Original Paper

Co-expression of microbial cellulases in transgenic wheat as a potential source for cellulosic ethanol production

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Abstract   Crop residues are produced in abundance in agriculture and forestry and are a potential source of lignocellulosic biomass for ethanol production. Recent research has included the heterologous expression in transgenic plants of genes encoding cellulases to produce fermentable sugars from plant biomass. In this study, transgenic wheat lines were generated co-expressing the genes for an endoglucanase 1 (E1) of *Acidothermus cellulolyticus* and cellobiohydrolase 1 (CBH1) of *Trichoderma reesei*. Both genes were under the control of a wheat Rubisco small subunit promoter (RbcS). Transgenic wheat leaves accumulated apoplast targeted E1 and CBH1 proteins at levels up to 1 and 0.5% respectively of total soluble protein as determined by immunoblotting. Transgenic plants co-expressing E1 and CBH1 were analysed by a 4-methylumbelliferyl-β-D-cellobioside (MUC) assay and enzymatic activity was detected up to 92 nmol 4-MU/mg tsp/min.

Key words: Cellobiohydrolase 1, endoglucanse 1, 4-methylumbelliferone, 4-methylumbelliferyl-β-D-cellobioside, total soluble protein.

The production of biofuels from agricultural cellulosic wastes as an alternative to corn (starch) based ethanol production has been the subject of significant interest (Mei et al. 2009; Torney et al. 2007). The growth in demand for corn for use in fuel ethanol production has increased the price of corn for food and feed uses in both the US and in other countries that import corn based products (Somerville 2006). It is estimated that annually somewhere between 10–50 billion tonnes of lignocellulose wastes from crop residues are available globally (Sticklen 2006), potentially yielding as much as 400 billion litres of ethanol per annum (Kim and Dale 2004).

A major roadblock in commercialising lignocellulosic based bioethanol is the cost associated with microbial fermentation of cellulases and feedstock pretreatment. Since 1980 research into microbial cellulase production has led to significant decreases in cost (Commandeur et al. 2003), but the costs associated with production of the cellulases to digest lignocelluloses are still high (Mei et al. 2009). Generating transgenic plants producing cellulases might be a cost effective alternative (Sticklen 2008). A further advantage of producing cellulases within plant biomass over microbial fermenters is that plants use solar energy whereas fermenters require significant input of energy (Mei et al. 2009).

A number of research groups have successfully expressed E1 or its truncated form in transgenic plants (Biswas et al. 2006; Chou et al. 2011; Dai et al. 2000, 2005; Hood et al. 2007; Mei et al. 2009; Oraby et al. 2007; Ziegler et al. 2000; Ziegelhoffer et al. 2001, 2009). However, the expression of CBH1 in transgenic plants has been less frequently reported (Dai et al. 1999; Harrison et al. 2011; Hood et al. 2007). There are no reports of the co-expression of E1 and CBH1 cellulases in transgenic plants. It was hypothesised that the expression of a truncated form of *E1* and *cbhI* containing only the coding region for the catalytic domain of the respective proteins may aid the heterologous expression of these enzymes in transgenic plants because of their resistance to degradation by endogenous plant proteases (Hooker et al. 2000). Furthermore synergistic cellulase action is required for the efficient breakdown of crystalline cellulose for production of fermentable sugars for bioethanol production (Baker et al. 1998), therefore expression of multiple cellulases, including CBH1, is vital if *in planta* cellulase expression is to be used as a tool for biofuel production.

Wheat is an attractive host to express recombinant cellulases, which could ultimately supplement industrially produced enzymes for biomass digestion. We report here the production of a number of transgenic wheat lines, via particle bombardment of immature embryos that express either or both apoplast-targeted E1

and CBH1. This is, to our knowledge, the first reported co-expression of endo and exo-cellulases in wheat, an important monocot crop and the largest worldwide source of agricultural residue.

Materials and methods

Wheat transformation vector

The Gateway® cloning bacteriophage lambda recombination system was used to construct the wheat transformation vector. Three pDONR vectors from the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) were used to clone the expression cassettes of *E1*, *cbh1* and a non-functional *gfp* fragment to form the entry vector. The genes were flanked by recombination sites and introduced into a plant destination vector provided by the Molecular Plant Breeding Cooperative Research Centre (MPBCRC), Melbourne, Australia.

Plant transformation

Immature embryos were bombarded with the wheat transformation vector (Figure 1) and transgenic wheat plants were regenerated from immature embryo-derived calli. Putative transgenic wheat shoots were initially analysed by PCR for the presence of the *E1*, *cbhI* and *bar* in genomic DNA. The bombardment was done at the MPBCRC (Vasil and Vasil 2006). The plant selectable marker *bar* and its corresponding selective agent phosphinothricin (PPT) were used to screen for putative transgenic plants. Genomic DNA (gDNA) was extracted from wheat shoots using approximately 100 mg leaf tissue using the DNeasy plant mini kit (Qiagen®). DNA from control and putative transgenic wheat shoots was screened by polymerase chain reaction (PCR) using gene specific primer pairs designed to the internal regions of *E1* (Forward (F) 5′acgcgacgagcgtctacccgcagacg3′, Reverse(R) 5′ggcgcgagatagccgtcttttac 3′), *cbh1* (F-5′acacgggcattggagg acacggaag3′, R-5′gaaagaggatccgccgaattctgcctc3′) and *bar* (F-5′gtctgcaccatcgtcaacc3′, R-5′gaagtccagctgccagaaac3′). PCR products were generated using a PCR Express ThermalCycler (ThermoHybaid®). DNA amplifications were carried out using GoTaq Green Master Mix (Promega®).

RNA isolation and RT-PCR analysis

RNA was isolated from transgenic wheat plants using a Trizol based method (Chomczynski and Sacchi 1987), but substituting with Trisure (Bioline). Wheat leaves (100–200 mg) were ground in liquid nitrogen to a fine powder and, without allowing them to thaw, ground leaf material was transferred to a 2 ml Eppendorf tube containing 1 ml Trisure solution (Bioline, Australia). The suspension was homogenized by shaking moderately for several seconds and incubated for 2–3 min at room temperature. The insoluble material was removed by centrifugation at 12,000×g for 10 min at 4°C. The cleared homogenate was transferred to a fresh tube and 0.2 ml chloroform added for each 1 ml of initial Trisure. The mixture was shaken for 15 s and incubated for an additional 2–3 min at room temperature. The samples were centrifuged for 15 min at 12,000×g at 4°C. The upper, aqueous phase (550–600 μl) was removed without disturbing the interface and transferred to a fresh Eppendorf tube and 0.5 ml of chilled isopropanol was added. The tubes were inverted to mix the contents and incubated for 10 min at room temperature. The tubes then were spun at 12,000×g for 10 min at 4°C. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. The tubes were again spun in a microfuge at maximum speed for 15 min at 4°C. The supernatant was removed and pellets were air dried in a laminar flow hood. RNA was dissolved in 50 μl of diethylpyrocarbonate (DEPC) treated sterile water. RNA yield and concentration were quantified by a spectrophotometer at 260 nm. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed after genomic DNA elimination and cDNA synthesis, according to the manufacturer's instructions provided for the Qiagen Quantitect Reverse Transcription Kit. Please refer to Table S1 for *E1*, *cbh1*, *bar* and *actin* gene specific primers. Cycling conditions were an initial denaturation at 95°C followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s followed by a final extension step at 72°C for 4 min using GoTaq Hot Start Green Master Mix (Promega).

Western blot analyses

Wheat leaves were snap frozen in liquid nitrogen and ground to fine powder in a 1.5 ml microcentrifuge tube with a precooled conical plastic pestle. The leaf powder was resuspended in protein extraction buffer (50 mM sodium acetate pH 5.5, 100 mM NaCl, 10% v/v glycerol, 0.5 mM ethylenediaminetetraacetic acid (disodium salt), 1 mM phenylmethylsulfonyl fluoride) at a ratio of 2 μl per mg of sample (fresh weight). Soluble extracts were recovered from insoluble debris after centrifugation at 14,000 rpm for 10 min. Total soluble proteins (TSP) extracted from the leaf tissue
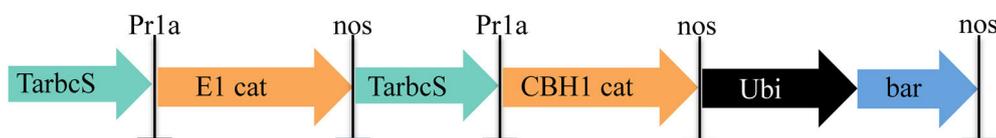

Figure 1. Plant transformation vector for expression of *E1* and *cbh1* catalytic domains (cat) in wheat. The expression construct for an *E1* and *cbh1* catalytic domain (long orange arrows) is fused to the *Pr1a* transit peptide coding sequence (short black line) and is under the regulation of a *Triticum aestivum rbcS* promoter (*TarbcS*, short green arrows). The plant selectable marker gene, *bar* (short blue arrows) was included between the *ubiquitin* promoter (long black arrow) and *nos* terminator (short blue line).

were resolved by 12% precast gels for SDS-PAGE (ClearPAGE) and electroblotted onto nitrocellulose by using an iBlot (Invitrogen). Loadings of 5 and 10 μg of TSP were resolved by SDS-PAGE for E1 and CBH1 detection. The membrane was blocked with 1X TBS, 5% non-fat dry milk, 0.1% Tween-20 at room temperature for 1 h, and then incubated with primary antibody (mouse anti-E1) at 4°C overnight. The membrane was washed three times with 1X TBS containing 0.1% Tween-20, each time for 10 min and incubated with secondary enzyme conjugate anti-mouse or anti-rabbit IgG: HRP (Promega®) at room temperature for 1 h. Immunodetection was accomplished with the ECL system (Amersham Pharmacia Biotech). The primary antibody used for E1 detection was a mouse polyclonal [provided by the National Renewable Energy Laboratory (NREL), Colorado] (1:2500 dilution) directed against E1 catalytic domain (E1cd). The correct size prediction of *in planta* expressed CBH1 is not clear in the literature. Therefore, the design and production of a CBH1 antibody was produced commercially using anti-peptide antibody production method against a single epitope of CBH1 catalytic region (Mimotopes Pty. Ltd., Victoria, Australia). The catalytic domain is 436 amino acids (aa) in length and the best predicted suited single epitope was found between 193–206 aa. The 14 aa long synthetic peptide sequence (CPRDLKFINGQANV) is located towards the N terminus of the catalytic domain and was used to inoculate rabbits. The primary antibody dilution used in this study to predict the correct size of the catalytic domain was (1:2500). All western blot images were analysed using the MyImageAnalysis software version 1.1 (Thermo Fisher Scientific Inc., Rockford, IL).

MUC assay

Cellulase activity from transgenic plant extracts was estimated by the MUC activity assay. Assays were carried out in a 96-well black microtitre plate; 10 μl of protein extracts were mixed with 100 μl reaction buffer (50 mM sodium acetate pH 5.0 containing 0.5 mM of substrate 4-methylumbelliferyl-β-D-cellobioside (MUC) (Mei et al. 2009; Ziegler et al. 2000). The plates were covered with adhesive lids and incubated at 50°C in the dark for 60 min. The reaction was stopped with the addition of 100 μl of stop buffer (0.1 M glycine, pH 10.3). The fluorophore 4-methylumbelliferone (MU), the product of both E1 and CBH1 hydrolysis of the substrate MUC was measured at 465 nm using a POLARstar Omega spectrofluorometer (BMG Labtech) at an excitation wavelength of 360 nm. Background fluorescence contributed by each extract was subtracted and the activity of each sample was calculated using a 4-MU standard curve constructed with 4-MU in the range 2 to 150 nmol.

Results

Regeneration and molecular analyses of E1 and CBH1 co-expressing transgenic plants

Genomic DNA was extracted from a total of 92 shoots that had regenerated on medium containing
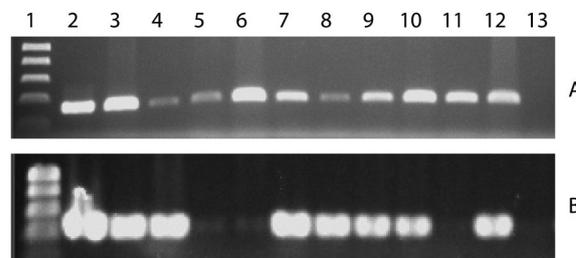

Figure 2.   PCR analysis of wheat genomic DNA for *E1* (A) and *cbh1* (B). (A) Lane 1=DNA marker (HyperLadder1 kb); Lane 2=Entry clone containing *E1* cat (327 bp); Lanes 3–12=Transgenic wheat plants PCR positive for *E1 cat*; Lane 13=non-transformed wheat plant (B) Lane 1=DNA marker (HyperLadder1 kb); Lane 2=Wheat transformation vector containing *cbh1* (437 bp); Lane 3–12=Transgenic wheat plants PCR positive for *cbh1 cat* and Lane 13=non-transformed wheat plant.

phosphinothricin as selection for *bar* expression. The PCR results indicated there were 11 transgenic plants with *E1*, 12 with *cbh1* and 56 in which both the genes appeared to have been incorporated into the plant genome. A representative sample of plants analysed by PCR shows the amplification of *E1* and *cbh1* at the expected sizes of 327 and 437 bp. The absence of the amplification products in the non-transformed control plants was also observed (Figure 2). In addition, transgenic wheat lines expressing cellulases did not show any deleterious effect in comparison to the wild type (Figure S1). There were no major differences in the length, width and weight of seeds collected from T_0 plants in comparison to non-transformed seeds (Figure S2).

Validation of gene expression in transgenic wheat plants using RT-PCR

RT-PCR analysis of wheat plants co-transformed with *E1* and *cbhI* was performed. A representative sample of plants showed generally lower levels of mRNA for *cbh1* (Figure 3). A high level of mRNA expression for *E1* was observed in plants that co-expressed *cbh1* (Figure 3). As expected no expression was observed in non-transformed plants. An *E. coli* expression vector (pNAV129) that contained both the genes was used a positive control.

Testing of commercially synthesized anti-CBH1 antibody

In order to test the functionality of the commercially derived anti-CBH1 antibody several constructs encoding CBH1 were expressed in *E. coli*. The native *T. reesei* full length *cbh1* sequence was cloned by PCR as an *Nco*I and *Sal*I fragment into pUC120 creating pNAV103 and the sequence encoding the catalytic domain was cloned as an *Nco*I and *Sal*I fragment into the pUC120 backbone creating pNAV161. Both gene sequences were under the control of the *lac* promoter. The parent vector pUC120
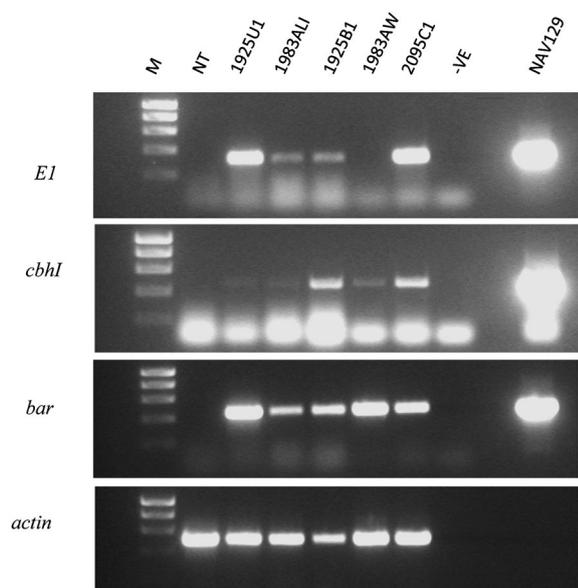
Figure 3. Validation of differential gene expression in transgenic wheat plants using RT-PCR. Lane 1=M: DNA size marker (Hyperladder1); Lane 2=non-transformed (WT) wheat plant; Lanes 3–7=transgenic wheat plants 1925U1, 1983AL1, 1925B1, 1983AW1 and 2095C1 showing mRNA levels for both *E1* and *cbh1*; Lane 8=no template; Lane 9=blank well and Lane 10=positive control plasmid template, pNAV129, containing expression cassettes of *E1* and *cbh1*.
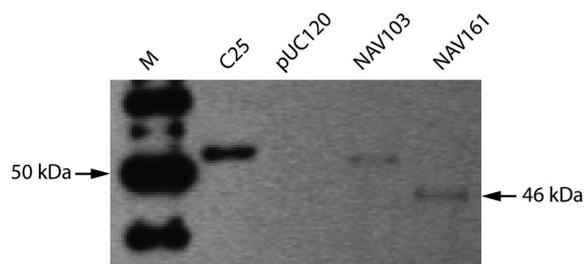

Figure 4. Immunodetection of CBH1 catalytic domain in *E. coli*. Lanes; M=250 kDa Unstained molecular marker; C25=25 ng of full length CBH1 purified from *T. reesei* (ca. 52.2 kDa); pUC120=negative control, protein extract from *E. coli* containing an empty cloning vector; NAV103=*E. coli* protein extract containing recombinant full length CBH1 protein and NAV161=*E. coli* protein extract containing recombinant catalytic domain (46 kDa) of CBH1.

with no insert was used as a control. All three vectors were transformed into *E. coli*.

Western blot analysis of protein extracts from *E. coli* using the commercial antibody raised against the 14 amino acid epitope within the catalytic domain of CBH1 revealed cross-reacting bands of varying molecular weights (Figure 4). Full length CBH1 purified from *T. reesei* (supplied by NREL) showed strong cross-reactivity, migrating above the 50 kDa molecular weight marker. This higher molecular weight of the fungal expressed protein is probably due to glycosylation as expression of the full length coding sequence of *cbh1* (NAV103) in *E. coli*, a prokaryotic host with no post-translational glycosylation system produced a cross-reacting band

that migrated at a slightly lower molecular weight. The catalytic domain construct when expressed in *E. coli* produced a cross-reacting band that migrated at a size consistent with the predicted molecular mass of 46 kDa. No cross-reacting bands were detected in extracts from *E. coli* with the pUC120 vector. The cross-reactivity and specificity of the CBH1 antibody and the migration of protein products from full-length and catalytic-domain constructs provide a reference for examination and quantification of CBH1 expressed in transgenic wheat.

Western blot analysis of transgenic wheat plants expressing only E1

The 11 transgenic wheat lines PCR positive for *E1* expressed a protein migrating at approximately 40 kDa that cross-reacted with the E1 specific antibody. These bands co-migrated with the immuno-reactive band of the truncated E1 purified from *A. cellulolyticus* (Figure 5A). No cross reacting band was observed in the non-transformed control plant extract. The levels of E1 accumulation in leaf tissues was estimated based on the known amount of E1 standard loaded. The accumulation of E1 in the TSP extracts of these transgenic plants was estimated to be in the range of 0.2–1%. The highest accumulation was observed in line 1925B1 (Figure 5A).

MUC activity assay of wheat plants expressing only E1

The E1 enzyme activity in immunoblot positive transgenic wheat plants was estimated by the MUC-based fluorometric assay. The activity levels in leaf extracts were expressed in nmol 4-MU/mg TSP/min (Figure 5B). The highest activity achieved was in extracts from transgenic wheat line 1925B1, which correlated with the highest level of E1 of approximately 1% based on the immunoblot.

Western blot analysis of transgenic wheat plants expressing CBH1 protein only

The twelve transgenic lines shown to contain the *cbh 1* sequence, but not *E1* were examined by immunoblot using the commercially produced anti-CBH1 antibody. To validate the antibody's use against transgenic wheat samples, an initial western blot analysis of 25 ng purified CBH 1 protein (C25), control non-transformed tissue and two transgenic wheat lines 1983AI1 and 1983BZ1 revealed a strongly cross-reacting band at slightly lower molecular weight compared to the purified CBH1 in all samples (Figure 5C), but clearly too large to be the CBH1 catalytic domain. However, the second, lower molecular weight cross-reacting band migrated well ahead of both the full length CBH1 native protein and the cross-reacting band present in the non-transformed plants and transgenic lines. Relative to migration of the molecular weight marker this band has a molecular weight of
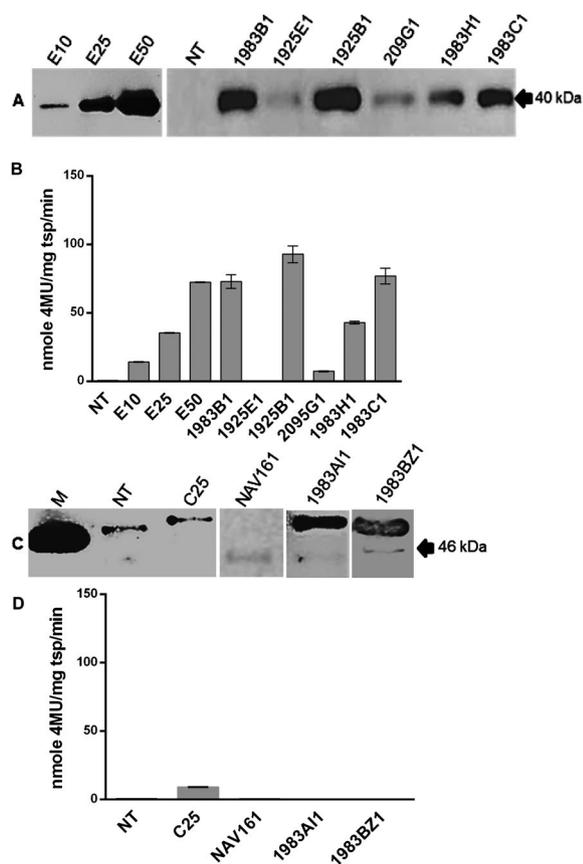
Figure 5. Immunodetection and enzymatic activity of E1 or CBH1 protein expressed separately in wheat plants. (A) E10, 25 and 50=10, 25 and 50 ng purified E1 cellulase from *A. cellulolyticus*; NT=Non-transformed plant protein extract; 1983B1-C1=Transgenic plant protein extracts containing recombinant E1 cellulase (40 kDa); (B) Enzyme activity of plant extracts. NT=Non-transformed plant; E10-50 ng=Purified E1 cellulase from *A. cellulolyticus*; 1983B1-1983C1=E1 cellulase enzymatic activity in selected transgenic wheat lines; (C) M=Molecular marker (250 kDa unstained (Bio-Rad) showing 50 kDa band size); NT=Non-transformed plant protein extract; C25=25 ng of full length CBH1 purified from *T. reesei* (ca. 52.2 kDa); NAV161=*E. coli* protein extract containing recombinant CBH1 catalytic domain (46 kDa) and 1983AI1-BZ1=Transgenic plant protein extracts containing recombinant catalytic domain of CBH1 and (D) shows the enzymatic activity of a non-transformed plant, purified CBH1 from *T. reesei*, *E. coli* expressed CBH1 catalytic domain and transgenic wheat lines expressing only CBH1 catalytic domain against the substrate MUC. NT=Non-transformed plant protein extract; 25 ng of full length CBH1 purified from *T. reesei*; NAV161=*E. coli* protein extract containing recombinant CBH1 catalytic domain and 1983AI1-BZ1=Transgenic wheat lines containing recombinant catalytic domain of CBH1. Each error bar represents±SE (*n*=3).

approximately 46 kDa, the predicted size of the CBH1 catalytic domain. The 46 kDa cross-reacting band did not appear in non-transformed samples but this protein was detected in 4 out of the 12 transgenic lines examined (data not shown). Based on comparison of the band intensities relative to known CBH1, the accumulation of CBH1 in transgenic leaf tissue was estimated to be 0.1–0.2% of the TSP. The transgenic wheat line showing the highest levels of CBH1 protein was 1983BZ1 that accumulated 0.2% of the TSP.
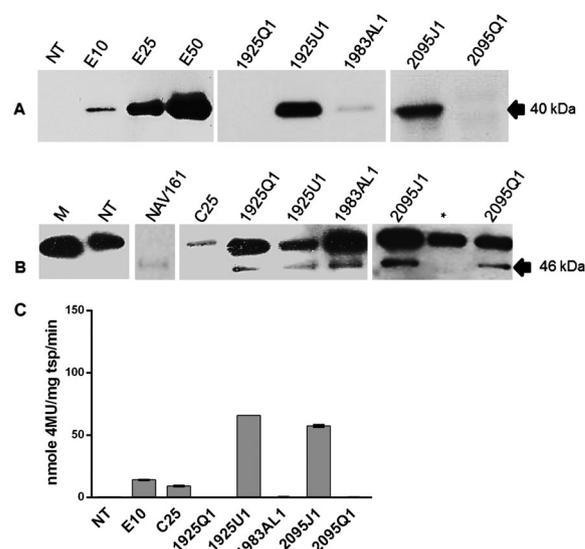

Figure 6. Immunodetection and enzymatic activity in transgenic plants expressing both E1 and CBH1 proteins. (A) NT=Non-transformed plant protein extract; E10, 25 and 50=10, 25 and 50 ng purified E1 cellulase from *A. cellulolyticus*; 1925Q1-2095Q1=Transgenic plant protein extracts containing recombinant E1 cellulase (40 kDa); (B) M=Molecular marker (250 kDa unstained (Bio-Rad) showing 50 kDa band size); NT=Non-transformed plant protein extract; C25=25 ng of full length CBH1 purified from *T. reesei* (ca. 52.2 kDa) and 1925Q1-2095Q1=Transgenic plant protein extracts containing recombinant catalytic domain (46 kDa) of CBH1 protein and (C) NT=No enzymatic activity detected in non-transformed plant; E10=E1 purified cellulase from *A. cellulolyticus* showing enzymatic activity relative to those detected in the immunoblots; C25=Enzymatic activity detected in purified CBH1 cellulase from *T. reesei* and 1925Q1-2095Q1=Cellulase enzymatic activity in transgenic wheat expressing both the genes.

MUC activity assay of transgenic wheat plants expressing only CBH1

The CBH1 expression was analysed in transgenic lines using the fluorometric assay in the same manner as E1 enzyme activity was determined (Figure 5D). The CBH1 accumulated in transgenic plants did not show any activity against the MUC substrate as the enzymatic activity was similar to the levels observed in the non-transformed plant extracts.

Western blot and enzymatic analysis of transgenic wheat expressing both E1 and CBH1

Total soluble protein extracts were prepared from transgenic plants containing both *E1* and *cbh1* and were examined in immunoblots using anti-E1 and CBH1 specific antibodies. A representative sample of the *cbh1/E1* double transgenics (Figure 6A and B) showed that the anti-CBH1 antibody produced a non-specific cross-reacting band of varying intensity in all wheat extracts. A band migrating at 46 kDa, the predicted size of the CBH1 catalytic domain, was present in 9 of the double transgenic lines. A representative sample of 5 is shown with the intensity varying (Figure 5B), from the

Table 1. Total cellulase in g tonne^{-1} in transgenic wheat calculated based on western blot analysis.

Sample	ng of E1 in 10000 ng TSP	ng of CBH1 in 10000 ng TSP	E1 to CBH1 ratio	Total cellulase (E1+CBH1 in 10000 ng TSP)	Total cellulase g tonnes^{-1} wheat biomass
1983B1	100	nd	na	100/nd	652
1925B1	100	nd	na	100/nd	652
1983H1	50	nd	na	50/nd	326
1983C1	80	nd	na	80/nd	523
1983BZ1	nd	25	na	nd/25	163
1983AL1	10	30	1:3	40	267
1925U1	60	25	12:5	85	556
2095J1	50	50	1:1	100	652

*nd, not detected; na, not applicable.

highest expressing lines 2095J1 and 1983AL1, to lower level in 2095Q1 to very low, barely detectable in 1925U1 and Q1. CBH1 is estimated to be around 0.1–0.5% TSP in these lines. The 5 plants that expressed CBH1 also expressed E1 at the predicted size of 40 kDa (Figure 6A) and three lines were found to be enzymatically active when compared to the non-transformed plant (Figure 6C), but clearly most if not all of this activity would be due to the E1 present in the extracts.

Discussion

In this study, we report for the first time the co-expression of E1 and CBH1 cellulases in transgenic wheat, a commercially important broad acre crop. Microprojectile bombardment of immature embryos of wheat (*Triticum aestivum* cv. Bobwhite) with the plant transformation vector consisting of *E1*, *cbh1* expression cassettes, and the *bar* herbicide resistance selectable marker gene, resulted in the regeneration of 92 phosphinothricin resistant shoots. PCR screening showed that 79 of the 92 phosphinothricin resistant plants contained either one or both *E1* or *cbh1*, 11 being *E1* single transgenics, 12 *cbh1* single transgenics and 56 co-transformed with both cellulase genes. Despite the advantage of stringent selection for resistant plants, the generation of 13 escapes in the experiment indicated that the *bar* selection system seems to be leaky in some cereal transformations (Grootboom et al. 2010).

E1 accumulated at levels up to 1% of TSP in leaves of transgenic wheat, which is in agreement with levels of recombinant E1 in transgenic plants in most published reports, (Biswas et al. 2006; Dai et al. 2000, 2005; Mei et al. 2009; Oraby et al. 2007; Ziegler et al. 2000) apart from the very high E1 levels reported in transgenic maize seed (Hood et al. 2007). The expression of the coding region for the catalytic domain of CBH1 in plants has not previously been reported. However, there are only two reports of CBH1 expression; firstly Dai et al. (1999) reported expression in tobacco leaf and callus tissue and Hood et al. (2007) reported the expression of CBH1 in transgenic maize seed. Levels of CBH1 catalytic domain

transgene product accumulating in wheat leaf tissue in this study was up to approximately 0.5% of the total soluble protein which is about five fold higher than levels reported by Dai et al. (1999) in tobacco.

Hood et al. (2007) reported extraordinarily high CBH1 accumulation in seeds, with levels of nearly 18% of TSP. However, this level is based on an activity estimate, but close examination of their CBH1 immunoblot shows the highest expressing line to have CBH1 closer to 0.5% of TSP, which is more consistent with levels estimated in our study. We believe that estimates of both E1 and CBH1 in transgenic plants based on activity assays with synthetic substrates such as MUC (Hood et al. 2007) and MUL (Harrison et al. 2011) overestimate actual protein levels compared to immunoblot quantitation.

Baker et al. (1998) suggested that in order to achieve digestion of crystalline cellulose a ratio of 1:4 of endocellulase to exocellulase is necessary and optimal. The best ratio achieved in this study was 1:3 (Table 1). Table 1 also shows the transgenic plants producing both E1 and CBH1 could theoretically produce 652 g of recombinant cellulase tonne^{-1} of wheat straw. This is to our knowledge the first study reporting co-expression of an endoglucanse and cellobiohydrolase in plants from the nuclear genome. However, E1 has consistently been reported to accumulate to higher levels in leaves than CBH1 in transgenic plants to date. Thus there remains a significant challenge to obtain ratios and CBH1 activity levels that will provide a cost effective solution to the needs for saccharification.

This study reports the co-expression of constructs encoding the catalytic domain of the *A. cellulolyticus* E1 endocellulase and the *T. reesei* CBH1 exocellulase in transgenic wheat plants. The recombinant proteins were directed to the apoplast of the plant cell. The accumulation of E1 protein was up to 1% of TSP and of the CBH1 was up to 0.5% of TSP. Expression of cellulases specifically in green tissues in transgenic wheat is expected to avoid accumulation of the recombinant proteins in seeds. The E1 protein showed enzymatic activity against the soluble substrate MUC but no enzymatic activity could be detected in transgenic wheat

plants expressing CBH1. Consistent with earlier reports, there is low level of *in planta* accumulation of CBH1 in leaves of transgenic plants, and achieving higher levels of *cbh1* expression in transgenic plants remains a challenge.

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Author Contributions

Hashmath I Hussain designed and performed the research, analysed data and co-wrote the paper. Kim Stevenson performed the research. Gregory D Nugent, David M Stalker and Trevor W Stevenson designed the research, analysed data and co-wrote the paper.

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Fig. S1 Representative images of transgenic and non-transformed wheat plants showing no deleterious effects.

Pots (1, 2): Transgenic wheat lines 1983B1 and 1983AI1 expressing only E1 and CBH1 respectively; (3, 5) transgenic wheat line 1925Q1 expressing both E1 and CBH1 and 1983BZ1 expressing only CBH1 and pot (4) non-transformed plant.

Fig. S2 Phenotypic analysis of T0 seeds.

Transgenic and non-transformed plants were grown to seed set and samples of at least 10 seeds measured for weight, width and length. (A and B) Seed length and width and (C) seed weight. Each error bar represents ± S.E. (n=10).
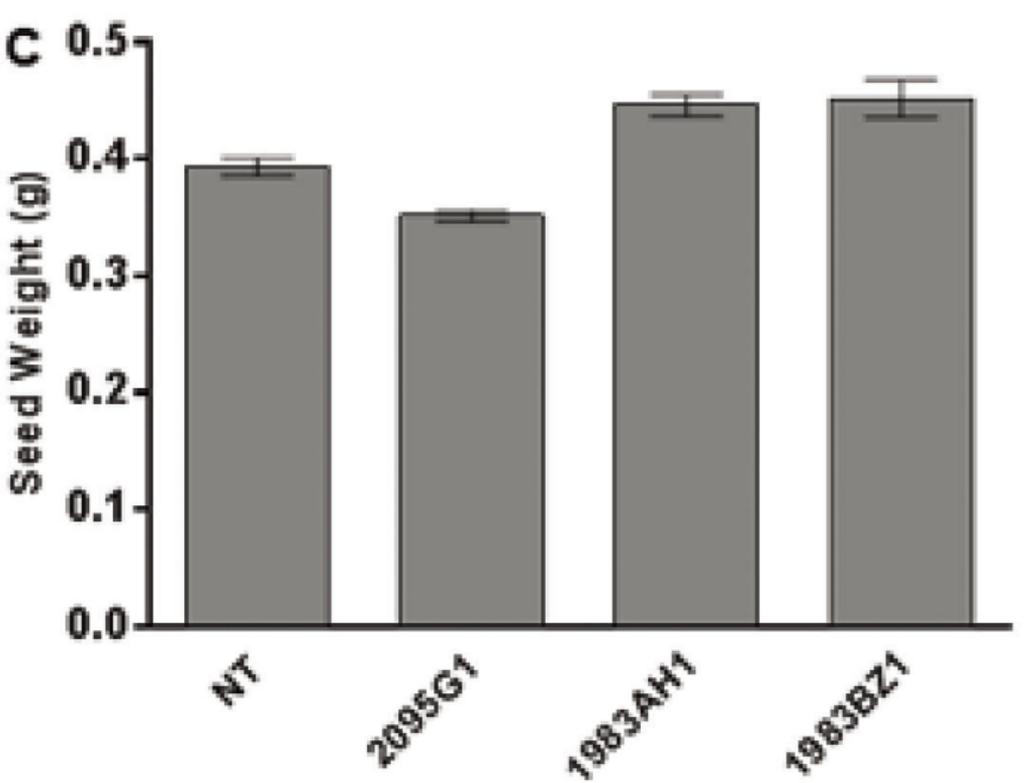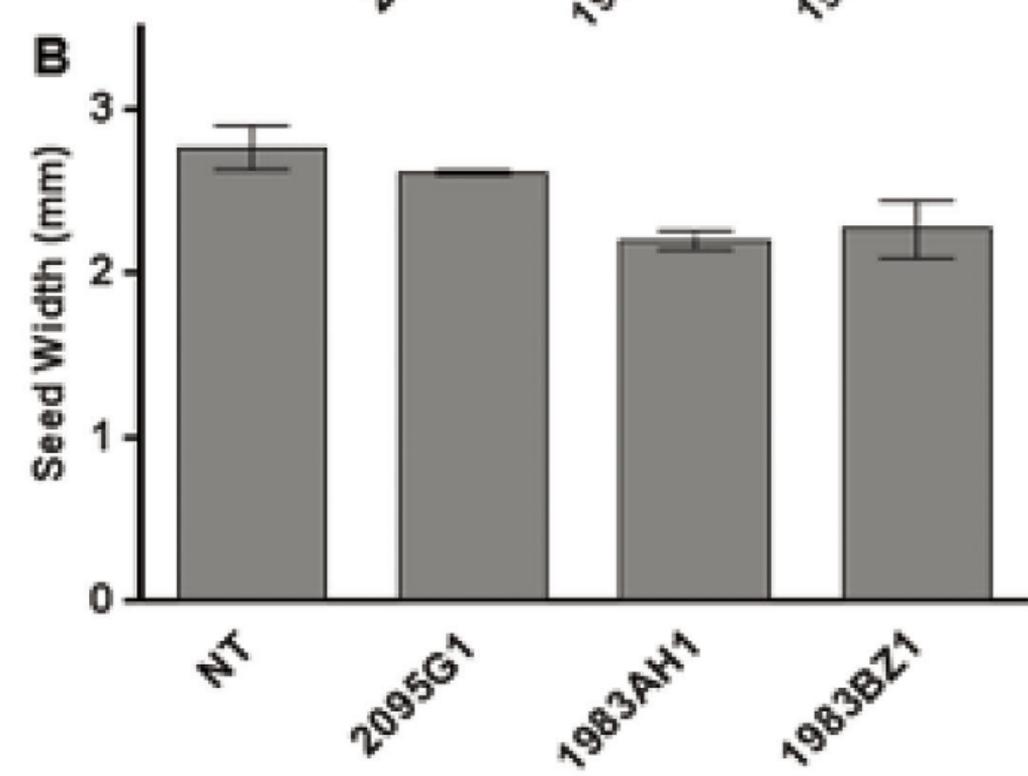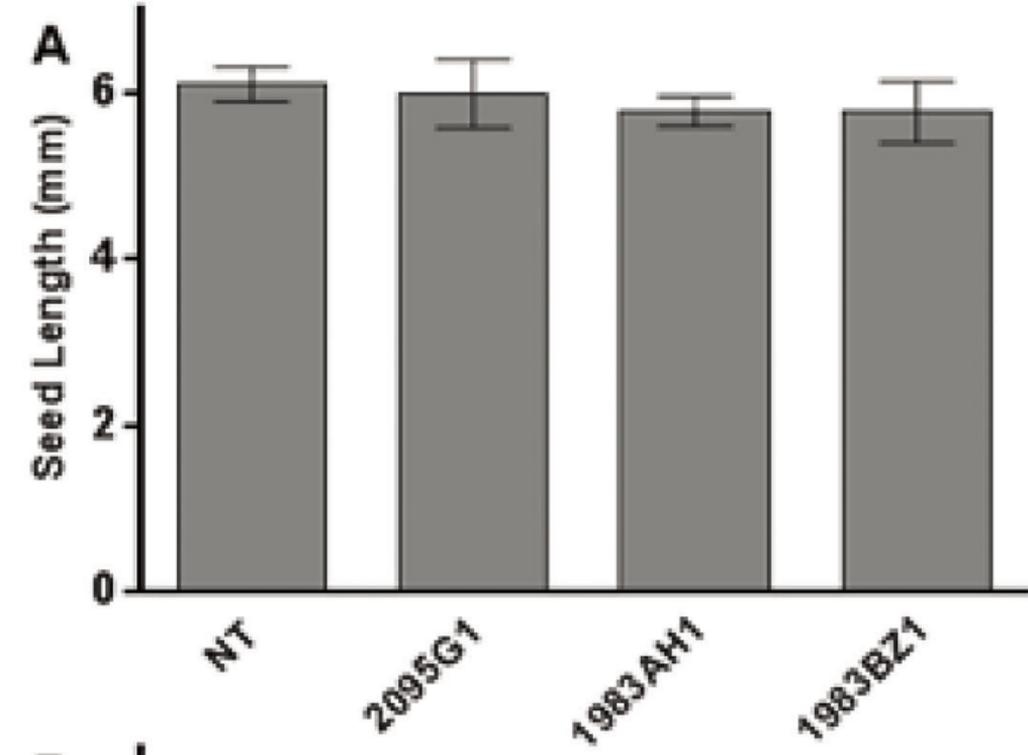
Table S1 Gene specific primers used for RT-PCR analysis
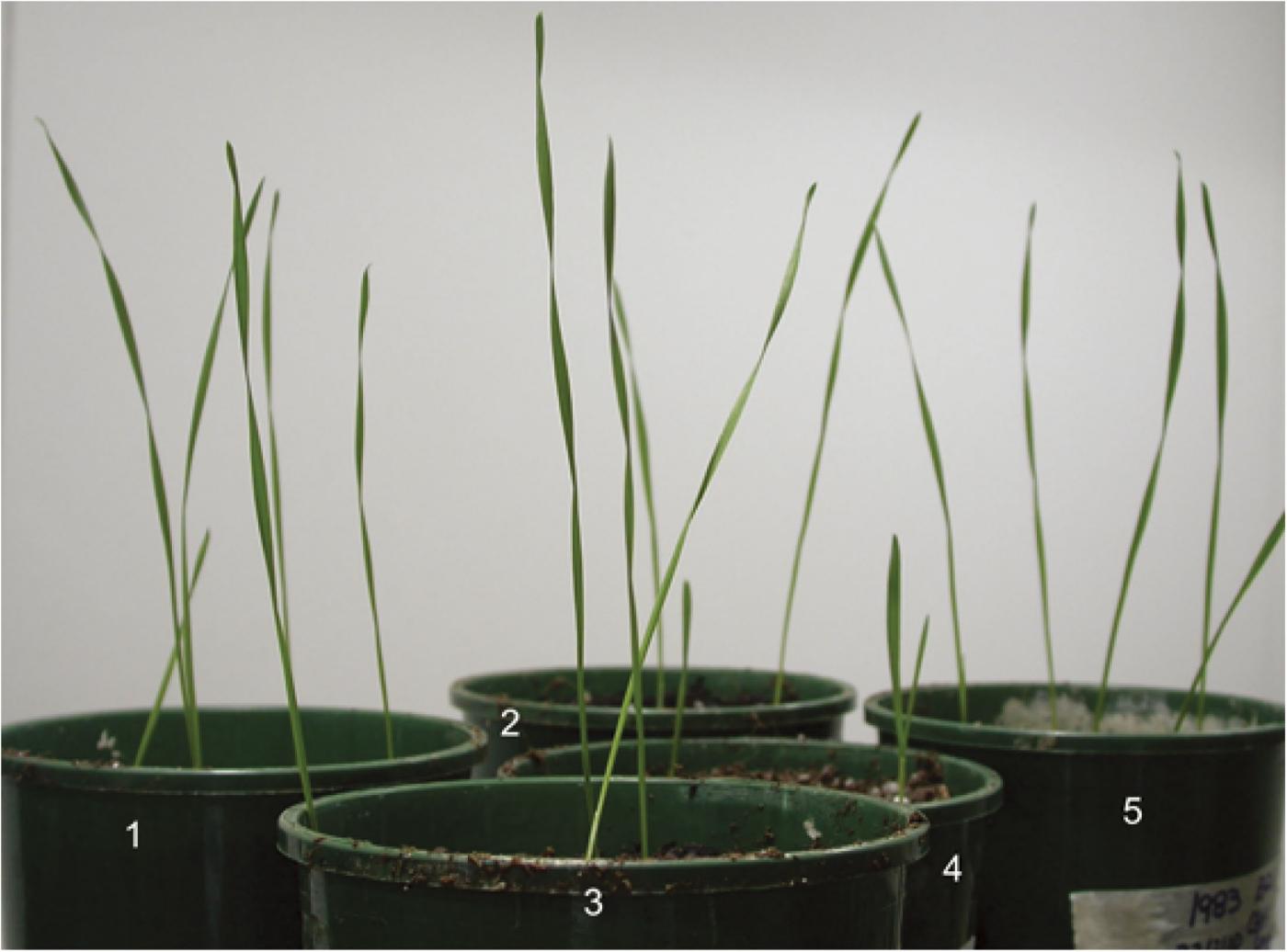
Name	Primer sequence (5' to 3')	Tm
E1RTFor	ACGCGACGAGCGTCTACCCGCAGACG	67
E1RTRev	GGCGCGAGATAGCCGTCTTTTAC	59
Bar Forward	GTCTGCACCATCGTCAACC	53
Bar Reverse	GAAGTCCAGCTGCCAGAAAC	54
TaActinF	GACCCAGACAACTCGCAA	54
TaActinR	CTCGCATATGTGGCTCTTGA	52
CBH 6* (CHB 15)	AGTACCATGGCTTGATCACAGTCGGCCTGCACTCCAATCGG	72
CBH2	CATCCGCGTCCATAGACACGAAGTAGAG	63