was used to cover the 96 well plate prior to heating to avoid evaporation. The reading was done with 100 µl sub-samples using a UV spectrophotometer at 540 nm. The readings were compared to glucose standards, and the actual percent AFEX pretreated corn stover conversion into glucose equivalents was calculated. In these assays, the enzyme and substrate blanks were included, and all reactions were done in triplicate to measure accuracy. T0 transgenic E1, CBH I and cellobiose were self-pollinated for production of T1 plants, and seeds were collected for further analyses.

III. RESULTS

1. Plant genetic engineering followed by confirmation of transgene integration and expression

Herbicide resistant transgenic corn plants were produced from immature embryoderived cell lines biolistically co-bombarded with each of the three constructs (pE1ER, pCBH-IApo, and pBG1Vac) containing the cellulase genes and one of the two constructs containing the bar gene (pDM302 and pGreen). We also produced CBH I independent transgenic tobacco plants via the *Agrobacterium* transformation system because most independent transgenic CBH I corn lines were lost prior to seed development. Polymerase chain reaction (PCR) analysis of herbicide resistant plants confirmed the presence of E1 gene in plants (data not shown), and Northern blotting confirmed the E1 transcription (Fig. 2a) in leaves of PCR positive plants. The production of heterologous E1 protein was confirmed via Western blotting using monoclonal E1 antibody (Fig. 2a).

A total of 30 mature independent CBH I transgenic corn lines were produced. Prior to death of some of these plants, PCR analysis of CBH I confirmed the presence and Northern blotting confirmed the transcription of CBH I transgene in corn plants (Fig. 2b). In addition, PCR confirmed the presence, and Western blotting confirmed the production of heterologous CBH I protein in tobacco plants (Fig. 2c).

A total of 35 mature independent corn cellobiase transgenic lines were produced. PCR analysis confirmed the presence, and Northern blotting confirmed the transcription of cellobiase transgene in corn plants (Fig. 2d).



Figure 2. Molecular analyses of E1, CBH I and cellobiase. Wt means wild-type untransformed control plant leaf. (a) E1 Northern blot analysis (top. Tob. E1; E1 heterologous tobacco) and Western blot analysis (bottom). (b) CBH I PCR analysis (top) and Northern blot analysis (bottom). (c) Tobacco heterologous CBH I Northern blot analysis (top) and Western blot analysis with 6xhistidine antibody (bottom). (d) Cellobiase PCR analysis (top) and Northern blot analysis (bottom).

2. Biological activities of heterologous cellulases

Biological activity of each of the heterologous cellulases is shown in Figure 3. In Figure 3a, enzymatic activity of E1 was measured in leaves of transgenic corn plants. One unit of E1 activity is defined by measuring the amount of 4MU released from reaction of one mg of plant total soluble protein (TSP or crude protein extract) added into one mM of 4MUC in one minute. Figure 3a confirms no activity in the wild-type control leaf while leaves from different independent transgenic E1 lines show different levels of activities, with line 19e showing the highest (205 nmol 4MU/mg TSP/min).

Enzymatic activity of CBH I was measured in leaves of transgenic corn and transgenic tobacco plants (Fig. 3b and 3c). In Figure 3b, one unit of CBH I activity is defined

by measuring the amount of 4MU released from reaction of one mg of crude protein added into one mM of 4MUC in one hour. Although wild-type control plant leaf shows a small amount of CBH I activity, transgenic corn leaves (61a and 61b) show 1.5 to 2.5 times greater activity as compared to their wild-type control plant leaf. In Figure 11c, we used one unit of CBH I activity as defined by measuring the amount of 4MU released from reaction of one picomole (pmol) of crude protein added into one mM of 4MUC in one hour. Transgenic tobacco leaf (line 1-3) shows 25 times greater activity than its wild-type control tobacco plant leaf (Fig. 3c). Overall, the activity of heterologous CBH I was much lower in transgenic corn than transgenic tobacco.

In Figure 3d, enzymatic activity of cellobiase was measured in leaves of transgenic corn. In Figure 3d, one unit of cellobiase activity is defined by measuring the amount of pNP released from reaction of one mg of crude protein added into one mM of $pNP\beta G$ in one minute. Figure 3d confirms that the wild-type control plant leaf had no activity while different independent transgenic corn cellobiase lines show different levels of activities, with line 3-1 showing the highest (5.475nmol pNPU/min).

We must indicate that the units for measuring the tobacco (Fig. 3c) and corn (Fig. 3b) heterologous CBH I are very different. While corn heterologous CBH I was measured in nmol, tobacco heterologous CBH I was measured in pmol due to its low activity.



For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 3. Heterologous E1, CBH I and cellobiase enzymatic activity assays. (a) Corn heterologous E1 activity. (b) Corn heterologous CBH I activity. (c) Tobacco heterologous CBH I activity. (d) Corn heterologous cellobiase activity. TSP means plant total soluble protein or crude protein extract. Mean \pm standard deviation (P<0.05, n=3).

3. Carboxymethyl cellulose (CMC) conversion using heterologous cellulases

Carboxymethyl cellulose (CMC) substrate conversion into low molecular weight reducing sugars was performed using the corn crude protein containing heterologous E1 or cellobiase. Figure 4a shows that the four corn E1 transgenic lines tested have significantly higher CMC conversion as compared to the wild-type control corn plant. Figure 4a shows that the crude protein containing corn-produced heterologous E1 tested displays higher CMC conversion capacities, and Figure 4b shows that the crude protein containing heterologous cellobiase



displays higher cellobiose conversion as compared to the wild-type control crude protein.

Figure 4. Glucose conversion assays of heterologous E1 using carboxymethyl cellulose (CMC) (a) and cellobiase using cellobiose (b) as substrate. Mean \pm standard deviation (P<0.05, n=3).

4. Multicellulase enzyme mix ratio optimization for CMC and AFEX-pretreated corn stover conversion

It has been well documented that different cellulases work together synergistically to decrystallize and hydrolyze cellulose, and also much more CBH I enzyme is required for optimal conversion. Therefore, different ratios of E1:CBH I (1:4, 1:10 and 1:15) based on total protein concentration were used in the hydrolytic conversion of soluble cellulose CMC to glucose. The total proteins were extracted from E1 and CBH I transgenic tobacco plants, respectively. Figure 5a shows that the ratio of 1:4 of E1:CBH I was the most effective ratio in cellulose-to-glucose conversion.

The ultimate goal of producing hydrolytic enzymes in plants is to use them in actual cellulosic biomass conversion. Therefore, various combinations of corn-produced E1, CBH I and cellobiase enzyme isolates were tested on AFEX pretreated corn stover representing 1 % glucan in 24 hr hydrolysis reaction. Figure 5b shows the amount of reducing sugars estimated by dinitrosalicylic acid (DNS) assay, and the best ratio of E1:CBH I:cellobiase tested appears

to be a 1:4:1, with release of nearly 1 g/L glucose equivalents. Although the biological activities of CBH I was relatively low, the conversion activity of the three plant-produced crude heterologous enzymes at 1:4:1 ratio shows similar conversion effectiveness as compared to the commercial enzyme Spezyme CP (SCP), meaning that the heterologous enzyme mixtures have the potential to substitute or at least be used as supplements to commercially available cellulase mixtures.

Since the heterologous multicellulase enzyme mix shows efficient conversion of pretreated corn stover, it is worthwhile to have estimations of heterologous cellulase productions in mature corn stover dry matter versus corn silage. Table 1 represents the amount of heterologous cellulases which could have been produced per ton dry mature corn stover versus corn silage.

Using densitometry analysis, the heterologous E1 protein production was estimated to be up to 2% of transgenic corn leaf crude protein. Based on our calculations, the heterologous E1 could be produced up to 400 grams per ton of dry mature corn stover and 752 grams per ton of corn silage.

The heterologous cellobiase protein produced was estimated up to 3.11% of transgenic plant leaf crude protein extract. Based on our calculations, the heterologous cellobiase could produce up to 622 grams per ton of dry mature corn stover and at 1165 grams per ton on corn silage.

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Figure 5. Heterologous multicellulase ratio optimization. SCP means commercial Spezyme CP (a mixture of endo and exo-glucanase) mixed with commercial β -glucosidase (NovozymeTM 188).

| Table 1. | Estimation | of heterologous | cellulase | productions | in c | dry mature | corn | stover | versus |
|------------|------------|-----------------|-----------|-------------|------|------------|------|--------|--------|
| corn silag | ge. | | | | | | | | |

| Heterologous Cellulase | Transgenic lines | % cellulase in crude protein extract | Approximate Heterologous Cellulases (g) / ton dry mature corn stover | Approximate Heterologous Cellulases (g) / ton corn silage | |
|---------------------------|---------------------|---|--|--|--|
| | 5a | 2.0 | 400 | 752 | |
| | 19e | 0.2 | 33 | 75.2 | |
| F1 | 21 | 0.2 | 33 | 75.2 | |
| E1 | 21c | 0.3 | 67 | 112.8 | |
| | 21g | 0.7 | 133 | 263.2 | |
| | 3-1 | 3.11 | 622 | 1165.6 | |
| Cellobiase | 9-18 | 2.2 | 436 | 827.2 | |
| | 9-2 | 1.8 | 368 | 676.8 | |
| | 2-1 | 1.6 | 314 | 601.6 | |
| | 10-24 | 0.9 | 182 | 338.4 | |

IV. DISCUSSION

1. Corn-produced heterologous multi-cellulases as a value-added biobased product

The demands for cellulosic biofuels as petroleum alternatives have surged within last few decades. Despite efforts made to date to increase the productivity of cellulase-producing microbes through genetic engineering, the high costs of microbial cellulase enzyme production still impede the commercialization of cellulosic ethanol industries. The production of microbial E1and CBH I in different plants have already been reported (Sticklen, 2007), and human and corn cellobiase genes have been expressed in tobacco (Reggi et al., 2005; Kiran et al., 2006).

A. cellulolyticus E1 is thermostable which helps it to endure the relatively high temperature of pretreatment processes (example; AFEX pretreatment), and shows high specific affinity to cellulose derivatives such as CMC (Sakon et al., 1996) which was used in our studies for E1 enzymatic activity tests.

In this report, we have targeted the *A. cellulolyticus* E1 into corn ER. Our recent report (Mei et al., 2008) indicated that the ER targeting is suitable for the accumulation of heterologous E1 because of the fact that ER is the first site for protein synthesis and is known to contain a series of molecular chaperones such as the ER Luminal Binding Protein (BiP) needed during protein folding, assembly and preventing the transport of immature protein molecules (Wrobel et al., 1997; Randall et al., 2005; Sticklen, 2009b).

We have also targeted *T. reesei* CBH I into corn apoplast because this sub-cellular compartment is a free diffusional space outside of the plasma membrane which can accumulate large quantities of foreign proteins. The filamentous fungus *T. reesei* is considered to be the most efficient cell wall degrading microbe, encoding for only 10 cellulolytic enzymes including cellobiohydrolases (Claeyssens et al., 1990; Wang et al., 2008). About 80~85% (40 g/L) of genetically modified *T. reesei* extracellular proteins is

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cellobiohydrolases, among which 50~60% are CBH I (Durand et al., 1988). In fact, due to its importance, CBH I enzyme quantity has been increased up to 1.5 fold via genetic engineering of *T. reesei* (Miettinen-Oinonen et al., 2005).

We have targeted cellobiase, into corn vacuoles because vacuoles occupy 30-90% (depending on plant maturity) of the cell volume, and therefore more heterologous proteins may accumulate in mature transgenic plants. We selected the cellobiase gene from bovine rumen *B. fibrisolvens* H17c (Lin et al., 1990) because its enzyme assists in enabling the conversion of cellulosic matter of silage feed into energy in rumen.

2. Using biologically active crude heterologous cellulases for saccharifying cellulosic biomass

It would have been ideal to use mixtures of pure E1, CBH I and cellobiase as positive controls in Figure 3. However, we only had pure E1 available in our laboratory. Figure 3 shows the biological activities of heterologous E1, CBH I and cellobiase. We used commercial pure microbial E1 (provided by National Renewable Energy Laboratory; NREL) as positive control in Figure 3a. We also used a commercially available pure E1- CBH I mixture (SCP) and an impure commercial microbial cellobiase as positive control (Fig. 5b).

Corn plants contain exo-glucanase genes and therefore exhibit background exoglucanase activities (Huber and Nevins, 1982). It is also possible that wild-type tobacco plants have exo-cellulase activities. These might be the reasons that the wild-type corn (Fig. 3b) and tobacco (Fig. 3c) plants have shown some exo-glucanase biological activities. Also, corn contains endo-glucanase (Hatfield and Nevis, 1986) and β -glucosidase (cellobiase) genes.(Kiran et al., 2006) The reason that the wild-type corn plants did not show any biological activity of E1 (Fig. 3a) or cellobiase (Fig. 3d) might be because either these two genes were not transcribing in to produce these enzymes when we harvested the plant leaves for analysis, or the amount of activity of these endogenous cellulases were not sufficient for detection. The activity assay for detecting E1 and CBH I were the same.

In Figure 3a, we show the biological activity of E1 in nmol 4MU/mg TSP per minute. However, in Figure 3b, we show the biological activity of E1 in nmol 4MU/mg TSP per hour because the heterologous E1 had much more activity as compared to the heterologous CBH I, and therefore less time is needed for the analysis of the heterologous E1. For the activity assay, we used EDTA in our extraction buffer for production of E1 and CBH I crude proteins. Considering that EDTA is known to partially inhibit the biological activities of cellulases (Chinedu et al., 2008), the biological activity of heterologous cellulases produced in plants in our studies might have been much more, should we have used an alternative to EDTA in our extraction buffer.

To calculate the biological activity of each heterologous cellulase in unit, we used equal amount of crude plant proteins, substrates and incubation time. There is an inconsistency between data presented in Table 1 and Figure 3. In Figure 3a, the 21g column (the column related to crude protein of independent transgenic corn line) should have been higher than the 19e column because we used higher percentage of E1 in 21g. This inconsistency might be due to the fact that non-measureable factors such as expansins and other cell wall loosening proteins in crude protein extracts of different independent transgenic lines might have been different in 21g as compared to 19e.

3. Crude heterologous cellulase mix ratio

At present, a naturally produced mixture of endo-glucanase, exo-glucanase and cellobiase is extracted from microbes and added to pretreated corn stover for enzymatic hydrolysis. When NREL mixed pure microbial E1 and CBH I and added the mixture to the pretreated corn stover in different ratios, a ratio of 1:17 (E1-CBH I) resulted in highest level

of fermentable sugars produced (communication with Dr. Michael Himmel of NREL). Therefore, one of our research goals was to find the optimal ratio of plant-produced heterologous cellulases on AFEX-pretreated corn stover for production of fermentable sugars.

We learned that a ratio of 1:4 of the crude E1 to CBH I was needed for production of the highest level of glucose. Crude cellulases are advantageous over using purified cellulases because plant crude proteins contain other useful molecules that cause cell wall loosening. For example, expansins (Shieh and Cosgrove, 1998; Cosgrove et al., 2002; Li et al., 2002) break hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides without having any hydrolytic activity (Brotman et al., 2008). Both the amino acid sequence and the role of plant expansins are similar to those of *T. reesei* swollenin which is reported to weaken filter paper (cellulose) and disrupt other cellulosic materials such as cotton fibers (Saloheimo et al., 2002).

In our studies, we produced three different cellulases in three sets of independent transgenic plants, and then mixed all three plant crude proteins in a ratio of 1:4:1 (E1:CBH I: cellobiase) for conversion of AFEX-pretreated corn stover into fermentable sugars because this ratio was most effective under our experimental conditions.

4. Field level estimation of corn-produced heterologous cellulases

We extrapolated the amount of heterologous cellulases that could be produced in the field per ton of mature dry corn stover

(http://www1.eere.energy.gov/biomass/pdfs/Biomass%202007%20Overview_Web.pdf) versus corn silage (http://www.gov.mb.ca/agriculture/crops/specialcrops/bii01s02.html) based on data produced from our greenhouse studies (Table 1). With these calculations, transgenic corn reported here could have produced up to 400 grams of E1 and 622 grams of cellobiase per ton of dry mature corn stover (third column) and up to 752 grams of E1and 1165.6 grams

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of heterologous cellobiase per ton of corn silage (fourth column).

5. Single cellulases gene transfer versus gene stacking

We chose to produce each cellulase enzyme in one set of transgenic corn plants instead of using transgene stacking because we wished to assure the possible effect of each transgene on plant health. We have started cross-breeding of these cellulase producing corn plants to combine transgenes (to be reported elsewhere). Gene stacking in transgenic plants might be a good option, should one be able to control the ratio of production of heterologous cellulases produced in the same plant or to balance the ratio by adding certain cellulases. The idea of gene stacking comes from bacterial cellulosome. Cellulosome is a large extracellular enzyme complex in certain anaerobic bacteria which break down cellulose. Unlike our transgenic plants that carry different heterologous cellulases in different sub-cellular compartments, cellulosomes are produced in microbial cytosol as bacteria do not contain subcellular compartments. Cellulosome contains nine different cellulases on the same structural base which is a "scaffolding protein" containing cellulose binding domains (Doi and Kosugi, 2004). The idea of assembling several cellulases as gene stacking on a structural base could be applicable to crop plants should the optimum ratio be achieved.

Expression of multi-gene assembly also works when genes are translationally fused and transferred to chloroplast genome. Plant chloroplasts can be genetically engineered with several coding sequences controlled by a single promoter, a phenomenon that cannot occur in nuclear transgenesis as presented here. The authors hope that the challenges associated with efficient chloroplast transgenesis of cereal crops including corn will soon be resolved, because translationally fused cellulases might be even more efficient for cell wall degradation than the heterologous cellulase mix produced in our studies. For example, when the fusion cellulase (CelYZ) produced from fusion of artificial heterologous endo 1,4-glucanase (CelZ) and exo1,4-glucanase (CelY) genes, regulated by tetA promoter/operator was successfully produced in *Escherichia coli*, the hydrolytic activity of such fusion protein was three- to four-fold higher than the sum of the activity of the combined CelZ and CelY due to the intra-molecular synergism of the fused cellulases in hydrolysis of crystalline cellulosic matter (Riedel and Bronnenmeier, 1998). If this were transformed corn chloroplasts, we would have been more beneficial, extract the fusion cellulase, and add to pretreated lignocellulosic matter for enzymatic hydrolysis, a cocktail of 12 heterologous hydrolytic enzymes were produced in tobacco via chloroplast transgenesis (Verma et al., 2010).

6. Quest for alternatives to production of microbial cellulases

According to a National Research Council report of the U.S. National Academies (Kirk et al., 2004) the chloroplast transgenesis platform has the major advantages of (1) relatively higher heterologous protein production, (2) reducing or preventing of transgene flow via pollen grain transfer in most flowering plants due to maternal inheritance of chloroplast genome, and (3) plastid genome is normally transferred via heterologous recombination allowing the site-specific insertion of transgenes in chloroplast genome, helping with reducing of unintended phenotypic effects of transgenes.

V. Conclusion

We have produced biologically active *A. cellulolyticus* E1, *Trichoderma reesei* 1,4- β cellobiohydrolases I (CBH I) exo-cellulase and bovine rumen *Butyrivibrio fibrisolvens* cellobiase in corn plant endoplasmic reticulum (ER), apoplast (cell wall areas) and vacuole respectively. Results show that the ratio 1 : 4 : 1 (E1 : CBH I : cellobiase) of crude heterologous cellulases is ideal for converting ammonia fiber explosion (AFEX) pretreated corn stover into fermentable sugars required for biofuel productions.

Corn plants that express all three biologically active heterologous cellulases within their cellulosic biomass to facilitate conversion of pretreated corn stover into fermentable sugars is a step forward in the quest for alternatives to the present microbial cellulase mix production system for cellulosic biofuels.

The nuclear transgenesis presented here and that of chloroplast transgenesis for production of multiple heterologous cellulases in tobacco (Verma et al., 2010) are expected to advance the field of cellulosic biofuels by reducing the costs associated with production of cellulases in microbial systems. This is because plants access solar energy for protein production while microbial bioreactors require chemical energy inputs.

The research presented here is indeed a step forward in the quest for commercialization of biomass crop-produced heterologous cellulases as an alternative or supplement to current microbial-based cellulase production for cellulosic biofuels (Sticklen, 2010).

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CHAPTER II

Production of recombinant human saliva secretory leukocyte protease inhibitor (rhsSLPI) in plants for preclinical testing

I. LITERATURE REVIEW

1. Human Secretory Leukocyte Protease Inhibitor (hSLPI)

1.1 Secretory Leukocyte Protease Inhibitor (SLPI)

Human secretory leukocyte protease inhibitor (SLPI) is a low-molecular weight (11.7 kDa) serine protease inhibitor that is non-glycosylated, basic and cysteine rich. SLPI consists of 107 amino acids with a high affinity for the neutrophil serine proteases including elastase, proteases 3 and cathepsin G (Moreau et al., 2008). SLPI is naturally expressed in a variety of cell types including serous cells of salivary glands, phagocytic cells, lung epithelial cells, pulmonary vascular endothelial cells, parotid tissue and hepatocytes (Eisenberg et al., 1990; Farquhar et al., 2002). In addition, SLPI is also found in various human mucous fluids such as parotid secretions, cervical mucus, seminal plasma and ascites.

SLPI has been known by several different names, depending on the tissue of origin. These include anti-leukoprotease (Boudier et al., 1987), HUSI-1, protease inhibitor whey acidic protein 4 (WAP4), mucus protease inhibitor, seminal protease inhibitor, WAP four-disulfide core domain 4 (WFDC) and human seminal fluid inhibitor (Schiessler et al., 1976).

Human SLPI was originally isolated from bronchial secretions (Ohlsson and Tegner, 1976; Ohlsson et al., 1977) and human cervical mucus (Schill et al., 1978). SLPI protein was also isolated from a human parotid (Thompson and Ohlsson, 1986) and its primary structure was configured. The gene sequencing was completed in 1986 by the same research group (Stetler et al., 1986; Thompson and Ohlsson, 1986). Shown in Figure 6A, human SLPI consists of two structurally homologous domains; one having a transglutaminase substrate (Domain 1, N-terminal) and the other (Domain 2; C-terminal) an elastase inhibitory domain. Each domain contains eight cysteines, resulting in four disulfide bonds. The disulfide bonding and helical structure of each domain make SLPI a very stable protein. Also, the acid stability of SLPI allows the protein to maintain its function in an acidic environment such as the human mouth.

The WFDC domain of SLPI is composed of about 40 amino acid residues that form a relatively flat structure. The peptide chain is folded into a central two-stranded β -sheet surrounded by two peptide strands that are joined at one end of the molecule, forming an inhibitory loop. This loop binds to the active site and inhibits the target proteases (Grutter et al., 1988; Tsunemi et al., 1996).

The WFDC domain plays an important role in innate immunity and inflammation by inhibiting serine proteases such as elastase and cathepsin G. The target specificity of SLPI against proteases is not restricted to only endogenous proteases secreted by proinflammatory cells, but also exogenous proteases which are derived from microorganisms (Clauss et al., 2005). Because of its broad spectrum anti-protease activity, SLPI has been considered a potent pharmaceutical to control human pathogens.



Figure 6. Diagram of SLPI amino acid structure showing domain orientation, 8 disulfide bonds indicated as a line.

1.2 Origin and evolution of SLPI

In humans, the SLPI gene is located on chromosome 20q12~13.2 and consists of four exons and three introns spanning approximately 2.65 kilobases (kb). It appears to be a relatively nonpolymorphic and stable gene that can be modulated at both transcriptional and translational levels. Promoter analysis with fragments of up to 1.2 kb of the 5' flanking region of the SLPI gene demonstrated a high promoter activity in a 131 base pair (bp) fragment relative to the transcription start site (Stolk and Hiemstra, 1999). In addition, it is known that a locus on human chromosome 20 contains 14 genes which form the WAP four-disulfide core (WFDC) domain. The biological role of this locus is the regulated inhibition of the activity of a broad range of microbial proteases (Weldon and Taggart, 2007).

SLPI is a member of the trappin (<u>TR</u>ansglutaminase substrate and W<u>AP</u> motifcontaining <u>ProteIN</u>) gene family which has been identified in mammals. SLPI is derived from a precursor trappin-2. Trappins are 2.0 kb genes composed of three exons. The first exon encodes a signal peptide flanked with few amino acid residues. The second exon is responsible for most of the mature protein displaying protease inhibition. The third exon encodes the 3'-untranslated region (Schalkwijk et al., 1999). Through the RNA processing, human trappin-2 is made up of two domains, the transglutaminase substrate domain and the WAP motif. As mentioned above, exon 2 plays an important role in protease inhibition. The wide range of inhibitory activity against many proteases was acquired as a result of evolutionary adaptation. The evolution was accelerated by diverse molecular evolutionary events such as gene duplication, exon shuffling and gene conversion etc. (Schalkwijk et al., 1999). The SLPI gene also evolved from an ancestral WAP gene. A single WAP gene underwent duplication and each WAP gene has since evolved into two domains.

1.3 Properties of the transglutaminase substrate domain

The trappin family is defined by two features of their termini. The N-terminal consists of a repeated sequence of Gly-Gln-Asp-Pro-Val-Lys (GQDPVK) that can be used as a transglutaminase substrate. This hexapeptide acts as an anchoring motif via transglutaminase cross-linking. Transglutaminase can efficiently recognize and bind to both single hexapeptides and hexapeptide repeats. Using single amino acid substitution analysis, two unique repetitive sequences of glutamine and lysine residues in the N-terminal appear to play important roles as acyl donor and acceptor sites for the formation of transglutaminase-mediated isopeptide. The length of the transglutaminase substrate domain varies among species and family members (Schalkwijk et al., 1999). The N-terminal domain stabilizes protease-inhibitor interactions and contains binding sites critical for transglutaminase and heparin binding (Ying et al., 1994).

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1.4 Properties of the four-disulfide core domain

Like the N-terminal of SLPI, the C-terminal domain contains a four-disulfide core or whey acidic protein (WAP) domain which is responsible for binding to target proteases. WAP is the most abundant protein in rodent milk (Zeeuwen et al., 1997; Schalkwijk et al., 1999). Because of the eight conserved cysteine residues in the Ctermini, the C-terminal remains structurally more compacted by forming four disulfide bonds. The disulfide bonds enable the tertiary structure to be more stable. The production of SLPI in a prokaryote system is largely influenced by the proper folding of these disulfide bonds.

The active center of the SLPI C-terminal confers a broad spectrum of inhibitory activity against most serine proteases while elafin, a member of human trappin-2, shows limited inhibitory activity of elastin-degrading serine protease (Schalkwijk et al., 1999).

1.5 The trappin gene family in human; elafin

Another low-molecular weight protein, elafin, is found in human mucous fluids including lung secretions and is often compared to SLPI in terms of its biological and structural similarities. Elafin consists of 57 amino acids. Elafin is derived from a precursor called trappin-2 or pre-elfin via proteolysis. Trappin-2 consists of 95 amino acids, including a transglutamase substrate domain in five different regions at its Nterminal. Elafin is also called skin-derived anti-leukoprotease (SKALP) and was first purified in 1990 (Moreau et al., 2008). Like SLPI, Elafin possesses two distinctive structures which are described above, including a four disulfide core or whey acidic protein (WAP) domain which is responsible for protease inhibition and a unique N- terminal domain that enables it to cross link to extracellular matrix proteins by transglutaminase. The C-terminal contains four disulfide bonds that create the inhibitory loop.

1.6 Regulation of SLPI expression

The induction of SLPI can be specifically triggered by several exogenous factors resulting from microbial infections. The regulation of SLPI gene expression is heavily dependent upon chemical mediators such as bacterial lipopolysaccharides (LPS), interleukin-1 (IL-1), tumor necrosis factor (TNF), neutrophil elastase and defensins. The regulation of serine protease inhibitors, such as SLPI and elafin, is tightly regulated by the fluctuations of cytokines, which are responsible for initiating, regulating and terminating the inflammatory response. Therefore, the SLPI gene is triggered by the upregulation of these factors. Conversely, the gene expression of protease inhibitors decreases with the reduction of the level of these cytokines (Sallenave, 2000).

Furthermore, the expression and biological activity of SLPI can also be regulated by several environmental factors. One study has shown that cigarette smoke induced a 50% drop in the inhibitory activity of human recombinant SLPI (Cavarra et al., 2001). This study underscores the important role of SLPI in lung and human health. Moreover, a recent study indicated that the concentration of SLPI decreases in salivary secretions with age, particularly in elderly males, thereby increasing the risk of oral mucosal disease in elderly people (Shugars et al., 2001). These studies collectively suggest that SLPI functions as an inducible regulator of lung, heart, liver, and skin inflammation as well as the immune response.

2. Defensive roles of SLPI

2.1 Roles in innate immunity

SLPI, also known as antiprotease, is induced acutely and promptly at the site of inflammation and involved in the innate immune system. Destructive proteases released from viruses are responsible for tissue damage, resulting in organ failure in human. As compared to other human salivary factors, SLPI is an essential component for the regulation of proteases which are produced by a variety of phagocytic inflammatory cells including neutrophil and activated macrophages. SLPI is constitutively expressed in digestive, respiratory and reproductive tracts in human organs. The physiological concentration of SLPI in saliva is 4 - 24 ug/ml and 10 ug/ml in lung epithelial surfaces (McNeely et al., 1995; Taggart et al., 2001). The concentration of SLPI in human organs varies depending upon the environment. One study showed that the concentration of SLPI is stimulated in oral epithelial cells in response to HIV-1 infection and the increased amount is heavily associated with the prevention of the further spreading of the virus. The spread of HIV-1 through target tissues is the result of an imbalance between the amount of proteases and protease inhibitors. The infection site begins to secrete numerous antiproteases which neutralize excess proteases and protect host tissues from further detrimental effects. This phenomenon demonstrates that SLPI plays an important role in innate mucosal immunity (Jana et al., 2005).

SLPI's ability to compensate for excess protease has been demonstrated in other disease systems. For instance, psoriasis and emphysema are diseases caused by imbalances of elastase and its inhibitor. It has been observed that SLPI and elafin are

expressed at much higher levels at the site of infection than in healthy tissues. In general, SLPI and elafin are induced and secreted from the site of inflammation in response to several chemical mediators (LPS, IL-1 and TNF etc.). The detrimental proteolysis caused by these diseases was significantly diminished by the induced SLPI and elafin (Williams et al., 2006).

Neutrophil elastase is an important protease enzyme that when expressed aberrantly can cause emphysema. This involves the breakdown of the lung structure increasing the airspaces. SLPI has a high affinity to elastase. The proteolytic effect of elastase was largely diminished by SLPI treatment in vitro and the reverse correlation between SLPI dosage and elastase proteolytic activity was proved by its low dissociation constant and favorable inhibition kinetics (Sallenave, 2000). Interestingly, microbial pathogens have actively evolved to overcome innate human defense mechanisms especially those mediated by antiproteases. While SLPI exhibits a broad spectrum of inhibitory activity to diverse proteases such as cathepsin G, trypsin, chemotrypsin and chymase, it can be also cleaved by the cysteine proteases cathepsin B, S and L, thereby losing its biological functions (Taggart et al., 2001).

Human SLPI is well known as a primary defense mechanism in skin injury (Wingens et al., 1998) and wound healing (Ashcroft et al., 2000). This protease inhibitor has been shown to be an important endogenous mediator in both skin and oral mucosal wound healing (van Bergen et al., 1996). The molecular mechanisms mediated by SLPI promote cellular proliferation in the wound area. For example, fibroblast-mediated collagen gel contraction, an in vitro model of dermal scarring, was inhibited by SLPI.

Recent research proposes an additional beneficial effect of SLPI treatment

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following spinal cord injury. SLPI shows an early protective role after spinal cord contusion injury and prevents secondary tissue damage in mice with over-expression of SLPI (Ghasemlou et al., 2010). The major role of SLPI in vivo is to protect local tissue from spreading secondary microbial infection to neighboring tissue cells. This role as a protective barrier is achieved not only as a result of anti-inflammatory activity but also because of its antiprotease and anti-microbial functions (Weldon and Taggart, 2007).

2.2 Roles in adaptive immune system

It has been proposed that SLPI and elafin also play an important role in the adaptive immune system. For example, the active production of elafin induced by the onset of inflammation in epithelial cells leads to a modification of dentritic cell (DC) phenotypes. Dendritic cells are immune cells that present antigens to other immune cells. By altering the DC morphology, these immune cells are activated and migrate to the lymph node where they interact with T cells and B cells to initiate and recruit the adaptive immune response (Williams et al., 2006).

Although SLPI and elafin were initially discovered as a consequence of their antiprotease activity, their potential as a potent medicine with multiple functions must be highlighted. Table 1 shows many of their biological activities implicating that SLPI and elafin as essential regulators in innate and adaptive immunity as well as the inflammatory response (Sallenave, 2000; Williams et al., 2006).

2.3 Anti-microbial activity of SLPI

2.3.1 Anti-viral functions of SLPI

In addition to the roles previously described, SLPI has also been shown to have anti-viral activity. The investigation of the HIV-1 BaL strain in human adherent monocytes (Konopka et al., 1998) and primary strains of HIV-1 and HIV-2 in lymphocytes has confirmed the inhibitory activity of SLPI against HIV strains in different target tissues (Shugars et al., 1997). Furthermore, SLPI has been shown to have anti-viral effects in both influenza and Sendai viruses as well (Beppu et al., 1997).

The human immunodeficiency virus type 1 (HIV-1) is rarely transmitted via mouth salivary secretions (Cavarra et al., 2001). In saliva, there are more than 10 endogenous inhibitors of HIV-1 including SLPI, amylase, cystatins and defensins. However, Freel et al. (1999) demonstrated that when SLPI is filtered out of saliva, the saliva sample lost most of its inhibitory activity against HIV-1. However, modest inhibition of HIV-1 remained, suggesting the presence of other anti-HIV-1 agents in oral secretions. These studies conclude clearly that SLPI is a major anti-HIV-1 component as SLPI blocked greater than 90% of HIV-1 infectivity at endogenous concentrations (1-10 ug/ml). It appears to be collaborating with other endogenous viral inhibitory factors (Shugars et al., 1999).

Recent studies support the anti-HIV-1 role of SLPI. Jana et al. (2005) demonstrated that brief exposure of human oral kerationcytes, the predominant cell type in the epidermis, to HIV-1 triggered SLPI mRNA expression, resulting in increased SLPI production. It suggests that virus-induced SLPI up-regulation may protect the oral cavity from HIV infection (Jana et al., 2005). Additionally, increased SLPI levels

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significantly reduce the rate of mother to child transmission of HIV-1 during birth possibly due to the presence of SLPI in vaginal fluids, which may block entry of the virus into infant cells (Pillay et al., 2001). Higher concentrations of SLPI in human saliva correlated with reduced rates of HIV-1 transmission.

In addition to the protective role of SLPI against HIV-1, SLPI also inhibits herpes simplex virus (HSV) infection in vitro by binding to epithelial cell surface and preventing viral infection. Interestingly, HSV facilitates its transmission by downregulating SLPI gene expression (Fakioglu et al., 2008). The presence of HSV might result in an increased risk of HIV acquisition and transmission.

SLPI interferes with HIV-1 transmission by disrupting the formation of bridge between HIV-1 and human innate immune cells or human target cell surfaces. The antiviral role is accomplished by a number of possible mechanisms. SLPI may form a complex with human scramblase, which is a membrane protein required for the movement of membrane phospholipids, thereby interfering with the fusion of the HIV-1 virus with the host cell membrane (Tseng and Tseng, 2000). SLPI may also interfere by blocking the entry of HIV-1 via binding to the phospholipid-binding protein annexin II required to promote HIV-1 infection of human macrophage cells (Ma et al., 2004). Finally, SLPI possibly interferes with the interaction between the HIV protein gp120 and the target cell surface (Lin et al., 2004; Campo et al., 2006).

2.3.2 Anti-bacterial functions of SLPI

SLPI, the third most abundant anti-microbial protein in upper airways, shows anti-microbial activity in vitro against *Escherichia coli*, lung pathogens such as

Pseudomonas aeruginosa, Staphylococcus aureus as well as the skin pathogens, *S. epidermis* and *Streptococcus sp.* (Taggart et al., 2001). The anti-microbial activity of SLPI has been shown to reside in the N-terminal domains of the protein while the C-terminal is responsible for protease inhibition. The SLPI-mediated inhibitory mechanism is largely unknown, but may include binding of the protease inhibitor to bacterial mRNA and DNA. Recent research suggests that the anti-microbial activity is mediated by the cationic charge of SLPI, which allows it to interact with the anionic microbial cell membrane (Williams et al., 2006)

2.3.3 Anti-fungal functions of SLPI

In addition to anti-bacterial activities, SLPI has a fungicidal activity. SLPI inhibits the growth of several fungi, such as *Aspergillus fumigates* and *Candida albicans*. Fifty percent fungicidal activity was shown at the concentration of 5.8 uM SLPI in the incubation of *A. fumigates* in liquid media and 50 % fungicidal activity was shown at 10 uM SLPI in *C. albicans* yeast cells. Interestingly, the fungicidal action only affected *A. fumigates* when it was grown in liquid media, and did not affect the airborne conidia (metabolically quiescent) (Tomee et al., 1998). SLPIs inhibitory function is also ascribed to the N-terminus (Tomee et al., 1997). The mechanism by which SLPI inhibits microbial pathogens is still unclear, but it might also be related to the interaction of the anionic cell membranes with the cationic SLPI.

2.4 Therapeutic potential of SLPI

SLPI is currently available for a wide array of therapeutic applications (e.g., HIV-1 vaccine) and is also being considered for the treatment of inflammatory diseases in different organs and tissues because of its broad spectrum of anti-microbial activity against pathogenic bacteria fungus and viruses. Due to this broad target specificity, SLPI may be a potent cure for patients with multiple infections or in those patients with unidentified infections.

Abnormal production of several proteases including elastase, cathepsin G and protease 3 may be involved in various forms of cystic fibrosis, adult respiratory distress syndrome (ARDS), asthma, chronic obstructive pulmonary diseases (COPD) and pulmonary fibrosis (Moreau et al., 2008). In particular, uncontrolled elastase can cause especially deleterious effects in lung tissue. In ARDS patients, the level of SLPI is markedly increased in diseased areas and also shows increased levels of SLPI in early and late phase asthma patients. In the early 1990s, when the importance of the therapeutic use of SLPI first arose, clinical trials of SLPI treatment in patients with cystic fibrosis showed a significant reduction in both elastase activity and interleukin 8 (IL-8) levels in epithelial lining fluid. The SLPI treatment had effectively limited airway inflammation in these patients (McElvaney et al., 1992).

To control abnormally produced serine proteases such as elastase, SLPI is a potent regulator as it is a naturally occurring human protein and is therefore free from immune system attack. SLPI, a low-molecular-mass protease inhibitor which has the advantages of size, diffusibility and high target specificity, could reduce the elastasederived symptoms (Hunninghake et al., 1981). In addition, SLPI may be retained in the lung for at least 24 hr and an increase in SLPI concentration may result from even a single dose of SLPI. Moreover, SLPI may enhance biological activity in vivo without causing any adverse effects on the patients because it is derived from humans. Although SLPI may prove therapeutically important, proteases released from *Staphylococcus aureus* and *Pseudomonas aeruginosa* in cystic fibrosis may inactivate the biological activity of SLPI (Sponer et al., 1991).

Resistance to conventionally used antibiotics is becoming more prevalent among all microorganisms. This makes seeking alternative medicines more and more important. Using SLPI as an "alarm antiprotease" may be a feasible solution. The antimicrobial function is mostly due to the interaction with the cell membrane, changes to which significantly impact the survival of the microorganisms. In contrast to conventional antibiotics, the occurrence of resistance is unlikely. Furthermore, the transglutaminase substrate domain in SLPI may also be important for its anti-microbial activity. This domain enhances the interaction of alarm proteases with microbial cell membranes, hence potentially prolonging the period of activity in the target microbes (Williams et al., 2006).

Although still in its infancy compared to other pharmaceuticals, due to limitations such as short half-life and poor accessibility of the recombinant product to target areas, the potential of SLPI as an anti-viral therapy warrants public attention (Sallenave, 2000). Several efforts have been made to overcome the limitations in drug delivery. For instance, most recombinant SLPI is degraded before it reaches the diseased areas in cystic fibrosis patients. Alternatively, gene therapy might be a feasible option to avoid this degradation. Using an adenovirus as a vector to deliver therapeutic transgenes into target tissues, the gene can be directly transformed and will maintain adequate levels of the therapeutic transgenes. In rodent models, several molecules such as IL-12, TNF and IL-10 were successfully expressed using an adenovirus vector system and the gene expression was drastically improved. Even though technical challenges still remain, it suggests the potential application of SLPI.

In addition to the therapeutic potential, SLPI can be used as a biomarker. SLPI shows a unique cleavage pattern with treatment of chymase, which is one of the major proteases released during mast cell degranulation in asthmatic and allergic responses in the airway epithelium. The unique cleavage pattern of SLPI can be used as a biomarker of chymase activity and SLPI may be useful to pre-examine the onset of the diseases in respiratory tracts (Belkowski et al., 2008).

Because of its broad spectrum target range SLPI may prove to be a useful agent to inhibit microbial produced-proteases and their detrimental effects in human body. As a part of the human immune systems, it would provide a tool necessary for the battle against unconquered diseases.

| Property | SLPI | Elafin | |
|---------------------------------------|---|---|--|
| Family name | Four disulfide core | Four disulfide core/trappin | |
| Molecular mass (kDa) | 11.7 | 9.8 | |
| Spectrum of protease inhibition | HNE, trypsin, chymotrypsin, tryptase, chymase, cathepsin G | HNE, pig pancreatic elastase, protease-3 | |
| Regulatory stimuli | LPS, IL-1, TNF, HNE | IL-1, TNF, HNE | |
| Biological properties | Anti-bacterial, Anti-fungal Anti-viral (HIV transmission,) Anti-inflammatory Inhibition of inflammatory Inhibition of inflammatory Inhibition of NF-kB activation Inhibition of mast cell histamine release Inhibition of C5a production in the inflamed lung Priming of innate immunity Inhibition of the neutrophil mediated down-regulation of C5a-induced activities in other PMSs Tissue repair Augmented macrophage production of TGF-b/IL-10 Improved cutaneous and oral mucosal wound healing | Anti-bacterial Anti-inflammatory -Inhibition of inflammatory infiltrate recruitment -Inhibition of NF-kB activation Priming of innate immunity -Chemotaxis of neutrophil -Inhibition of the neutrophil- mediated down-regulation of C5a-induced activities in other PMSs -Enhancement of LPS response in vivo and in vitro Tissue remodeling and cellular differentiation - Involvement in salivary gland development Augmentation of anti-viral adaptive immunity | |
| Gene therapy potential | Anti-HIV drugs Currently being explored | Adenovirus-elafin protects against lung injury (rodent-model) | |

Table 2. The characteristics of secretory leukocyte protease inhibitor (SLPI) and Elafin(Sallenave, 2000; Williams et al., 2006)

II. INTRODUCTION

Tremendous demands for the development of safe vaccines against AIDS have been raised since the breakthrough of the disease in early 1980's. It is estimated that approximately 33.4 million people were living with HIV in 2008 worldwide and 2 million people died of AIDS-related illnesses. Surprisingly, 2.1 million are children under 15 years of age (Global summary of the HIV/AIDS epidemic, WHO, 2009). In addition, 2.7 million people were newly infected with HIV in 2008. The number is still steadily increasing in developing countries while the number of AIDS patients in Europe and the United States slows down due to greater public awareness and education. Nevertheless, poor economic conditions in Africa and developing countries limit the use of precautionary medicines, resulting in the spread into public.

To date approximately 95 million people suffer worldwide from wounds resulting in tissue loss. Wounds and tissue loss become more critical when chronic and are the main reasons for the prolonged stay of the elderly in hospitals after surgery. This results in an additional \$1.5 billion in healthcare costs in the United States alone (http://www.bioportfolio,com/LeadDiscovery/PubMed-100301.html). Treatment of wounds and tissue loss become even more critical during times of wars. According to the Department of Defense records, the number of American soldiers wounded in Iraq excluding causalities and all wounded in Afghanistan and elsewhere during the last two years is more than 15,000. An additional 83,623 individuals were wounded in all terrorist attacks worldwide since 1968 (National Memorial Institute for the Prevention of Terrorism). Current therapy to treat wounds and related tissue loss are synthetic (i.e. do not occur naturally in the human defense system) and are mostly used to treat skin

surface injuries.

SLPI has been considered as one of the potent tools to control human HIV because of the fact that HIV is seldom spread through the oral cavity. HIV-1 resistance is attributed to a series of anti-microbial factors present in the oral fluids including not only SLPI but also defensins, salivary agglutinin and thrombospondin (Rocha et al., 2008). HIV-1 transmission through oral-genital sexual intercourse is far less than through genital-genital contact (Campo et al., 2006).

As a feasible vaccine to treat AIDS, SLPI was genetically modified for production in microbes. The microbe system is still requiring extensive denaturation and renaturation of protein due to the high levels of disulfide formation. The major advantage of human SLPI over other relevant biotech drugs is that SLPI is human-derived, and therefore is not expected to trigger an allergic response.

At least 20 grams of SLPI is needed in order to conduct a pre-clinical test (Dr. Sticklen communication with Dr. Andrew Badley, Director, HIV Research Center, Mayo Clinic, Rochester, MN). Further pre-clinical tests have not been achieved due to the high costs (\$245/100ug) of human SLPI production in the *Escherichia coli* protein expression system. Reasons for the high costs include the fact that production of non-glycosylated cationic SLPI requires extensive denaturation and renaturation processes to refold the disulfide-rich SLPI into its normal biologically active form. Also, it is reported that intracellular expression of SLPI causes severe reductions in overall bacterial nucleotides and protein synthesis, resulting in declined bacterial viability (~25 %), presumably due to interference of ribosome-mRNA interactions (Miller et al., 1989). To overcome limitations with production of human SLPI in *E. coli*, SLPI was transformed in insect

cells using the baculovirus promoter, and the results showed that SLPI could be produced in insect cells while sustaining its biological activity with much higher cellular viability (~90 %) than microbe-produced SLPI (Gray et al., 2002b).

Transgenic plants have successfully produced a number of recombinant protein biopharmaceuticals, such as insulin, erythropoietin, α -interferon, human serum albumin, as well as glucocerebrosidase and granulocyte–macrophage colony-stimulating factor (Giddings et al., 2000). Plants are considered as an ideal biofactory for the production of drugs because of the economic and qualitative benefits. Plant systems are relatively cheaper and yields are relatively high. In addition, any recombinant protein can be targeted in different plant organelles depending upon the properties of the protein (Goddijn et al., 1997). For example, Kusnadi et al. estimated that the cost of producing recombinant proteins in plants could be 10- to 50-fold lower than their production using *E. coli* fermentation (Kusnadi et al., 1997).

The goal of this research is to develop a large scale plant production system of biologically active rhSLPI that can be used for preclinical testing. The objectives are 1) to determine an ideal plant sub-cellular compartment for a stable rhSLPI production 2) to develop a purification method that will satisfy pre-clinical testing requirements and 3) to examine the anti-protease and anti-microbial activities of rhSLPI.

III. MATERIALS AND METHODS

1. Expression vector constructs

A set of sub-cellular targeting plant expression vectors (ImpactVectorTM) containing recombinant human SLPI (rhSLPI) were produced and transformed into tobacco (*Nicotiana tabacum* L. cv Samsun) and corn (Hi II) plants.

These vectors are specifically designed for plant transformation and employ the rubisco small subunit (RbcS1) promoter from *Asteraceous chrysanthemum* and 1 kb of the RbcS1 terminator sequence. Each one utilizes a different targeting sequence: cytoplasm, secretory pathway, endoplasmic reticulum (ER), chloroplast and mitochondria. In addition, the vectors have a cmyc-tag allowing identification of expressed proteins using commercially available monoclonal antibodies and six histidines for protein purification using a nickel column (Fig. 7 and Table 3).



Figure 7. Plant expression vectors containing the rhSLPI gene regulated by the greentissue specific promoter and targeting sequences specific for cytoplasm, apoplast, ER, chloroplast and mitochondria. *P-RbcS1*: Ribulose bisphosphate carboxylase promoter from the *Asteraceous chrysanthemum*. SPS: signal peptide sequences, *T-RbcS1*: Ribulose bisphosphate carboxylase small unit terminator from the *Asteraceous chrysanthemum*.

| Table 5 . Impact vector series for plant sub-centular targeting | Table 3. ImpactVector TM | series for plant sub-cellular | targeting. |
|--|----------------------------------|-------------------------------|------------|
|--|----------------------------------|-------------------------------|------------|

| | IV1.1-tag | IV1.2-tag | IV1.3-tag | IV1.4-tag | IV1.5-tag |
|--------------------------------|----------------------|--------------------------------------|--|-----------------------------|------------------------------------|
| Target organelle | Cytoplasm | Apoplast (Secreted expression) | ER | Chloroplast stroma | mitochondrial matrix |
| Origin of signal peptide | No signal peptide | Sea anemone equistatin | Sea anemone equistatin with KDEL retention signal | Chrysanthemum morifolium | Yeast CoxIV secretion signal |

2. Plants transformation

2.1 Agrobacterium-mediated transformation

In vitro cultured wild-type tobacco plants (4-8 week old) were used for Agrobacterium-mediated transformation. Each sub-cellular targeting vector was inserted into a pBINPLUS binary vector and transformed using Agrobacterium tumefaciens strain LBA4404 (Invitrogen, Carlsbad). For tobacco transformation, healthy expanded leaves from 4-5 wk old plants were cut into the size of $0.6 \sim 0.8$ cm squares and incubated in regeneration media (RM; 4.43 g/L MS with vitamin, 2.0 mg/L BAP, 0.5 mg/L IAA, 8 g/L bacto-agar, pH 5.6) containing suspended agrobacteria at 28 °C with gentle shaking at 100 rpm for 30 min. Then, after four days of co-cultivation in dark room conditions, Agrobacterium infected explants were washed with liquid RM including 1000 mg/L timentin for 3 min and washed with liquid RM for 3 min twice. The washed explants were then blotted onto sterile filter-paper to remove excess bacteria and transferred into selection RM (SRM; 4.43 g/L MS with vitamin, 2.0 mg/L BAP, 0.5 mg/L IAA, 300 mg/L timentin, 100 mg/L Kanamycin, 8 g/L Bacto-agar, pH 5.6). The explants were subcultured to fresh SRM every two wks. Once the callus formed shoots and stems (~8 wks), the whole explants were transferred to selection rooting medium (4.43 g/L MS with vitamin, 300 mg/L timentin, 100 mg/L kanamycin, 2.5 g/L GELRITE[®] Gellan Gum, pH 5.6). The shoots were then sub-cultured with a 16 hr photoperiod for 3 wks. The rooted explants were transferred to potting soil and grown under greenhouse conditions.

2.2 Corn transformation

Highly proliferating, immature-embryo-derived Hi II embryogenic corn calli

were co-bombarded via the Biolistic[™] gun with a 1:1 ratio of the each rhSLPI-subcloned ImpactVector[™] series with a pDM302 (McElroy et al., 1990b) containing the *bar* gene encoding phosphinothricin acetyltransferase (PAT) as a selectable marker. The *bar* gene is regulated by the rice actin 1 (Act1) promoter and *nos* terminator. In vitro cultured phosphinothricin (PPT) resistant callus was selected based on standard procedures (Biswas et al., 2006). The herbicide-resistant plants were acclimated in a growth chamber, and then transferred to a greenhouse until maturity.

3. Integration and transcriptional analysis of rhSLPI

PCR analyses were performed on transgenic corn and tobacco plants to confirm the presence of transgenes using the primer, SLPI_NcoI-F: 5'-

CCATGGGATCTGGAAAGTCCTTCAAA-3' (Tm: 58.8°C)

SLPI_BgIII-R: 5'-AGATCTCCAGCTTTCACAGGGGAAA-3' (59.9 °C). Northern blotting was performed to confirm the transcriptional level of transgenes. Total RNA was isolated from putatively transgenic and untransformed wild-type control plants using Trizol reagent following the manufacturer instructions (Invitrogen, Carlsbad). RNA gel blot analysis was carried out with following modifications of our previous procedure (Biswas et al., 2006).

4. Preparation of crude plant protein extracts and Western blotting

Proteins were extracted from untransformed wild-type and transgenic plant tobacco leaf tissues using 2 x SDS protein extraction buffer (100 mM Tris-Cl pH 6.8, 200 mM DTT, 4 % SDS, 20 % glycerol). For crude protein extraction, two leaf discs (0.5 cm in diameter) were collected and ground in 100 ul of 2 x SDS protein extraction buffer. The Invitrogen NuPAGE® Bis-Tris Discontinuous Buffer System with a 10 % NuPAGE® Novex Bis-Tris Pre-Cast Gel was used for Western blotting of the transgenic plants according to the manufacturer instruction (Invitrogen, Carlsbad) with human SLPI polyclonal antibody, goat IgG (R&D systems, Minneapolis), human SLPI monoclonal antibody, Mouse IgG (R&D systems, Minneapolis) and histidine monoclonal antibody (R&D systems, Minneapolis). The signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Rockford).

5. SLPI Purification by dialysis

Prior to the purification, crude extracts from tobacco leaves were centrifuged at 14,000 rpm for 10 min and the supernatant was filterized using 2.0 um filter. Recombinant human SLPI was dialyzed using Slide-A-Lyzer® G2 Dialysis Cassette (Thermo scientific, Rockford) to remove low molecular weight contaminants according to manufacturer's protocol. Briefly, the dialysis cassette was hydrated in dialysis buffer for 2 min and 2.5 ml of tobacco leaf crude extracts were injected into the cassette and removed most of the air. Then, the cassette was dialyzed for 2 hours at room temperature or 4 °C; change the dialysis buffer and dialyze for another 2 hours; change the dialysis buffer and dialyze overnight. The dialyzed tobacco leaf crude extracts were collected from the cassette using a syringe.

6. Biological activity tests

The purified rhSLPI was tested for biological activity against the proteases α chymotrypsin. In a 96-well plate, four ug of purified rhSLPI was added to wells containing 90 ul of reaction buffer (0.1M Tris-HCl, 0.01 M CaCl₂, pH 7.8). Ten ul of 1uM α -chymotrypsin was added to wells. The final volume was adjusted into 110 ul with reaction buffer and pre-incubated at 37 °C for 20 min. Ten ul of substrate (0.4mM N-succinyl-Ala-Ala-Pro-Phe-nitroanilide was added and the absorbance monitored at 405 nm to determine the substrate degradation activity of the proteases using Gen5 (Biotek, Winooski).

IV. RESULTS

1. Recombinant human SLPI gene constructs

Figure 8 shows the rhSLPI nucleotide and amino acid sequences. The theoretical pI/MW of the rhSLPI protein is 7.83 / 19.8 kDa. The amino acid sequences also include a signal peptide (3.7 kDa), rhSLPI (11.9 kDa) and the rest of the C-terminal (2.6 kDa) containing

c-myc, six histidines and an endoplasmic reticulum retention signal (KDEL).



Figure 8. Recombinant human SLPI nucleotide and amino acid sequences. ImpactVectorTM 1.3-tag (IV1.3-tag) contains signal peptides (3.7 kDa), rhSLPI (11.9 kDa), c-myc, polyhistidines and retention signal (2.6 kDa).

2. Tobacco transformation and molecular analyses

More than 30 different tobacco lines from each gene construct were generated from *Agrobacterium*-mediated transformation. The regenerated tobacco tissues were subjected to a series of molecular analyses to confirm the rhSLPI gene integration and transcriptional expression. Figure 9A shows 45-day old regenerated tobacco tissues used for PCR analysis. The expected size (337 bp) of rhSLPI product was observed (Figure 9B).



Figure 9. Tobacco regeneration and PCR analysis. A. 45 day old regenerated tobacco tissues putatively expressing rhSLPI gene. B. PCR amplication of the 337 bp fragment

for rhSLPI confirms the presence of the gene.

3. Transcription analysis

Figure 10 showed the different transcription levels of rhSLPI putatively produced in five different sub-cellular compartments in transgenic tobacco plants. Recombinant human SLPI putatively targeted into different sub-cellular compartments showed the high transcription level while wild-type tobacco showed no transcription of rhSLPI.



Figure 10. Northern blot analysis of rhSLPI in transgenic tobacco lines. A total 5 ug of total RNA extracted from each ImpactVectorTM series was used for the analysis. Different levels of rhSLPI transcription are shown in transgenic tobacco plants. Wt: wild-type tobacco; Cytoplasm: rhSLPI targeted into cytoplasm; Apoplast: rhSLPI targeted into apoplast; ER: rhSLPI targeted into endoplasmic reticulum; Chloroplast: rhSLPI targeted into chloroplast; Mitochondria: rhSLPI targeted into mitochondria.

4. Recombinant human SLPI protein analyses

More than 10 independent transgenic lines from each set of ImpactVectorTM series were analyzed to confirm the production of rhSLPI protein using a human SLPI polyclonal antibody (R&D systems, Minneapolis). The rhSLPI putatively targeted into tobacco apoplast (cell wall membrane area) consistently showed the expression of rhSLPI protein (Fig. 11 and Fig. 12). Among 14 independent tobacco transgenic lines putatively targeting the heterologous protein into apoplast, 13 transgenic lines showed the high expression of rhSLPI protein (>90%).



Figure 11. Western blot analysis of rhSLPI produced in different sub-cellular compartments in transgenic tobacco lines. Two leaf disc (0.5 cm in diameter) from 15-week old transgenic tobacco plants were used for the samples preparation. SM: protein standard (MagicMark^{XP}, Invitrogen); Wt: wild-type tobacco; 1.1: rhSLPI targeted into cytoplasm; 1.2: rhSLPI targeted into apoplast; 1.3: rhSLPI targeted into endoplasmic reticulum; 1.4: rhSLPI targeted into chloroplast; 1.5: rhSLPI targeted into mitochondria. Ten ng of rhSLPI produced in *E.coli* was used as positive control.



Figure 12. Western blot analysis of rhSLPI. The confirmation of rhSLPI protein production in more transgenic lines targeting rhSLPI into apoplast. Two leaf disc (0.5 cm in diameter) from 15-week old transgenic tobacco plants were used for the samples preparation. SM: protein standard (MagicMark^{XP}, Invitrogen); Wt: wild-type tobacco; SLPI (apoplast): SLPI targeted into plant cell apoplast. Fifty ng of rhSLPI produced in *E.coli* was used as positive control.

There was no IgG conjugated protein standard for the range below 20 kDa for the Western blot analysis, therefore MagicMark^{XP} (the smallest size is 20 kDa) was used as a protein size marker in Figure 13B. The Novex Sharp protein standard, which ranges from 3.5 kDa to 230 kDa, was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) commassie blue staining to identify protein size more accurately on the protein gel. Even though the rhSLPI protein expression was not distinctively visualized in the SDS-PAGE gel, the gel clearly showed the rhSLPI protein (marked with red asterisk). The amount might be speculated by the comparing with the positive control (100 ng of *E. coli* produced rhSLPI). The SDS-PAGE positive tobacco line was also used for a Western blot analysis using rhSLPI specific polyclonal antibody and the protein expression was confirmed (Fig. 13B). To reiterate, both protein analyses confirmed the rhSLPI protein expression of tobacco transgenic lines and their approximate sizes in SDS-PAGE gel (rhSLPI produced in *E. coli* : ~15 kDa and rhSLPI produced in tobacco: ~14 kDa).



Figure 13. SLPI protein analyses in transgenic tobacco callus (45 days old). A. SDS-PAGE. SM: protein standard (Novex Sharp protein standard, Invitrogen); 100 ng rhSLPI: positive control; Wt: wild-type tobacco; SLPI apoplast: SLPI targeted into plant cell apoplast. B. Western blot analysis. SM: protein standard (MagicMark^{XP}, Invitrogen); Wt: wild-type tobacco; SLPI apoplast: SLPI targeted into plant cell apoplast.

В.

A.
5. rhSLPI purification from tobacco leaf crude extracts

Prior to the rhSLPI biological activity assay, tobacco leaf crude extracts containing rhSLPI was dialyzed using a Slide-A-Lyzer G2 Dialysis Cassettes, 2 KDa molecular-weight cutoff (Thermo Scientific, Rockford) to remove low molecular weight contaminants including tar and nicotine etc. Figure 14 indicated that the amount of rhSLPI was not reduced after the dialysis procedure. As a consequence of two purification steps (centrifugation followed by filtration and dialysis), leaf tissue debris, micro-molecules (< 2 kDa) and salt compositions were efficiently removed from tobacco leaf crude extracts.



Figure 14. Western blot analysis with purified rhSLPI. SM: protein standard (MagicMark^{XP}, Invitrogen); Wt: wild-type tobacco; SLPI-1: rhSLPI targeted into apoplast (line 1); SLPI-2: rhSLPI targeted into apoplast (line 2); BD: before dialysis: AD: after dialysis.

6. Recombinant human SLPI antiprotease activity assay

To determine whether rhSLPI produced in transgenic lines retains its antiprotease activity, the dialyzed rhSLPI protein was pre-incubated with 1mM serine protease α -chymotrypsin at 37 °C for 20 min. Then, 0.4 mM protease substrate N-succinyl–Ala–Ala–Pro–Phe q-nitroanilide was added to the mixtures and the absorbance was measured at 405 nm at the indicated time.

Figure 15A shows the absorbance of the mixtures including α -chymotrypsin, Nsuccinyl–Ala–Ala–Pro–Phe q-nitroanilide and each sample proteins (2 ug of *E.coli* purified rhSLPI, two negative controls and 4 ug of rhSLPI-1 and rhSLPI-2 at indicated time. Two negative controls show high absorbance because α -chymotrypsin was not inhibited. Contrary, *E. coli* purified rhSLPI and two rhSLPI crude extracts added mixtures show low absorbance because the α -chymotrypsin was inhibited by *E. coli* purified rhSLPI and rhSLPI crude extracts. Simultaneously, yellow fluorescence was observed from negative control added wells while low yellow fluorescence was released from *E.coli* purified rhSLPI and rhSLPI crude extracts added wells (data not shown). We thus conclude that the rhSLPI produced in two transgenic lines (rhSLPI-1 and rhSLPI-2) exhibits significant antiprotease activities against the serine protease.



Figure 15. rhSLPI serine protease inhibition assay. A. Purified tobacco produced rhSLPI was preincubated with serine proteases chymotrypsin and the absorbance was monitored at the 5 min interval. B. Absorbance at 405 nm monitored at 5 min after adding substrate N-succinyl–Ala–Ala–Pro–Phe q-nitroanilide to the mixtures. Mean \pm standard deviation (P<0.05, n=6)

V. DISCUSSION

Due to the limited levels of SLPI production in human body fluids including saliva, previously human SLPI was heterologously expressed in *E.coli*. However, this system still requires expensive denaturation and renaturation processes because rhSLPI is not properly folded in *E. coli*, increasing its production costs (\$275/100 ug of SLPI; R&D system, Minneapolis). Recombinant human SLPI has also been produced in insect cells using baculovirus promoter (Gray et al., 2002a). However, the level of production is very low.

In this study, rhSLPI was produced in tobacco using an apoplast targeting gene construct under a green-tissue specific promoter. The major goal of this study has been to produce rhSLPI in large-scale as a bioenergy crop co-product using free solar energy.

Although all different sub-cellular targeted rhSLPI lines showed transcription of the relevant gene (Fig.10), Western blotting showed rhSLPI being produced only in apoplast (Fig. 11 and 12). The reason for production of rhSLPI protein in apoplast might be that this compartment may avoid protein degradation which caused by plant derived proteases. For further confirmation of apoplast production of rhSLPI, either *in situ* hybridization using rhSLPI monoclonal antibody followed by confocal laser microscopy, or isolation of apoplast followed by Western blot analysis of apoplast is necessary.

It is well known that tobacco leaf crude extracts contain not only macromolecules, but also micro-molecules such as water soluble sugars, amino acids, vitamins, salts, and other toxic compounds such as tar and nicotine (Fantozzi and Sensidoni, 1983). Therefore, the contaminants which are below 2 kDa molecular weight were

removed from tobacco leaf crude extracts by dialysis. The Western blotting (Fig. 14) indicates that there is no significant content loss of rhSLPI after the dialysis procedure.

Dialyzed tobacco produced rhSLPI was tested to determine whether the heterologously produced rhSLPI retain antiprotease activity against serine protease α chymotrypsin. In previous research, rhSLPI inhibited the activity of serine protease α chymotrypsin which breaks down a peptide substrate, N-succinyl-Ala-Ala-Pro-Phe qnitroanilide. Once the substrate is cleaved, yellow fluorescence (nitroanilide) is released under an alkaline condition (Delmar et al., 1979; Gray et al., 2002b). Fluorescence at 405 nm was used as an indicator of the activity of serine protease. Figure 15B indicating that tobacco produced rhSLPI-1 (A_{405 nm}: 0.5803) and rhSLPI-2 (A_{405 nm}: 0.4798) show statistically increase in antiprotease activity compared to the negative controls (Wt; A₄₀₅ nm: 2.2802 and buffer only; A_{405 nm}: 2.2585). Since the accurate rhSLPI concentration of dialyzed crude extracts was not measured in this experiment, it is hard to conclude that rhSLPI-2 has a higher inhibition activity over rhSLPI-1 line. However, when the equivalent amount of total crude extracts (40 ug) of wild-type, rhSLPI-1 and rhSLPI-2 was used for protease inhibition assay, it was concluded that the protease inhibition assay of dialyzed tobacco produced rhSLPI also retained serine protease activity.

For further future analyses, mass spectrometry (MS) will be performed to validate the chemical structure and mass of rhSLPI molecule. Most importantly, intensive purification methods will be employed to reach the level of protein purity required for pre-clinical testing. In addition, anti-viral activity against human pathogens such as simplex herpes virus and HIV will be examined to determine if plant-produced rhSLPI

could be used to develop a feasible vaccine for these and potentially other important diseases.

VI. Conclusion

We have produced genetically engineered tobacco plants heterologously producing rhSLPI using an *Agrobacterium*-mediated transformation technique. The green-tissue specific promoter enables the *rhSLPI* gene to be produced in the green tissues such as leaf and stem while the apoplast signal peptides target the gene outside the cell wall membranes (apoplast). Although the presence of rhSLPI in leaf apoplast was not experimentally confirmed, only the purified rhSLPI extracted from apoplast-targeted tobacco leaf tissues showed the protein expression as well as serine protease inhibitor activity.

The research presented here demonstrates the feasibility of producing rhSLPI in a non-microbial organism for the first time. This represents a major step forward in the de velopment of a large scale production system of the potent vaccine rhSLPI in crop plants.

CHAPTER III

Down-regulation of *Zea mays* cinnamoyl-CoA reductase via RNA interference to improve biofuels production

I. LITERATURE REVIEW

1. Lignin in plant cell walls

The plant secondary cell wall is mainly composed of crystalline cellulose, varying mixtures of hemicelluloses and lignin. Lignin, the most abundant biopolymer (cellulose being the first) on earth is a complex mixture of phenylpropanoid polymers that are formed via the oxidative coupling of hydroxycinnamyl alcohol monolignols including para-coumaryl, coniferyl, and sinapyl alcohols, synthesized via the shikimic acid and phenylpropanoid pathways (Boerjan et al., 2003; Ralph et al., 2004). Each of these residues results from separate but interconnected pathways (Sticklen, 2007). Manipulation of each of the lignin biosynthesis pathways is expected to modify plant lignin level, composition and structures.

In corn plants, lignin is covalently cross-linked to hemicellulose (e.g., glucuronoarabinoxylans in primary cell walls) via ferulate and diferulate bridges (Ralph et al., 1995). The lignin-hemicellulose complexes are associated with cellulose microfibrils through hydrogen bonding and Van der Waals forces, resulting in a complex matrix which confers the integrity and strength of the secondary cell walls. The mechanical strength of plant cell walls is largely influenced by the type of lignin subunits. Guaiacyl (G) units make plant cell walls softer, while syringly (S) units are responsible for the stiffness of cell walls. The S/G ratio varies greatly among hardwoods (Novaes et al., 2010). Lignin is also considered detrimental to the effectiveness of paper, pulp and biofuel industries because it interferes with the enzymatic digestion of cellulose microfibrils (Vermerris et al., 2010).

2. Lignin biosynthesis

A schematic drawing of the biosynthesis of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohol, starting from phenylalanine are shown in a review article (Sticklen, 2007). The monolignols are the result of sequential chemical reactions of several enzymes (Vanholme et al., 2008). Briefly, following deamination of phenylalanine, successive hydroxylation reactions of the aromatic ring occurs, followed by phenolic *O*-methylation and conversion of the side-chain carboxyl to an alcohol group. It has long been believed that the hydroxylation and methylation reactions take place at the level of the cinnamic acid and that *p*-coumaric, ferulic, and sinapic acids are subsequently converted to the corresponding monolignols by the sequential action of 4-coumarate:CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) (Boerjan et al., 2003).

The three monolignols are further polymerized by a process of lignification. Most of the enzymes and multiple intermediates described below are differentially expressed in the response to environmental cues (Lauvergeat et al., 2001) and also exhibit different kinetics and substrate preferences (Harding et al., 2002). It is also known that lignin biosynthesis pathway enzymes are associated with other functional and plant defense responsibilities, such as protecting plants from pathogens and insects (Ragauskas et al., 2006).

3. Biological function of lignin

In plants, lignin plays important roles in plant growth, development and survival. It provides mechanical strength by strengthening of cell walls where they are cross-linked

with hemicellulose. The polymerized monolignols also help the plant vascular system to conduct water and nutrients without diffusion by conferring hydrophobicity to cell walls. In addition, lignin fortifies plant secondary cell walls, thereby limiting invasion by various plant pathogens (Vanholme et al., 2010).

4. Brown-midrib (bm) natural mutants

There are naturally occurring corn mutants defective in lignin biosynthesis, named brown-midrib (bm) mutants because of the brown-coloration of midrib. Four bm mutants (bm1, bm2, bm3 and bm4) have been reported. The bm1 mutant appears when the cinnamyl alcohol dehydrogenase gene is mutated (Halpin et al., 1998a), while the bm3 mutant has a mutation in the *COMT* gene (Zuber et al., 1977). The mechanisms controlling the other two mutants are unknown. However, a gene-specific cell wall macro-array demonstrated that under-expressed genes in the bm2 mutant are closely associated with several functional groups including phenylpropanoid metabolism, transport and trafficking, as well as transcription factors and regulatory genes. Also, because lower guaiacyl monolignol content is shown in the bm2 mutant, the bm2 mutation could affect a regulatory gene involved in the regulation of coniferaldehyde. The bm4 mutant shows over-expression of phenylpropanoid and methylation genes, which are also important in the lignification of plant cell walls (Halpin et al., 1998b).

5. Lignin down-regulation via genetic engineering

While playing such important roles in sessile plants, densely lignified monolignols limit industrial uses of plant biomass because of their indigestibility. Pretreatment processing is required to open up plant cell wall complexes chemically and/or physically and to remove lignin. Based on methods used, pretreatment processes cost approximately \$1.15 to \$2.20 to produce a gallon of bioethanol (Eggeman et al., 2005). High pretreatment costs are considered the major barrier to commercialization of cellulosic biofuels, despite the annual availability of one billion tons of lignocellulosic biomass for biofuels production.

Plant lignin concentrations have been reduced via genetic engineering. A series of mutants recently gained public attention because of their possible use in bioethanol production due to their reduced lignin content. The lignin biosynthetic pathway has been successfully modified in alfalfa (Chen and Dixon, 2007), poplar (Leple et al., 2007), *Pinus radiata* (Wagner et al., 2009), tobacco (Chabannes et al., 2001a), Arabidopsis (Goujon et al., 2003) and corn (Piquemal et al., 2002).

For example, the down-regulation of the 4CL gene of quaking aspen (*Populus tremuloides*) resulted in a 45 % decrease in lignin content with a 15 % increase in cellulose without apparent damage to plant growth and development (Hu et al., 1999). It is believed that such compensation occurred because the quantitative or qualitative changes of one cell wall component often result in alteration of other cell wall components (Boudet et al., 2003).

In addition, the down-regulation of CAD in poplars has caused an increase in less conventional syringyl units and free phenolic groups (Lapierre et al., 2004). The downregulation of phenyl ammonia lyase (PAL), which is crucial to convert phenylalanine to cinnamic acid, largely affects the regulation of the entire monolignol biosynthesis pathway (Elkind et al., 1990; Bate et al., 1994). It is believed that this effect could be further enhanced by multiple gene knock-out mutations (Ragauskas et al., 2006). In alfalfa, modifications in lignin content and structure have been made to improve digestibility of this crop by ruminate animals (Jung et al., 1999). In addition, cell wall lignin structure in transgenic tobacco has been modified by down-regulating a 4-hydroxycinnamate 3-hydroxylase (C3H) gene, resulting in an increase in the proportion of para-hydroxyphenyl units relative to the normally dominant guaiacyl to syringyl (G: S) ratio (Ralph et al., 2006).

Studies on lignin down-regulation in Arabidopsis using an allelic series of C4H mutants showed that the strongest mutant allele caused stunted and sterile plants, while the weaker mutant alleles led to relatively normal plants with slightly reduced lignin content (Schilmiller et al., 2009). This implies that an optimal lignin reduction level has to be determined when plants are subjected to lignin down-regulation (Vanholme et al., 2010).

II. INTRODUCTION

There are two major steps associated with conversion of lignocellulosic biomass into fermentable sugars for biofuel productions. The first step is the production of hydrolysis enzymes which convert the feedstock crop cellulose into fermentable sugars. The second step is the expensive pretreatment process (\$1.15~ \$2.15 per gallon of ethanol) used to chemically and physically break down the recalcitrant feedstock lignocellulose complex into more reactive intermediates and to disrupt the lignin structure so that the cellulase enzymes can access the cellulose.

Pretreatment of lignocellulosic biomass is necessary prior to enzymatic hydrolysis, because the enzymes' access to cellulose is restricted by lignin-hemicellulose interference. Pretreatments (e.g., AFEX) break the lignin seal, disrupt the crystalline structure of macro- and microfibrils, and increase the pore volume and available surface area. These physicochemical changes allow the enzymes to penetrate into lignocellulosic fibers, rendering them amenable to enzymatic hydrolysis (Sticklen, 2007; Chundawat et al., 2010).

It is believed that the reduction of lignin content or the modification of lignin structure in feedstock crops can reduce the high costs associated with pretreatment processes (Ragauskas et al., 2006; Sticklen, 2006, 2009a).

The corn genome has been mapped, and from the corn genomic sequences (MaizeGDB), two corn CCR genes, *ZmCCR1* and *ZmCCR2*, were identified and cloned and characterized previously (Pichon et al., 1998). Cinnamoyl-CoA reductase (E.C.1.2.1.44; Accession no.: X98083) catalyses the conversion of the three hydroxycinnamoyl-CoA esters (*p*-coumaroyl-CoA, feruloyl-CoA, sinapoyl-CoA) into

their corresponding cinnamyl aldehydes. The goal of this project has been to examine the possible ways that lignin content of corn stover can be reduced to decease the needs for pretreatment processes.

It is one of the essential genes involved in constitutive lignifications. Therefore, in this research, I analyzed the effect of the corn cinnamoyl-CoA reductase gene when the gene is down-regulated via a RNA interference technique.

III. MATERIALS AND METHODS

1. dsRNAi gene constructs for ZmCCR1_RNAi

The corn gene CCR (Accession no.: X98083) involved in lignin biosynthesis was selected for lignin down regulation in corn. The cDNA for ZmCCR1 (X98083) gene was purchased from Arizona Genome Institute. The detail method is described below. For down regulation of each of the above, the C-terminal sequence of ZmCCR1 was chosen to design dsRNAi construct because of its specific and more unique sequences. For example, for CCR gene, large inverted fragments (523 bp) were amplified using two primers flanked with a restriction enzyme recognition site for *BgIII* and *NcoI* in 5'end. The large inversed fragments were then subcloned into a pGEM[®]-T Easy vector (Promega, Madison) via T4 DNA ligase (1hr at room temperature). The pGEM[®]-T Easy vector, including the inversed large fragments, was digested with NotI and NcoI and then the excised fragments were ligated into an ImpactVectorTM (Plant Research International, Netherlands) containing a green-tissue specific promoter (Rubisco). Also, another set of two primers were designed with BglII in the forward primer and SacI in the reverse primer in order to amplify an approximately 285 bp fragment from the C-terminal of the gene. The small fragments were also subcloned into pGEM[®]-T Easy vector. The small fragments were produced from $pGEM^{\mathbb{R}}$ -T Easy vector via digestion with two restriction enzymes (*BglII* and *SacI*) and then ligated into the ImpactVectorTM system containing large inversed fragments. This resulted in an approximately 285 bp inverted repeat sequence and an approximately 238 bp spacer in the middle of the construct (Fig. 16). Table 4 shows the primer sequences used to design the dsRNAi construct.

| CCR_RNAi | P-RbcS1 | CCB | Spacer | $CCR \longrightarrow$ | T-RbcS1 |
|----------|---------|-----|--------|-----------------------|---------|
| | | | | | |
| pDM302 | Act-5 | 5' | bar | Nos-T | _ |

Figure 16. RNAi plasmid constructs for the down regulation of the ZmCCR1 in Impactvector 1.1. *P-RbcS1*: Ribulose bisphosphate carboxylase promoter from *Asteraceous chrysanthemum*. *T-RbcS1*: Ribulose bisphosphate carboxylase small unit terminator from *Asteraceous chrysanthemum*. pDM302 containing the bar gene selectable marker sequences regulated by rice actin promoter and nos terminator (Mcelroy et al., 1990a).

Table 4. Primer sequences used for RNAi gene constructs.

| CCR primers | Inversed large fragment (523 bp) | CCR-748F-BglII: 5'- <u>AGATCT</u> ACATCCTCAAGTACCTGGAC-3' CCR-1271R-NcoI: 5'- <u>GCATGG</u> TTTACACAGCAGGGGAAGGT-3' |
|----------------|--|--|
| | Small fragment (285 bp) | CCR-986F-BgIII: 5'- <u>AGATCT</u> GGAAGCAGCCGTACAAGTTC-3' CCR-1271R-SacI: 5'- GAGCTCTTTACACAGCAGGGGAAGGT-3' |

* Underlined sequences indicate restriction enzyme recognition sites used for ImpactVector TM1.1 (cytoplasmic expression) cloning.

2. Corn genetic transformation

Highly proliferating, immature-embryo-derived Hi II embryogenic corn calli were co-bombarded using the BiolisticTM gun with a 1:1 ratio of the *ZmRNAi_RNAi* subcloned into ImpactVectorTM 1.1 (cytoplasm expression) and the pDM302 plasmid, (McElroy et al., 1990b) which contains the *bar* gene encoding phosphinothricin acetyltransferase (PAT) to be used as a selectable marker. The *bar* gene is regulated by the rice actin 1 (*Act1*) promoter and *nos* terminator. Using in vitro culture, phosphinothricin (PPT) resistant callus was selected based on standard procedures (Biswas et al., 2006). The herbicide resistant plants were acclimated in a growth chamber, and then transferred to a greenhouse until maturity. Fertile T0 plants were self-pollinated and seeds were harvested 35-45 days after pollination, when they had reached maturity.

3. Microscopy

3.1 Brightfield microscopy

All microscopic imaging was conducted in collaboration with the Center for Advanced Microscopy at Michigan State University. Bright field images were collected on an Olympus IX81 inverted microscope (Olympus Corporation, Center Valley, PA) configured with an MBF Bioscience CX9000 camera (Williston, VT) using the 20x UPlanFL N (0.5NA) air and the 40x UPlanFL N (1.3NA) oil objectives. The images were recorded using a Zeiss (Jena, Germany) PASCAL confocal laser scanning microscope with a 488-nm excitation mirror, a 560 nm emission filter, and a 505 to 530 nm emission filter. Image analysis was performed using Laser scanning microscope PASCAL LSM version 3.0 SP3 software.

3.2 Confocal fluorescence microscopy

Confocal fluorescence images were collected using an Olympus FluoView FV1000 confocal laser scanning microscope configured with an IX81 inverted microscope (Olympus Corporation, Center Valley, PA). Toluidine blue O fluorescence (blue) was excited with the 405 nm diode laser and fluorescence emission from 430-470 nm was collected. Red was excited with 543 nm Helium Neon laser and fluorescence

emission was collected with a 560 nm long pass filter. For comparison, bright field images (gray) were also collected with the 543 nm laser line. All images were collected with the 40x UPlanFL N (1.3NA) oil objective.

3.3 Scanning Electron Microscopy (SEM)

Samples were fixed at 4°C for 1-2 hr in 4% glutaraldehyde buffered with 0.1 m sodium phosphate at pH 7.4. Following a brief rinse in the buffer, samples were dehydrated in an ethanol series (25%, 50%, 75%, and 95%) for 10~15 min. at each gradation and with three 10 min. changes in 100% ethanol. Samples were critical point dried in a Balzers Model 010 critical point dryer (Balzers Union Ltd., Balzers, Liechtenstein) using liquid carbon dioxide as the transitional fluid. Samples were mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, West Chester, PA). Samples were coated with gold (approximately 20 nm thickness) in an Emscope Sputter Coater model SC 500 (Ashford, Kent, England) purged with argon gas. Samples were examined in a JEOL JSM-6400V (lanthanum hexaboride electron emitter) scanning electron microscope (JEOL Ltd., Tokyo, Japan). Digital images were acquired using analySIS Pro software version 3.2 (Olympus Soft Imaging Solution Corp., Münster, Germany).

4. Klason lignin analysis

Klason lignin is examined by measuring the remaining ash content (acid insoluble lignin) after hydrolyzation of other cell-wall components using strong acids. The procedure is based on the Klason method with modifications (Hatfield et al., 1994).

Briefly, leaf mid-rib samples were lyophilized and biomass moisture content of the milled corn stover samples was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). Because of the small quantity of sample involved, the biomass was not able to be extracted prior to compositional analysis. Otherwise, the lignin and structural sugars were determined as outlined in the standard NREL protocol (NREL, 2008). The acid insoluble lignin analysis method was modified to use 47 mm, 0.22 µm pore-size, mixed-cellulose ester filter discs (Millipore Corp., Bedford, MA) during the filtration step instead of fritted crucibles. Due to problems with burning, these discs with the filtered lignin residue were dried overnight in a desiccator prior to weighing, rather than in a vacuum oven. The soluble sugars released from the untreated enzymatic hydrolysis control sample (without enzymes) were quantified and subtracted from the sugars released during two stage acid hydrolysis to give the structural sugar content of the corn stover samples.

5. Hemicellulose compositional analysis

Three mg of corn mid-ribs were freeze-dried and hydrolyzed with trifluoroacetic acid (TFA). After the acid hydrolysis, the soluble parts were reduced by adding sodium borohydrate and acetylated by adding acetic anhydride. Errors bars are standard deviation of biological triplicates.

6. Enzymatic hydrolysis of lignin down-regulated AFEX-pretreated corn stover

Prior to enzymatic hydrolysis, ammonia fiber explosion (AFEX) was conducted at 90 °C, 0.6 g H_2O / g dry biomass for a 5 minute residence time following heat-up to the set temperature. Two ammonia loadings were tested for each sample: 1.0 and 1.5 g NH_3 / g dry biomass.

For each pretreated sample and untreated control, enzymatic hydrolysis was conducted in duplicate in 20 mL screw-cap vials at 1% glucan loading with a total volume of 15 mL. Samples were adjusted to pH 4.8 using a 1M citrate buffer solution. Accelerase[®]1000 (Genencor Division of Danisco US, Inc, New York, USA) cellulase at 5.5 mg protein g^{-1} glucan and Multifect[®] Xylanase (Genencor Division of Danisco US, Inc, New York, USA) was loaded at 0.5 mg protein g^{-1} glucan. A lower enzyme loading was chosen compared to the industry standard in order to expose greater differences between the transgenic lines. Samples were placed in a New Brunswick Scientific (New Jersey) incubator shaker and hydrolyzed at 50 °C and 200 rpm for 72 hr. The hydrolysates were sampled at 24 hr and 72 hr, after which samples were heated at 90 °C for 15 min, cooled and centrifuged at 15.000 rpm for 5 min. The supernatant was filtered into HPLC shell vials using a 25 mm, 0.2 µm polyethersulfone syringe filter (Whatman Inc, New Jersey). Samples were then stored at -20°C until further sugar analysis (Garlock et al., 2009).

An HPLC system was used to determine the monomeric glucose and xylose concentrations of each sample following enzymatic hydrolysis. The HPLC system consisted of a Waters (Massachusetts, USA) pump, auto-sampler and Waters 410

refractive index detector, equipped with a Bio-Rad (Hercules, California, USA) Aminex HPX-87P carbohydrate analysis column with an attached de-ashing guard column. Degassed HPLC grade water was used as the mobile phase, at 0.6 mL/min, with the column temperature set at 85 °C. Injection volume was 10 μ L with a run time of 20 min per sample. Mixed sugar standards were used to quantify the amount of monomeric glucose and xylose in each hydrolysate sample.

IV. RESULTS

1. Regeneration of ZmCCR1 RNAi corn lines

More than 30 mature independent *ZmCCR1_RNAi* transgenic corn lines (T0) were produced. Among them, transcription levels of 10 independent lines were examined after co-bombardment of the *ZmCCR1_RNAi* plasmid. Based on the Northern blot analysis, 6 different lines (1a, 1b, 1c, 4c, 6a and 6b) showed significantly reduced *ZmCCR1* gene expression levels (Fig. 17). Remaining corn lines showed similar level of transcription levels to wild-type corn.

We generated more than 100 *ZmCCR_RNAi* corn lines (T1) as a result of selfpollination of the first generation (T0) plants. To examine whether the *ZmCCR1_RNAi* gene construct was vertically transferred, their transcriptional levels were analyzed by a series of Northern blot experiments. Considering the Mendelian inheritance, the 2nd generation results were fairly consistent with that of first generation. Figure 18 shows that the progenies generated from self –pollination of 1a, 1b and 1c reduced levels of *ZmCCR1* genes. Distinctively, several different T1 lines, 1b (19, 20, 23, 27, 31, 34, 39, 44, 46, 47 and 48) and 1c (10, 19, 24, 29, 38, 42, 43, 46) showed a significant transcriptional reduction of corn *ZmCCR1* gene.

Figure 19 shows the presence of *bar* gene in *ZmCCR1_RNAi* transgenic corn lines 1b and 1c. The herbicide resistant gene was co-bombarded with dsRNAi gene constructs. The corn transgenic lines presenting both *ZmCCR1_RNAi* and *bar* gene (1b-3, 1b-4 and 1c-4, Ic-5) were selected to generate second transgenic lines for further analysis.



Figure 17. Transcriptional analysis of wild-type corn and *ZmCCR1_RNAi* transgenic corn lines (T0). A. The level of gene expression of wild-type and *ZmCCR1_RNAi* lines. B. RNA loading control (15ug of total RNA extracted from corn leaves).



Figure 18. Transcriptional analysis of wild-type and *ZmCCR1_RNAi* transgenic corn lines (T1). Seeds were collected from T1 transgenic lines and then grown under greenhouse conditions. Fully matured leaves were used for RNA extraction. 15ug of total RNA was used as a loading control.



Figure 19. PCR analysis of *ZmCCR_RNAi* lines to examine the integration of bar gene for transgenic corn selection. SM; Size marker (100bp ladder, Invitrogen), H₂O and Wt corn leaf; negative control, pDM302; plasmid containing *bar* gene.

2. Phenotypic analysis

The seed germination, growth and development of *ZmCCR1_RNAi* corn lines (T0-T2) were monitored and no detrimental effects were found under growth chamber and greenhouse conditions. Table 5 reports the height and diameter of stems measured from 13 cm from base line of wild-type corn and T2 transgenic lines. There is no significant difference between wild-type corn and *ZmCCR1* down-regulated corn plants.

The 2nd and 3rd (T1 and T2) ZmCCR1_RNAi transgenic corn lines appeared to be

not defective in seed germination, growth and germination, while approximately 5 % of T0 transgenic lines showed abnormal growth, such as early flowering and stunting. The abnormalities of T0 *ZmCCR1_RNAi* lines might have been caused by the significant reduction of lignin in plant cell walls. Due to the loss of strength in main stems and leaves, they were not able to stand upright. Also, curly leaves and meristems were observed in poor growth corn lines (data not recorded). Here, we also assume that there was a significant reduction in lignin content as a consequence of interference in transcription mechanism.

| | Wild-type (Hi-II) | ZmCCR1_RNAi-1c |
|---------------|-------------------------|---------------------------------|
| Height (cm) | $153.92 \pm 6.37 (n=5)$ | $159.2 \pm 14.41 \text{ (n=8)}$ |
| Diameter (cm) | $2.78 \pm 0.82 (n=5)$ | 2.26 ± 0.36 (n=8) |

Table 5. Comparison of plant height and diameter between wild-type corn and $ZmCCR1_RNAi$ -1c transgenic lines (T2). Plant height was measured from the soil base line to the top of the tassel and stem diameter was measured 13 cm distal from the soil base line. Mean \pm standard deviation (P>0.05).

In matured corn (~8 weeks old, T1 generation), six out of 20 *ZmCCR1_RNAi* transgenic lines showed obvious brown discoloration in the leaf mid-rib, stems and husks without any other abnormality under greenhouse conditions (23°C, 16 hr light:8 hr dark). Figure 20 shows brown coloration of the leaf mid-rib, stem and husk.



Figure 20. Phenotypic analysis of wild-type (Hi-II) and *ZmCCR1_RNAi* transgenic corn line (T1). Brown coloration was seen in *ZmCCR1* down-regulated corn leaves, stems and corn husks.

3. Histological assay

To examine whether there are any structural changes in plant cell walls, a histological assay was carried out using *ZmCCR1_RNAi-c* lines (T1). The cross-sectioned corn leave were fixed in 10% Neutral Buffered Formalin, processed on an automated vacuum infiltrating tissue processor, embedded in paraffin and sectioned on a rotary microtome at 4 - 5 microns. Sections were then placed on adhesive slides, dried at 56°C , deparaffinized and hydrated to distilled water followed and then stained with 2% phloroglucinol-HCl (Fig. 20A) or 0.05% toluidine blue O (Fig. 21B) to visualize lignified cell wall areas. Figure 21A shows a cross-sectioned leaf mid-rib and there is no significant differences found in xylem vessel, phloem and sheath cells except for sclerenchyma fibers. The arrows indicate sclerenchyma fiber cell walls with less lignified cell walls (thinner and bright blue) compared to wild-type corn (thicker and dark blue), demonstrating that lignin was reduced in sclerenchyma fibers of *ZmCCR1_RNAi-1c* line.

Figure 21C is an enlarged version of A and B with detail descriptions. To reiterate, no differences were found in size or primary and secondary cell wall structures in leaf midribs between wild-type (Honey'n Pearl Hybrid sweet corn) and *CCR1* down-regulated transgenic 1c. However, lignin reduction is apparent in the cell walls of sclerenchyma fibers.



Figure 21. Histological assay (brightfield images) of wild-type (Honey'n Pearl Hybrid sweet corn) and *ZmCCR1_RNAi-1c* leaves (T1); each leaf was cross-sectioned via rotary microtome. The cross-sectioned corn leaves were stained with 0.05% toluidine blue O or 2% phloroglucinol- HCl for 1 min to visualize secondary xylem tissues. The red arrowhead indicates the cell walls of sclerenchyma fibers of the leaf midrib.

In addition, confocal fluorescence microscopy was employed to observe lignin deposits in the leaf mid-rib. In Figure 22, wild-type (Hi-II) was compared to the *ZmCCR_RNAi*-1c lines, showing similar structural changes in sclerenchyma cell walls. The images confirmed the no structural changes in vascular bundles including xylem and phloem, but showed the lignin reduction in sclerenchyma fibers in *ZmCCR1_RNAi-1c-6* line (red arrowhead).



Figure 22. Confocal fluorescence images of wild-type corn (Hi-II) and Zm*CCR1-RNAi-1c-6* leaves (T1); microtome-sectioned corn leaves were stained with toluidine blue O (TBO). Upper panels represent wild-type corn plants at 40x magnification. Bottom panels represent the *ZmCCR1_RNAi-1c-6* transgenic corn line. The red arrowhead indicates the cell walls of sclerenchyma fibers of the leaf midrib.

Figure 23 shows scanning electron microscope (SEM) images of *ZmCCR1* downregulated corn midrib compared with that of wild-type (Hi-II). No differences were found in major xylem vessel, phloem and sheath cells in both corn lines while sclerenchyma fibers in *ZmCCR1_RNAi* showed thinner cell wall (red arrow head in left bottom) compared to that of wild-type (red arrow head in right bottom). The major vascular system showed no defects in function for either water and nutrient transfer or mechanical support of stems. However, the SEM images confirmed the reduction of lignin deposition in the cell wall area of sclerenchyma fibers.



Figure 23. Scanning electron microscopy (SEM) of *ZmCCR1* down-regulated transgenic corn leaf midrib as compared to that of wild-type (Honey'n Pearl Hybrid sweet corn) non-transgenic control plant. The red arrow indicates sclerenchyma fibers.

4. Lignin measurement

We have learned that *ZmCCR1_RNAi-1c* lines showed both reduced transcription and lignin deposition which may affect the cell wall thickness of sclerenchyma fibers. Therefore, lignin measurement will enable us to confirm the actual reduction in corn transgenic lines. Acid (H_2SO_4)-insoluble Klason lignin was measured as a difference in weight of the insoluble residue before and after ashing a 450°C for 6 hr.

Figure 24 shows the amount of acid-insoluble Klason lignin (g/kg con stover) in wild-type corn and ZmCCR1_RNAi transgenic corn lines (T1). Acid-insoluble Klason lignin from all ZmCCR1_RNAi 1b transgenic lines did not differ from those of wild-type corn. However, ZmCCR1-RNAi 1c-4-6 showed a statistically significant reduction in lignin composition. Each line shows 8.1%, 7.0% and 8.7% lignin reduction compared to that of the wild-type corn plant.



Figure 24. Acid-insoluble lignin contents of wild-type corn and *ZmCCR1_RNAi-1c* transgenic corn lines (T1). The lignin content was measured via the Klason method. The red asterisk indicates that the line is significantly different from the lignin content of wild-type corn. Mean \pm standard deviation (P<0.05, n=3).

5. Crystalline cellulose assay

To determine whether crystalline cellulose production is increased when lignin production is decreased in *CCR1* down regulated transgenic corn plants, the amount of leaf cellulose was examined via Gas Chromatography (GC). Figure 25 shows that only two lines (*ZmCCR1b-6* and *1c-6*) have increased levels of crystalline cellulose. However, when this assay was repeated, the differences in the amount of cellulose were not found (data now shown). It is hard to conclude that there are compensatory effects on cellulose content when lignin content is decreased.



Figure 25. Anthrone crystalline cellulose assay. Crystalline cellulose content was measured in wild-type corn and *ZmCCR_RNAi* lines (T1). Three mg total of mid-ribs was extracted from leaves for this assay. Compared with wild-type, *ZmCCR1b-6 and CCR1c-6* showed statistically significant increases in cellulose content in their leaf mid-ribs.

6. Hemicellulose composition analysis

To examine change in hemicellulose components in $ZmCCR_RNAi$ lines (T1), gas chromatography (GC) analysis was performed. Six major hemicelluloses were analyzed in wild-type corn and transgenic lines. Figure 26 shows that three hemicelluloses (arabionose, xylose and galactose) were detected in GC analysis. Among them, xylose was the most predominant in this analysis because xylose is one of the main components of hemicellulose, which comprises about 30% of plant biomass. Glucose was slightly increased in $ZmCCR1_RNAi$ corn lines but it was not statistically significant. In other hemicelluloses, no statistical differences were found in the amount of hemicellulose after lignin down-regulation either (P>0.05, n=3). The hemicellulose composition analysis was repeated with the same samples (data not shown) and the results were consistent.



Figure 26. Hemicelllulose compositional analysis of wild-type corn and *ZmCCR_RNAi* transgenic corn lines (T1) via Gas Chromatography (GC). The main peaks from the chromatograms were integrated, identified based on retention times and fragment ion signatures, and expressed as mol percentage (P>0.05, n=3).

7. Enzymatic conversion of AFEX-pretreated corn stover to fermentable sugars

Figures 27 and 28 show the two reducing sugars (glucan and xylan) percentage conversion after AFEX pretreatment. The only statistical difference between the sugar conversions for any of the lines was for the 24 hr glucan conversion (Fig. 27). The control line had a significantly lower glucan percent conversion compared to the 1c-4 and 1c-6 lines at the 1.0 g NH₃ loading level (P<0.05, n=8). However, there was no difference compared to the 1c-5 line. There was no statistical difference between any of the lines for 72 hr glucan percent conversion or for 24 hr and 72 hr xylan percent conversions (Fig. 27). There was also no statistical difference between any of the percent conversions for the untreated samples.

In Figure 28, the wild-type corn control line, as well as 1c-4 and 1c-6 all gave higher percent conversions at the higher ammonia loading level after 72 hr of hydrolysis. The 1c-5 line percent conversions were fairly constant across both ammonia loadings.


Figure 27. Percent glucan conversion for AFEX-pretreated (90°C, 5min) corn stover at different concentrations of ammonia loading (UT; no NH₃ treatment, 1.0; 1.0g NH₃/g dry biomass 1.5; 1.5g NH₃/g dry biomass). The AFEX-pretreated corn stover was hydrolyzed by Accelerase (cellulase; added 5.5 mg/g glucan) and Multifect Xylanase (added 0.5 mg/g glucan). % glucan was sampled twice at 24 hr and 72 hr. Mean \pm standard deviation (P<0.05, n=8).



Figure 28. Percent xylan conversion for AFEX-pretreated (90°C, 5min) corn stover at different concentrations of ammonia loading (UT; no NH₃ treatment, 1.0; 1.0g NH₃/g dry biomass 1.5; 1.5g NH₃/g dry biomass). The AFEX-pretreated corn stover was hydrolyzed by Accelerase (cellulase; added 5.5 mg/g glucan) and Multifect Xylanase (added 0.5 mg/g glucan). % xylan was sampled twice at 24 hr and 72 hr. Mean \pm standard deviation (P>0.05, n=8).

V. DISCUSSION

The development of alternative renewable energy has been emphasized because of the shortage of fossil fuels. Seeking natural and renewable energy resources is also necessary for reducing air pollution, for which burning of fossil fuel products is a major contributor. To resolve the limitations of fossil fuels, several efforts have been made using renewable resources in recent decades. One possibility is to utilize lignocellulosic biomass, which is annually produced in the U.S. at levels of 1 billion tons (DOE, 2005). It is expected that the U.S. dependency on foreign oil can be significantly reduced if alternative energy generated from renewable lignocellulosic biomass is utilized for the production of biofuels (DOE, 2010). However, the utilization of lignocellulosic biomass is impeded due to the high costs associated with the production of microbial cellulases and pretreatment processes.

Pretreatment processes are required to break down and open up complex plant cell wall structures enabling hydrolytic enzymes to access biomass and convert it into fermentable sugars (Mei et al., 2009b). Lignin is the second most abundant polymer in plant biomass following cellulose. Lignin is covalently linked to hemicellulose as well as other polysaccharides, enhancing cell wall integrity and limiting water permeability (Hatakeyama and Hatakeyama, 2010).

Lignin contents vary in different plant species (3-13% in crop residues, 18-30% in wood residues). The accessibility of microbial cellulases to plant cell wall polysaccharides is determined by the degree to which they are associated with phenolic polymers (Kuhad et al., 1997). There is a negative correlation between lignin content and the conversion rate to fermentable sugars from lignocellulosic biomass. This correlation

is likely due to the properties of lignin such as hydrophobicity (Kudanga et al., 2010), chemical heterogeneity, and absence of regular hydrolysable intermonomeric linkages (Yin et al., 2000).

Recent studies have demonstrated that the lignin formation and its degradability can be influenced by various monolignol substitutes. By substituting with methyl caffeate, caffeoylquinic acid, or feruloylquinic acid to monolignol, lignin degradability can be significantly improved in plant cell walls (Grabber et al., 2010).

In the research presented here, the research focused on ZmCCR1 that is one of the major genes associated with in lignin biosynthesis pathway (Fig. 14) to accelerate deconstruction of lignocellulosic biomass into fermentable sugars that can be converted to biofuels.

For down-regulation of the *ZmCCR1* gene, the *ZmCCR1_RNAi* gene construct (Fig. 16) was designed under the control of rubisco small subunit promoter from *Asteraceous chrysanthemum*, which is 8 times stronger than the commonly used CaMV-35S promoter. The expression cassette preserved 1 kb of the native RbcS1 terminator sequence, which may contribute to the improved performance relative to similar RbcS promoters derived from other plants (Outchkourov et al., 2003).

Figure 17 and 18 show the range of transcriptional reductions of the *ZmCCR1* gene throughout the T0 and T2 generations. No significant differences were found in height and stem diameter between wild-type corn and *ZmCCR1_RNAi* transgenic lines. Under greenhouse conditions, transgenic corn lines showed normal growth patterns when compared to wild-type corn plants (Table 5). This implies that *ZmCCR1_RNAi* transgenic

plants would provide enough biomass with the benefit of less lignin for pretreatment processing.

The co-bombarded pDM302 plasmid containing the bar gene that was used for plant selection was also confirmed using PCR analysis (Fig. 19), meaning that transgenic plants have the benefit of being herbicide resistant.

The T0 *ZmCCR1_RNAi* lines did not show any brown coloration, but six out of 15 transgenic corn lines showed obvious brown coloration in leaf mid-rib, stems and husks (Fig. 20). It is well known that key intermediates (e.g., phenylalmine) involved in lignin biosynthesis pathways are closely associated with anthocyanin synthesis (Whetten and Sederoff, 1995).

Brown-midrib is one of naturally occurring phenomena that result when a lignin biosynthesis-related gene is defective or less functional (Ambavaram et al., 2010; Voelker et al., 2010; Voelker et al., 2011). Unlike other naturally occurring *bm* mutants, the brown coloration seen in the transgenic lines also occurred in the stems and corn husks but without any detrimental effects on plant biomass (Table 5).

Also, brightfield microscope images indicated that the sclerenchyma cell wall thickness of *CCR* down-regulated leaves was much less than those of the wild-type control non-transgenic plants (Fig. 21). There was no change in the cell wall thickness of the main xylem vessels, phloem or sheath cells, which could explain why the *ZmCCR1_RNAi* transgenics grew normally in terms of plant height and stem diameter (i.e. no biomass change). Along with brightfield images, confocal fluorescence images (Fig. 22) also confirmed a lignin reduction in sclerenchyma fiber cell walls in comparison to wild-type corn (Hi-II).

In order to visualize only lignified cell wall areas, two stains were used with microtome-sectioned corn leaf midribs. Briefly, phloroglucinol stains lignified cells red (purple chromophore) upon reaction with the hydroxycinnamaldehyde groups present in the lignin, while toluidine blue O (TBO) stains lignified cell walls green/blue and non-lignified cell walls purple.

In this experiment, the Klason lignin method was used to measure lignin content. Usually, lignin concentrations are determined by two lignin methods – the Klason method and the acid detergent lignin method (ADL). These two methods are positively correlated, and the Klason lignin method always presents a greater value than the acid detergent lignin concentration (Jung et al., 1997). Figure 24 shows the amount of acid-insoluble Klason lignin (g/kg con stover) in wild-type corn and *ZmCCR1_RNAi* transgenic corn lines (T1). The range of lignin reduction varies in transgenic corn. As expected, the *ZmCCR1_RNAi-1c* (T1) lines representing high transcription reduction showed an approximately 8 % reduction in lignin content. Lignin reduction in this research was consistent with microscopy of *ZmCCR1* versus wild-type control corn leaves.

In the research presented here, the composition of crystalline cellulose and hemicelluloses were determined in order to examine any energy compensation shifts in other cell wall components. Increase in crystalline cellulose of *ZmCCR1* down-regulated corn plants were expected because similar compensation effects have been observed in cellulose-deficient Arabidopsis mutants (CESA3). The Arabidopsis mutants exhibited ectopic lignification via the jasmonate and ethylene signaling pathways (Cano-Delgado et al., 2003).

Crystalline cellulose composition was increased in two transgenic lines, *ZmCCR1*-1b-6 and 1c-6. Although *ZmCCR1_RNAi-1c-5* and *1c-6* transgenic lines showed higher hemicellulose, no statistically significant changes were found in hemicellulose composition. This result may be because the slight lignin reduction was not sufficient to trigger additional hemicellulose synthesis.

The level of increase in glucan and xylan after AFEX-pretreatment (Fig. 27 and 28) show that the approximately 8 % lignin reduction may have increased the percent conversion of these two fermentable sugars, assuming an increase in percent conversion appears because less physical barriers (i.e., lignin) are present in plant secondary cell walls.

Previously, it was demonstrated that transgenic tobacco lines with significantly reduced CCR expression also showed an increase of other cell wall constituents such as glucose, xylose and wall-bound phenolic compounds (e.g., sinapic and ferulic acids) (Chabannes et al., 2001b). Figure 27 shows percent glucan conversion after enzymatic hydrolysis and AFEX pretreatment. As described in the results, the only statistical difference between the sugar conversions for any of the lines occurred for the 24 hr glucan conversion. The increased glucan conversion in 1c-4 and 1c-6 corn lines at 24 hr with 1.0 g/NH₃ loading could be as the consequence could be due to energy shift from lignin synthesis to carbohydrate synthesis in transgenic plant, and possibly because of less needs for AFEX pretreatment for production of glucan and xylan.

As a next step, lignin compositional analysis is necessary to examine if guaiacyl to syringyl (G: S) ratio has been changed. Also, the proportion of para-hydroxyphenyl units *in ZmCCR1_RNAi* lines would be examined as well. In addition, since all

experiments described in this chapter have been performed in a greenhouse facility, as further analyses, the lignin down-regulated homozygous *ZmCCR1_RNAi* lines will require field testing to examine whether the mutants are capable of normal growth under natural environment conditions. Therefore the field test is crucial to confirm the feasibility of the genetically-altered down-regulated lignin corn for biofuel production.

VI. Conclusion

We have genetically reduced the lignin content in corn plants via an RNA interference technique. The reduced lignin mutants grow similarly to wild-type corn plants except for displaying a brown- coloration in the leaf mid-rib, husk and stem. In addition, the mutants show no severe pathogen infection under greenhouse condition. Defective lignin deposition was only found in sclerenchyma fibers in leaves, not in the major vascular bundle system (xylem, phloem etc.). A minor reduction in lignin content (<10%) might enable the mutants to be more resistant to abiotic and biotic stresses while a reduction of more than 20% lignin content generally causes a loss of biomass (Voelker et al., 2010; Voelker et al., 2011). Results show that corn with reduced lignin content induces an increase in crystalline cellulose but not in hemicellulose. Lastly, the reduced lignin corn mutant showed an increase in the glucan conversion rate after AFEX-pretreatment.

Milder pre-treatment conditions such as reduced temperature, pressure and ammonia loading would be possible on the lignin down-regulated corn plants, compared to conventional pre-treatment conditions required for processing corn plants with normal lignin levels. In addition to the decreased level of lignin, the increased crystalline sucrose level is also advantageous for biofuel production. Taking advantages of these properties, the high costs required for expensive pre-treatment processes could be lowered making biofuel production more commercially competitive. Genetic engineering of corn for lignin down-regulation could be a step forward towards reducing the high costs of pretreatment processes.

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