DOE/ER/12194--T2

OVERVIEW

The goal of this project is to facilitate conversion of plant biomass to usable energy by developing transgenic plants that express genes for microbial cellulases, which can be activated after harvest of the plants. In particular, we want to determine the feasibility of targeting an endoglucanase and a cellobiohydrolase to the plant apoplast (cell wall milieu). The apoplast not only contains cellulose, the substrate for the enzymes, but also can tolerate large amounts of foreign protein. To avoid detrimental effects of cellulase expression in plants, we have chosen enzymes with high temperature optima; the genes for these enzymes are from thermophilic organisms that can use cellulose as a sole energy source.

During the past year (year 2 of the grant), we have focused our efforts on testing expression of endoglucanase E1, from *Acidothermus cellulolyticus*, in the apoplast of both tobacco suspension cells and *Arabidopsis thaliana* plants. Using the plasmids we constructed during the first year of the grant, we have obtained transgenic cells and plants that contain the gene for the E1 catalytic domain fused to a signal peptide sequence. We constructed this gene so that the fusion protein will be secreted into the apoplast. Our results are quite exciting. The enzyme is made in large quantities and is secreted into the apoplast. More importantly it is enzymatically active when placed under optimal reaction conditions (high temperature). Moreover, the plant cells and intact plants exhibit no obvious problems with growth and development under laboratory conditions. We have also continued our work to improve binary vectors for *Agrobacterium*-mediated transformation, to determine activity of E1 at various temperatures, and to investigate the activity of the 35S Cauliflower Mosaic Virus promoter in *E. coli*. Details of our findings are presented below.

PUBLICATIONS

We now have one paper in press and several in preparation (summer, 1998 as target date for completion).

Hennegan, K. P. and K. J. Danna (1998) pBIN20: An improved binary vector for *Agrobacterium*-mediated transformation. *Plant Mol. Biol. Reporter* (in press, June issue)

Ziegler, M. and K.J. Danna (in preparation) Thermostable endoglucanse E1 from *Acidothermus cellulolyticus* retains activity when expressed in the apoplast of tobacco BY-2 suspension cells (tentative title). We plan to submit this paper to *The Plant Cell.*

Ziegler, M. and K.J. Danna (in preparation) Expression of thermostable endoglucanase E1 from *Acidothermus cellulolyticus* in the apoplast of transgenic *Arabidopsis thaliana* plants (tentative title). We plan to submit this paper to *Plant Physiology.*

Ziegler, M. and K.J. Danna (in preparation) Cellulase zymogram assays: Inclusion of substrate in gels eliminates need for overlays. We plan to submit this paper to *BioTechniques.*

Jensen, E. and K.J. Danna (in preparation) Rapid screening of Agrobacterium colonies for presence of binary vectors. We plan to submit this paper to *BioTechniques.*

DISCLAIMER

PLASMIDS USED FOR PLANT EXPRESSION

General Features: The expression plasmids we have developed for plant transformation have the following elements:

35S CaMV promoter - Ω - Pr1a - transgene - polyA signal

The 35S CaMV promoter drives constitutive transcription in plants. The Ω sequence, a translational enhancer from tobacco mosaic virus, increases the efficiency of translation. The Pr1a sequence is an amino terminal signal peptide from a tobacco protein fused to the transgene protein sequence in frame. The signal peptide directs the protein to the endoplasmic reticulum (ER) where the signal peptide is removed. By default, proteins that enter the ER will be secreted into the apoplast. The polyA signal at the 3' end of the gene ensures proper mRNA processing.

Specific binary vectors used for *Agrobacterium*-mediated transformation contain a selectable marker for selection of transformed plants as well as border sequences needed for mobilization of the DNA. In addition, we have employed a reporter protein, the green fluorescent protein (GFP), to determine whether our targeting scheme is effective.

Improvement of binary vectors: We have completed our improvement of binary vectors, pOCA28 and pBI121(derived from pBIN19) which we need for *Agrobacterium*-mediated transformation. We added a polylinker that contains sites for 45 enzymes. Based on the deduced sequence, our vector pBIN20 (a derivative of pBI121) has a total of 30 unique restriction enzyme recognition sites, 21 of which are located in the multi-cloning site that we added. As this vector will be the most useful in the research community, we submitted a paper about it (now in press; see publications).

Construction of plasmids for expression of cellulases in plants: We have constructed expression cassettes that contain the regulatory elements listed above with either *Xho*I or *Sac*I as convenient cloning sites for insertion of cellulase genes. We now have a series of small *E. coli* plasmids that contain transgenes for the following proteins: E1 holoenzyme (includes the catalytic domain, linker and cellulose binding domain); E1 catalytic domain; CBHI catalytic domain; GFP with an ER retention signal; and GFP without an ER retention signal. For each of these, we have transferred the expression cassette region to our improved binary vector (renamed pBIN20) and made stocks of both *E. coli* and *Agrobacterium* that carry the binary plasmid. The latter can be grown and used directly for plant and plant cell transformation.

CELLULASE ASSAYS

We now have purified CBHI and E1 enzymes as well as specific antibodies against each enzyme, thanks to Dr. Steve Thomas at NREL. These will serve as positive controls for both biochemical assays of cellulase activity as well as Western blots to detect the proteins. An example of a gel diffusion assay to measure endoglucanase activity is shown in Figure 4 in a later section.

We have also set up a quantitative assay using 4-methylumbelliferyl β-D-cellobioside (MUC) as a substrate. When cleaved by a glucanase, this substrate releases the fluorigenic compound MU, which can be quantitated with a

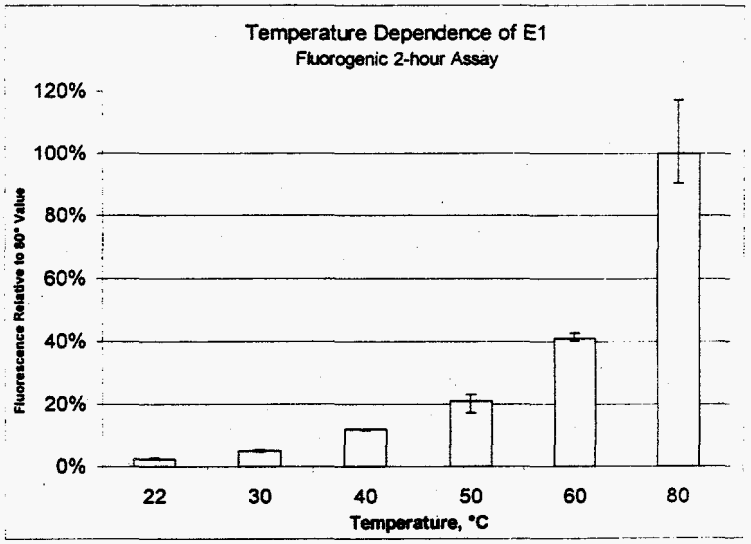

Figure 1

fluorimeter. Figure 1 shows the relative activity of E1 at various temperatures. The source of enzyme was culture medium of *E. coli* expressing the E1 gene from our plasmid pE1. We assayed E1 activity over a temperature range of 22-80°C for 2 hours by monitoring enzymatic release of fluorescent MU. The activity of E1 at 22°C is <3% that at 80°C. Thus, E1 activity should be minimal at the growth temperature of many plants.

TARGETING PROTEINS TO THE APOPLAST

To test our expression cassettes for effective targeting of proteins to the apoplast (cell wall), we used GFP as a reporter protein. Using *Agrobacterium*-mediated transformation, we transformed tobacco BY-2 cells with our binary vector containing GFP fused to the signal peptide Pr1a; the GFP should be secreted into the apoplast. As a control for this experiment, we used a binary plasmid from J. Haseloff; this plasmid contains GFP fused to an amino terminal signal peptide and a carboxyl terminal ER retention signal (HDEL). The control protein is localized to the endoplasmic reticulum (ER). We selected transformed calli on agar plates containing kanamycin and subcultured the clones as both callus cultures and suspension cultures in liquid medium. Transformed calli fluoresce bright green under a handheld mineral light (UV). To determine intracellular localization of protein, we examined cells microscopically, using both epifluorescence and laser scanning confocal microscopes. Figures 2 and 3 show confocal images of transformed cells.

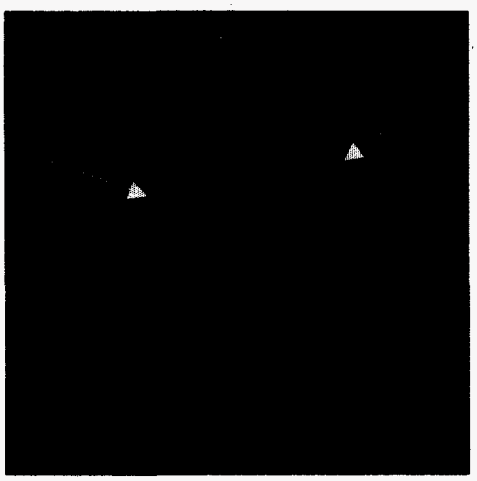

Figure 2

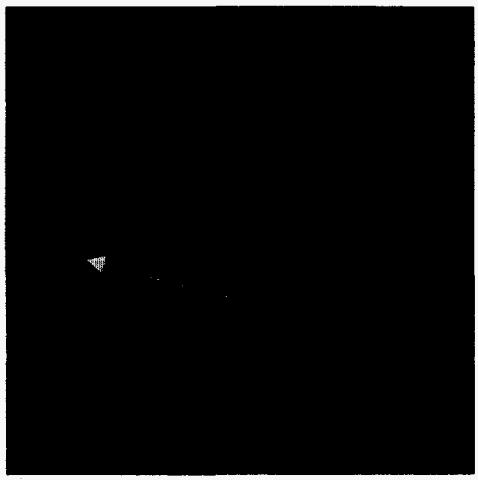

Figure 3

Figure 2 shows a confocal image of a pair of adjacent BY2 cells expressing ER-localized GFP. Confocal images scan a thin section through a cell. The nuclear envelope appears faintly in the upper cell ( ) and the periplasmic ER is prominent in both cells. The intercellular space ( ) corresponding to the apoplast (cell wall) is devoid of GFP.

Figure 3 shows a confocal image of several adjacent cells expressing the apoplast-targeted GFP. Again, GFP appears in the ER, since it must travel through the ER before it is secreted. The space between adjacent cells ( ) is filled with GFP illustrating that GFP is targeted properly to the apoplast. This image has a higher magnification than that in Figure2.

TARGETING CELLULASE E1 TO THE APOPLAST OF BY-2 CELLS

After confirming that our targeting strategy resulted in secretion of GFP in our controls, we transformed BY-2 cells with the plasmids that contained either the E1 holoenzyme or the E1 catalytic domain for apoplast targeting. In three attempts, we obtained no transformants with the holoenzyme, whereas we obtained numerous transformants with the catalytic domain alone (note that the catalytic domain of the enzyme retains enzymatic activity under optimal reaction conditions *in vitro*). This result suggests that either the cellulose binding domain (CBD) of E1 is itself deleterious to plant cells or that it is the combination of the CBD with the catalytic domain that is deleterious (*e.g.*, a tethered catalytic domain might perturb the cell wall more than a freely diffusible catalytic domain).

We picked several microcalli that were transformed with the gene for the apoplast-targeted E1 catalytic domain for further characterization. We wanted to determine whether the transformed cells express the E1 protein, whether E1 retains enzymatic activity, and whether the cell lines grow normally.

Do the transformants synthesize E1 protein? To answer this question, we made extracts of transformed cells and subjected them to Western blot analysis. The proteins in the extract were separated by size on an SDS-polyacrylamide gel and then transferred to a membrane. The membrane was then probed with specific antibody for E1 and then with a secondary antibody that is tagged to give a visible color. At each step, unbound antibodies are removed by washing. Figure 4 shows the result of this experiment.

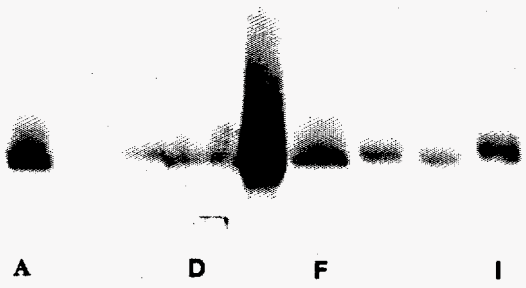

Figure 4. Western blot of BY-2 calli expressing apoplast-targeted E1. Lanes A,C,D, and F-I contain 4 micrograms of total protein from extracts of seven independent transformants. Lane B (negative control) contains 80 micrograms of total protein from non-transformed BY-2 cells. Lane E contains 1 microgram of purified E1 catalytic domain. The molecular weight of the bands is about 40,000 kD, as expected for the catalytic domain of E1. All of the transformants express a protein that migrates with the same mobility as purified E1 and that is specifically recognized by anti-E1 antibody. The negative control (overloaded by a factor of 20) contains no proteins that interact with anti-E1 antibody.

Conclusion: BY-2 cells transformed with the gene for apoplast-targeted E1 catalytic domain synthesize E1 protein.

Do the transformants secrete active E1 enzyme? To answer this question, we grew calli on nutrient agar that contained the E1 substrate CMC. After 24 hours, we removed the calli to a fresh plate for maintenance, heated the original plate (with CMC) to 80°C for one hour to activate any enzyme that the cells had secreted into the agar, and then stained the agar with Congo Red. Because Congo Red stains CMC, areas of the plate that contained active E1 (*i.e.*, where the substrate would be degraded) would appear as clear haloes. Figures 5 and 6 show the results.

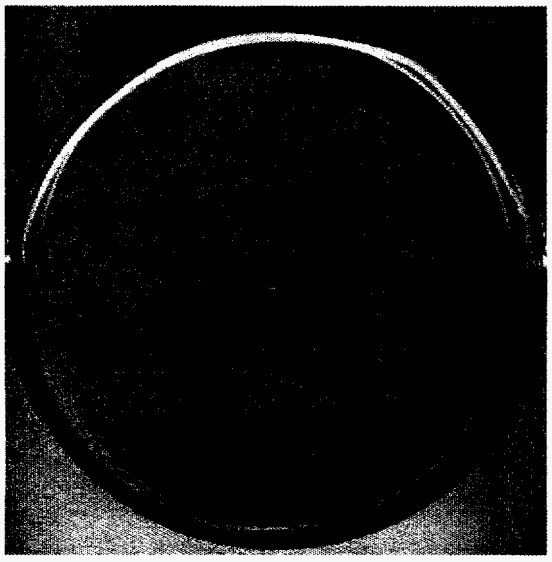

Figure 5

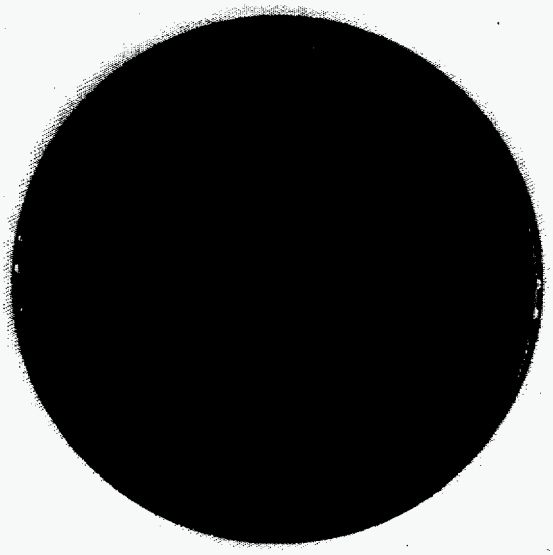

Figure 6

Figure 5. Plate showing calli grown on CMC agar for 24 hours at 26°C. Green boxes indicate two negative control calli that expressed GFP but no E1. The other calli were transformed with the gene for apoplast-targeted E1.

Figure 6. Calli were removed from the plate shown in Figure 4. The plate was incubated at 80°C for one hour and then stained with Congo Red. Green boxes surround the areas on which the negative control were located.

Large haloes surround calli that express E1. The sizes of the haloes vary because each callus represents an independent transformant and the levels of expression of the transgene can vary. In this kind of assay, nontransformed BY-2 calli cause a slight clearing only at sites directly in contact with the callus. E1 that is secreted into the apoplast diffuses into the agar, and the size of the halo is proportional to enzyme concentration.

Conclusion: BY2 cells transformed with apoplast-targeted E1synthesize **active** enzyme and secrete it.

Do transformants grow normally? To answer this question, we established suspension cultures of each of four independent transformants and determined growth curves in parallel with non-transformed BY-2 cultures. After seeding cultures, we incubated them at 26°C with constant agitation at 120 rpm. We sampled 0.5-ml aliquots of each suspension at various time after seeding, pelleted the cells in a calibrated tube, and determined the packed cell volume. Because these cells grow in clumps, cell counting with a hemacytometer is impossible. Figure 7 summarizes the data.

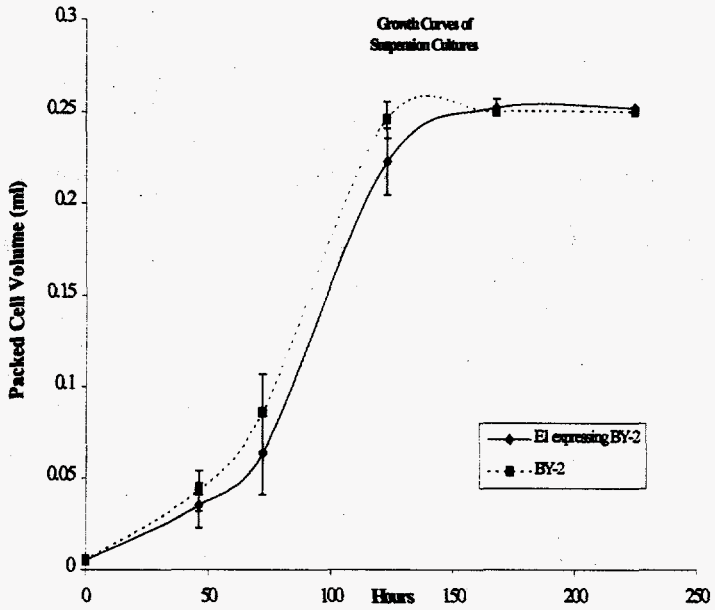

Figure 7. Growth curve of non-transformed BY-2 suspension cells and of BY-2 cells transformed with apoplast-targeted E1. Data from five cultures of non-transformed cells were averaged. Data from four independent transformants run in duplicate were averaged (eight samples total per point). The time after seeding at which the aliquots were removed is indicated in hours. None of the individual cultures deviated significantly from the average.

Conclusion: By-2 cells expressing E1 in the apoplast do not exhibit an altered growth pattern.

TARGETING E1 TO THE APOPLAST OF *ARABIDOPSIS THALIANA* PLANTS

Having demonstrated that plant suspension cells can express active E1 in the apoplast, we wanted to test whether intact plants could tolerate similar levels of expression. Our ultimate goal is to use transgenic plants for large-scale production of enzyme. Because it is easily grown and transformed, *Arabidopsis thaliana* is an ideal plant for this feasibility study. It is a fast-cycling brassica.

We used the same binary plasmid as for the previous experiments to transform *Arabidopsis*. When plants begin to bolt, the inflorescences are dipped for five seconds into a slurry of Agrobacterium that carries the plasmid. The pots are laid on their sides for 24 hours and then the plants are allowed to continue growth normally. Seeds are collected and germinated on selection plates that contain kanamycin. Non-transformed seedlings do not grow whereas transformed seedlings turn green and form roots. Transformants are subcultured onto a fresh selective plate and are eventually placed in soil.

To test for expression of E1 in our transformants, we have thus far performed two kinds of experiment. The first is a Western blot analysis of protein extracts made from the transformants to detect E1 protein. The second is a zymogram assay in which protein extracts are run on a non-denaturing gel that contains CMC, the enzyme substrate. The gel is incubated at high temperature to activate the enzyme and is stained with Congo Red to detect clear areas that contain activity. In both assays, we included negative controls of nontransformed plants. Results of these two experiments are shown in Figures 8 and 9.

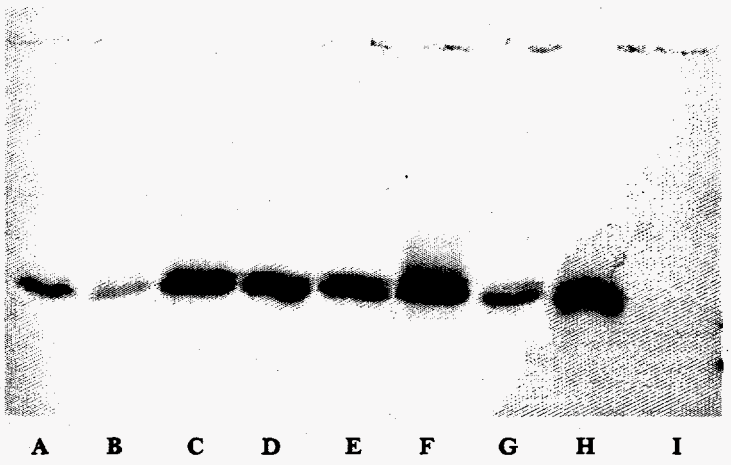

Figure 8. Western blot of extracts from transformed *Arabidopsis thaliana* plants. Lanes A-F contain one microgram of total protein from six independent transformed plants. Lane G contains 15 nanograms of purified E1. Lane H contains 150 nanograms of purified E1. Lane I (negative control) contains 40 micrograms of protein from non-transformed *Arabidopsis* plants.

Conclusion: Transformed *Arabidopsis* plants synthesis apoplast-targeted E1 catalytic domain.

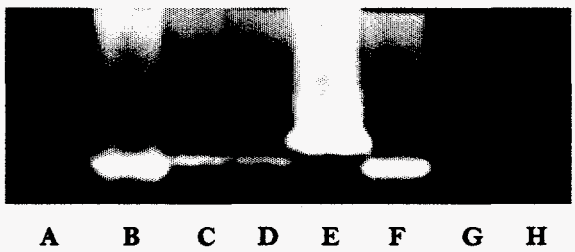

Figure 9. Zymogram assay of extracts from transformed *Arabidopsis* plants expressing apoplast-targeted E1 catalytic domain. Extracts were run in a native gel that contained CMC. The gel was heated for one hour at 80°C, and remaining substrate was stained with Congo Red. Clear areas indicate enzyme activity. Lane A (negative control) contains 40 micrograms of nontransformed plant extract. Lanes B-D and F-H each contained one microgram of extract from a transformed plant. Lane E contained 5 micrograms of purified E1 enzyme as a positive control.

Conclusion: The E1 enzyme produced in transformed *Arabidopsis* plants is active.

The transformed plants did not exhibit any abnormal growth or developmental problems when grown at 22°C.

ACTIVITY OF CAULIFLOWER MOSAIC VIRUS 35S PROMOTER

The CaMV35S promoter has high activity in plant cells but no reported activity in *E. coli.* We have followed up on our initial observation that the promoter is active in E. coli by constructing a series of plasmids to determine levels of activity. We have a positive control containing a bacterial promoter; a negative control with the Pr1a signal peptide sequence in place of a promoter; the double 35S promoter; and the single 35S promoter. Each plasmid has the kanamycin resistance gene whose expression would be controlled by the promoter or control sequence. Bacteria harboring the positive control plasmid or the one containing the double 35S promoter grown on kanamycin agar, but the latter requires a longer period of time to form colonies. Bacteria containing either of the other two plasmids as well as the bacterial strain without a plasmid fail to grow. Using ELISA assays, we have measured the amount of protein corresponding to the kanamycin resistance gene and found that the level of expression with the double 35S promoter is less than 3% that of the bacterial promoter.

GOALS FOR THE NEXT YEAR

1) **Continue characterization of transformed BY-2 cells and Arabidopsis thaliana plants expressing the E1 catalytic domain.** We want to determine the amount of E1 protein by ELISA, to determine the specific activity of the protein, and to determine whether the protein is glycosylated. We also want to determine the number of loci in our transformed plants and to obtain homozygous plants (from the second generation) that will breed true. In addition, we will determine if the plants can grow at elevated temperatures since the enzyme will have greater activity and will confirm the apoplast localization by performing apoplast extractions as well as immunofluorescence assays.

2) **Continue with our work to express the CBHI catalytic domain in BY-2 cells and *Arabidopsis* plants.**

3) **Follow up on our observation that the CBD of E1 is apparently deleterious. Is it the CBD alone or the CBD plus catalytic domain?** We will link GFP to the CBD and determine whether this fusion protein can be expressed in the apoplast.

4) **Continue determination of the activity of the CaMV 35S promoter in E. coli. We need to normalize extracts for plasmid copy number and repeat the ELISA experiments.**

5) **Scale up our experiments by expressing E1 and/or CBHI in tobacco plants and perform pilot biomass conversion assays.**