Note

Overproduction of Hyperthermostable β-1,4-Endoglucanase from the Archaeon Pyrococcus horikoshii by Tobacco Chloroplast Engineering

Yoichi NAKAHIRA, Kazuhiko ISHIKAWA, Kunisuke TANAKA, and Takashi SHIINA

1 Venture Business Laboratory, Ehime University, 3 Bunkycho, Matsuyama, Ehime 790-8577, Japan
2 Graduate School of Life and Environmental Sciences Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan
3 Phytoculture Control Co., Ltd., Jyoto-ku, Osaka 536-0008, Japan
4 Biomass Refinery Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 3-11-32 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan
5 Proteo-Science Center, Ehime University, 3 Bunkycho, Matsuyama, Ehime 790-8577, Japan

Received May 22, 2013; Accepted July 2, 2013; Online Publication, October 7, 2013

One of the most cost-effective methods of producing industrial enzymes is by the use of transgenic plants. We demonstrated successful high-level expression of a hyperthermostable archaeal β-1,4-endoglucanase in mature tobacco leaves by transformation of chloroplasts by homologous recombination. The active recombinant enzyme was readily recovered not only from fresh but also from dried leaves.

Key words: chloroplast transformation; tobacco; lignocellulosic biomass; β-1,4-endoglucanase; Pyrococcus horikoshii

Lignocellulosic biomass is a renewable resource that can be converted into the fermentable sugars necessary for the production of biofuels and other industrial chemicals through the action of hydrolytic enzymes such as cellulases, but the cost of enzyme production is a major barrier to commercialization of lignocellulosic products. One possible way to produce enzymes cost-effectively is to use transgenic plants. In comparison with microorganism-based recombinant protein production, plant-based systems offer the prospect of massive, scalable production without requiring expensive equipment, allowing the industrial-scale production of low-cost enzymes. In addition, the residue of the transgenic plants remaining after enzyme extraction can be used as feedstock from which to generate fermentable sugars. Conventional nuclear genome transformation has been used to introduce bacterial and fungal cellulase genes into plants in order to produce cost-efficient industrial enzymes, but the recombinant enzymes were reported to accumulate at unexpectedly low levels, up to only a few percent of total soluble protein (TSP). An alternative approach to the achievement of high-level enzyme production in plants is through chloroplast transformation. In contrast to nuclear transformation, highly polyploid chloroplast genomes allow the integration of thousands of copies of transgenes per cell, which can result in extremely high expression of foreign proteins in plants, up to 5–20% of TSP. Here we achieved high levels of expression of a hyperthermostable β-1,4-endoglucanase from the archaeon Pyrococcus horikoshii (EGPh; GH family 5) by tobacco chloroplast engineering. This enzyme is capable of hydrolyzing cellulose at about 100 °C, a favorable characteristic for applications in biomass utilization.

To construct a chloroplast transformation vector for the expression of EGPh, the open reading frame of EGPh was amplified from pETEGDSC plasmid by PCR with primers 5'-CCATGGAAAATACAATCATA- TCAAACACCG-3' and 5'-TCAAAGAACCTTTTGGAA- CAACTATC-3'. The amplified DNA fragment was subcloned into pGEM-T vector (Promega). From the resulting plasmid, a 1.2-kb Ncol–NotI DNA segment including EGPh was excised and recloned into the corresponding sites of pMIK1 (Nozoe and Shiina, unpublished results), which harbors the tobacco psbA promoter followed by its 5'-untranslated region (UTR) (tobacco chloroplast DNA nucleotides 1,595–1,811: GenBank accession no. Z00044), a multiple cloning site, and the 3'-UTR of tobacco rps16 (tobacco chloroplast DNA nucleotides 4,939–5,091). The resulting EGPh expression cassette plasmid was digested with SacI and KpnI, and the excised 1.6-kb DNA segment was ligated into the corresponding sites of pKH3, generating chloroplast transformation vector pCEG1 (Fig. 1A). Transformation of tobacco (Nicotiana tabacum cv. Xanthi) chloroplasts was done using the Biolistic® PDS 1000/He particle delivery system (Bio-Rad), as described by Svab and Maliga (1993). Transgenic shoots were selected on a solid Murashige and Skoog (MS) agar medium containing 3% sucrose, 0.1 mg/L of 1-naphthaleneacetic acid, 1 mg/L of N⁶-benzyladenine, 1 mg/L of thiamine, 100 mg/L of inositol, and 200 mg/L of spectinomycin dihydrochloride. Spectinomycin-resistant shoots were transferred to MS agar medium containing 3% sucrose and 500 mg/L of spectinomycin dihydrochloride for rooting. The resulting chloroplast-transformed lines were further subjected...
to several rounds of regeneration in order to ensure that all of the chloroplast DNA copies carried the transgene. Site-specific integration of the target DNA was confirmed by DNA gel bloting. The probe, covering trnV and its flanking region, was generated with a PCR DIG Probe Synthesis Kit (Roche) with wild-type tobacco and its flanking region, was generated with a PCR DIG Probe Synthesis Kit (Roche) with wild-type tobacco total DNA as template with primers 5'-CCAGAAAT-ATAGCCATCCCTGGCCCTC-3' and 5'-TCTGAT-GAATTTGTTGGCACCAGTCTCATC-3'. As shown in Fig. 1A, the sizes of the signals for the SacII-digested DNA fragments were expected to be 1.5 kb for the wild-type chloroplast and 4.5 kb for the transformed chloroplast DNAs. There was no detectable signal of the 1.5-kb band in the chloroplast-transformed lines (Fig. 1B). This indicates that all copies of the chloroplast genome were transformed (homoaplasmic) in these transgenic lines (CEG1-1 and CEG1-2). Two independent homoplasmic lines were obtained through 12 biolistic bombardments of tobacco leaves. The homoplasmic T1 lines were grown and fertilized for seed collection. Plants of the T1 progeny were used in all subsequent experiments. Since two lines showed similar phenotypes and high levels of accumulation of the target enzyme, we mainly used CEG1-1 in further analysis.

The chloroplast-transformed (CEG1-1) and wild-type tobacco plants were grown in soil at 25 °C under 100 µmol photons/m²/s white fluorescent light under a light cycle (16 h light and 8 h dark). Although the CEG1-1 plants exhibited a somewhat pale-green color and a slower growth rate than the wild-type plants, the height of the transgenic plants was comparable to that of the wild-type plants at the beginning of the flowering stage (Fig. 2).

To examine the expression of the EGPh protein, TSP was prepared from young (upper) and mature (non-senescent lower) leaves. Two-hundred mg of fresh leaves was homogenized in 1 mL of buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol, and a protease inhibitor cocktail (Roche). We obtained 1.8–3.5 mg of TSP from 200 mg of fresh leaves. The extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue (CBB) staining. Predominant bands, corresponding to the approximately 40 kDa EGPh protein, were detected in extracts from both young and mature CEG1-1 leaves, but not in ones from the wild-type plants (Fig. 3A). A polyclonal antibody raised against EGPh specifically recognized the corresponding bands (Fig. 3B), indicating that the 40-kDa protein was the EGPh gene product. The levels of EGPh accumulation were quantified by immunoblot analysis by comparison with a dilution series of purified C-terminal 6 × His-tagged EGPh (EGPh-His) (Fig. 3B). Three independent experiments indicated that EGPh accounted for up to approximately 25% of the TSP from the mature leaves. Heat treatment of the TSP at 85 °C for 20 min resulted in denaturation and decreased solubility of most of the native proteins, which were readily removed by centrifugation. By contrast, more than half of the chloroplast-expressed EGPh protein was resistant to heat denaturation (Fig. 3A). These results indicate that large amounts of soluble hyperthermostable EGPh accumulated in the tobacco chloroplasts in the mature leaves.

In order to test whether the chloroplast-expressed EGPh protein was functional, we examined its hydrolitic activity with carboxymethyl cellulose (CMC) as
Fig. 3. High Accumulation of Archaeal Hyperthermostable β-1,4-Endoglucanase in Mature Leaves of Chloroplast-Transformed Tobacco.
A. SDS–PAGE of TSPs extracted from young (Y) and from mature (M) leaves of adult plants. TSPs from wild-type (WT) and from chloroplast-transformed (CEG1-1) plants were resolved by SDS–PAGE and stained with CBB (indicated as “TSP”). Each lane represents 5 μL of TSP. TSPs were further incubated at 85 °C for 20 min and centrifuged at 20,000 g for 5 min to remove denatured insoluble proteins. Samples (5 μL) of the resulting supernatants were analyzed by SDS–PAGE (“Heated”). Two μg of C-terminal 6 × His-tagged EGPh (EGPh-His), which was expressed in E. coli and purified with a TALON® Disposable Gravity Column (Chromtech), was used as control. The 40-kDa band corresponding to EGPh protein is indicated by an arrow on the right. The EGPh-His protein was slightly larger than the EGPh from the transgenic plants due to the presence of the C-6 × His tag. B. Immunoblot analysis to detect the EGPh protein. The indicated amounts of TSP prepared from mature leaves of CEG1-1 were analyzed. TSP extracted from wild-type tobacco leaves (WT) was loaded as control. A dilution series of purified EGPh-His was analyzed as reference. Fifty ng of TSP was estimated to contain approximately 12.5 ng of EGPh protein. C, SDS–PAGE analysis of TSP extracted from fresh and from dried leaves of chloroplast-transformed plants. Mature leaves from CEG1-1 were harvested and divided into two groups. One was used immediately for TSP preparation (“Fresh”), and the other was dried at 60 °C for 48 h and then used for TSP preparation (“Dried”). TSP preparation from the dried leaves was done after rehydration of the samples with water equivalent to the amount of weight loss during desiccation. The “Heat” samples were prepared as described in Fig. 3A. For each sample, 5 μL was separated by SDS–PAGE and subjected to CBB staining.

In this assay, aliquots containing 500 ng of TSP from CEG1-1 mature leaves were added to 400 μL reaction mixtures containing 100 mM acetate buffer (pH 5.5) and 0.5% (w/v) CMC. The reactions were incubated at 85 °C for 10 min, and the resulting reducing sugar was measured by a modification of the Somogyi-Nelson method.33) To calculate the amount of sugar generated by the chloroplast-expressed EGPh protein, the amount of background sugar in TSP from wild-type leaves was subtracted from the amount generated by TSP from transgenic plants. One unit of enzyme activity was defined as 1 μmol/mL of glucose equivalent released per min in the reaction mixture. The CMC hydrolytic activity of the leaf extracts of the transgenic plants was calculated to be 20.5 ± 2.0 U/mg of TSP. Considering the levels of EGPh protein in the mature leaves (approximately 25% of the TSP), the specific activity of the plant-derived recombinant EGPh was estimated to be 82.0 ± 8.0 U/mg of protein (20.5 ± 2.0 U/mg of TSP was divided by 0.25 μg of EGPh protein/mg of TSP), which corresponds to approximately 60% of the specific activity of the purified EGPh-His protein (141.9 ± 6.6 U/mg of EGPh-His). The activity of EGPh-His was not inhibited in the leaf extracts from the wild-type plants, suggesting that tobacco leaves contain no inhibitory factors (data not shown). Presumably, this apparent reduction is due to the presence of a certain amount of inactive EGPh in TSP from mature leaves.

We examined the stability of the recombinant EGPh proteins in the dried leaves. EGPh was recovered efficiently from leaves that were desiccated at 60 °C for 48 h (Fig. 3C). TSP prepared from fresh and from dried leaves was further subjected to heat treatment (85 °C for 20 min) to obtain partially purified EGPhs (indicated as “Heat” in Fig. 3C). We performed a CMC hydrolytic assay with 100 ng of heat-treated EGPhs, and found that the EGPh prepared from the dried leaves (120.5 ± 2.6 U/mg of protein) had CMC hydrolytic activity comparable to that prepared from fresh leaves (126.9 ± 3.3 U/mg of protein). This indicates a potential for cost-effective storage of the chloroplast-expressed enzyme as dried plant material at room temperature. In addition, the specific activity of the heat-treated EGPh (126.9 ± 3.3 U/mg of protein) from fresh leaves was higher than that in TSP (82.0 ± 8.0 U/mg of protein), suggesting that inactive form(s) of EGPh in mature leaves were readily removable through heat treatment.

This study indicates that a hyperthermostable archaeal β-1,4-endoglucanase can be expressed efficiently in tobacco chloroplasts, and that the active enzyme can be readily recovered not only from fresh but also from dried leaves. In addition to our work, other recent research has also illustrated high expression of cell wall-degrading enzymes derived from bacteria and fungi by chloroplast engineering.3–11) These studies together provide a basis for cost-effective production of biomass-degrading enzyme cocktails in plants.

Acknowledgment

This work was supported in part by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) (to Y.N.), and by the Program for Academic Contribution to Region (ACTR) of Kyoto Prefectural University (to T.S.).
References