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Comparative Study of Anti-Apoptotic Genes, *Bcl*-2 and *P35* for the Suppression of Apoptosis Induced in Suspension Culture of Transformed *Trichoplusia ni* BTI Tn 5B1-4 Cells

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To delay the onset of apoptosis in the culture, transformed Tn 5B1-4 cells harboring anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, have been established and analyzed for their anti-apoptotic ability in suspension culture using spinner flasks. In the suspension culture at agitation speeds of 100 rpm and 200 rpm, the cell growth of cell clone expressing *Bcl*-2 protein was much higher than other two clones and the maximum cell density of the clone was 6.0×10^6 cells/ml and 6.2×10^6 cells/ml at day three of the incubation. On the other hand, the cell growth of cell clone expressing baculovirus protein *P35* was much higher than other two clones in suspension culture at agitation speed of 300 rpm and the maximum cell density of the clone was 6.1×10^6 cells/ml at day three of the incubation. Based on the pattern of genomic DNA laddering and the microscopic observation of apoptotic bodies, the more apoptotic bodies are induced in Tn 5B1-4 control cell clone at higher agitation speed. This result shows that the shear stress can be a main factor in inducing apoptosis in spinner flask culture. At low agitation speed, cell clone expressing *Bcl*-2 was more effective in delaying the onset of apoptosis than the cell clone expressing *P35*. On the other hand, at high agitation speed, cell clones expressing baculovirus *P35* was more effective in delaying the onset of apoptosis than the cell clone expressing *Bcl*-2. Therefore, anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, can play a distinct role depending on agitation speed in the suspension culture.

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Introduction

Apoptotic cells follow a progressive morphological change that is characterized by plasma-membrane blebbing, cell shrinkage, chromatin condensation, as well as nuclear degradation, and results in fragmentation into apoptotic bodies (Wyllie *et al*., 1980). This effector/degradation phase is common to all apoptotic processes. The family of cysteine aspartate proteases, or caspases, currently comprises ten different members, and constitutes one of the most important classes of gene products acting in this process. In the two major mammalian apoptotic pathways, the mitochondria pathway and the cell surface death receptor pathway, caspase-9 and caspase-8 are two key initiator caspases that can be, respectively, activated when cellular or environmental apoptotic signals are received. Then the following caspase activation cascades can activate downstream effector caspases such as caspase-3 or caspase-7 trigger the apoptotic execution phase (Budihardjo *et al*., 1999; Green, 2000).

To date, anti-apoptotic genes such as *bcl*-2, *ced*-9, cowpox virus *crm*A, baculovirus inhibitor of apoptosis (*iap*), and *p35*, when overexpressed, prevent apoptosis induced by a variety of apoptotic agents in different systems (Vucic *et al*., 1997). The *bcl*-2 family includes those that promote cell survival by inhibiting adaptors needed for activation of the caspases, whereas other members, such as Bax and Bak, of this family promote apoptosis (Reed, 1997; Adams and Cory, 1998). Membrane-bound localization of *bcl*-2 (mainly in mitochondria) helps to support the anti-apoptotic

property of this protein as a free radical scavenger and/or its ability to interact with other proteins such as cytochorome *c* involved in apoptotic induction (Kharbanda *et al.*, 1997; Green and Reed, 1998; Rossé *et al.*, 1998).

Anti-apoptotic *P35* protein is a potent inhibitor of the caspases, which accounts for its broad anti-apoptotic activity (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). During infection of *Spodoptera frugiperda* SF21 cells with the prototype baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), viral-synthesized *P35* is cleaved by and inhibits a novel insect caspase (*S. frugiperda* caspase) (Bertin *et al.*, 1996). Stoichiometric interaction of active caspase with *P35* protein blocks protease activity by a mechanism that requires *P35* cleavage at residue Asp^{87} (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). In addition to its ability to protect baculovirus-infected insect cells from apoptosis, ectopic expression of *p35* blocks apoptosis involving diverse death signals, e.g., developmental death, growth factor withdrawal, or DNA damage, in many different cell types and species (Villa *et al.*, 1997).

To date, an attachment-dependent cell line, *Trichoplusia ni* BTI Tn 5B1-4 cell has been shown to be superior to Sf9 or Sf21 lepidopteran cell lines for expression of both cytoplasmic and secreted glycosylated proteins (Wickham *et al.*, 1992; Davis *et al.*, 1993; Wickham and Nemerow, 1993; Lee *et al.*, 2001). Stably transformed Tn 5B1-4 cells have also been used for the expression of useful proteins as an alternative to the baculovirus expression system. And optimization of medium composition and bioreactor culture system for Tn 5B1-4 cells have been also described (Chung *et al.*, 1993; Wickham and Nemerow, 1993; Dee *et al.*, 1997). However, the suppression of apoptosis in the suspension culture of *Trichoplusia ni* BTI Tn 5B1-4 (Tn 5B1-4) in spinner flasks has not been investigated. Therefore, an attempt to maintain high-cell density culture was made by delaying onset of apoptosis during the spinner flask culture of Tn 5B1-4 cells. If the apoptosis of Tn 5B1-4 cells in the spinner flask culture can be delayed by over-expression of anti-apoptotic genes, this will be very useful for biotechnological applications. For this, We have established transformed Tn 5B1-4 cells using anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, and analyzed for the function of those genes in spinner flask culture of stably transformed Tn 5B1-4 cells.

Materials and Methods

Cell line, plasmids and enzymes

Trichoplusia ni BTI Tn 5B1-4 (Tn 5B1-4) cells were grown at 27°C in Nunc (T-25; Roskilde, Denmark) flasks in Sf900II-SFM (serumfree medium; Gibco BRL, Grand Island, NY, USA) medium. The Tn 5B1-4 cells were a gift from Dr. Granados at Boyce Thompson Institute for Plant Research (Ithaca, N.Y., USA). The plasmid pIZT/V5-His (3.3 kb; Invitrogen, Carlsbad, CA, USA) contains an OpIE2 promoter, a V5 epitope tag, a polyhistidine region, and a zeocin resistance gene under the control of the EM7 promoter. *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3) containing 50 μg zeocin/ml (Invitrogen) with agitation at 37°C. We used DNA restriction enzymes from Promega (Madison, WI, USA) or Takara (Shiga, Japan) according to the manufacturers instructions.

Construction of expression plasmids containing anti-apoptotic genes

Bcl-2 gene was derived from pB4 (ATCC 79804). Plasmid pIZT/*bcl*-2/V5-His (Fig. 1) was constructed by inserting a *EcoR*I fragment of pB4 to pIZT/V5-His (Invitrogen). Also, baculovirus *p35* sequence was amplified from *Autographa californica* nucleopolyhedrosis virus (strain L-1) DNA by PCR using oligonucleotide primers. The sense primer was 5′-GGTACCATGTGTGTAATTTTTC-3′ and the antisense primer was 5′-GCGGCCGCCTT-

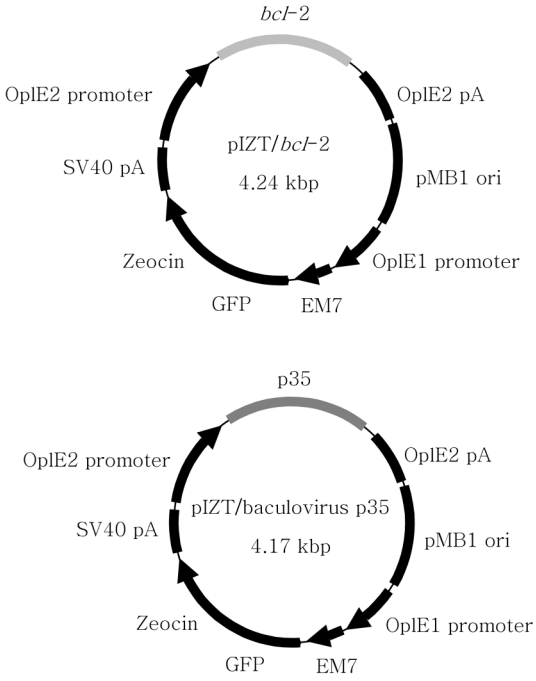

Fig. 1. Construction of anti-apoptotic genes expression plasmids.