New Negatives in Plant Science 3 (2016) 10–18

Contents lists available at ScienceDirect

New Negatives in Plant Science

journal homepage: www.elsevier.com/locate/neps

# Failure to over-express expansin in multiple heterologous systems

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ARTICLE INFO

*Article history:*
Received 22 January 2016
Received in revised form 8 March 2016
Accepted 18 March 2016

*Keywords:*
*Arabidopsis*
tobaccoTobacco
Cucumber expansin
Transient expression
Bialaphos selection
Bamboo expansin

ABSTRACT

*Background:* Expansin has been proposed to be an enhancer of cellulase activity in the deconstruction of biomass for sugars for industrial applications. However, the expansin protein is present in plant tissue only in minute quantities for promoting growth. Thus, producing adequate amounts of expansin for applications in industry will require a heterologous system that will over-express an expansin gene to produce large quantities of expansin protein. Development of a production system requires a facile, rapid assay. However, because no straightforward assay for expansin protein exists, we attempted to make milligram quantities of the protein in a fast or transient system for anti-expansin antibody preparation for use on Western blots or in ELISA assays.

*Results:* We tested the expression of the cucumber expansin gene in several heterologous systems including *Escherichia coli* and transient *Nicotiana benthamiana* leaves with limited success. We also had limited success in transiently expressing an alternative expansin gene from bamboo in *N. benthamiana* leaves. In order to determine if expansin over-expression is limited to a seed system, *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. coli*, transient tobacco leaves, and *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.



## 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 *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 *E. coli* and the transient *Nicotiana benthamiana* (wood tobacco) system using a fusion with a poly-histidine tag. As reported here,

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http://dx.doi.org/10.1016/j.neps.2016.03.002

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 the *Xba*I/*Sac*I 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 (bphyem105j06, 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 *Xba*I/*Sac*I 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 Streatfield 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 pBIB-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, 0.3g/L $MgSO_4{\cdot}7H_2O$, 0.15 g/L KCl, 0.01 g/L $CaCl_2$, 0.0025 g/L $FeSO_4 \cdot 7H_2O$, 2 mL/L 1 M $NaH_2PO_4$ pH 7.0, 10 g/L glucose) containing 100 μg/mL kanamycin, 100 μg/mL streptomycin, and 200 μM acetosyringone. 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

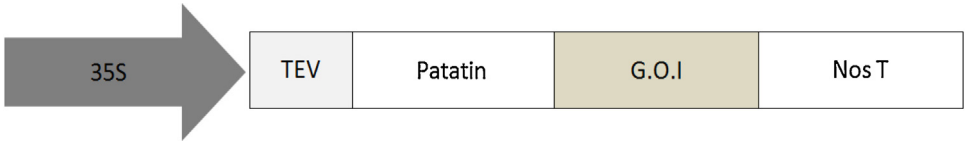


**Fig. 1.** Vectors used for plant transformation in A) tobacco and B) *Arabidopsis*. Abbreviations: 35S, constitutive promoter from the cauliflower mosaic virus (CaMV); TEV, tobacco etch virus leader sequence; patatin, signal sequence from the potato storage protein, patatin; GOI, gene of interest—in this case expansin; NosT, nopaline synthetase gene terminator from *Agrobacterium tumefaciens.*