Failure to over-express expansin in multiple heterologous systems

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1. Introduction

Expansin is a plant cell wall loosening protein that is active in the growing region of most plants. Its mode of action is not clearly understood but it is believed that expansin induces cell wall expansion by cleaving cell wall polymer connections (i.e., hydrogen bonds). Because of this unique activity, expansin may lower the depolymerization barrier of biofuel production through synergistic activity with cellulase. Indeed, expansin was shown to increase cellulase activity through a synergistic effect and this activity has been tested with small amounts of expansin in bench scale experiments \[1\].

In the native plant cell, expansin accumulation is very low. For example, in cucumber hypocotyls, expansin exists at one part protein per 5000 parts cell wall (on a dry mass basis) and induces wall extension with these very small amounts \[2\]. One challenge with the low concentrations of expansin in native tissues is that it is difficult to obtain enough protein to do activity assays \textit{in vitro} or to make antibodies in order to perform Western blots or enzyme-linked immunosorbent assays (ELISAs). Moreover, any attempt to study or exploit its synergistic activity with cellulase in commercial scale applications will require obtaining sufficient expansin from a recombinant heterologous system.

The cucumber expansin gene has been expressed in transgenic maize seed \[3\]. In order to screen the seed from the 375 transgenic maize plants, we planned to develop an ELISA assay. To accomplish this, we attempted to express the expansin gene in a heterologous system to obtain sufficient protein for immunizing rabbits to produce polyclonal antibodies. We utilized two common expression systems, the Gateway cloning system for protein expression in \textit{Nicotiana benthamiana} \(N.\ \textit{benthamiana}\) leaves, and transient expression in \textit{Escherichia coli} \(E.\ \textit{coli}\). The Gateway system has been used successfully for transient expression of expansin protein in maize plants, we planned to develop an ELISA assay. To accomplish this, we attempted to express the expansin gene in a heterologous system to obtain sufficient protein for immunizing rabbits to produce polyclonal antibodies. We utilized two common expression systems, the Gateway cloning system for protein expression in \textit{Nicotiana benthamiana} \(N.\ \textit{benthamiana}\) leaves, and transient expression in \textit{Escherichia coli} \(E.\ \textit{coli}\). The Gateway system has been used successfully for transient expression of expansin protein in maize plants, but it is not clear whether expansin accumulates in sufficient quantities for a recombinant heterologous system.

Results: We tested the expression of the cucumber expansin gene in several heterologous systems including \textit{Arabidopsis thaliana} seeds and \textit{Nicotiana benthamiana} leaves with limited success. We also had limited success in transiently expressing an alternative expansin gene from bamboo in \textit{N. benthamiana} leaves. In order to determine if expansin over-expression is limited to a seed system, \textit{Arabidopsis thaliana} seeds were tested. Although all positive and negative controls behaved as expected, none of these common systems expressed the expansin gene well.

Conclusions: Over-expression of cucumber expansin in three heterologous systems, \(E.\ \textit{coli}\), transient tobacco leaves, and \textit{Arabidopsis} seeds was unsuccessful. The cause of this failure is not known. These results confirm the necessity of experimentally exploring several heterologous systems for protein production in order to find one with utility.

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neither of those methods yielded sufficient protein for continued effort. Subsequently we attempted to resolve the low native expansin concentration issue first by identifying a source of expansin that may be more active or more abundant. We identified bamboo as being one of the fastest growing species of plant known. The Moso bamboo (Phyllostachys pubescens) is a perennial monocot plant, which is the most widely farmed bamboo species in China [4]. Its vegetative phase lasts about 100 years before flowering [5]. Moreover, this bamboo grows about one hundred centimeters per day under optimum conditions [6]. Because of this extremely high growth rate, we hypothesized that expansin from Moso bamboo must be highly active or abundant and thus desirable. Hence, bamboo expansin was cloned to test its transient expression in a plant system. However, as was true for cucumber expansin, the Moso bamboo expansin gene had limited expression through Agrobacterium tumefaciens-mediated transient transformation of N. benthamiana.

Finally, we attempted to express the cucumber expansin gene in Arabidopsis thaliana, again because it was a fast but facile expression system. We predicted that seed-based expression would be more stable [7] and thus used a seed-preferred promoter in these efforts. The results of this series of attempts at recombinant expansin expression in heterologous systems are reported here. None of the systems successfully produced amounts of expansin that were greater than what is seen in native plant tissues.

2. Materials and methods

2.1. Cucumber expansin gene

The cucumber expansin cDNA (accession no. U30382) was synthesized (Integrated DNA Technologies, Coralville, IA,) and amplified with primers which had restriction sites and histidine tag overhang to amplify the cDNA without the endogenous cucumber expansin signal sequence (Supplementary Table 1).

2.2. Bamboo expansin gene

A bamboo expansin EST sequence was identified via a tBLASTn search in GenBank using the cucumber expansin (accession #U30382) amino acid sequence as a query. The EST sequence (accession #FP094161) of the Phyllostachys pubescens cDNA clone (bphyem10506, full insert sequence) showing DNA sequence identity of 76% with cucumber expansin was chosen. The coding sequence and signal peptide were annotated (Supplementary Fig. 1) by aligning with the cucumber expansin sequence. The bamboo expansin genomic sequence was amplified from total genomic DNA, which was extracted with the DNeasy mini kit (Qiagen, Valencia, CA) from growing leaves (2–4 cm long) of Moso bamboo. Expansin specific primers, which had restriction sites and a six-histidine tag at either the 5’ or 3’ end, were used (Supplementary Table 1). The primers were also designed to exclude the endogenous expansin signal sequence.

Total RNA was extracted for cDNA preparation from leaves (as above) using the Maxwell® 16 Tissue LEV Total RNA Purification Kit (Promega, Madison, WI) and used for bamboo expansin cDNA synthesis. cDNA synthesis was conducted with two step RT-PCR using the RETROscript™ Reverse Transcription Kit (Ambion, Austin, TX) and the same primers as used for genomic DNA amplification.

2.3. Vector construction

Six expression cassettes were constructed, four using genomic DNA and cDNA of the bamboo expansin gene and two with the synthesized cucumber expansin gene (Fig. 1). All amplicons were cloned into the pBluescript II SK(+) vector. This vector harbors the cauliflower mosaic virus (CaMV) 35S promoter, the tobacco etch virus (TEV) leader sequence, the patatin signal sequence and the nopaline synthase (NOS) terminator (Fig. 1). Genes were cloned into this vector between the signal sequence and terminator as an XbaI/SacI fragment. The resulting clones were sequenced (Supplementary Fig. 2).

All vectors for Arabidopsis transformation have been described elsewhere and were used without modification. Vectors BCA, BCG, and BCJ are described by Yoon et al. [3] (Table 1 and Fig. 1). The pPGN7547 GUS vector was described by Streetfield et al. [8]. The E1 cellulase expression vector, pPGN9101 (BCH), was described in Hood et al. [7].

2.4. Transient tobacco transformation

Expression vectors were prepared by transforming Agrobacterium tumefaciens strain LBA4404 with the p8BiB-Kan vector harboring each expression cassette. Transient transformation via vacuum infiltration was performed following a protocol described by Medrano et al. [9]. In brief, bacterial strains were incubated in 100 mL YEP medium containing 100 μg/mL kanamycin and 60 μg/mL streptomycin for 48 h at 28 °C, 220 rpm. Prior to infiltration, A. tumefaciens cells were induced by resuspending the cells collected via centrifugation (5000 X g for 10 min) in the 400 mL induction medium (20 mM MES pH 5.5, 0.3 g/L MgSO4·7H2O, 0.15 g/L KCl, 0.01 g/L CaCl2, 0.0025 g/L FeSO4·7H2O, 2 mL/L M NaH2PO4 pH 7.0, 10 g/L glucose) containing 100 μg/mL kanamycin, 100 μg/mL streptomycin, and 200 μM acetylsyringone. This induction medium containing induced A. tumefaciens cells, was used to vacuum infiltrate the four to six-week-old N. benthamiana plants. The beaker containing the induced culture was placed in a vacuum chamber, then all aerial parts of the plant were submerged in the culture and the plant was infiltrated by applying a vacuum for 30–60 s at maximum pressure (~25 in. Hg) then the vacuum was quickly released. To ensure infiltration, this procedure was repeated and plants were returned to the growth chamber to incubate for 48–96 h.

2.5. Immunoblotting of tobacco leaf protein

Crude protein extracts were prepared for immunoblot analysis from 0.5 g of tissue sample with SDS extraction buffer (150 mM Tris–HCl pH6.8, 30% glycerol, 6% SDS and 5 mM EDTA) then each
sample was desalted with a Cornings® Spin-X® UF Centrifugal 10,000 molecular weight cut-off (MWCO) polyethylenesulfone (PES) Membrane (Corning, Inc., Corning, NY) just before use. SDS-PAGE was conducted with the Novex® NuPAGE® SDS-PAGE Gel System (Invitrogen, Carlsbad, CA) using 12% Bis-Tris minigels. Minigels were stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad, Richmond, CA) to confirm efficient separation. Proteins in unstained gels were electrophoretically transferred using an XCell II™ Blot Module (Invitrogen, Carlsbad, CA) to an Immobilon-NY+ nitrocellulose membrane (Millipore, Bedford, MA). Transfer was performed in a solution of 48 mM Tris, 250 mL of ice-cold 10% glycerol two times. After a centrifugation, the pellet was resuspended in 1 mL ice cold 10% glycerol and 50 mM sodium acetate buffer pH 9.5. The anti-expansin antibody (1:1000) was added to blocking buffer and the blot was incubated in this solution for 1 h. The membrane was washed with PBS twice for 5 min each and Tris-buffered saline (TBS) twice for 5 min each. The blot was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, 1:10,000) in 10% horse serum in TBS for 1 h. The membrane was washed four times for 5 min each in TBS. Chemiluminescent detection was conducted with CDP-Star (Roche Diagnostics, Indianapolis, IN), Nitro Block II (Tropix, Bedford, MA) and CDP-Star detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5). The membrane was washed with CDP-Star detection buffer twice for 2 min each then placed in a sealable plastic bag. Detection reagent for 1 h. The membrane was washed with PBS twice for 5 min each and Tris-buffered saline (TBS) twice for 5 min each. The blot was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, 1:10,000) in 10% horse serum in TBS for 1 h. The membrane was washed four times for 5 min each in TBS. Chemiluminescent detection was conducted with CDP-Star (Roche Diagnostics, Indianapolis, IN), Nitro Block II (Tropix, Bedford, MA) and CDP-Star detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5). The membrane was washed with CDP-Star detection buffer twice for 2 min each then placed in a sealable plastic bag. Detection reagent for 1 h. The membrane was washed with PBS twice for 5 min each and Tris-buffered saline (TBS) twice for 5 min each. The blot was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, 1:10,000) in 10% horse serum in TBS for 1 h. The membrane was washed four times for 5 min each in TBS. Chemiluminescent detection was conducted with CDP-Star (Roche Diagnostics, Indianapolis, IN), Nitro Block II (Tropix, Bedford, MA) and CDP-Star detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5). The membrane was washed with CDP-Star detection buffer twice for 2 min each then placed in a sealable plastic bag. Detection reagent (3 mL CDP-Star detection buffer, 150 μL Nitro Block II, 30 μL CDP-Star) was poured on the membrane and the bag sealed. After incubation for 5 min, chemiluminescent signal was detected with Kodak BioMax XAR film (Sigma, Rochester, NY) and the film was developed with an SRX-101A Medical Film Processor (Konica, Shinjuku-ku, Tokyo, Japan). The protein marker for SDS-PAGE was Precision Plus Protein™ Dual Color Standards (Bio-Rad). The molecular mass was calculated by measuring relative mobility of protein bands on the gel. The expansin immunoblot protocol and antibody were kindly provided by D. Cosgrove (The Pennsylvania State University).

2.6. Enriched cucumber expansin preparation

Enriched cucumber expansin extract was prepared following a protocol described by McQueen-Mason et al. [10] with minor modifications. In brief, 300 g of ~3 cm hypocotyl regions of etiolated cucumber seedlings were collected on ice and frozen in liquid nitrogen. Frozen tissues were ground with a mortar and pestle then the wall fragments were resuspended with 20 mM HEPES pH 6.8, 2 mM EDTA, 3 mM sodium metabisulphite at 4 °C and extracted with gentle stirring for 30 min. Tissues were collected by filtration using Miracloth (Calbiochem, San Diego, CA) and washed twice with the same buffer. Expansin crude extract was prepared from the pellet by three extractions with 20 mM HEPES pH 6.8, 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulphite at 4 °C for 1 h each. Supernatants were pooled and expansin protein was precipitated with 60% ammonium sulfate. Ammonium sulfate pellets were stored at −80 °C and were resuspended with 50 mM sodium acetate buffer pH 5.0 and desalted with a Cornings® Spin-X® UF Centrifugal Concentrator 10,000 MWCO PES Membrane (Corning, Corning, NY) just before use.

2.7. Bacterial transformation

For Arabidopsis transformation, the original maize expression vectors had to be moved from A. tumefaciens EHA101 (used for maize) into A. tumefaciens GV3101. EHA101 strains of A. tumefaciens with the constructs of interest were used to purify the plasmid using the QIAprep Spin Miniprep kit. Bacterial transformations of resulting binary plasmids were performed by either the freeze thaw [11] or electroporation method. For electroporation, electrocompetent A. tumefaciens GV3101 was prepared as follows. A 500 mL culture of the strain was grown in YEP with rifampicin (50 mg/L) and gentamycin (50 mg/L) at 28 °C for 2 days. The culture was centrifuged at 12,000 X g for 15 min at 4 °C and resuspended in 500 mL of ice-cold 10% glycerol, centrifuged and resuspended in 250 mL of ice-cold 10% glycerol two times. After a final centrifugation, the pellet was resuspended in 1 mL ice cold 10% glycerol and 50 μL aliquots were frozen in liquid nitrogen and stored at −80 °C.

Frozen cells were thawed on ice and 50 ng of each plasmid of interest was added and mixed in a pre-cooled electroporation cuvette of 2 mm Gap (Fisher Biotech). The cuvette was placed in the electroporation chamber (Bio-Rad Micropulse™) at 2.5 kV for 6 milliseconds, 500 μL of YEP medium was immediately added and the culture incubated at 28 °C for two h, shaking at 120 rpm. The cells were plated on YEP medium with spectinomycin (100 mg/L), rifampicin (50 mg/L) and gentamycin (50 mg/L) at 28 °C for 2 days. The resulting GV3101 strains of A. tumefaciens were used for A. thaliana transformation.

2.8. Arabidopsis plant material

Arabidopsis thaliana accession Columbia wild type seeds (CS-6000) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus OH USA). Plants were grown under the

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Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>Gene of Interest</th>
<th>Antibiotic selection (bacteria)</th>
<th>Selection (plant)</th>
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<td>pPGN9094 (BCA)</td>
<td>Cucumber EXP A (Cell wall targeted)</td>
<td>Spectinomycin</td>
<td>Bialaphos</td>
</tr>
<tr>
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<td>pPGN9099 (BCG)</td>
<td>Cucumber EXP A (ER targeted)</td>
<td>Spectinomycin</td>
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<td>pPGN9100 (BCJ)</td>
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<td>GV3101</td>
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following conditions: 23 °C temperature, 65% relative humidity, 16:8 h light/dark photoperiod, and 100 – 120 μmol/m²/s light intensity.

2.9. Toxicity test with bialaphos

In order to know the best concentration for transgenic plant selection with bialaphos, a kill curve with various concentrations of herbicide was performed. Murashige and Skoog (MS) [12] medium was made with different concentrations of bialaphos (0.5, 1, 1.5, 2, 4, 6, 8, 10, 20, 30 mg/L). The optimal concentration was 4 mg/L.

2.10. A. tumefaciens mediated Arabidopsis transformation

*A. thaliana* was transformed by the floral dip method [13] with the constructs of interest in A. tumefaciens GV3101: pPGN9094 (BCA), pPGN9099 (BCG), pPGN9100 (BCC), pPGN9104 (BCH) or pPGN7547 (GUS). Untransformed Arabidopsis seeds were sterilized with 70% (v/v) sodium hypochlorite containing 0.05% (v/v) Tween 20 for 15 min. Seeds were washed 6 times with sterile water. Finally, the seeds were transferred to a petri dish containing Murashige and Skoog (MS) medium, consisting of 3% sucrose, at pH 5.7. The medium was supplemented with appropriate antibiotics. This culture was grown overnight at 28 °C in YEP medium with 50 mL culture plus antibiotics. The 50 mL culture was grown to maturity. One or two rosette leaves were collected from plants that grew in MS medium with bialaphos. The explants were incubated in 70% ethanol for 10 min followed by 50% (v/v) acetic acid containing 0.05% (v/v) Tween 20 for 15 min. Seeds were washed 6 times with sterile water. Finally, the seeds were transferred to a petri dish containing Murashige and Skoog (MS); which consists of MS salts, MS vitamins, and 3% (w/v) sucrose, at pH 5.7. The medium was supplemented with 0.04% (w/v) MgSO₄·7H₂O. Plates containing the seeds were vernalized for 3 days at 4 °C. After seedling establishment, plantlets were transferred to soil and grown under the above conditions until they reached flowering stage.

A 4 mL culture of A. tumefaciens strain GV3101 with the binary vector was grown overnight at 28 °C in YEP medium with appropriate antibiotics. This culture was then used to transfer a 50 mL YEP culture plus antibiotics. The 50 mL culture was grown until the OD₆₀₀ was approximately 1.0. The culture was centrifuged at 13,000 g for 20 min at 4 °C, and the pellet was resuspended in 500 mL of 5% (w/v) sucrose solution with the surfactant Silwet L-77 (Helena Chemical Co., Collierville, TN, USA) at 0.02% (v/v). Unopened flowers from an average of forty plants were immersed in the A. tumefaciens solution for 15 min, after which plants were then placed in a tray with a sealed transparent lid to maintain high humidity for 48 h. Plants were allowed to grow and produce seeds for three weeks after transformation. Seeds were collected 2–3 weeks later. These seeds were considered the T₀ generation.

2.11. Screening of primary transformants

T₀ seeds were sterilized as previously described and plated on MS medium supplemented with 4 mg/L bialaphos. After 2 weeks, herbicide resistant seedlings were transferred to soil and plants were grown to maturity. One or two rosette leaves were collected for DNA extraction. Genomic DNA samples were prepared using the CTAB method [14]. Presence of the transgene of interest was established via PCR using gene specific primers (expansin, bialaphos, 35S and GUS) and genomic DNA as a template. PCR reactions were performed using Promega Master Mix (Promega, Madison, WI, USA) following the manufacturer’s instructions. One hundred ng of genomic DNA was used. The conditions for PCR were optimized as follows: (1) 94 °C for 5 min; (2) 94 °C for 1 min; (3) 55 °C for 1 min; (4) 72 °C for 1 min; (5) repeat 30 cycles of steps 2 through 4; (6) 94 °C for 5 min; and (7) 6 °C for 10 min. The primers used for these analyses were: bialaphos forward (5’-GG-ACT CAC ACA TTA TTA TAG AG-3’), expansin forward (5’-ACC TTC TAC GGC GGT GGT GA-3’), expansin reverse (5’-CAT CCA GTG CAC CCC TT-3’), pSSS forward (5’-ECT GTT GGG TCC AT-3’), pSSS reverse (5’-TCC CGG TAA AGA CTG GCC AAG-3’), GUS forward (5’-CAT GAA GAT GGC GAC TTA CG-3’), GUS reverse (5’-ATC CAC GCC GTA TTG GC-3’).

2.12. Histochemical localization of β-glucuronidase (GUS) by staining

Explants (leaves, flowers and fruits) were cut from plants that grew in MS medium with bialaphos. The explants were incubated in fresh phosphate buffer pH 7.0 with 4% formaldehyde at room temperature for 30 min as previously described (http://microscopy.tamu.edu/lab-protocols/GUS_Localization_in_plants.pdf). Explants were washed several times with cold phosphate buffer for 1 h, then vacuum infiltrated with the X-Gluc substrate solution. Tissues were incubated in darkness at room temperature overnight until a distinct blue staining appeared. The stained explants were incubated in 70% ethanol until the chlorophyll was removed. The X-Gluc reaction consisted of 1 mg 5-bromo-4-chloro-3-indolyl β-d-glucuronide in 100 μL of methanol, added to 1 mL phosphate buffer, 20 μL 0.1 M potassium ferrocyanide, 20 μL 0.1 M potassium ferricyanide, 10 μL 10% (w/v) solution of Triton X-100 (Fisher Scientific, Pittsburg, PA), and 850 μL of water.

2.13. Immunoblotting of E1 in Arabidopsis thaliana seeds

Total soluble protein was extracted from 20 mg *Arabidopsis* seeds from plants that were positive for PCR by the bialaphos gene. Seeds were placed into a 2 mL Precellys tube with six 2.8 mm steel balls and 500 mL of 50 mM sodium acetate buffer pH 5. Samples were cooled on ice for 15 min then centrifuged at 4 °C. The supernatant was transferred to a fresh tube and spun again at 12,000 X g for 1 min. The supernatant was transferred to a fresh tube and spun again at 12,000 X g for 1 min. Protein assays were performed to determine total protein and 20 μL of each sample was loaded onto the protein gel for the western blot. Antibodies were prepared previously against E1 purified protein that was expressed in the *E. coli* Gateway vector system [16]. Separated proteins from SDS-PAGE were transferred to PVDF membrane (Millipore) and blocked 2 h with 5% BSA in Tris-buffered saline (TBS). The blocked membrane was incubated with primary antibody, anti-E1 [7] (1:2,500) for 2 h and with secondary antibody, goat anti-rabbit alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO, USA), (1:5,000) for 2 h. Color detection carried out using NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) reagent.
3. Results and discussion

3.1. Attempts to express expansin in E. coli

Multiple attempts were made to move the cucumber expansin gene into Gateway vectors for E. coli expression. Although the gene was cloned into the vector (determined by PCR, data not shown), little to no expression was detected even after induction with L-arabinose. We have successfully used this method previously to express cellulase genes (an endoglucanase and an exoglucanase) to produce protein for generating antibodies [16]. We hypothesized that perhaps the gene was not compatible with a prokaryotic system, so we proceeded with a fast, transient expression system in a plant.

3.2. Transient expression in tobacco for bamboo and cucumber expansin genes

The cucumber expansin gene was synthesized using its native codons and a histidine tag and restriction enzyme sites were added on either the 5'- or 3'-end. A cDNA of the bamboo gene was cloned using RNA isolated from young tissue of growing moso bamboo shoots. In addition, the genomic clone of the gene was isolated by PCR amplification of moso bamboo DNA. Several expression vectors were generated for tobacco transient expression (Fig. 1) because vector construction elements have been shown to affect the amount of protein recovered from this system [9]. The bamboo expansin cDNA clone (Supplemental Fig. 1, 2A) showed 12 nucleotide differences and 8 nucleotide indels compared to the

<table>
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<th># Plants recovered</th>
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Fig. 3. Recovery of herbicide resistant Arabidopsis plants after floral dip transformation. A) Arabidopsis wild type seeds plated on Murashige and Skoog medium without selection; B) WT seeds plated on MS media with 4 mg/L of bialaphos; C) Seeds of plants transformed with the BCA construct plated on MS media with 4 mg/L of bialaphos; D) Seeds of plants transformed with the BCG construct plated on MS media with 4 mg/L of bialaphos; E) Seeds of plants transformed with the BCJ construct plated on MS media with 4 mg/L of bialaphos; F) Seeds of plants transformed with the GUS construct plated on MS media with 4 mg/L of bialaphos. Representative plates are shown. Several GUS positive transformants were recovered.
published coding region of the EST sequence (Supplemental Fig. 2C). The sequence differences between the synthesized cDNA and the EST sequence may be caused by the inaccuracy of the reverse transcription reaction. Alternatively, there may be two native bamboo genes. The size of the bamboo expansin gDNA was 975 bp (Supplemental Fig. 2B) including two introns but without the native signal sequence. The bamboo gDNA sequence was perfectly matched with the published EST sequence.

Multiple infiltrations of *N. benthamiana* were made to test expression of the bamboo and cucumber expansin genes in this system. In most infiltrations of this species with heterologous genes, enough protein can be recovered to view on Coomassie blue stained gels. However, in this case, no bands different from controls could be detected. When tested on Western blots with anti-expansin antibodies, limited expression was detected. In fact it was no higher than what can be seen in concentrated extracts from native cucumber tissue (Fig. 2). Two different size proteins (30 kDa and 33 kDa) are detected with the antibodies. We see this often in Fig. 4.

![Fig. 4](image)

**Fig. 4.** Results of PCR screening of *Arabidopsis* plants recovered after transformation with expansin, GUS, or E1 constructs. Three putative transformants were recovered after transformation with the BCG construct, as illustrated in (A) these were PCR negative for expansin and for bialaphos. On the other hand multiple transformants were recovered after transformation with the GUS construct; 17 of 29 putative transformants were PCR positive for the GUS insert (B). Multiple bialaphos resistant plants were obtained after transformation with the E1 construct; 8 of 31 lines were positive for the presence of the 3SS promoter (C). WT = wild type, P = positive control (plasmid), Φ = negative control (all PCR reagents except DNA).

3.3. Stable expression of expansin in *Arabidopsis thaliana* seeds

Because of our difficulty with *E. coli* and *N. benthamiana* expression, we decided to test a third common expression system, stable expression in *A. thaliana* seeds. We used bialaphos selection, which has been tested previously in *A. thaliana* [17]. We began by determining the best concentration for selection and concluded that 4 mg/L was best (data not shown). We proceeded to test transformation and selection with a plasmid that contained the maize ubiquitin promoter and the β-glucuronidase (GUS) gene (Fig. 1, Tables 1 and 2). This plasmid was transformed into the GV3101 *A. tumefaciens* strain, which is routinely used in our laboratory for *A. thaliana* floral dip transformation. From the seeds recovered from the transformation, 2475 were cleaned and plated onto MS medium containing 4 mg/L of bialaphos (Fig. 3F). From these, 29 herbicide-selected seedlings were recovered, transferred to soil, and analyzed by PCR for the presence of the gene. As shown in Fig. 4B, 17 of 29 selected plants were positive by PCR for the GUS gene. Progeny from these plants were germinated on MS medium with bialaphos, grown in soil, and several plant parts subsequently stained for GUS activity to ensure function of the maize (monocot) promoter in this dicotyledonous plant (Fig. 5). Clearly, the maize ubiquitin promoter is highly active in *Arabidopsis* leaf, inflorescence and silique.

Based on these positive results, the expansin gene constructs were tested for seed-based expression in *A. thaliana*. We had the seed-preferred promoter constructs for maize transformation with the protein targeted to three subcellular locations and utilizing bialaphos selection as for the GUS construct (Fig. 1 and Table 1). Each of the three vectors was used for three separate transformation experiments. Seed recovered from the floral-dipped plants was plated on bialaphos-containing medium (4 mg/L; Fig. 3, Table 2). Out of more than 18,000 seeds screened from these 9 transformation experiments, three small, weak plantlets were recovered from the BCG vector. However, not a single transgenic plant was recovered as shown by PCR (Fig. 4A).

We hypothesized that expansin may be potentially lethal to this seed expression system. Alternatively, perhaps any *Arabidopsis* seed with a recombinant protein expressed from the globulin-1 promoter from maize would be lethal. To test this hypothesis, we transformed *Arabidopsis* with an additional construct expressing a globulin-1 promoter driven heat stable cellulase, E1 (Table 1), an enzyme that has been successfully expressed in *Arabidopsis* previously [18]. Thirty-two plants were recovered in a single experiment using this vector and of these, eight were positive by PCR (Table 2, Fig. 4C). Seeds recovered from these of the selected plants showed accumulation of the E1 protein by Western blot assay (Fig. 6).

Several studies have been published describing expression of an expansin gene in its native host. Brummel and colleagues [19] over-expressed the tomato fruit-specific expansin gene in tomato and showed that the fruits were considerably softer during development than wild type controls. Cho and Cosgrove over-expressed a leaf-specific native gene, *AtEXP10*, in *Arabidopsis* and showed that enhanced leaf and petiole growth occurred as well as facile leaf abscission [20]. Ma and co-authors over-expressed the rice expansin gene in rice using the constitutive 35S promoter resulting in pleiotropic effects including increased root growth, increased plant height, increased leaf number and larger leaves [21]. Yan and colleagues [22] over-expressed a root-preferred expansin gene (*AtEXP2*) from *Arabidopsis* and showed that transgenic *Arabidopsis* seeds germinated much sooner than controls. Over-expression of this gene also provided tolerance to osmotic stress during germination.

Coh and colleagues [23] expressed the cucumber expansin gene in *Arabidopsis* using an inducible promoter. Their
conclusions were that the effects of expansin expression on growth are tissue, temporal and organ growth pattern dependent, suggesting a role of expansin in directional cell expansion. The authors also concluded that excess expansin may impede normal activities of cell wall modifications, resulting in growth promotion and/or repression depending on the amount of protein accumulating. Li and co-authors [24] showed that constitutive over-expression of the wheat EXPB gene in tobacco and Arabidopsis caused primarily faster growth, earlier flowering and shorter plants. When using a stress inducible promoter, however, these pleiotropic effects were reduced, and interestingly, these plants had increased tolerance to water stress. Physiologically, wheat genes expressed in Arabidopsis showed similar functions to their homologous counterparts from Arabidopsis, suggesting conserved function [25]. Although these several studies expressing either a native gene or a heterologous gene have shown expansin expression in transgenic systems, they all showed that growth abnormalities and pleiotropic effects occurred without high accumulation of the expansin protein in the transgenic plants. The Hood laboratory has expressed the cucumber expansin gene in transgenic maize seed without any apparent growth abnormalities [3]. Our goal was to develop a system that would overexpress expansin so that copious amounts of the protein could be produced for utilization in the manufacture of biobased products. However, a high throughput assay for expansin was needed to screen these plants. Thus, we determined that E. coli or transient protein expression was necessary. This paper describes our multiple unsuccessful attempts to overexpress expansin in a fast heterologous system in order to produce a sufficient amount of recombinant protein for assay controls and for antibody generation.

Why were these attempts to produce expansin unsuccessful? Although these experiments do not directly address the mechanism of low expression, we hypothesize that the protein itself is having a negative effect on the tissue of choice. Each of the constructs utilized—promoter and signal sequence cassettes, has been used to express successfully other genes of interest including the lectin binding domain of ricin and several human enzymes [9, 26, 27] in transient tobacco, and the Gateway system for E. coli expression of cellulases [16]. Indeed, the cucumber and bamboo constructs were expressed, albeit at a very low level, in N. benthamiana, much too low for producing protein for antibodies. Moreover, the globulin-1 promoter construct was highly active in maize [3], and a nearly identical construct was active in A. thaliana for the E1 cellulase reported here. Thus, it is unlikely that the lack of expansin expression was due to inactive constructs.

4. Conclusions

Industrial enzymes are generally required in large quantities and at low unit prices to assist with low production costs of commodity chemicals. The maize recombinant enzyme production system was chosen for expression of expansin as an accessory protein to cellulases for biomass conversion [3]. These plants could not be screened without generating a sensitive assay that would detect protein activity or presence in a single seed. Therefore, we attempted to exploit several heterologous protein expression systems that have been successfully utilized to express foreign proteins from multiple organisms to generate protein for production of antibodies. However, as demonstrated in the studies reported here, no expansin protein was recovered from the E. coli

Fig. 5. GUS staining of: A, transgenic inflorescence; B, transgenic leaf; C, transgenic silique; D, control silique; E, control inflorescence; F, control leaf.
system, the transient tobacco system, or stably transformed Arabidopsis seeds.

Acknowledgements

This project was supported in part by Department of Energy grant DE FG08 88025 to EH. The Arkansas Biosciences Institute is appreciated for partial support for supplies. JPYC’s stipend is supported by the Molecular Biosciences PhD program at Arkansas State University.

We gratefully acknowledge Dr. Daniel Cosgrove (The Pennsylvania State University) for providing the cucumber expansin gene and the anti-expansin antibodies used in this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neps.2016.03.002.

References
