Transgenic ferritin overproduction largely enhances thermochemical
pretreatments in *Arabidopsis*

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ABSTRACT

Reducing the severity of thermochemical pretreatment by incorporating iron ions as co-catalysts has been shown to enhance the sugar yield from dilute acid pretreatments and enzymatic saccharification. However, current approach of soaking iron containing acid solutions onto milled biomass prior to pretreatment is time-consuming and subject to diffusion limitations. Here, we overexpressed soybean ferritin protein intracellularly in *Arabidopsis* plants (referred to as FerIN) under the control of the 35S promoter for the purpose of accumulating iron ions in *Arabidopsis* plants. The transgenic *Arabidopsis* plants accumulated iron during growth under both normal and iron-augmented watering conditions. Prussian blue staining showed punctuate staining of iron predominantly on the interior surfaces of cell lumen in FerIN plants. The harvested transgenic biomass showed enhanced pretreatability, in that it released 13-19% more glucose and xylose than empty vector control plants. The data indicated a positive correlation between iron concentration and sugar release during pretreatment of transgenic biomass.

Keywords: iron co-catalyst; ferritin; iron accumulation; transgenic *Arabidopsis*; biomass saccharification; dilute acid pretreatment; Prussian blue staining; transmission electron microscopy

1. Introduction

Hydrolyzing biomass by thermochemical pretreatment with dilute acid has been studied for many years, and yet deconstruction of plant cell walls remains one of the key obstacles for the economical production of lignocellulosic biofuels today. The incorporation of iron ions as co-catalysts in dilute acid pretreatments to enhance the yields in pretreatment and enzymatic digestibility is a promising strategy for increasing the effectiveness and reducing the cost of pretreatment [1-3]. The iron is thought to target multiple chemical bonds in plant cell wall polymer networks, including the C-O-C and C-H bonds in cellulose, during dilute acid pretreatment [4]. It also increases the solubilization and enzymatic digestion of both cellulose and xylan to monomers. In addition, electron microscopy analysis revealed the delamination and fibrillation of the cell wall by iron ions [4]. Furthermore, a recent study showed that iron ion catalyst enhances the selectivity of acid hydrolysis of amorphous over crystalline region of celluloses under augmented hydrolysis conditions [2]. However, the current approach of adding iron ions into milled biomass by soaking or spraying prior to pretreatment is less than ideal due to equipment costs, water usage, and diffusion limitations. To overcome these process limitations, we are pursuing genetic approaches for engineering metal catalyst accumulation in bioenergy crops.

Ferritin is a highly conserved protein known for iron-storage and plays an essential role for iron homeostasis in animals, plants, and microorganisms. As a globular protein complex, ferritin consists of 24 subunits capable of binding up to 4,300 atoms of iron per ferritin molecule [5]. Heterologous ferritin has been expressed in *Arabidopsis* [6, 7], grapevine [8], lettuce [9], maize [10], rice [11-13], and tobacco [14-17]. The expression of soybean and pea ferritin genes, under the control of an endosperm-specific glutelin promoter, has been shown to enhance iron

and zinc accumulation (as high as 3 to 4 times higher than the level of the control) in the seeds of transgenic rice [11-13]. Furthermore, the expression of a soybean ferritin gene under the control of the CaMV 35S promoter was reported to increase the expression of at least one of the endogenous ferritin genes in transgenic tobacco plants, and the iron concentrations in transgenic tobacco leaves were about 1.5-fold higher than that in non-transformed plants [14]. In addition, the transgenic tobacco plants had enhanced ferric chelate reductase activity, iron transport in the root, and photosynthesis, which cumulatively resulted in significantly greater plant height and fresh weight than the non-transformant [14]. Interestingly, ferritin overexpression in plants has been found to protect plants from free iron toxicity, photoinhibition, and to reduce oxidative stress [7, 8, 15, 16, 18]. Consistent with the above physiological effects, ferritin overexpression driven by the CaMV 35S promoter was also found to enhance the growth of transgenic tobacco and lettuce plants [9, 14].

However, these past efforts incorporating ferritin into transgenic plants focused on either increasing iron content in seeds for improved nutritional value, or investigating its physiological roles in plants. No attempts have been made to use the transgenic ferritin plant approach to make the plant biomass more convertible to simple sugars for biofuel development. The objective of this study was to use *Arabidopsis* as a model plant to test the approach of improving biomass conversion efficiency by increasing iron content in plant stems by the use of intracellularly expressed ferritin. Our hypothesis is that at the senescent stage, the intracellularly accumulated iron will be released from the broken cells of stems at late growth into the internal (luminal) surface of cell wall, which would facilitate the interaction between iron co-catalyst and plant biomass during the pretreatment and lead to increased biomass pretreatability. This study reports the development of transgenic *Arabidopsis* plants that overexpressed heterologous soybean

ferritin protein, which allowed the plants to accumulate metals during growth. The resultant biomass released more glucose and xylose after dilute acid pretreatment than the controls, demonstrating that this concept works for *Arabidopsis*. The improvement in pretreatment was much larger than the enhancements found for plant biomass subjected to the exogenous addition of metal co-catalysts by soaking.

2. Materials and methods

2.1. Pretreatments of corn stover with ferritin or iron nanoparticles

The iron nanoparticles tested include: (1) ferrihydrite, an iron (III) oxide with an empirical formula of Fe_2O_3 (catalog no. 310050, Sigma; powder size $<5\ \mu\text{m}$), (2) magnetite, an iron (II, III) oxide with an empirical formula of Fe_3O_4 (catalog no. 310069-25G, Sigma; powder size $<5\ \mu\text{m}$), and (3) ferritin from equine spleen (catalog no. F4503, Lot# 079K7001, Sigma; 56 mg protein/mL in 150 mM NaCl buffer) and its apoferritin form as a control (catalog no. A3641, Lot# 109K7013V; 48 mg protein/mL). In order to eliminate a potential problem with the high NaCl concentrations used as protein stabilizer, the commercial ferritin and its apoferritin control in NaCl solutions were dialyzed for 24 h against three changes of 30 mM NaCl buffer.

Biomass-acid-iron nanoparticle mixtures were prepared according to [Supplementary Table S1](#) and set up in inside an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) with an inert atmosphere consisting of 5% hydrogen and 95% nitrogen. The reaction vessels consisted of 65-mL heavy walled glass pressure tubes with screw-on Teflon caps and O-ring seals (Ace glass # 8648-30 tube with #5845-47 plug; Ace Glass Inc, Vineland) to reduce the leaching of metals into solution during pretreatment. Stock solution of 0.5% H_2SO_4 solution was

freshly sparged with nitrogen gas for 5 min before use. The acid was added to all the tubes to give 0.5 wt% acid and 10 wt% solids to liquid ratio. The 65-mL glass pressure tubes were sparged with N₂, sealed, and allowed to settle overnight for impregnation of the biomass. The glass reactor tubes were placed into a pre-warmed two-gallon Parr reactor (Parr Instrument Co., Moline, IL, USA). Steam was admitted into the Parr reactor to heat the tubes to 150 °C for 20 min. A thermocouple was inserted into the middle of a tube to monitor temperature during pretreatment. The pretreatment time was measured from when the temperature reached reaction temperature until cooling water was applied. The time to reach pretreatment temperature was approximately 10 min and the tubes were cooled below 100 °C in less than one minute. Condensate was drained from the Parr reactor during pretreatment via a steam trap. Cooling water was admitted to the Parr reactor to cool the tubes at the end of reaction time.

After pretreatment, the tubes were transferred to the anaerobic chamber to avoid any atmospheric contact that may affect the oxidation state of the iron inside the pretreated slurries. The glass pressure vessels were opened and the pretreated slurries were pipetted into 1-mL syringes fitted with 0.45 µm nylon filters and (10 µl) used for ferrous (Fe²⁺) and ferric (Fe³⁺) analysis as described below. The remainder of the contents were used for measurement of released sugars using HPLC, as described previously [4]. Five replicates were run for each of the dilute acid pretreatments of corn stover supplemented with model Fe nanoparticles.

2.2. Measurement of Fe²⁺ and Fe³⁺ concentrations in pretreatment liquors

The above filtered pretreatment hydrolysate liquors were used for the determination of Fe²⁺ and total iron concentrations using QuantiChrom iron assay kit (Bioassay System, Hayward, CA)

according to the manufacturer's instructions. The Fe^{3+} concentrations are calculated by subtracting the measured Fe^{2+} concentrations from the total ($[\text{Fe}^{3+}] = [\text{Fe}] - [\text{Fe}^{2+}]$). The assays were conducted inside the anaerobic chamber under an inert atmosphere.

2.3. Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as the parent line for *Agrobacterium*-mediated transformation with soybean ferritin gene. Col-0 seeds were germinated on ½ MS agar medium containing 1% sucrose. After 2-d incubation at 4 °C, the medium plates were placed in a growth chamber with 16 h light ($140 \mu\text{mol E m}^{-2} \text{s}^{-1}$) and 8 h dark cycles at 24 °C for 5 d, then the seedlings were transferred to pots containing Metro-Mix 360 soil (SunGro Horticulture, Canada) and placed under light shelves of ArabiSun Lighting System (Lehle seeds, Texas, USA) with 16 h light ($170 \mu\text{mol E m}^{-2} \text{s}^{-1}$) and 8 h dark cycles at 24 °C.

2.4. Gene synthesis and construct

The diagram for the mature form of soybean ferritin (SferH-1; GenBank accession no. M64337) is illustrated in **Figure 1A**. As transit peptide (TP) is only needed for delivering the ferritin precursor to plastids [17], the TP-deleted mature form of SferH-1 was used in this study for expression in *Arabidopsis*. The nucleotide sequence encoding mature protein of SferH-1 was codon optimized for *Arabidopsis*, synthesized with a BglII restriction site at the 5'-end and a BstEII site at the 3'-end by GenScript (Piscataway, NJ), and cloned into *E. coli* vector pUC57.

For the preparation of expression construct, the synthesized gene cloned in PUC57 was cut with BglII-BstEII, and linked to BglII-BstEII cut binary vector pCAMBIA1305.1 (GenBank

accession no. AF354045) for intracellular ferritin expression. The vector carries the hygromycin B resistance gene (hpt II), driven by CaMV35Sx2, the CaMV35S promoter with duplicated enhancer region, as the plant selection marker. Ferritin gene is driven by the CaMV35S promoter ([Figure 1B](#)).

2.5. *Agrobacterium*-mediated transformation and homozygous transgenic plant selection

The above construct and the empty vector pCAMBIA 1305.1 were separately introduced into competent cells of *A. tumefaciens* strain C58 using a freeze-thaw method [19], using rifampicin (10 µg/mL) and kanamycin (50 µg/mL) as selection markers for positive colonies. The positive colonies were confirmed by PCR analysis for the presence of heterologous ferritin gene using the ferritin transgene specific primers (as listed in [Supplementary Table S2](#)). Floral dip transformation method [20] was used to transform *Arabidopsis* Col-0 with *A. tumefaciens* strain C58 harboring the above constructs. The transformed plants were grown to maturation to collect T1 seeds, which were used for further selection of homozygous transformants as described in [Supplementary Materials and Methods](#).

2.6. Genomic DNA and total RNA isolation, reverse transcription and real time RT-PCR

Extraction of genomic DNA and total RNA from plant shoots, the reverse transcription and real time RT-PCR analysis are described in [Supplementary Materials and Methods](#).

2.7. Plant growth with iron fertilization and determination of iron accumulation

Transgenic plants were grown with 2 mM Fe-ethylenediaminedi(*O*-hydroxyphenylacetic) acid (Fe-EDDHA) as iron fertilizer to test their capacity for iron accumulation, as previously reported

[7]. The pots with transgenic and empty vector control seedlings were randomly placed in the greenhouse and watered with either distilled H₂O (referred to as H₂O-only thereafter) or 2 mM Fe-EDDHA (referred to as iron-fertilized thereafter) twice a week, with 16 h light (200 to 300 $\mu\text{mol E m}^{-2} \text{ s}^{-1}$), 8 h dark cycles at 24 °C. Plants were harvested at the senescent stage, and rinsed three times with ddH₂O so that no surface residual iron would affect the biomass iron content measurement. Dry shoot samples were then ground to pass through a 20-mesh (1 mm) screen (Wiley knife mill; Thomas-Wiley, Philadelphia), and used to measure the iron concentration by nitric acid digestion and inductively coupled plasma - optical emission spectroscopy (ICP-OES) [6, 21, 22]. Five plants (i.e. replicates) were planted for each transgenic line.

2.8. Total protein extraction, anti-soybean ferritin antibody preparation, and Western blot

Total protein extraction from plant shoots, the preparation of anti-soybean ferritin antibody, and the Western blot analysis are described in [Supplementary Materials and Methods](#).

2.9. Prussian blue staining for subcellular iron accumulation

Samples of senesced stem segments (approximately 0.5-cm long and cut from stem base at one cm above the soil surface) were immersed in freshly prepared Prussian blue staining buffer (2% potassium ferrocyanide, 2% hydrochloric acid) for ~10 min at room temperature in a container under vacuum for staining combined with simultaneous degassing. The stems were then gently washed and hand-sectioned to a thickness of approximately 500- μm . The hand-cut sections were either directly viewed with a Nikon SMZ1500 stereomicroscope (Nikon Instruments, Inc., Melville, NY) at $\times 11.25$ magnification, or viewed with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan), for which the sections were positioned on glass microscope slides, and

one drop of immersion oil was placed on the stem sections. Bright-field images were captured under x 100 objective using a SPOT RTKE CCD camera (Diagnostic Instruments, Sterling Heights, MI) equipped on the Nikon E800 microscope platform.

2.10. Dilute acid pretreatment and enzymatic hydrolysis of transgenic plant biomass

Transgenic plants were harvested, air-dried, ground to pass through a 20-mesh (1 mm) screen using a Wiley Mill, and then tested for total sugar release through dilute acid pretreatment. Dilute acid pretreatment was carried out in 2-mL HPLC vials containing 100 mg ground plant biomass and one mL 0.5% H₂SO₄. The 0.5% H₂SO₄ stock solution was freshly sparged with nitrogen gas for 5 min before adding into the vials with ground biomass. The individual vials were sealed with septum/aluminum caps, and allowed to settle overnight for solvent equilibration and impregnation of biomass, then put into a pre-warmed two-gallon Parr reactor (Parr Instrument Co., Moline, IL, USA). The Parr reactor was heated with steam to 150 °C for 20 min before being cooled by flushing with cooling water. The vials were uncapped and the pretreated slurries were pipetted into 1-mL syringes fitted with 0.45 µm nylon filters. The filtrates were used for measurements of the released sugars using HPLC, as described previously [4].

For saccharification analysis the pretreatment residues were washed four times with deionized distilled H₂O (ddH₂O) with centrifugation between the washes. Enzymatic digestion of washed residues from each uncapped 2 mL HPLC vial was performed in 10 mL of 50 mM citrate buffer, pH 4.8, containing 2 mg GC220 cellulase protein (Genencor, Rochester, New York). No additional beta-glucosidase was added. The enzymatic reaction mixtures were transferred to 125-mL Erlenmeyer shake flasks with screw caps and incubated at 50 °C and 130 rpm for 5 d

according to NREL LAP 009 [23]. The glucose and cellobiose released was measured by HPLC, which is a common method for the determination of glucose and cellobiose after saccharification or pretreatment of biomass [24-27].

3. Results and discussion

3.1. Availability of ferritin iron as bio-catalyst in biomass pretreatment

Ferritin iron core consists of a polyphasic structure, wherein the majority of iron exists as ferrihydrite, mixed with the iron oxides magnetite and hematite [28, 29]. This form of ferric oxide nanoparticles raises the question about the chemo-availability of ferritin-contained iron ions for biomass pretreatment. To address this issue, experiments were performed to evaluate whether or not the impregnation by soaking of corn stover with horse spleen ferritin or two other types of commercially available preparations of iron nanoparticles, namely ferrihydrite and magnetite, can increase biomass pretreatability.

The results indicate that the rank order for iron nanoparticles in releasing soluble Fe ions is: ferritin > magnetite > ferrihydrite (**Table 1**). This indicates that ferritin-containing iron nanoparticles are better at releasing iron or interconverting Fe^{3+} and Fe^{2+} ions in pretreatment conditions than inorganic iron nanoparticles. This finding is consistent with previous studies indicating that ferritin-derived ferrihydrite is effectively maintained in an unaggregated state [30], likely facilitating release of the contained iron. Furthermore, the observation that magnetite released more Fe^{2+} than ferrihydrite can be attributed to the fact that magnetite is a mixture of Fe^{2+} and Fe^{3+} , whereas iron in ferrihydrite is solely in Fe^{3+} form.

For sugars release, the results indicate that the DA/ferrihydrite and DA/magnetite pretreatments of corn stover moderately enhance the release of xylose monomer and oligomers (Figure 2A). However, the release of glucose monomer and oligomers by DA/ferrihydrite and DA/magnetite pretreatments of corn stover was not significantly different from that by the DA only pretreatment control (i.e., similar to the results illustrated in the first stacked bar in Figure 2B). In contrast, the DA/ferritin pretreatment enhanced the release of monomeric and oligomeric glucose and xylose by 11% and 12%, respectively, compared with the DA/apoferritin pretreatment control (Figure 2 B-C). These results are consistent with the observation that Fe^{3+} from ferritin (referred to here as ferritin- Fe^{3+}) was more prone to release, and thus more chemically available than Fe^{3+} from ferrihydrite and magnetite (referred to here as ferrihydrite- Fe^{3+} and magnetite- Fe^{3+} , respectively). Note that the DA/apoferritin pretreatment control had no impact on the release of monomeric and oligomeric glucose and xylose, compared with the DA pretreatment – the “absolute” control (Figure 2 B-C). Overall, these results suggest a beneficial role for using ferritin as a biocatalyst for biomass pretreatment.

3.2. Effects of ferritin overexpression on plant growth and iron accumulation

Ten independently transformed T1 *Arabidopsis* plants expressing intracellularly targeted soybean ferritin protein (FerIN) were generated. Based on segregation analysis, three T3 transformants (FerIN-1a, -2a and -4b; referred to as FerIN(1), FerIN(2) and FerIN(3), respectively) were confirmed to be homozygous. PCR analysis of these T3 plants, using primers listed in Supplementary Table S2, confirmed the integration of the ferritin transgene into the genome as well as the presence of ferritin transcript in these *Arabidopsis* lines.

FerIN transgenic plants were examined under both H₂O-only spraying (i.e., non-Fe fertilizing) and Fe-fertilized conditions, with the representative transgenic line being shown in [Figure 3A and B](#), respectively. Under H₂O-only spraying condition, transgenic FerIN lines grew normally, with plant height comparable to that of the EV control plants. However, when the transgenic plants were grown under Fe fertilized condition, their average height was 42.8 cm, taller than the EV control (40.2 cm), but not statistically significant. The shoot biomass of iron-fertilized FerIN was 128 mg DW/plant, 11% higher than that of EV control ($p < 0.05$; Table 2), suggesting that the increase in biomass yield in FerIN can be mainly attributed to the possible increase in shoot diameter and/or possible more branches per transgenic plant, which should be further investigated in future studies. Our observation of increase in transgenic biomass is consistent with the reported increase in biomass yield of ferritin transgenic plants of *Arabidopsis* [6], lettuce [9], and tobacco [14].

The expression of the heterologous ferritin in FerIN transgenic *Arabidopsis* lines was tested first by Real-time RT (reverse transcription) PCR ([Figure 3C](#)), followed by Western blot analysis, using the total soluble proteins extracted from mid-pod stage shoot and the chicken IgY polyclonal antibody against the synthesized soybean ferritin peptide. The antibody tests showed that the prepared anti-soybean ferritin antibody specifically recognized the heterologous ferritin and had no cross-reactivity with *Arabidopsis* ferritin. The Western blot result confirms expression of soybean ferritin at both the transcriptional and protein levels in FerIN(1), FerIN(2) and FerIN(3) transgenic lines with the expected molecular mass of 26-kDa ([Figure 3D](#)). The blot was quantified by densitometry analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA), and are shown as bar graphs in Figure 3D. Note that there is a positive correlation between the transcriptional and protein levels of heterologous ferritin in the three

FerIN transgenic lines, among which the line FerIN(2) had the highest expression at both transcriptional and protein levels for heterologous ferritin (Figure 3 C-D).

ICP-OES showed that even under normal growth condition of H₂O-only spraying, iron content in the shoot tissues of FerIN plants was 1.4 times that of the EV control (100 versus 69 ppm), which suggests that the ferritin transgenic plants can be planted without iron supplementation and still hyper-accumulate iron from the soil (Figure 4, bars 1 to 4). Not surprisingly, FerIN plants under iron-fertilized condition accumulated even more iron than FerIN plants grown under the same iron-fertilized condition, up to 1.7 times that of the EV control (520 vs. 308 ppm; Figure 4, bars 5 to 8). The iron contents in the three FerIN transgenic lines were positively correlated with the protein levels of the heterologous ferritin in these lines, with FerIN(2) having the highest iron accumulation level (Figures 3D and 4).

3.3. Prussian blue staining for iron localization

To observe the iron localization across the whole stem section, the hand-cut section (approximately 500- μ m) of dry, senescent stem from iron-fertilized control and transgenic ferritin plants were stained with Prussian blue solution and rinsed with ddH₂O, followed by examination with a Nikon SMZ1500 stereomicroscope. The obtained images showed that overall, iron accumulated to higher levels across the stem section in FerIN transgenic plants compared to the empty vector control, especially in the primary xylem (xy) and interfascicular (if) fibre tissues (Figure 5 A-B).

To visualize the presence of iron at the cellular scale, the Prussian blue stained hand-cut cross-sections of FerIN transgenic and EV control plant shoots were also viewed with a Nikon Eclipse E800 microscope under x 100 objective lens. Overall, the “zoom-in” views in Figure 5

C-D correspond to the middle layer of the cross section from *Arabidopsis* stems, between the cortex (co) and the interfascicular (if) fibre tissue. Some faint blue regions were observed in the EV control (**Figure 5C**), which is expected since endogenous *Arabidopsis* ferritin proteins are also present in the host plant. In contrast, the FerIN plant showed positive, punctuate staining of iron that was predominantly localized to the interior surface of the cell lumen, or the lumen side of cell walls (**Figure 5D**, black arrows). This observation is consistent with our strategy to express heterologous ferritin in the cytoplasmic region of plant cells, which enabled adherence of the ferritin/iron complexes to the interior surfaces of cell lumena after plant senescence.

Note that the Prussian Blue staining in the transgenic line (**Figure 5D**) may look slightly diffused across lumen, cell walls, and different cell types, which can be attributed to the fact that the dry biomass stem samples were collected at senescent stage, at which most plant cells are broken, causing the leaking of some intracellular Fe into the extracellular space. In addition, the image of Panel D may not be perfectly focused due to the thickness of hand-cut stem section (approximately 500- μ m).

Nevertheless, the overall quality of presented images are at least comparable to published study of ferritin transgenic plant characterizing the localization of iron in rice seeds [11], and to another study examining the iron localization in the root section of *Arabidopsis frd3* mutant [31], both using the Prussian blue staining while the latter also used a relative thick section (70- μ m) of tissues.

We note that deeper studies are needed to further characterize the localization of the transgenic ferritin at the level of the cell walls, organelles, and ultrastructures during plant development. To be best of our knowledge, so far the reported transmission electron microscopy

(TEM) studies on plant and animal ferritins all used extracted and concentrated ferritins and failed to reveal observations at the ultrastructural level using TEM [32-36]. Further study and method-development are needed. Although progress toward this aspect of the work will help provide deeper insights into the ferritin localization at the subcellular level, the development and optimization of these methods is beyond the scope of this paper.

3.4. Ferritin expression in *Arabidopsis* plants improves biomass pretreatability

To evaluate the effectiveness of intracellularly expressed ferritin in enhancing the pretreatability of *Arabidopsis*, the harvested biomass was dried, ground, and subjected to DA pretreatment in 2-mL HPLC vials. The results showed that for glucose release, the biomass from H₂O-only sprayed and iron-fertilized FerIN plants released 18 to 19% more glucose ($p < 0.01$) than that from their respective EV controls (**Figure 6A**). Similarly, the xylose release from DA-pretreated biomass of H₂O-only sprayed and iron-fertilized FerIN plants was found to be 13 to 14% higher than their respective EV controls (**Figure 6B**). Not surprisingly, there is a positive correlation between the iron contents in the three FerIN transgenic lines and their sugar release data during DA pretreatment (**Figures 4 and 6 A-B**).

In contrast, glucose release during the saccharification of residues from H₂O-only sprayed and iron-fertilized FerIN plants was found to be only slightly higher (2 to 5%) than their respective EV controls (**Figure 6C**), indicating there is no inhibition of *in planta* accumulated iron on digestion of biomass residues. On another hand, the results also suggest that iron may be more tightly “embedded” into biomass which renders it hard to thoroughly rinse away. This condition may negatively affect Ctech2 activity. More iron-residue resistant enzymes should be screened and tested with iron enhanced biomass residues in future studies.

Potential use of ferritin overexpression biomass in other pretreatments

Recently published studies have reported that copper (II) 2,2'-bipyridine complexes (Cu(bpy)) can significantly enhance alkaline hydrogen peroxide (AHP) pretreatment of hybrid poplar heartwood and sapwood etc., and improve subsequent enzymatic glucose and xylose release at modest reaction conditions [37, 38]. Although in our study the biomass subjected to ferritin overexpression was designed for dilute acid pretreatment, published work suggests that some pretreatment specificity should be investigated in the future, including alkaline and oxidative pretreatments. Furthermore, steam explosion pretreatment is a technology that combines both physical destruction and chemical hydrolysis in degrading the structure of the lignocellulose. Steam explosion has a lower capital investment and environmental impact compared to other leading pretreatment technologies today [39, 40]. It would be equally intriguing to test the efficiency of ferritin overexpression in the steam explosion pretreatment [40]. However, the nature of steam explosion in combining physical destructive and chemical hydrolytic effects on lignocellulose makes it different from the DA pretreatment used in our study; the latter mainly applies the chemical effects in degrading the lignocellulose, which facilitates the exploration of the mechanisms involved with metal catalysts. In addition, the current steam explosion reactor available at NREL requires at least approximately 500 gram dry biomass for each run, which makes it difficult to produce sufficient quantities of transgenic *Arabidopsis* plants generated in this study (each *Arabidopsis* plant generates approximately 0.1 gram dry wt). To meet this challenge, studies are underway to express heterologous ferritin gene in bioenergy poplar, which will produce sufficient amounts of biomass for pretreatment screening.

383

384 **4. Conclusions**

385 This study demonstrates the successful intracellular expression of soybean ferritin protein in
386 *Arabidopsis* plants. These plants showed hyper-accumulation of iron during plant growth.
387 Prussian blue staining suggests punctuate localization of iron particles predominantly on the
388 interior surfaces of cell walls in FerIN plants. The harvested transgenic biomass had
389 significantly enhanced pretreatability and released more glucose and xylose after dilute acid
390 pretreatment. Whereas past efforts with ferritin transgenic plants focused entirely on plant seed
391 production or physiological benefits, our study demonstrated a value for ferritin expression for
392 biofuels production.

393

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Figure legends

Figure 1. Construct for intracellular expression of soybean ferritin in *Arabidopsis*. (A) Mature soybean ferritin H-1 (Sfer H-1), which was used for gene synthesis. EP: extension peptide; ABCD and E, 5 helices. (B) Expression construct showing ferritin gene replacing the catalase intron-GusPlus gene cassette in the vector of pCAMBIA1305.1 (www.cambia.org). The ferritin gene expression was under the control of cauliflower mosaic virus 35S promoter (CAMV35S prom), a strong and constitutive promoter, and the terminator was nopaline synthase (nos) polyA.

Figure 2. Cellulose and xylan conversion after dilute acid pretreatments supplemented with ferrihydrite, magnetite or ferritin. (A) Xylan conversion after dilute acid (DA), DA/ferrihydrite and DA/magnetite pretreatments. (B, C) Cellulose and xylan conversion after DA/apoferritin versus DA/ferritin pretreatments. Glucose (Glu), glucose oligomer, 5-hydroxymethylfurfural (HMF), xylose (Xyl), xylo-oligomers and furfural in the pretreatment liquors were measured by HPLC. Glucan and xylan content in insoluble solid residues of pretreated corn stover were measured by chemical analysis. The standard error bar at the right side of each bar represents either the stacking glucose plus glucose oligomer, or stacking xylose plus xylo-oligomer; the percentage value next to the standard error bar represents the increase % of released monomer plus oligomer sugars compared to the control pretreatment (DA or DA/apoferritin).

Figure 3. Growth of representative transgenic plants and Western blot analysis. (A) H₂O-only sprayed and (B) iron-fertilized ferritin overexpressed line FerIN(2) plants at mid-pod stage. (C) Transcriptional and (D) Western blot analysis of ferritin expression levels in stems of plants grown under H₂O-only spraying condition and collected at mid-pod stage. The illustrated transgenic lines: FerIN(1), (2) and (3) represent the transgenic line FerIN-1a, -2a, and -4b, respectively.

Figure 4. Iron contents in shoots from intracellular ferritin-overexpressing transgenic *Arabidopsis* plants (FerIN) harvested at the senescent stage. Data are presented as the mean ($\pm$

SEM) of 5 replicate plants for each of the transgenic lines, i.e., FerIN(1), FerIN(2) and FerIN(3). * and ** indicate statistical significance of $p < 0.05$ and $p < 0.01$, respectively. EV, empty vector control plant.

Figure 5. Iron detection in cross sections of dry, senescent stems from empty vector control and transgenic ferritin *Arabidopsis* plants. The plants were grown under the iron-fertilized conditions. The hand-cut cross sections (~ 500- μ m) were stained with Prussian blue solution and observed under stereomicroscope (upper panels) and optical microscope (lower panels), respectively. (A) and (C), empty vector (EV) control cross sections. (B) and (D), transgenic line FerIN(2) cross sections. Bright field light microscopy showing Prussian blue staining of EV control (C) and FerIN transgenic plant shoot tissues (D) reveals Fe clusters localized at the interior surface of cell lumen, i.e., or the lumen side of cell walls (black arrows). Note that the inner layer part, i.e., the pith (pi) of cross section illustrated in upper panels is a hollow space enclosed by interfascicular (if) fibre tissue in the dry, senescent stems of *Arabidopsis* plants. cl, cell lumen; co, cortex; cw, cell wall; ep, epidermis; if, interfascicular fibre tissue; ph, primary phloem; pi, pith; xy, primary xylem.

Figure 6. Pretreatability and digestibility of shoot biomass from intracellular ferritin-overexpressing transgenic *Arabidopsis* plants harvested at the senescent stage. (A) Total hexose sugar release from dilute acid (DA) pretreated shoot biomass. (B) Xylose release after DA pretreatment. (C) Total hexose sugar release by saccharification of pretreated biomass residues with GC220 cellulase; the data were converted to the amount of sugar released per 100 g original biomass subjected to DA pretreatment. The percentage values on the top bars of FerIN(1), FerIN(2) and FerIN(3) transgenic lines represent the increase % of released sugar compared to the corresponding empty vector (EV) control plants. Data are presented as the mean ($\pm$ SEM) of 5 replicates for each of the pretreatments. * and ** indicate statistical significance of $p < 0.05$ and $p < 0.01$, respectively. Note that the total hexose sugars presented in (A) and (C) include glucose and cellobiose, but not the soluble oligosaccharides; the latter are the minor forms of sugars released by DA or saccharification.

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Tables

Table 1. Release of total iron and Fe^{2+} in the liquor after dilute acid pretreatment of corn stover supplemented with ferrihydrite, magnetite, or ferritin, respectively. Note that the initial concentration of nanoparticle iron added to the pretreatment was 5 mM, and the conversion % of nanoparticle iron to soluble iron is calculated by dividing 5 mM by the total soluble iron in the liquor after pretreatment.

Pretreatment	[Fe] in liquor after pretreatment			
	Fe^{2+}	Total soluble Fe	Fe^{2+} / total soluble Fe	Conversion % of nanoparticle Fe to soluble Fe
	mM	mM		
0.5% H_2SO_4	ND	ND		
DA+ 5 mM ferrihydrite-Fe	1.03 ± 0.03	1.13 ± 0.02	92%	23%
DA+ 5 mM magnetite-Fe	2.58 ± 0.08	2.59 ± 0.07	100%	52%
DA+ 5 mM ferritin-Fe	3.78 ± 0.03	4.03 ± 0.04	94%	81%

Table 2. Plant height and shoot dry weight of iron-fertilized empty vector control and transgenic FerIN lines at the senescent stage. Values are presented as the mean ($\pm$ SEM) of 9 plants, by which three replicates for each transgenic line of FerIN(1), FerIN(2) and FerIN(3). The percentage values inside brackets are the increase in transgenic lines compared with the EV control; * indicates statistical significance of $p < 0.05$. DW, dry weight; EV, empty vector.

Plants	Height cm	Shoot biomass mg DW/plant
EV	40.2 $\pm$ 1.4	115 $\pm$ 11
FerIN	42.8 $\pm$ 1.5 ($\uparrow$ 6%)	128 $\pm$ 10 ($\uparrow$ 11%*)

A **B**