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Addition of 2,4-D Enhances Excretion of Barley Lectin in Transgenic Tobacco Cells of Vacuole Targeting Signal Deleted Mutant

[1]Asuko ISHIHARA, [1]Setsuko WAKAO, [1]Yasunori TANJI, [2]Xin-hui XING, [1]Katsutoshi HORI, [1]Kazuhiko MIYANAGA and [1]Hajime UNNO

[1]*Graduate School of Bioscience and BiotechnologyTokyo Institute of Technology 4259 Nagatsutacho, Midori-ku, Yokohama, 226-8501,Japan*
[2]*Division of Materials Science and Chemical Engineering Yokohama National University 156 Tokiwadai, Hodogaya-ku, Yokohama, 240-0067,Japan*
Corresponding author E-mail address: ytanji@bio.titech.ac.jp

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Abstract

A suspension culture of tobacco cells was transformed with a gene encoding barley lectin to obtain an efficient production system of lectin. Lectin excreting transgenic tobacco was constructed by transformation with barley lectin deleted of the C-terminal vacuole targeting peptide (CTPP). 2,4-Dichlorophenoxyacetic acid (2,4-D) was examined for its effect on the excretion of lectin at different concentrations from 0 to 5 mg l^{-1}. 2,4-D addition increased excretion efficiency, defined as the amount of excreted lectin per cell, but reduced the growth rate. Microscopic observations showed loosening of the cell wall, which is assumed to be one of the causes of the enhanced excretion. Decrease in extracellular lectin was observed in spite of the growth of cells, which was confirmed to be caused by a degradation process in the culture medium. These results suggest that an efficient production process of lectin would include a process of simultaneously removing culture medium with high lectin content before degradation occurs and maintaining the cells at high excretion efficiency.

1. Introduction

Lectin is a class of proteins existing in a wide range of plant species and other organisms. Though the functions of these proteins in plants are yet to be elucidated, their ability to recognize complex carbohydrates makes them a useful tool in many research fields (Loris *et al.*, 1998). Excretion of an object protein is of great industrial interest since it simplifies the recovery process of the protein. Native barley lectin (BL) is translocated through the endoplasmic reticulum to the vacuole according to the carboxyl terminal vacuolar targeting propeptide (CTPP). Deletion of this CTPP is known to result in the excretion of lectin to the medium in transgenic tobacco suspension cultures (Borrebaeck *et al.*, 1990, Schroeder *et al.*, 1993).

Many studies have shown plant hormone concentration to be involved in protein synthesis and secretion. In particular, lectin synthesis is reported to be under developmental regulation and accompanied by an increase in endogenous and exogenous auxin concentration (Borrebaek & Linsefors, 1985, James *et al.*, 1985). Plant hormone concentration is also reported to have an influence on the targeting of vacuolar proteins, such as by Kunze *et al.* (1998), who found that an increase in 2,4-Dichlorophenoxyacetic acid (2,4-D) resulted in the excretion of a vacuolar chitinase. In the present research, two approaches were taken to increase the productivity of BL in tobacco cells: first by deletion of CTPP to excrete BL, hence simplifying the recovery process, and second by addition of 2,4-D to enhance the excretion efficiency.

2. Materials and methods

2.1 Vector construction and transformation

The coding sequence of both the BL and CTPP-deleted BL (BLΔCTPP) genes were amplified by polymerase chain reaction (PCR) using pNVR2093 (offered by Professor N. V. Raikhel, Michigan State University) as the template. The PCR products were ligated to the *Xba*I/*Sac*I restriction sites to replace the β-glucuronidase (GUS) gene of the plant expression vector, pBI121 (Clontech), to create plasmids pBI121BL and pBI121ΔCTPP using

Escherichia coli HB101 as a host cell.

Tobacco BY-2 cells were transformed with the pBI121 derived constructs by *Agrobacterium*-mediated transformation (An *et al.*, 1985) using *Agrobacterium tumefacience* EHA105.

2.2 Transformed tobacco suspension culture

The transformed cells were cultured in a modified liquid Linsmaier-Skoog (LS) medium (Sekiya, 1991) with 0.2 mg l^{-1} 2,4-D and 3% (w/v) sucrose at 100rpm and 28 ℃ , and subcultured every 7 days with 4% (v/v) inoculum.

2.3 Time course experiments

The effect of 2,4-D on BL localization was investigated in cultures with 0, 0.2, 1, and 5 mg l^{-1} of 2, 4-D. Cells were inoculated to 100ml flasks containing 15ml medium with 0.1g-fresh weight (FW) of normally maintained transformants. For each sampling, cells were separated by filtration from the medium from individual flasks. The lyophilized medium was suspended in phosphate buffered saline (PBS) adjusted to pH7.4, as the extracellular fraction. The cells were homogenized in 2 volume of PBS and centrifuged at approximately 15000g for 10min. The resulting supernatant (soluble fraction) and pellet (insoluble fraction) together represent the intracellular fraction.

2.4 Analysis of BL degradation

The cells were removed from a 7-day-old culture of 0.2 mg l^{-1} 2,4-D by filtration. Degradation of extracellular BL was examined by further incubating the BL including medium under the same conditions. Another lectin, wheat germ agglutinin (WGA), was also examined for degradation in autoclaved and non-autoclaved medium to determine whether the degradation was biological.

2.5 Immunoblot analysis

Intra- and extracellular fractions were separated by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Since WGA is antigenically indistinguishable from barley lectin, anti-WGA polyclonal antibody and horseradish peroxidase-conjugated protein A were used as primary and secondary antibodies respectively, for immunoblot analysis following the method described by Wilkins *et al.* (1990). The immuno-detected BL was quantified by calculating the density of the scanned band with NIH Image 1.62 (National Institutes of Health).

3. Results and Discussion

3.1 Localization of BL

The localization of BL in BL-transformants and BLΔCTPP-transformants were examined in 7-day-old cultures with 0.2 mg l^{-1} 2,4-D cultures (**Fig.1**). In BL-transformants, two polypeptides corresponding to the 23-kD proprotein, which was synthesized as glycosylated form within the lumen of the endoplasmic reticulum (Mansfield *et al.*, 1988), and 18-kD mature subunit were detected in intercellular insoluble fraction (**Fig. 1A, lane 2**). But no BL product was detected in the extracellular fraction (**Fig. 1A, lane 3**). In BLΔCTPP-transformants on the other hand, 18-kD mature subunit of barley lectin was present in the culture medium (**Fig. 1B, lane 3**), indicating that the produced and

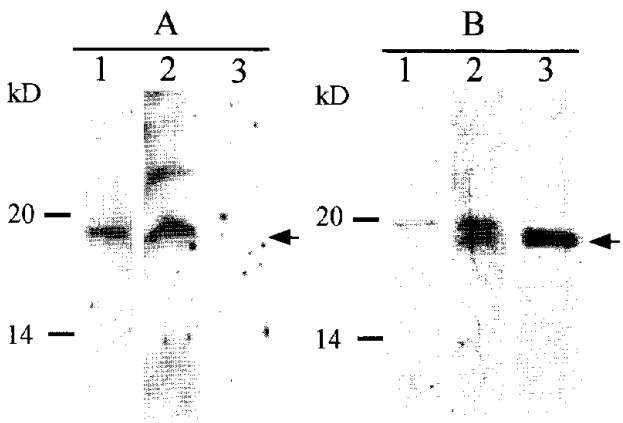

Fig. 1 BL localization in BL (A) and BLΔCTPP (B) transformants. Arrows indicate 18-kD, the size of mature BL. Lane 1: Intracellular soluble, lane 2: Intracellular insoluble, lane 3: Extracellular fraction. Molecular mass markers (in kilodaltons) are indicated on the right.

processed BL in the cell was excreted to the medium. Approximately 20-kD product was detected in the intracellular fraction in BLΔCTPP-transformants. The product was thought to be precursor protein with N-terminal signal peptide 26amino acid residues in length. Exhibited defect of processing in BLΔCTPP-transformants was not elucidated. Since no immuno reactive polypeptide was detected in non-transformed tobacco cells (data not shown), these results indicate that BL was successfully produced in tobacco cells by transformation with the BL gene and the deletion of CTPP enhanced excretion into the medium.

3.2 Effects of 2,4-D addition on lectin excretion

Lectin excreting cells cultivated in the usual 2,4-D concentration for callus (0.2 mg l^{-1}), exhibited normal growth consisting of lag, log, and stationary

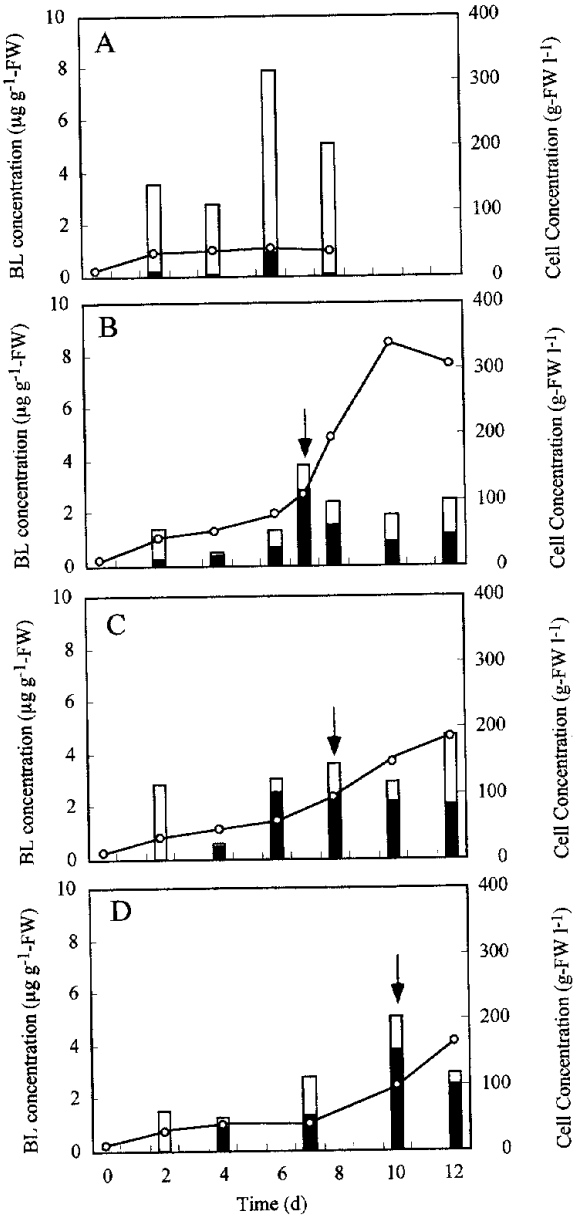

Fig. 2 Growth and BL localization of BLΔCTPP cells cultivated in 2,4-D concentrations of 0 (A), 0.2 (B), 1 (C), and 5 mg l^{-1} (D). (■) and (□) represent extracellular and intracellular fractions respectively. Arrows indicate where highest excretion efficiency was achieved.

phases (**Fig. 2B**), though with a lower rate than non-transformed cells. Growth rates of 1 and 5mg l^{-1} cultures (**Fig. 2C, 2D**) were lower than that of 0.2 mg l^{-1} culture, and 2,4-D free cultures (**Fig. 2A**) showing no growth. Optimal growth of cells was observed at 2,4-D concentration of 0.2 mg l^{-1}.

In 0.2, 1, 5 mg l^{-1} cultures, BL mainly accumulated intracellularly during lag phase, while highest excretion was observed at early log phase. Intracellular accumulation was observed throughout incubation time in 2,4-D free cultures. Maximum BL excreted to the medium was 0.89 $\mu g\ g^{-1}$ -FW at 6 days, 2.82 at 7 days, 3.77 at 10 days for 0, 0.2, 5mg

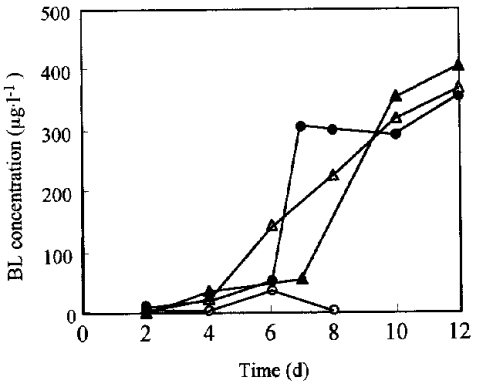

Fig. 3 Extracellular BL concentration of cultures in 2,4-D concentrations of 0 mg l^{-1} (○), 0.2 mg l^{-1} (●), 1 mg l^{-1} (△), and 5 mg l^{-1} (▲).

l^{-1} cultures, respectively. These results indicate that the addition of 2,4-D increased BL excretion efficiency, defined as the amount of excreted BL per cell.

Growth rate is of concern in considering productivity per cultivation. Extracellular BL converted to amount per volume-medium throughout the cultivation time is shown in **Fig. 3**. In 0.2 mg l^{-1} 2,4-D culture, a sharp increase was observed which correlated with the growth of cells (**Fig. 2B**). BL concentration stayed almost constant after reaching 304 $\mu g\ l^{-1}$ at 7 days incubation period. At higher 2,4-D concentration, increase was much slower, paralleling the low growth rate. The productivity for reaching the highest BL concentration per cultivation time was calculated, giving 43.5 $\mu g^{-1}\ d^{-1}\ l^{-1}$ at 7 days and 33.6 $\mu g^{-1}\ d^{-1}$ at 12 days for 0.2 and 5 mg l^{-1} 2,4-D.

3.3 Morphological changes by 2,4-D addition

It is known that 2,4-D is involved in the loosening of the cell wall by inducing expressions of cell wall degrading enzymes (Takeda *et al.*, 1996, Valero *et al.*, 1995). Transformed calli incubated in different 2,4-D concentrations were observed by microscopy (**Fig. 4**). Cells were long and singular in 2,4-D free culture, implying the abnormality of the cells, which usually grow by elongation and division to form aggregates of string-like cells. On the other hand, a higher density of round calli was observed at higher 2,4-D concentrations. The cause of cell roundness is assumed to be the loss of strength in the extracellular matrix, which is described as the loosening of the cell wall. The cells become unable to maintain their unequal distribution of pressure, hence altering their shapes. From reports on cell wall degrading enzymes being expressed by addition of auxin, 2,4-D is assumed to have induced such enzymes that promote loosening. It was speculated that BL that had already left the

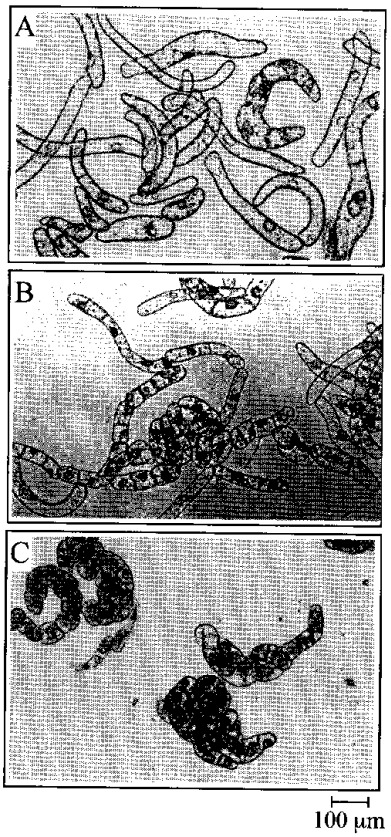

Fig. 4 Microscopic observation of 8d-old BLΔCTPP cells in 2,4-D concentrations of 0 (A), 0.2 (B), and 5 mg l^{-1}(C).

cell by deletion of CTPP but had been caught in the extracellular matrix, was released by cell wall degradation by 2,4-D addition, and it was this phenomenon that was seen as an increase of extracellular BL. Another possibility that cannot be ignored is that an increase in total protein synthesis including BL occurred that would naturally increase the amount of the detected extracellular BL. Amount of total protein synthesis was not examined in this research and remains to be investigated.

3.4 Degradation of extracellular BL

Extracellular lectin did not increase in 0.2 mg l^{-1} 2,4-D culture after 6 days despite the growth of cells. Degradation of BL was confirmed in the medium that was filtrated to eliminate new excretion from cells (**Fig. 5**). BL was rapidly degraded to 54% amount within 1 day when no BL was newly synthesized. To investigate whether this degradation process was of biological origin, WGA was added to this filtrated medium after autoclaving and its degradation was compared (**Fig. 6**). In the autoclaved medium, WGA remained nearly 100% for 2 days then gradually decreased. These results imply that the plateau of extracellular BL after 6 days in 0.2 mg l^{-1} culture was caused by a biological compound responsible for the degradation as well as a decrease in excretion efficiency of BL. This degradation process is quick, implying that further

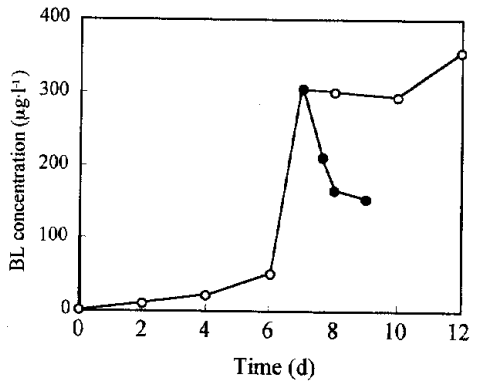

Fig. 5 Degradation of extracellular BL. Accumulation in control culture (○), degradation in cell removed medium (●).

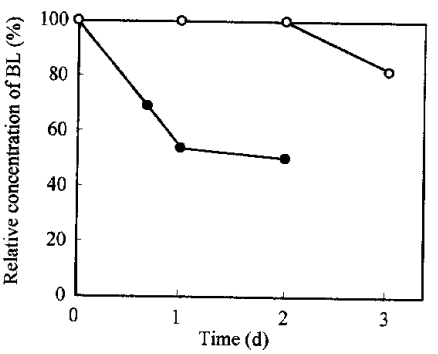

Fig. 6 Degradation of WGA in autoclaved (○) and non-autoclaved (●) medium.

incubation of excreted BL in the medium is unnecessary. These results suggested that continuous separation and purification of the excreted BL from the culture medium is necessary for efficient production of the BL in this system.

An efficient production and recovery process for BL with transgenic tobacco cells would be designed to cultivate under the optimum 2,4-D concentration for growth until the early log phase. Exchange of medium at this stage not only enables recovery of the highest concentration of extracellular BL before degradation occurs but also maintains the cells at the highest excretion efficiency.

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