FLUORESCENT PROTEIN REPORTER FOR MONITORING TRANSGENIC PLANT CELL CULTURES

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ABSTRACT

Green fluorescent protein (GFP) is a useful tool for studying online monitoring of recombinant protein in plant cell bioprocesses. Three forms of GFP were constructed for the study. They are secreted GFP, SEAP-GFP fusion and inducible GFP. The secretory GFPs were accumulated over the time course of the culture in the medium. The fluorescent spectrum of the spent medium mimics that of the pure GFP, indicating GFP as the predominant fluorescing compound in the medium. The fluorescence signal of the spent medium linearly correlated with the secreted GFP concentration. It was also demonstrated in bioreactor cultures where GFP production was positively correlated with online culture fluorescence and offline spent medium fluorescence. For SEAP-GFP fusion, both of decreased function from SEAP and GFP were found in their fusion form. The fluorescence exhibited a similar trend as cell growth in the bioreactor or flask. However, the linear correlations between the fluorescence with fusion protein and SEAP activity are only tenable before the cell reaches the growth peak because of fusion protein degradation. By using alc promoter system, GFP expression can be controlled with ethanol. The optimal dosage is between 0.1% and 1 % (v/v) for the tobacco suspension cells, with maximal fluorescent intensity occurring after 4 days. In conclusion, all above different styles of GFPs provide an efficient and versatile method to study plant bioprocesses.

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LITERATURE REVIEW

1.1. Plants and plant tissue cultures as biofactories of recombinant proteins

Plants are now widely considered as a cheap alternative to animal and microbial cell culture for large-scale production of recombinant proteins such as antibodies, vaccines, enzymes, and therapeutics. There are several advantages that make plants highly attractive for the purpose (Napier et al., 1998):

1. Large-scale production and processing of plant material is routine.
2. They modify proteins (in terms of glycosylation, prenylation, and so on) in the same way as other higher eukaryotes (Chrispeels et al., 1991).
3. Plants transmit the transgene encoding the heterologous protein in a Mendelian fashion.
4. The cost of agricultural biomass production is low compared with animal cell culture.
5. The risk of product contamination by mammalian viruses and other animal pathogens is reduced.

However, at the present time the biggest issues associated with plant-based production of recombinant proteins include low levels of foreign protein accumulation, expression instability, and regulatory uncertainty, particularly for proteins requiring approval as drugs for human use.

Besides whole-plants, plant tissues cultures, especially plant suspension cell cultures, grown in bioreactor are also being considered for foreign protein production

(Doran, 2000). Although the cost of generating plant biomass using tissue culture in bioreactor is more expensive than the cost of agriculture produced transgenic plants in field (Evangelista et al., 1998), in vitro cultures offer other advantages that could potentially render them economically competitive with whole plant systems. In bioprocess, the most expensive steps are in the downstream where heterologous protein must be recovered. When whole plant system is used, protein extraction and purification processes account for over 90% of the total production cost and require a series of unit operations such as milling, aqueous extraction, vacuum filtration, ultrafiltration, ion-exchange chromatography, hydrophobic interaction chromatography, and dialysis (Evangelista et al., 1998). Several of these steps can be eliminated using homogenous plant tissue culture methods if the heterologous protein is released into medium though secretory pathway and harvested from the liquid medium instead of extracting protein from whole plants. As plant culture medium is a relatively simple mixture of salt, sugar, and vitamins and contains little proteinaceous material, the prize of protein recovery and separation is low. Plant tissue culture also has the advantage of being more readily controlled and manipulated than crops in the field and obviate health and environmental issues concerned by some public. From a research point of view, in vitro culture systems are a convenient tool for studying the processes of heterologous protein synthesis, assembly, secretion, and turnover in plant cells and tissues.

1.2. Green Fluorescence Protein

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is widely used in recent years as a versatile reporter in biologic research since its gene was cloned (Prasher et al., 1992). It is small protein, only 27 kDa in size, and stable, retaining

fluorescence in temperatures up to 65°C, pH up to 11, and in the presence of various detergents such as 1% SDS (Bokman and Ward, 1981). GFP has 238 amino acids, undergoing posttranslational modification, and a tripeptide at position 65-67 is cyclized and oxidized to form a covalently attached chromophore (Chalfie et al., 1994; Heim et al., 1994). The mature GFP with intrinsic fluorescence has a barrel-like structure that encases the chromophore and acts as a natural shield for solvents (Yang et al., 1996; Ormo et al., 1996).

The utility of wild type GFP is not optimal for certain reporter gene applications, giving low fluorescent intensity and low expression in higher eukaryotes (Kainet al, 1995). To overcome the shortcomings of wild type GFP, a large collection of GFP mutants have been constructed in the past 10 years since its cDNA was cloned form *Aequorea Victoria* by Prasher et al. (1992). Most of these mutants have optimized codon and/or fluorophore mutations that alter the spectral properties of GFP for use in specific applications (Heim et al., 1994, 1995, 1996). Using PCR, Crameri (1996) and Davis and Vierstra (1998) constructed a series of mutants which can be correctly folded as soluble protein or with shifts in their spectra. Siemering et al. (1996) produced a much brighter mutant (GFP5) with dual excitation peaks (395 nm and 473 nm) of approximately equal amplitude, which can be visualized well with long wavelength UV of blue light. However, the most important GFP mutant, which solves the problem of poor expression in plant, is mGFP4 in which a plant recognized cryptic intron was removed (Haseloff et al., 1997). mGFP5er consists of the features of GFP5 and mGFP4, with additional ER targeting and retention sequences. The product of mGFP5er gene is targeted to and retained on the endoplasmic reticulum using an Arabidopsis basic chitinase sequence at

the N terminus and a HDEL polypeptide sequence at the C-terminus to decrease hypothesized cytotoxicity of GFP. Except GFP from jellyfish, recently a novel red fluorescent protein, dsRED, was isolated from reef corals (Discosoma sp.) with an excitation peak wavelength (553 nm) just above the excitation peak of chlorophyII (Matz et al., 1999).

1.3. Secretory pathway and GFP secretion in plant

All eukaryotes share the similar secretory pathway, in which soluble proteins are co-translationally inserted across the ER membrane during their synthesis on membrane-bound ribosomes, then a newly synthesized protein in the ER lumen is retained in, or directed to, a specific organelle within the protein transport pathway depending upon information encoded by specific amino acid sequences and/or by determinants formed from its three-dimensional structure as the protein folds into a stable conformation with the assistance of ER chaperone proteins. Golgi is the following organelle where the sorting of proteins destined for the vacuole in plant and yeast, or lysosomes in animals, or for the plasma membrane occurs in the trans-Golgi network (TGN). It has been proved that the export of protein from the endoplasmic reticulum (ER) to Golgi occurs in COP II- coated vesicles in yeast and mammalian cells. Secretory cargo is selected and concentrated into COP II vesicles during budding but ER residents are excluded (Barlowe et al, 1994). Retrograde transport from the Colgi to the ER and intra-Golgi transport is mediated by COP I-coated vesicles. One of the major differences between the secretory pathways of plant and animal cells is in the movement and localization of the Golgi stacks. Whereas in mature mammalian cells the Golgi stacks are organized on microtubules and remain clustered in a juxtanuclear position at the microtubule

organizing center, in plant cells the Golgi apparatus is dispersed into tens or hundreds of individual stacks that are highly mobile (Andreeva et al., 1998). The positive sorting information is required to transport proteins from Golgi complex to lysosomes in animals or vacuoles in plants (Neuhaus et al., 1998). In animal cells, enzyme sorted to lysosomes is phosphorylated in the cis-Golgi, yielding multiple mannose 6-phosphate (M6P) residues. M6P reporters in the trans-Golgi network bind the phosphorylated proteins and direct their transfer to late endosomes. Receptors and proteins dissociate in the late endosomes. Then the receptors are recycled to the Golgi or plasma membrane, and the lysosomal enzymes are transported to lysosomes (Lodish et al., 2000). However, in plants, protein sorting to vacuoles is quite different with protein sorting to lysosomes in animals. Three types of vacuolar sorting determinants with specific physical and functional characteristics have been identified in plants. One type was termed a N-terminal propeptides (NTPP) found in sweet potato prosporamin and barley proaleurain. It has a conserved amino acid sequence probably recognized by a sorting resceptor for their function and may function when placed elsewhere in the protein. A second type was termed C-terminal propeptides (CTPP) found in barley lectin and tobacco chitinase. In contrast, the CTPP have little or no discernable requirement for a conserved sequence. The last one was termed an internal determinant described for certain seed storage proteins (Neuhaus et al., 1998). An individual plant cell may contain at least two functionally and structurally distinct types of vacuoles: protein storage vacuoles and lytic vacuoles. A protein without specific information for retention or sorting to a particular secretory organelle will pass through the Golgi complex and be packaged into vesicles

that fuse with the plasma membrane and release the protein to the cell exterior. That is so-called default pathway (Denecke et al., 1990).

Fusion of GFP with a signal peptide, which would possibly lead to target to the ER or the Golgi apparatus, or secretion of GFP, has the potential of illuminating complex secretory pathways. In plant cells, a signal peptide-GFP-HDEL targeting to ER has been successfully expressed in *Arabidopsis thaliana* roots (Haseloff and Amos 1995). The modified GFPs have been reported to be localized in ER and Golgi complex (Boevink et al., 1998; Nebenfuhr et al., 1999) as well as in non-acidic vacuoles (Sansebastiano et al., 1998). However, attempts to secrete GFP/GFP-fusion through the default secretory pathway are not so successful like those of using GFP for organelle targeting. It has been shown in mammalian (HeLa) cell systems, that protein secretion proceeded faster than the GFP chromophore formation and thus the chromophore was not fully formed before GFP was secreted, rendering the secreted GFP non-fluorescent (Kaether and Gerdes 1995). By subjecting the HeLa cells to a temporary cold treatment (at 20°C), the rate of secretion was reduced, allowing sufficient time for proper protein folding that led to secretion of fluorescent GFP. This case exemplified that secreted GFP may not retain its fluorescence. Batoko et al. (2000) reported that secGFP, containing an N-terminal c-Myc-epitope tag, was delivered to the ER but did not accumulate unless transport from the ER was inhibited by treatment with BFA, whereas GFP-HDEL, containing N-terminal ER signal peptide and C-terminal HDEL peptide, was retained within the ER in the absence of BFA. Faint secGFP fluorescence was visible in the apoplast, which may arise at least in part from the lesser stability of the protein in this compartment compared with that in the ER. In addition, Sec GFP consistently accumulated in lesser amounts than did the ER-

localized GFP-HDEL, and its predominant form was a proteolytic product estimated to be ~2KD smaller than expected. In fact, in several recent reports, GFPs secreted from cultured tobacco cells or protoplasts were reported to be degraded and/or have lost their fluorescence (Di Sansebastiano et al. 1998; Mitsuhashi et al. 2000; Benghezal et al. 2000). Some plausible reasons i.e. choice of expression system, inclusion of interfering motif, or choice of signal peptides can explain the failure of achieving fluorescent GFP secretion.

1.4. GFP fusion protein

As described above, fusion of GFP to organelle-targeting signal peptides has lead to correct targeting of many different cellular compartments. Targeting of GFP-fusions to chloroplasts (Kohler et al., 1997a), mitochondria (Kohler et al., 1997b), cytoskeletal structures (Heinlein et al., 1995) and the nucleus (Grebenok et al., 1997) has provided new insights into diverse cellular processes. A fusion between GFP amd COP I complements a lethal COP I loss-of-function allele in *Arabidopsis*, suggesting that fusion to GFP does not necessarily compromise the function of the fusion partner (von Arnim et al., 1998). However, there are only few reports on the application GFP as a translational fusion partner for monitoring foreign protein expression in bioprocessing. GFP fluorescence can be used to quantitative monitor expression of target protein. In *Escherichia coli,* GFP was fused with the enzymes such as chloramphenicol acetyl transferase (CAT) (Albano et al., 1998) and organophosphorus hydrolase (OPH) (Wu et al., 2000). Both of them found that the fluorescence intensity was proportional to the enzyme activity in vivo GFP. The synthesized fusion protein was directly visualized due to the optical properties of GFP. In other cases, therapeutic protein was used as GFP's

partner. GFP/hIL-2 fusion protein, consisting of a hexahistidine aaffinity ligand (His6), the GFPuv, an enterokinase (EK) cleavages site, followed by the HIL-2, was successfully employed in *E.coli* (Cha et al., 2000), insect Sf-9/baculovirus (Cha et al., 1999a), insect *T.ni*larvae/baculovirus (Cha et al., 1999b), and insect *Drosophila* S2 cells (Shin et al., 2003). It has been proved that GFP fluorescence intensity had a linear relation ship with its target foreign protein fusion partner.

1.5. Inducible GFP expression using the alc promoter system

The ability to exogenously regulate expression of transgenes in higher plants has considerable advantages for both pure scientific and practical applications (Gatz, 1997). Several different methods of chemically regulating transgene expression in plants have been reported (Gatz, 1997). Among these, the alc regulon from *Aspergillus nidulans* (Gwynne et al., 1987; Lockington et al., 1985) provides a promising chemically regulated gene expression cassette for higher plants with the potential to be accepted for routine use in the field. It composed of two elements: the AlcR encoded transcription factor; and a promoter derived from AlcA, which encode alcohol dehydrogenase I (ADHI). For use in plants, the AlcR cDNA was expressed from the constitutively expressing CaMV35S promoter. A region of the AlcA promoter, including two AlcR binding sites, was fused to the CaMV35S minimal promoter at the TATA sequence. AlcR activates expression of the AlcA promoter specifically in the presence of various alcohols and ketones. This chimeric promoter has been used to regulate expression of a yeast cytosolic invertase in transgenic tobacco (Caddick et al., 1998). Details on the dose-response and time course of induction with enthanol for young seedings and mature plant have been studied using chloramphenicol acetyl transferase (CAT) as reporter gene (Salter et al., 1998). It has

been demonstrated that AlcR-mediated expression occurs throughout the plant in a highly responsive manner by linking the AlcA promoter to β-glucuronidase (GUS), luciferase (LUC) and green fluorescent protein (GFP) genes (Roslan et al., 2001). For biotechnological application, transformed plant cells that have an inducible promotor are suitable for the production of useful products since the growth phase and the production phase can be separated.

1.6. Research Objectives

As stated above, *in-vitro* plant tissue culture system and GFP are powerful tools for studying recombinant protein expression in plant systems. These tools are especially appealing since GFP can be used to monitor production of foreign protein and biomass. To achieve optimal conditions in the bioprocess for protein production, several key issues are investigated in this study:

1. To examine GFP as a secretory reporter and its applications in tobacco cell culture
2. To achieve monitoring of GFP-fusion protein expression
3. To study inducible GFP expression

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CHAPTER 2

CHARACTERIZATION AND MONITORING OF TRANSGENIC TOBACCO CELL CULTURE SECRETING FUNCTIONAL GREEN FLUORESCENT PROTEIN

ABSTACT

Green fluorescent protein (GFP) is a useful tool for studying protein trafficking in plant cells. This utility could be extended to allow online monitoring of recombinant protein secretion in plant cell bioprocesses. The aim of this study is two fold: (1) to demonstrate and characterize highly efficient secretion of fluorescent GFP from transgenic plant cell culture; and (2) to examine the utility of GFP fluorescence for monitoring recombinant protein secretion. The secretory GFP construct was made by fusing GFP with an *Arabidopsis* basic chitinase signal sequence. Extracellular GFP typically accumulated to *ca.* 12 mg per liter of spent medium after 10 to 12 days of culture in shake flasks using a Murashige and Skoog media containing 20 g l^{-1} sucrose. This is equivalent to ca. 50% of the total extracellular protein, or 65% of the total GFP expressed. Upon brefeldin A (BFA) treatment, GFP secretion was substantially reduced, and several bright spots with highly localized GFP accumulation were noted on the reticulate network. This type of intracellular GFP distribution is distinct from that seen in the cells expressing an endoplasmic reticulum-retaining GFP construct. Upon cold (4°C) treatment, GFP secretion was also reduced, although the pattern of intracellular GFP distribution appeared to be different from that seen in the BFA-treated cells. The fluorescence spectrum of the spent

medium mimics that of the pure GFP, indicating GFP as the predominant fluorescing compound in the medium. Over the course of the culture, fluorescence signal of the spent medium linearly correlated with the secreted GFP concentration. The interference on this correlation by the variation in medium pH could be easily compensated or accounted for by automatic pH control, while the interference from inner filter effects caused by the secreted GFP and components in the spent medium was found insignificant. Taken together, real-time monitoring of secreted GFP by online fluorescence detection should be feasible. This was further verified in bioreactor cultures where GFP production was positively correlated with online culture fluorescence and off-line spent medium fluorescence.

Keywords: green fluorescent protein, plant cell culture, protein secretion, sensing

2.1. INTRODUCTION

Plant cells cultured in bioreactors can serve as an effective expression system for the production of high-value recombinant proteins that require stringent quality control. A principal advantage of using bioreactor-based plant cell cultures, as opposed to transgenic plants growing in open fields, is that the bioreactor environment can be precisely controlled to achieve optimal conditions favoring protein production. To this end, it is important to be able to rapidly monitor recombinant protein products to achieve effective dynamic bioprocess control and optimization (Baker et al. 2002). Furthermore, in developing an effective transgenic plant cell bioprocess for recombinant protein production, it is highly desirable that the target protein be efficiently secreted into the medium to simplify product purification. Taken together, a bioreactor-based plant cell culture system that allows

efficient recombinant protein expression and secretion, and online monitoring of such protein products in real time, is highly valuable.

The cloning of the gene encoded for the green fluorescent protein (GFP), and the development of various mutants of GFP, offer new opportunities for developing innovative bioprocess sensing techniques based on this versatile reporter. GFP was isolated from the jellyfish *Aequorea victoria* and it has been commonly used as a reporter protein or biomarker for studying subcellular localization of proteins (Akashi et al. 1998; Yamaguchi et al. 1999) and protein trafficking (Imlau et al. 1999; Oparka et al. 1999). As for bioprocess applications, GFP has been utilized in several studies to monitor bacterial fermentations (Randers-Eichhorn et al. 1997; Poppenborg et al. 1997; DeLisa et al. 1999). GFP has also been used in detecting cellular stress levels in quiescent *E. coli* cells by fusing GFP to heat shock stress protein promoter elements (Cha et al. 1999a). An online flow injection flow cytometry system was developed by Zhao et al. (1999) for monitoring the GFP fluorophore formation kinetics and *E. coli* growth dynamics. Functional fusion of GFP to a broad range of protein partners at either N- or C- terminals has been reported (Poppenborg et al. 1997; Cha et al. 2000), and a direct quantitative correlation between the culture GFP fluorescence intensity and the functional activity of the fusion protein can often be established (Albano et al. 1998; DeLisa et al. 1999). In addition to monitoring bacterial fermentations, constitutively expressed GFP was used to monitor biomass density in animal cell cultures (Hunt et al. 1999). Affinity protein separation was optimized by tagging the target protein with GFP and tracking the fluorescence of the fusion protein (Poppenborg et al. 1997). When applied to a *Trichoplusia ni* insect larvae expression

system, Cha et al. (1999b) demonstrated that the optimal harvest time of human interlukin-2 (IL-2) could be determined to minimize proteolytic degradation using GFP-IL-2 fusion. Patkar et al. (2002) recently demonstrated fusion of GFP with a protein-based biopolymer enabled monitoring of the biopolymer accumulation using flow cytometry. Such GFP fusions also allowed rapid evaluation of induction strategies and expression systems.

Compared with the aforementioned expression hosts, studies on bioprocess applications of GFP in plant cell cultures have been scarce. In a previous study (Liu et al. 2001), we established a tobacco cell culture expressing a GFP modified with an N-terminal *Arabidopsis* basic chitinase ER-targeting signal sequence and a C-terminal ER retrieval/retention HDEL tetrapeptide. We demonstrated that total culture GFP fluorescence could be used for monitoring cell growth and GFP expression, and for determining the feed rate in a fed-batch tobacco cell culture. Furthermore, we noted that approximately 35% of the total GFP protein was secreted into the medium whereas the remaining GFP was mainly retained in the ER, due to the presence of the HDEL ER retrieval/retention tetrapeptide. Here we report enhanced secretion of GFP by removing the HDEL tetrapeptide. Secretory GFP expression was compared with expression of cytosolic and ER-retained GFPs. GFP secretion by the cultured tobacco cells was further characterized by using treatments known to affect protein trafficking. We also investigated the utility of GFP fluorescence in reporting recombinant secretory protein production in plant cell culture. Such utility was checked by examining the correlation between fluorescence intensities and GFP protein concentrations in the tobacco cell culture spent

media, and by comparing GFP production with online culture fluorescence and off-line spent medium fluorescence in bioreactor cultures.

2.2. MATERIALS AND METHODS

2.2.1 Plant expression vectors

The plasmid pBIN mGFP5-ER encoded a mGFP5 for targeting to the endoplasmic reticulum (Siemering et al. 1996; Haseloff et al. 1997). The plasmids pBIN 1010 and pBIN 1012 encoded mGFP5 for targeting to the cytosol and the cell wall, respectively (Scott et al. 1999). In all of the expression constructs mGFP5 was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

2.2.2 Tobacco transformation and selection

The plant expression constructs were transferred into *Agrobacterium tumefaciens* LBA4404 by electroporation. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) transformation and subsequent transformant selection were carried out essentially as described in Liu et al. (2001), which was based on the procedures reported by Horsch et al. (1985) with modifications of Fisher and Guiltinan (1995). Stem tissue from the young primary transformants was cut into small pieces and positioned on solid Murashige and Skoog medium (Murashige and Skoog 1962) containing Gamborg's vitamins (Gamborg et al. 1968) and 2% sucrose, and supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 300 mg l^{-1} kanamycin and 500 mg l^{-1} carbencillin for callus induction and culture.

2.2.3 Suspension cell culture

Generation and maintenance of tobacco suspension cell cultures were performed as previously described (Liu et al. 2001). Media and growth conditions were identical to those reported in Liu et al. (2001) except that Murashige and Skoog medium was used instead of Gamborg's B5 medium. In shake-flask time-course experiments, to each one-liter baffled-shake flask, 300 ml of culture medium was inoculated with a 15% (v/v) inoculum from a 7-day-old cell culture. The culture was sampled periodically for the time course determination of growth and protein production parameters.

2.2.4 GFP extraction and fluorescence determination

Extraction of cell suspension samples was performed as previously described (Liu et al 2001). The supernatant following centrifugation was filtered through a 0.2 μm filter and used for determining intracellular GFP. For extracellular GFP determination, a sample of cell suspension was centrifuged at 3200 g for 5 min and the supernatant filtered through a 0.2μm filter.

Off-line GFP fluorescence intensity measurements were obtained using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan) at an excitation wavelength of 473 nm and an emission wavelength of 507 nm. To minimize the interference of the inner filter effect (Srinivas and Mutharasan, 1987), samples for culture and intracellular fluorescence measurements were diluted, when necessary, to the linear fluorescence response range, and then the fluorescence of the diluted sample was linearly extrapolated. For medium fluorescence measurements, sample dilution was unnecessary since the inner filter effect was found to be inconsequential (data not shown) and the extracellular GFP concentrations in all samples were within the linear response range.

2.2.5 Western blot analysis

Samples were mixed with an equal volume of 2× reducing sample buffer, resolved on a 12% SDS-polyacrylamide gel as described by Laemmli (1970) and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). Purified recombinant GFP (Clontech, Palo Alto, CA) was used to quantify GFP levels. Western analysis was performed as previously described (Liu et al. 2001) using an anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Quantification of the immunoreactive GFP bands on the Western blots was done using SigmaScan Pro 5.0 image-analysis software (SPSS, Chicago, IL).

2.2.6 ELISA analysis

A sandwich ELISA assay was used to determine the GFP concentration. To a 96-well plate, each well was coated overnight with 100 μl of anti-GFP monoclonal mouse antibodies (Molecular Probes, Eugene, OR) at a dilution of 1:2000 by PBS. The coated plates were washed 3 times by PBST (PBS containing 0.05% Tween). The wells were blocked using 300 μl blocking buffer (1% BSA, 5% sucrose in PBS) for 1 hour, and then washed 3 times with PBST. 100 μl diluted sample was then added to each well, and incubated for 2 hours. The plates were then washed 3 times by PBST, followed by addition of 100 μl detection antibody (anti-GFP rabbit polyclonal antibody diluted 1:2000 with PBST containing 1% BSA). After 2 hours inoculation, the plates were washed 3 times again. To develop color, alkaline phosphatase conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1:5000 (with PBST containing 1%

BSA) was added into the wells and incubated for 2 hours. The plates were washed 3 more times and then 100 µl substrate solution (10 mg of *p*-nitrophenyl phosphate in 10 ml 1M DEA buffer) was added and allowed to react for 20-30 minutes. The absorbance was read at 405 nm. GFP concentration was determined based on a calibration curve using a recombinant GFP standard (Clontech, Palo Alto, CA). All the above procedures were performed under room temperature. Multiple dilutions were made for each sample to crosscheck the absorbance readings, which was found to be necessary in assuring accurate measurements.

2.2.7 DNA assay

Detection of DNA in the culture medium was based on a protocol described by Ison and Matthew (1997). Briefly, 10 ml of culture sample was centrifuged at 2700 g for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 8000 g for 10 min at 4°C to remove any residual insoluble material. A total of 2 ml of cold 0.4 M perchloric acid was added to 2 ml of supernatant and mixed gently. After centrifugation at 8000 g for 10 min at 4°C, the supernatant was discarded and the pellet resuspended in 1 ml of 0.2 M perchloric acid. The resuspension was heated at 100°C for 20 min followed by centrifugation at 10000 g for 10 min at 4°C. The supernatant was transferred to a quartz cuvette and the absorbance at 260 nm was recorded.

2.2.8 Other measurements

Cell dry weight and packed cell volume (PCV) were determined as previously described (Liu et al. 2001). Protein was determined using a protein assay reagent (Bio-Rad,

Hercules, CA) with bovine serum albumin as the standard (Bradford, 1976). Glucose in the medium was measured using a hexokinase/glucose-6-phosphate dehydrogenase assay kit (Sigma, St. Louis, MO).

2.2.9 BFA and cold treatments

A 10 ml sample of cell suspension was collected under vacuum on a nylon mesh (10 μm pore size) fitted in a Buchner funnel and the cells were washed three times with fresh medium. The cells were carefully transferred to a flask and resuspended in 10 ml of fresh medium. For the cold treatment, the culture was incubated with shaking at 100 rpm on a gyratory shaker in a cold room at 4°C. For the BFA treatment, a stock solution of BFA was prepared (10 mg ml^{-1} in methanol) and added to the medium at a final concentration of 50 $\mu g\ ml^{-1}$ (Boevink et al., 1999). Spent medium samples were obtained 2-6 hr following treatment for analysis of GFP fluorescence and cells were also examined by fluorescence microscopy to determine the cellular distribution of GFP.

2.2.10 Bioreactor culture and online monitoring

Transgenic tobacco cell line 7-1 was cultivated in a 3-liter stirred-tank bioreactor (BioFlo III fermenter, New Brunswick Scientific, Edison, NJ) with online monitoring of culture fluorescence and pH/dissolved oxygen control. In the bioreactor culture, glucose at 20 $g\ l^{-1}$ was used as the carbon source. The dissolved oxygen was controlled at 30% air saturation by automatically regulating the sparged gas composition using two mass flow controllers controlled by a PID controller. The PID algorithm was coded in LabVIEW (National Instruments, Austin, TX). The culture temperature was set at 25°C with an

aeration rate of 0.5 l min^{-1} and an agitation rate of 150 rpm. Culture pH was controlled at 5.5 by adding HCl or KOH solution *via* the BioFlo reactor's pH controller. The online fluorescence monitoring system consisted of a 100W Xenon lamp light source, a 470 nm interference filter, a bifurcated fiber optic cable attached to a 12-mm diameter light rod through a removable coupling, a monochrometer with a photomultiplier tube, and a constant amp lock-in amplifier. Except for the fiber optic cable and the light rod, which were purchased from Fiber Optic Components, Inc. (Sterling, MA), the rest of the fluorescence sensor components were obtained from Thermal Oriel (Stratford, CT). The light rod was inserted into the reactor through the head-plate. Fluorescence spectra (495-515 nm) were acquired online and analyzed using the Thermal Oriel TRACQ32 software. In this work online fluorescence intensity was defined by the area under the fluorescence peak between 495-515 nm and measured in arbitrary units. To this end, integrated intensity signal has been commonly used in culture fluorescence measurements (Li and Humphrey, 1992; Horvath and Spangler, 1992). During the bioreactor culture, fluorescence intensity of the spent medium was also measured off-line using the Hitachi F-2500 spectrofluorometer as described earlier.

2.3. RESULTS

2.3.1 Time-course study of GFP-expressing tobacco cell cultures

In our earlier study (Liu et al. 2001), we detailed the characterization of a transgenic tobacco cell line denoted as 1-13, which expresses a plant-codon-optimized GFP (mGFP5, Haseloff et al. 1997) engineered to carry an ER retrieval/retention HDEL tetrapeptide at the carboxyl terminus, and an *Arabidopsis* basic chitinase signal sequence at

the amino terminus. The GFP expressed in the 1-13 culture is found to distribute evenly throughout the ER network. Here two additional cell lines are introduced. Cell line 6-15 expresses the mGFP5 gene without the HDEL or the chitinase signal sequence. Cell line 7-1 expresses the mGFP5 gene containing the chitinase signal but without the HDEL tetrapeptide. Time course studies were conducted in shake flasks to compare the performance of these cell lines in terms of GFP expression, secretion, fluorescence, and cell growth. Since most of the results for cell line 1-13 were similar to those reported previously (Liu et al. 2001), only the data for cell lines 6-15 and 7-1 are presented here. Growth characteristics and time courses of GFP fluorescence are presented in Fig. 2.1. Time courses of total and extracellular GFP protein concentrations, as determined by Western blot and densitometry analysis, are shown in Fig. 2.2. For the 1-13 culture, related data can be found in Appendix A and Liu et al (2001).

By comparing the results in Figs. 2.2 and 2.3 with the 1-13 culture data given in Appendix I, 7-1 culture gave the highest medium fluorescence and extracellular GFP concentration (reaching 12 μg ml^{-1} of spent medium), while 1-13 culture showed the highest culture fluorescence and total GFP concentration (reaching 30 μg ml^{-1} of culture). The time course of GFP concentrations showed a similar trend as that of the corresponding fluorescence intensities. In calculating the total GFP concentration in the culture (GFP_{total}, mg GFP per liter of culture), the following formula was employed to account for the changes in the abiotic phase volume due to changes in PCV throughout the cultivation:

$$GFP_{total} = GFP_{cell} + (1-PCV) \cdot GFP_{medium} \quad (1)$$

where GFP_{cell} stands for the intracellular GFP concentration and is expressed as mg GFP per liter of culture, while GFP_{medium} stands for the extracellular GFP concentration and is expressed as mg GFP per liter of medium. The presence of secreted GFP in the spent medium was further demonstrated by Western blot as shown in Fig. 2.3. We also found that the fluorescence emission spectrum of the 7-1 spent medium mimics that of the pure mGFP5 (data not shown), indicating GFP as the predominant fluorescing compound in the medium. The secreted GFP in the 7-1 culture accounted for more than 48% of the total GFP expressed during the most part of the culture and it reached *ca.* 50% of the total extracellular proteins in the spent medium. Taken together, our data indicated that GFP is efficiently secreted by cell line 7-1 and the secreted GFP remains fluorescent. For cell line 7-1, since the secreted GFP is the dominant form of GFP, culture fluorescence is found to follow a similar trend as that of the medium fluorescence (Fig. 2.4).

2.3.2 Correlation of GFP concentration with fluorescent intensity

From the above shake flask experiments, the time course of GFP concentrations was found to exhibit a similar trend as that of the corresponding fluorescence intensities. In these initial flask experiments, GFP concentration was quantified by western blot and subsequent densitometry analysis against pure GFP standards. The precision of such an analysis is affected by the quality of the western blot and the sensitivity and reproducibility of the image analysis method used. For the latter factor, repeated scans of the same western blot membrane are typically required to obtain reliable quantification of the immunoreactive protein bands, as also being well reported in other studies (Wu et al 2000). To cross check with the results of GFP concentrations determined using western blot and

densitometry analysis, ELISA was applied to determine GFP concentration in two subsequent runs of 7-1 shake-flask cultures that were conducted in parallel to further characterize the correlation between the fluorescence signals of the spent medium and the extracellular GFP concentration. The results from both runs were comparable and data from one experiment are presented in Fig. 2.5. Since solution pH is known to affect GFP fluorescence, and in the shake-flask cultures pH was not controlled, the raw fluorescence data were also normalized to pH 5.5 based on the following equation (Kneen et al. 1998):

$$F_{5.5} = F_{pH} \cdot \frac{1+10^{n_H(pKa-pH)}}{1+10^{n_H(pKa-5.5)}} \qquad (2)$$

where F_{pH} and $F_{5.5}$ represent the fluorescence intensity at the corresponding medium pH and the corrected fluorescence intensity at pH 5.5, respectively; *pKa* is the pH corresponding to 50% fluorescence maximum and n_H is the Hill coefficient which is proportional to the slope of fluorescence versus pH at *pKa*. Using a 6×His-tagged mGFP5 gene expressed in *E. coli* and purified using immobilized metal affinity columns, the *pKa* and n_H values for mGFP5 protein were determined to be 4.7891 and 0.7117, respectively (Paramban 2002). From Fig. 2.5, linear correlations with slightly different slopes could be found between GFP concentrations and either corrected or uncorrected spent medium fluorescence, covering a GFP concentration range typically seen in our transgenic tobacco cell cultures. This linear correlation supports the utility of GFP fluorescence for monitoring secretory GFP or GFP-fusion protein production in plant cell cultures. In addition, for a given GFP fluorescence, the corresponding GFP concentration determined using ELISA is

comparable with that determined using western blot and densitometry analysis (*cf.* Figs. 2.2 and 2.5).

2.3.3 Effect of BFA and cold treatments on GFP secretion

GFP secretion by cell line 7-1 was further examined by subjecting the culture to BFA- and cold- treatments, both are known to affect protein trafficking. The results from 4-h treatments of 7-, 9-, and 11- day old 7-1 culture with either 50 $\mu g\ ml^{-1}$ of BFA or incubation at 4°C are presented in Fig. 2.6. Untreated 7-1 cultures were used as controls. The results are presented as percentage inhibition (*i.e.* % reduction of GFP fluorescence compared with the control). Both treatments are shown to reduce GFP secretion without effecting cell viability. This result provides further evidences that the presence of extracellular GFP in the 7-1 culture was a result of specific secretion. In addition, comparable degree of inhibition was seen with either BFA or cold treatment. However, cellular GFP distributions were found to be very different between these two treatments as indicated in Fig. 2.7. The treatments are apparently more effective on younger (7-d culture) as opposed to older cultures (9 and 11 d cultures).

2.3.4 Online monitoring of GFP production in the batch reactor

Monitoring of secreted GFP production in the tobacco cell culture was then examined in a bioreactor culture with online measurement of culture GFP fluorescence intensity using a fiber-optic sensor. Cell line 7-1 was cultivated in a stirred-tank bioreactor for 11 days. As seen in Fig. 2.8, cell growth is closely correlated with glucose consumption. Time course of secreted GFP production (estimated based on scanning the

immunoreactive bands on the GFP Western blots) exhibited a similar trend as that of on-line culture fluorescence (expressed as the integral of fluorescence spectra from 495 to 515 nm), as well as off-line spent medium fluorescence. Among the three modes of quantifying on-line GFP fluorescence intensity, the results based on the peak fluorescence reading and on the integral of the fluorescence emission spectra from 495 to 515 nm gave similar trends as that of GFP concentration. On-line fluorescence intensity estimated based on the peak differentials (i.e. the difference in fluorescence intensity between the peak and the reading at 495 nm) is more scattering and indicates a different trend than that of GFP protein concentrations (Fig. 2.9). Western blot analysis of the reactor medium samples revealed a major band with the same size as the standard GFP (Fig. 2.10). Protein degradation was observed after 10 days (Fig. 2.10). Figure 11A shows that the correlation between off-line medium fluorescence and medium GFP concentration was linear throughout the entire time course, with a high linear regression coefficient (r^2) of 0.94. But after corrected for pH, r^2 value became worse, reaching only 0.70 (Fig. 2.11B). The linear correlation between on-line fluorescence and medium GFP concentration was also high (r^2=0.92; Fig. 2.11C). Thus, on-line fluorescence measurement is suited for tracking the quantity of GFP products in the medium.

2.4. DISCUSSION

A primary objective of this work is to determine whether, and to what extent, functional GFP that retains its fluorescence property can be properly secreted into the medium of culture tobacco cells. This is a precondition for using GFP as a reporter of secreted recombinant protein production. All eukaryotes share similar features in their

protein secretory pathway. Synthesis of the majority of the secretory proteins starts on the free ribosomes in the cytosol. The ribosome is directed to the ER membrane via an ER-targeting signal sequence typically located at the N-terminus of the protein, and initiates transport of the nascent polypeptide across the ER membrane (Lodish et al. 2000). Proteins destined for secretion are then typically moved from the rough ER, through the ER-to-Golgi transport vesicles, Golgi cisternae, transport vesicles, and finally to the cell surface and excreted (Lodish et al. 2000). This is commonly referred to as the default pathway (Denecke et al., 1990).

It has been shown in mammalian (HeLa) cell systems, that protein secretion proceeded faster than the GFP chromophore formation and thus the chromophore was not fully formed before GFP was secreted, rendering the secreted GFP non-fluorescent (Kaether and Gerdes 1995). By subjecting the HeLa cells to a temporary cold treatment (at 20°C), the rate of secretion was reduced, allowing sufficient time for proper protein folding that led to secretion of fluorescent GFP. This case exemplified that secreted GFP may not retain its fluorescence. In fact, in several recent reports, GFPs secreted from cultured tobacco cells or protoplasts were reported to be degraded and/or have lost their fluorescence (Di Sansebastiano et al. 1998; Mitsuhashi et al. 2000; Benghezal et al. 2000). Di Sansebastiano et al. (1998) reported that when a secretory form of GFP5 (fused to the *Arabidopsis* basic chitinase signal sequence) was transiently expressed in tobacco protoplasts, GFP was not accumulating inside the cell, but it was not detected in the medium either. It was speculated that the GFP might be secreted, but got degraded or diluted. Benghezal et al. (2000) reported the ER-localization function of the C-terminal di-

lysine motif (KKXX) of a tomato type-I membrane protein in *Arabidopsis* plants and tobacco BY-2 cell cultures using GFP as a reporter. When a mutated motif (NNRY) was fused to the C-terminus of a secretory GFP variant (GFPS65T fused with the *Arabidopsis* basic chitinase signal sequence), it resulted in secretion of GFP after proteolytic removal of the transmembrane domain. The 28-kDa GFP detected in the culture medium of transformed BY-2 cells was however found to loss its fluorescence properties (Benghezal et al. 2000). Mitsuhashi et al. (2000) created a secretory GFP (SP-GFP) by fusing the signal peptide of pumpkin 2S albumin with sGFP-TYG (Chiu et al. 1996). Fluorescence was detected on the ER-network and nuclear envelope of the BY-2 cells expressing the SP-GFP. A 28-kDa GFP was detected in the cell extract. However, in the culture medium an 18-kDa product of degradation was detected along with only a very small amount of 28-kDa GFP, indicating that the co-translational cleavage of the signal peptide of SP-GFP produces GFP that is secreted into the medium to be degraded. Plausible reasons for not being able to achieve fluorescent GFP secretion in prior studies may results from the choice of expression system (*e.g.* protoplasts used in transient expression are known to release increased amount of proteolytic enzymes that may degrade secreted GFP), inclusion of interfering motif (*e.g.* the NNXX membrane anchor), or choice of signal peptides.

In this study we have achieved efficient secretion of mGFP5 that retains its fluorescence property in stably transformed tobacco cell cultures. Presence of GFP in the medium is due to specific secretion rather than non-specific leakage is directly supported by the findings that a significantly reduced level of extracellular GFP was seen in cultures

of transformants expressing cytosolic GFP or ER-retained GFP (GFP-HDEL). Further proofs were provided by data from the BFA and cold-treatment experiments. BFA inhibits protein secretion, but does not affect protein synthesis (Klausner et al. 1992). After 2 hours of BFA treatment, medium fluorescence in the treated 7-1 culture already showed a substantial decrease compared with the control, indicating reduced protein secretion, while cellular distribution of GFP remained relatively unchanged. Upon 4-6 hours of 50 μg ml^{-1} BFA treatment, bright fluorescent granules began to emerge in the treated cells (Fig. 2.7). This pattern of GFP accumulation is very different from that of the cells expressing the HDEL-tagged GFP (Fig. 2.7). The latter resembled the GFP accumulation pattern reported by Boevink et al. (1998, 1999) in which tobacco leaves infected with a secretory GFP viral vector accumulated GFP fluorescence evenly throughout the ER network upon treatment with 10 μg ml^{-1} BFA. For the BFA-treated 7-1 cells reported here, the observed bright fluorescent granules bear more resemblance with aggregates of disintegrated Goligi-stacks (Fig. 2.7). The difference between our observation and that of Boevink et al. (1998, 1999) is likely to be caused by the different BFA concentrations used, while such effect could be further augmented by the different plant materials employed in the studies (*i.e.* stably-transformed cultured cells *vs.* virally-transfected leaves). In plant, depending on the BFA concentration, secretory traffic may be blocked either between the ER and the Golgi or between Golgi and the trans-Golgi network (TGN), leading to different morphological responses to BFA treatments (Staehelin and Driouich, 1997). At low BFA concentrations (under 10 μg ml^{-1}), Golgi-to-TGN transport is significantly inhibited, and as a result the protein secretion was shown to be blocked, but without breakdown of the Golgi stacks,

while a loss of TGN cisternae, and increase in the number of trans-like Golgi cisternae, and the accumulation of large numbers of trans-Golgi-derived vesicles in the adjacent cytoplasm were seen (Staehelin and Driouich, 1997). Whereas at high BFA concentrations (> 20 μg ml^{-1}), ER-to-Golgi transport is also blocked, but the budding of Golgi vesicles carries on, leading to the disintegration of the Golgi stacks (Staehelin and Driouich, 1997). For older (9 and 11 d) cultures, the BFA treatment is not as efficient as for the younger (day 7) cultures. Perhaps this was due to the fact that for older cultures the anterograde transport of vesicles from the ER to the Golgi apparatus (Klausner et al. 1992) was not as active as the younger cultures and hence less inhibition was observed. A similar observation has been reported by Sharp and Doran (2001) in the expression of a monoclonal antibody in tobacco cell suspension culture upon BFA treatment. Cold treatment at 4°C also inhibited post-ER transport of GFP, rendering reduced GFP secretion. In this case there were no bright fluorescence bodies as seen in the BFA-treated cells probably because the ER and Golgi were not disturbed as in the case of BFA treatment.

While we anticipated the majority of the GFP to be present intracellularly in cultures of cell lines 6-15 and 1-13, we did detect fluorescence and GFP protein in the spent media of both cultures (Figs.1-3; Appendix I, Fig. 1, 2; and Liu et al. 2001). Spent medium samples from all three cell lines were assayed for DNA content, which serves as a simple indicator for cell breakage. DNA was found in the medium samples from all three cell lines at comparable concentrations (data not shown), indicating certain levels of cell breakage did exist in all three cell lines. For the 1-13 culture, most of the GFP are likely

organelle- or vesicle-bound (Liu et al. 2001), and thus should not be released into the medium as soluble GFP upon natural cell breakage occurred during the cultivation. As such, the GFP detected in the medium is most likely a result from specific secretion. Over-expression of GFP led to saturation of the HDEL-receptors and subsequent escape into the secretion pathway is widely reported (Jones and Herman, 1993; Crofts et al. 1999). In addition to the Western blot data shown in Fig. 2.3, we further characterized the extracellular GFP in the 1-13 medium using a higher resolution 15% gel (as opposed to the 12% gel used to gather data shown in Fig. 3) and we found that the extracellular GFP has a similar molecular weight as the GFP standard, suggesting cleavage of the HDEL tail (Appendix I, Fig. 6). A recent study by Okomoto et al. (1999) also found that prior to maturation of the enzyme, the KDEL ER-retention sequence of a mung bean cycteine protease was post-translationally removed while the protein was still in the ER or immediately after its exit from the ER. We noted that the level of GFP secretion in cell line 1-13 was decreased substantially compared to that was observed in 2000 and as reported in Liu et al. (2001), even though the total expression level remained unchanged. For 6-15, the percentage of secreted GFP in the 6-15 culture was surprisingly high, which was accounted for more than 65% of the total GFP expressed during the most part of the culture. While cell breakage is believed to partially account for the GFP appearance in the medium of cell line 6-15, we could not exclude existence of other nonclassical secretory pathways, considering the relatively high level of extracellular GFP detected in the 6-15 culture (Appendix I, Fig. 2A, 3B, 5). In mammalian cells, a cytosolic protein has been shown to release by a nonclassical secretory pathway (Lindstedt et al, 1993).

By directing the GFP into the secretory pathway, the overall GFP production level was increased relative to that of cells expressing the cytosolic GFP. The overall GFP production level could be further increased by using the HDEL ER-retention signal, as seen in cell line 1-13 that expressed an ER-retained GFP (Liu et al. 2001; Appendix I, Fig. 3A). Various molecular chaperones that help folding of nascent proteins reside in the ER. The lumen of ER thus provides a stable environment for correct processing of GFP, rendering higher overall GFP expression. Tagging with the HDEL sequence however led to formation of more than one form of GFP inside the cell, as indicated by the multiple immunoreactive bands on the Western blots (Liu et al. 2001; Appendix I, Fig. 4), suggesting differential processing of the HDEL-tagged GFP may have led to sorting to different organelles (Haseloff et al. 1997).

In nature, high levels of GFP are well tolerated in jellyfish photocytes, in which the protein is found sequestered in cytoplasmic granules (Davenport and Nichol 1955). GFP without any added targeting signal (as in cell line 6-15) was found throughout the cytoplasm and nucleoplasm of transformed plant cells (Haseloff et al. 1997). Haseloff et al. (1997) thus postulated that GFP could be a source of fluorescence-related free radicals, and the lack of suitable GFP sequestration might cause toxicity or trigger cellular degradation of the protein, which led to the poor expression of cytosolic GFP. In this study we also found that the cytosolic GFP construct gave the lowest overall GFP expression among the three constructs examined, although all three cell lines used the same promoter. In addition, cell line 6-15 showed a lower level of biomass accumulation compared with the other two cell lines. Conversely, when using a viral vector, Boevink et al. (1999) reported

that the cytosolic construct gave the highest GFP expression compared with secreted or ER-retained constructs in infected tobacco leaves. The high expression of the cytosolic GFP in this case may be due to the fact that GFP was present as a fusion protein with the viral coat protein and was sequestered in viral particles accumulated to high levels in the cytosol of the transfected leaves.

In a previous study (Liu et al. 2001) we have demonstrated the use of whole culture GFP fluorescence to estimate cell concentrations in transgenic tobacco suspension cultures expressing mGFP5-ER, and the application of total culture GFP fluorescence to determine the feed rate in a fed-batch tobacco cell culture. Here we report the use of medium fluorescence measurement to report secreted GFP production. The secreted mGFP5 was found stable and not prone to degradation in the tobacco cell culture medium. The fluorescence spectrum of the spent medium mimics that of the pure mGFP5, indicating GFP as the predominant fluorescing compound in the medium. Over the course of the culture, fluorescence signal of the spent medium linearly correlated with the secreted GFP concentration as shown in Fig. 2.5. Two factors could affect this correlation; one is the variation in medium pH and the other the inner filter effect resulting primarily from light absorption/scattering (at excitation and emission wavelengths) by certain medium components, and self-absorption of the emitted fluorescence by GFP. The pH interference was corrected here using eq. (2) with the *pKa* and Hill coefficients of mGFP5. Upon correcting the pH effect, the r^2 value of the linear correlation was found to alter only slightly (from 0.971 to 0.965), along with a higher slope. Note that since GFP fluorescence increases with increased pH up to about pH 8, the slope of the correlation shown in Fig.

2.5 should decrease if the fluorescence is normalized to a pH higher than 5.5. The results shown in Fig. 2.5 indicate that the correlation between fluorescence and GFP concentration remained linear despite pH variation typically encountered in plant cell cultivation.

The most perceptible way to use GFP for reporting recombinant protein secretion is to fuse the GFP with a protein of interest. Provided secretion of such fusion proteins is feasible, monitoring of fusion protein secretion could be achieved by means of online GFP fluorescence sensing described in the present work. Considering the small size of GFP and the reported ability of cultured plant cells to secrete proteins as large as 155 kDa (Sharp and Doran 2001), the addition of the GFP tag is not expected to hamper secretion of most secretory proteins. In the event direct GFP fusion hampers protein secretion, modifications or alternatives to protein fusion may be sought. One possibility would be to express the protein of interest and GFP as separate genes, but from the identical promoter and with the same ER-targeting signals. If a constant ratio between GFP fluorescence and target protein concentration could be established, the independent GFP could be used for fluorescent monitoring. Another plausible approach is to incorporate, between the protein of interest and GFP, a cleavage site that is recognizable by specific ER-resident peptidases. It is conceivable that the target protein and GFP would be separated during co-translational processing in the ER lumen and both proteins are then sent to the default secretion pathway.

In an ideal bioreactor setup for monitoring secreted GFP production, one may install a filter for *in-situ* collection of cell-free spent medium which is then subject to online measurement of the fluorescence signal. For cell line 7-1, since the secreted GFP is

the dominant form of GFP, culture fluorescence is found to follow a similar trend as that of the medium fluorescence (Fig. 2.4). Furthermore, it is easier to detect, online, culture fluorescence than medium fluorescence, since no *in-situ* culture filtration is necessary. Consequently, we measured culture fluorescence online, in lieu of medium fluorescence, in the bioreactor experiment reported here. Although the presence of plant cell particles may introduce inner filter effect, the online culture fluorescence signal was shown to follow closely the time course of secreted GFP concentrations, as indicated in Fig. 2.8. Particularly, online fluorescence measurement correctly indicated the peak of secreted GFP product. Both offline and online fluorescence have a very accurate linear correlation with GFP concentration (Fig. 2.11A, C). Also, the slope of the plot of offline medium fluorescence vs. GFP is closed to that of flasks, which indicate this linear correlation is reliable. In this study we used a bifurcated optic fiber bundle connected to an optical light rod for online detection of culture fluorescence. Such optical probe operates in the backscatter configuration. Horvath and Spangler (1992) compared the performance of a similar light-rod sensor with a flow-through cuvette system for online monitoring of natural fluorophores in yeast fermentation. The cuvette measured fluorescence in the right-angle geometry. Horvath and Spangler (1992) concluded that the light-rod system is superior to the cuvette system in that it not only offers a higher sensitivity (and broader dynamic range of response), but also less susceptible to inner filter effects at high cell densities. This is due, in part, to the fact that the light rod measures fluorescence in the backscatter configuration with a variable path length. At low biomass densities the path length will be long (depending on the clearance between the light rod and the bottom of the

reactor), as optical density increases the path length shortens, maintaining light intensity and linear response at higher concentrations (Horvath and Spangler, 1992). The light rod system has another benefit in that it is made of fused silica and hence can be autoclaved with the bioreactor. Since the optical fibers can be detached from the light rod easily, the optic fibers can be removed during the autoclave. This makes sterile operation of the bioreactor an easier task. In addition, the light rod is compatible with essentially any bioreactors without the need to retrofit the bioreactor since it can be installed by simply inserting through the reactor head-plate or side-ports. Besides online culture fluorescence, offline measurement of medium fluorescence (taken in a cuvette using a spectrofluorometer) also closely correlated with the secreted GFP concentration. The bioreactor results reported here serve as the first experiments in establishing online GFP monitoring in plant cell bioreactors, further characterization and improvement of the monitoring system is currently underway in our laboratory.

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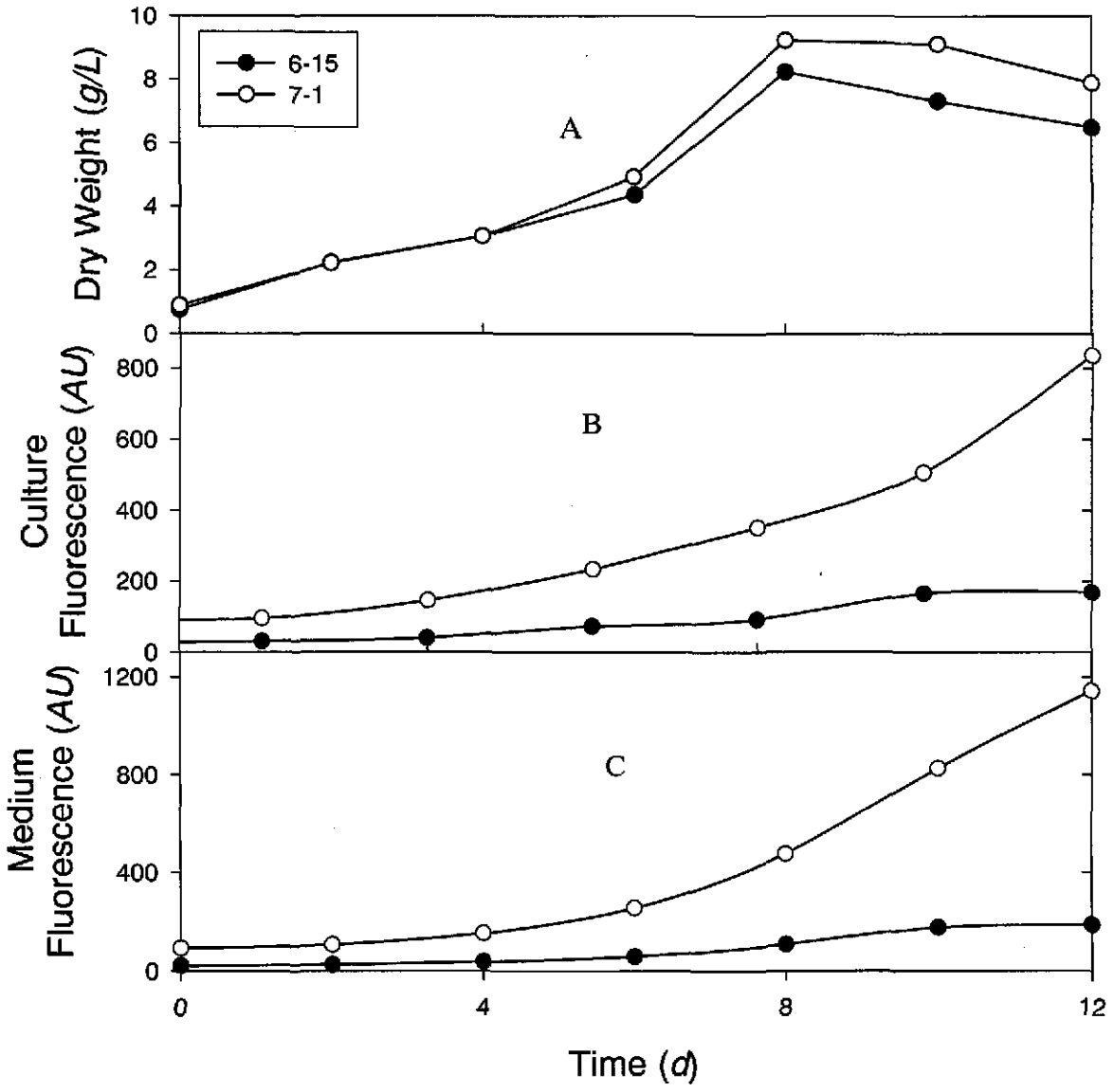

Figure 2.1: Time course for cell growth and fluorescence. A. Dry weight. B. Fluorescent intensity in 1 ml culture. C. Fluorescent intensity in 1 ml spent medium. Cell dry weight was determined by using cells from 5 ml culture in an oven at 50°C until a constant weight was reached. Fluorescence of 1 ml sample was measured at an excitation wavelength of 473 nm and emission wavelength of 507 nm using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan)

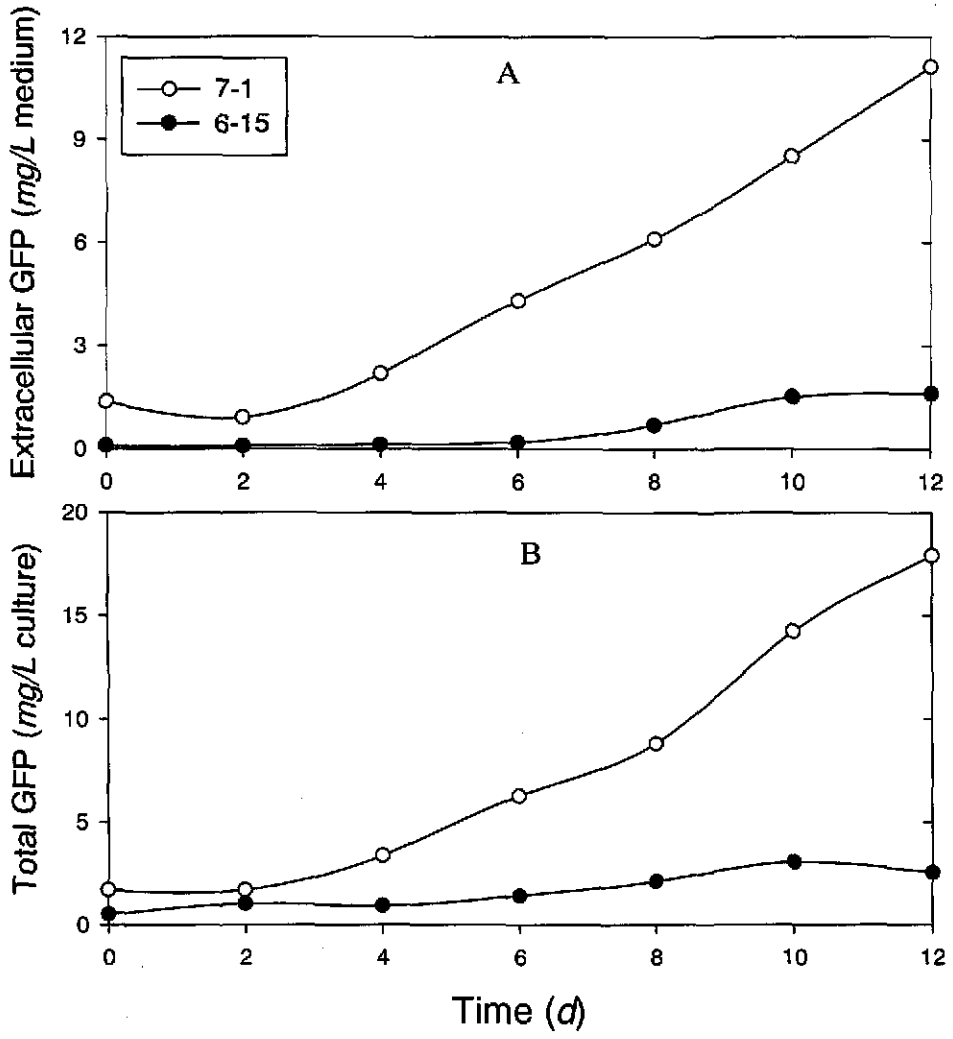

Figure 2.2: GFP concentration in the medium (A), and culture (B). GFP concentration was come from the analysis of western blot intensity. Intra and extra-cellular sample was used to calculated culture GFP concentration.

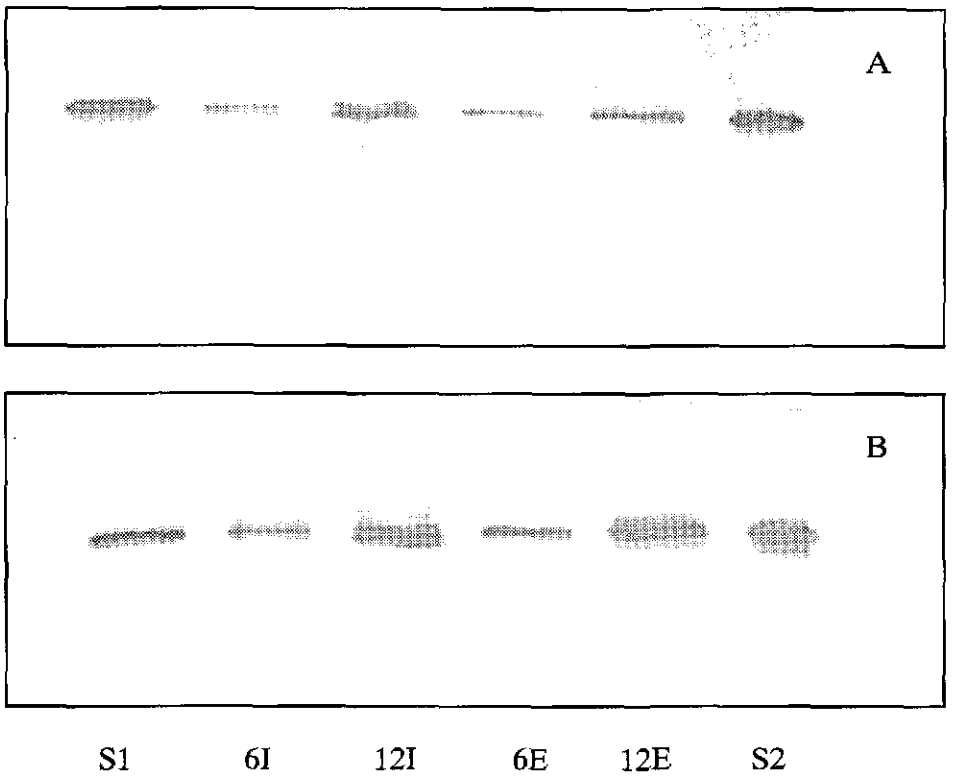

Figure 2.3: Western blots of intracellular and extracellular proteins of cell line 6-15 (A) and 7-1 (B). All lanes were loaded with equal volume of the extraction, except GFP standard. GFP standards, S1 and S2, have 10 µl containing 20 ng and 50 ng GFP. 6 and 12 represents the 6^{th} and 12^{th} culture. I and E represent intracellular and extracellular proteins.

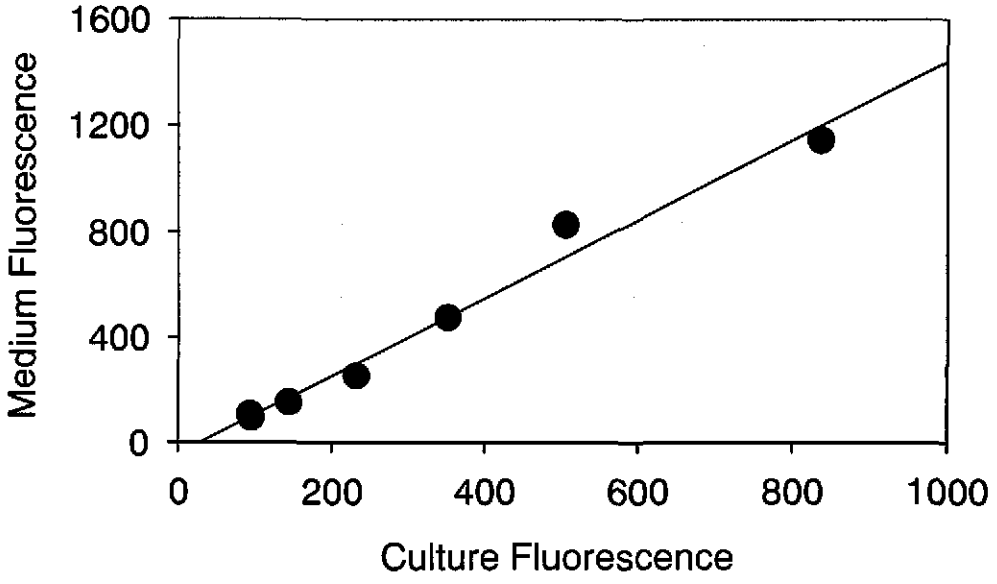

Figure 2.4: Medium fluorescence vs. culture fluorescence.

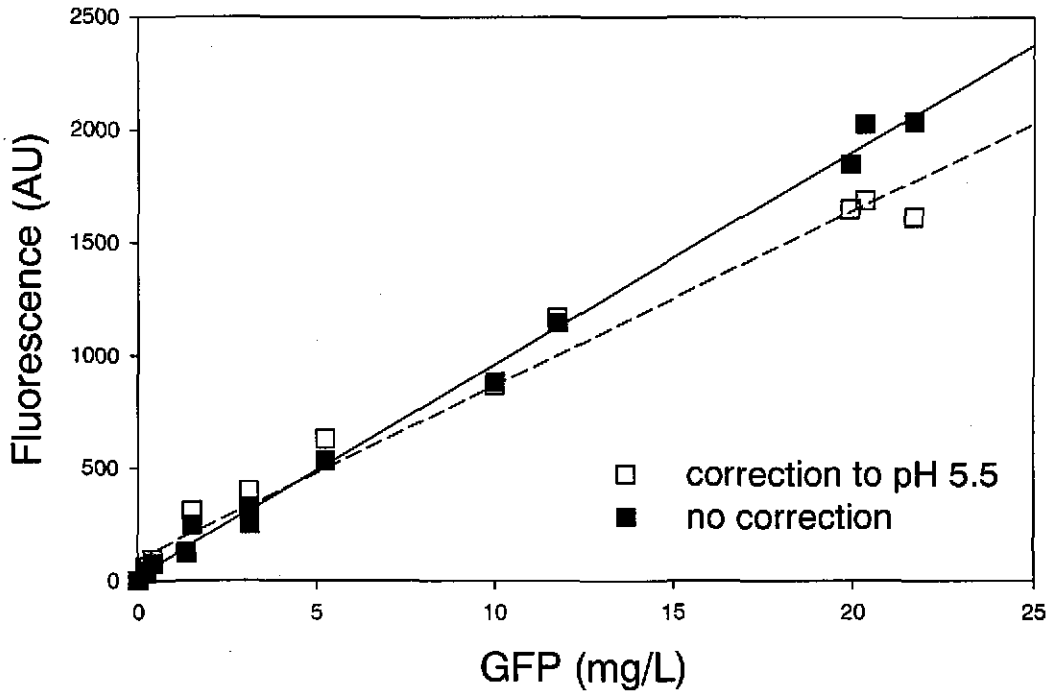

Figure 2.5: Correlation of GFP concentration to the fluorescent intensity in the 7-1 medium. The GFP concentration was determined by using ELISA. Monoclonal anti-GFP from mouse was coated on the plates. Then, anti-GFP from rabbit IgG as detective antibodies was used to bind with GFP. Goat anti-rabbit conjugated enzyme was applied to develop color. Standard GFP (Clontech) was used in each plate to build up the standard curve for determining GFP concentration.

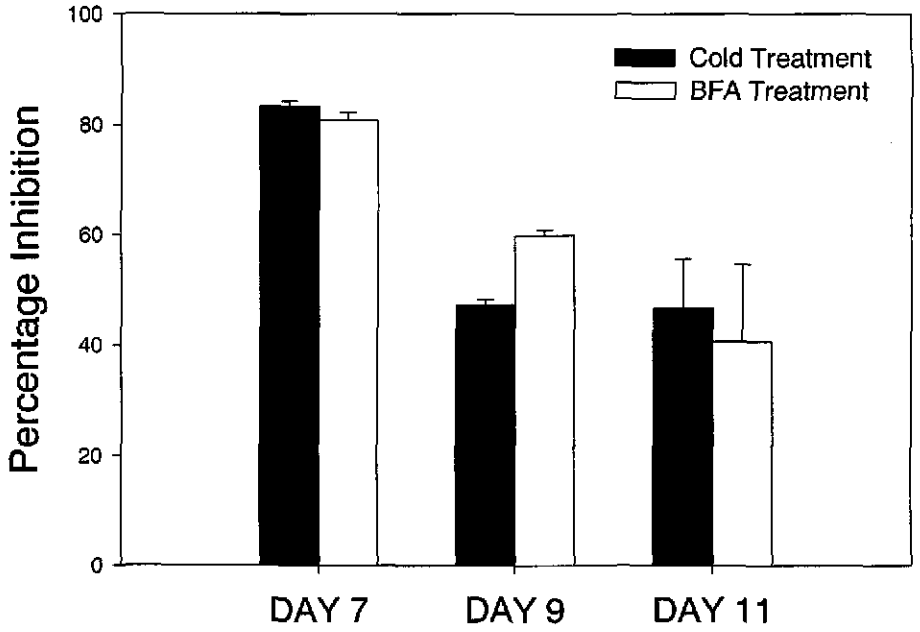

Figure 2.6: Effect of blocking the secretory pathway. For the BFA treatment, a stock solution of BFA was prepared (10 mg ml^{-1} in methanol) and added to the medium at a final concentration of 50 μg ml^{-1}. Spent medium was obtained 2-6 hr following treatment for analysis of GFP fluorescence and measured with spectrophotometer. Percentages are the fluorescent intensity with and without treatment.

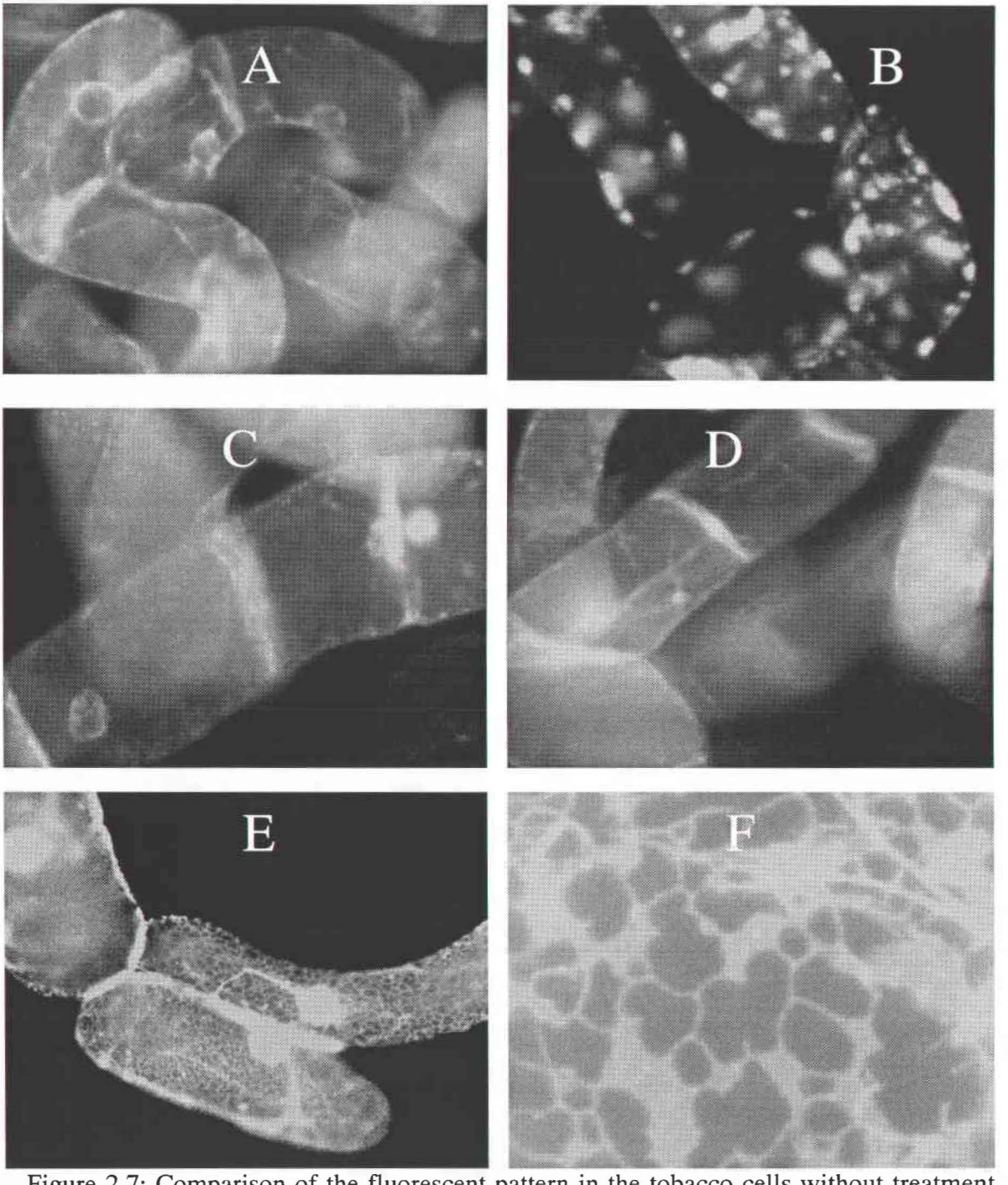

Figure 2.7: Comparison of the fluorescent pattern in the tobacco cells without treatment (A), with BFA for 4 hours at day 7(B), 4°C for 4 hours at day 9 (C), 4°C for 6 hours at day 9 (D), 1-13 cells (E), ER in 1-13 cells (F). Cells were examined by fluorescent microscopy to determine the cellular distribution of GFP.

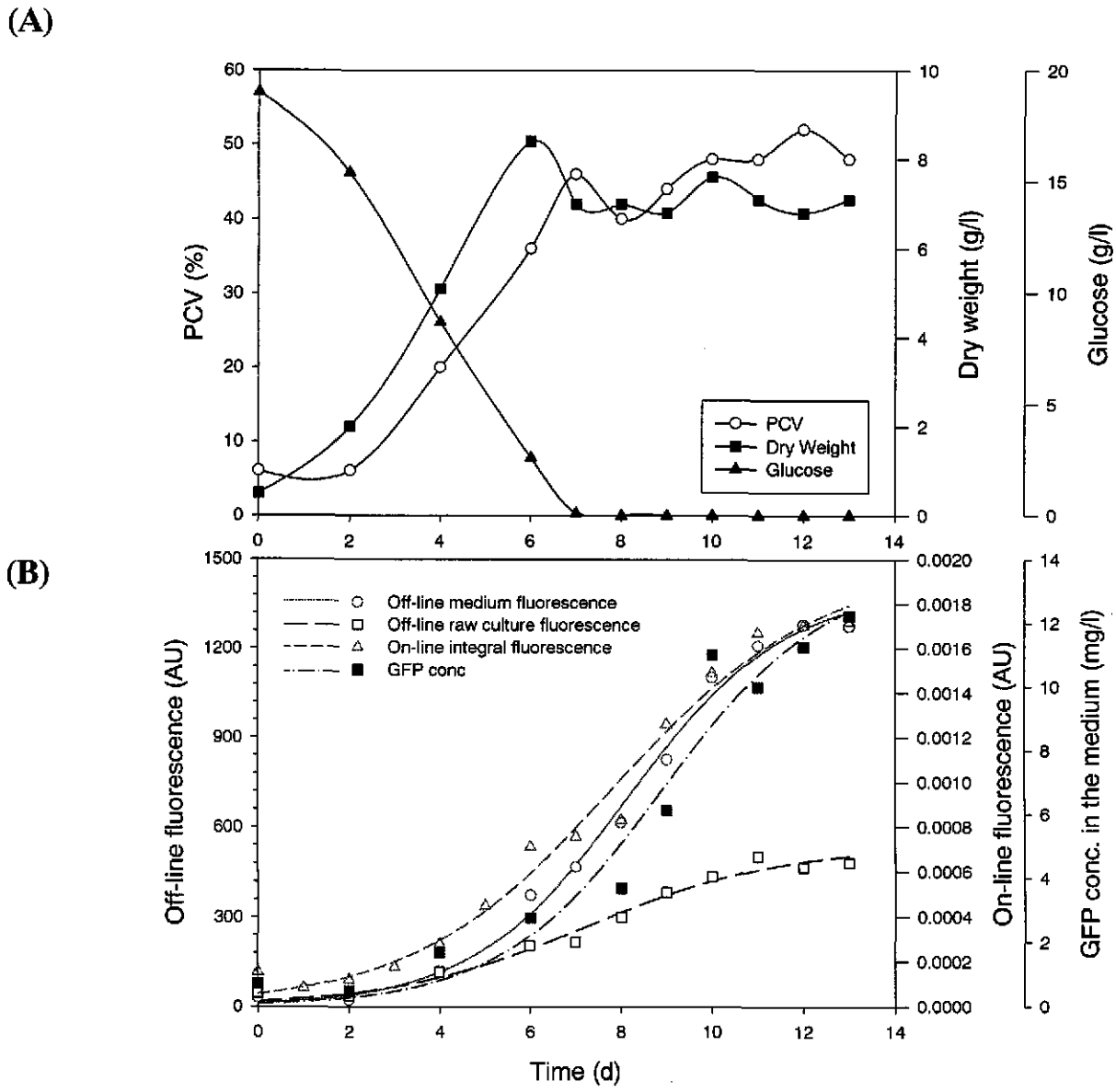

Figure 2.8: Time course of 7-1 bioreactor batch culture. (A). Cell growth and glucose consumption. (B). GFP concentration in the medium and corresponding on-line and off-line GFP fluorescence signals. GFP concentration was determined using western blot and densitometry analysis. Medium and raw culture fluorescence signals were measured offline using the Hitachi F-2500 spectrofluorometer. For the latter, the culture samples taken from the bioreactor were measured directly in the spectrofluorometer without correcting for the inner filter effect caused by the high biomass concentrations.

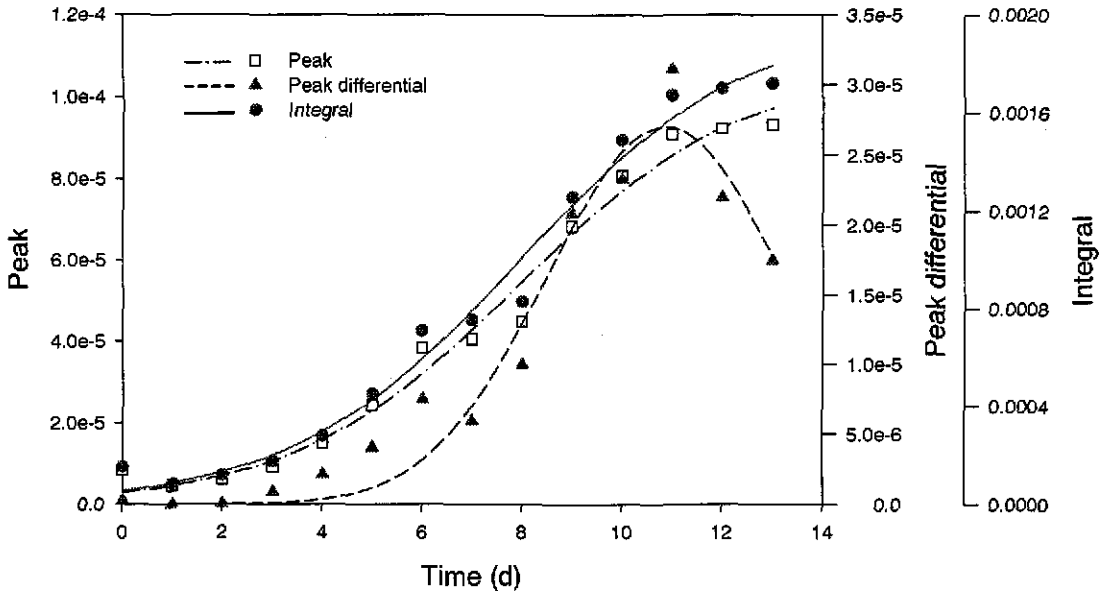

Figure 2.9: Comparison of three modes for quantifying online fluorescence: Peak: peak fluorescence reading of the emission spectra covering 495 to 515 nm; Peak difference: the difference of fluorescence intensity readings between the peak and the reading at 495 nm; Integral: integral of the emission spectra between 495 and 515 nm.

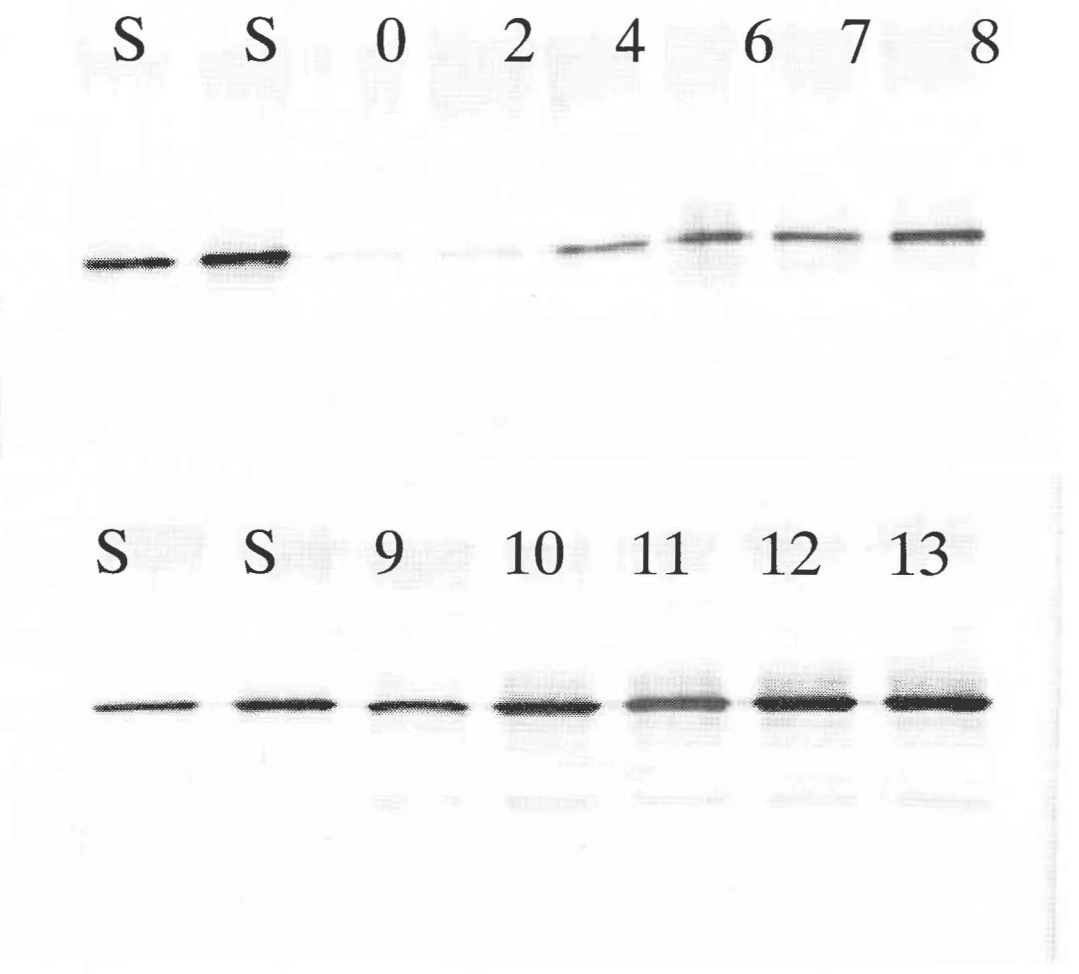

Figure 2.10: Western blot analyses of extracellular protein produced by cell line 7-1 in the reactor. A polyclonal rabbit anti-GFP antibody was used as the primary antibody and a goat anti-rabbit antibody was used as the secondary antibody. 0-9 shows the time course of the experiment with 7.5µl protein sample in each lane. S represents the GFP standard.

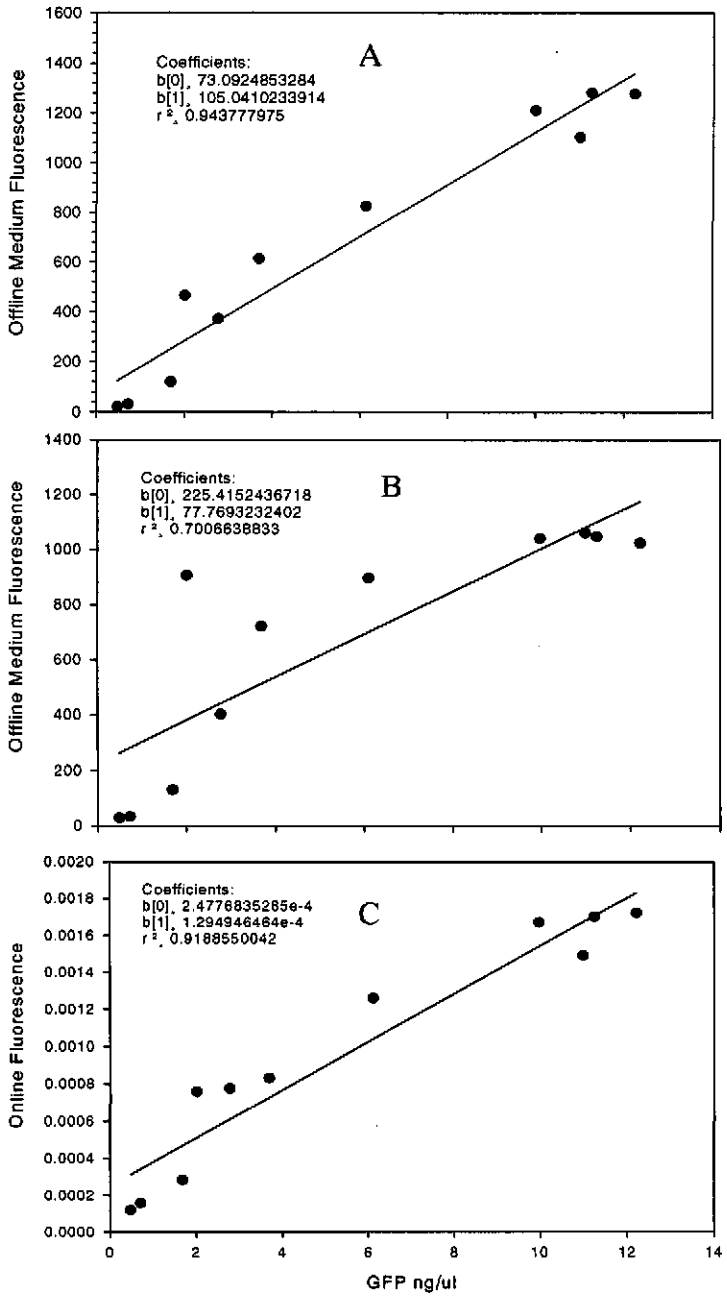

Fig2.11: Plots of GFP concentration vs. Fluorescence. A. GFP vs. Offline medium fluorescence. B. GFP vs. offline medium fluorescence corrected pH. C. GFP vs. online fluorescence.

CHAPTER 3

MONITORING SECRETED HUMAN PLACENTAL ALKALINE PHOSPHATASE PRODUCTION IN TOBACCO SUSPENSION CULTURES USING GREEN FLUORESCENT PROTEIN FUSION

ABSTRACT

Green fluorescence protein (GFP) has become a widely used reporter in many areas of biological research. Monitoring foreign protein expression via GFP fusion is also very appealing for bioprocess application. In this study we report the constitutive expression in stably transformed tobacco cell cultures a fusion protein that links the secreted human placental alkaline phosphatase (SEAP) with GFP. The SEAP-GFP fusion protein is efficiently sorted and retained in the endoplasmic reticulum by using an *Arabidopsis* basic chitinase signal sequence and a HDEL ER-retention signal. Four different extraction buffers were examined to determine the best condition for extracting the fusion protein. The fusion protein was found more stable at pH 8 than at pH 5 upon heat treatment at 65°C. Through non-denaturing gel assays, the SEAP-GFP fusion protein was shown to display both enzyme activity and fluorescence. The specific enzyme activity and fluorescence of the fusion protein, though, were found to be lower than those of SEAP and GFP, respectively. The molecular weight of the fusion protein was estimated to be approximately 103 kDa, indicating glycosylation of SEAP. Degradation of the fusion protein was evident especially after the culture entered the stationary phase or after the sugar was depleted. Before the cell growth reaches its peak, a linear correlation exists between the SEAP activity and either on-line or off-line GFP fluorescence. This study demonstrated the usefulness of GFP fusion as a tool to monitor

foreign protein production in the plant cell cultures. Such capability enables real-time analysis and hence timely control and optimization of foreign protein production in transgenic plant cell culture processes.

3.1. INTRODUCTION

Fusion protein strategy is a popular method in biological research. Fusions between the green fluorescence protein from *Aequorea Victoria* and specific passenger proteins have been widely applied in protein targeting researches. The advantages of GFP-based fusion strategy are visualization, no cofactor for fluorescence and rarely impeding protein function due to the small size of GFP. There have been several cases that successfully employed GFP-based fusion strategy to target fusion proteins to many different cellular compartments in plants, e.g., chloroplasts (Haselof et al., 1995), non-acidic vacuoles and Golgi complex (Sansebastina et al., 1998). Except studying protein targeting, fusion protein strategies have also been used in bioprocesses for better expression (Goeddel et al, 1979), purification (Hochuli et al., 1988) as well as monitoring (Albano et al., 1998; Wu et al., 2000). Due to GFP owes its incomparable advantage, GFP-based fusion strategy is considered as an idea method in the bioprocess monitoring, which allow GFP as a reporter to keep track of the expression of its partner protein in both downstream and upstream process. GFP was fused with the enzymes such as CAT (Albano et al., 1998) and OPH (Wu et al., 2000) in *Escherichia coli*,. The synthesized fusion protein was directly visualized due to the optical properties of GFP. Both of them found that the fluorescence intensity was proportional to the enzyme activity in vivo GFP. In other cases, therapeutic protein was used as GFP's partner. GFP/hIL-2 was successfully expressed in insect *Drosophila* S2 cells (Shin et al., 2003).

Alkaline phosphatase (AP) is frequently assayed in diagnostic laboratories and there is a very simple, fast, and inexpensive colorimetric assay is available for the determination of AP levels in solution (McComb and Bowers, 1972). Human placental alkaline phosphatase (PLAP, EC 3.1.3.1), normally expressed only by cells derived from the placentae of higher primates, has properties significantly different from the other isozymic forms observed in cultured cells. PLAP is unaffected by the presence of 10 mM homoarginine, an efficient inhibitor of other AP isozymes. But the most important different property is the high thermo stability of PLAP that tolerates heat treatments at 65°C for 5 minutes, which effectively eliminates background AP activities (Stigbrand, 1983; Berger et al., 1987). However, The disadvantage of the enzyme as a reporter gene is the expression at the membrane of the cells. To overcome the shortage, Berger et al. (1988) produce a gene that encodes a secreted form of human placental AP, truncating the PLAP gene by the C-terminal 24 aa. The mutant was termed SEAP that also can be quantified within the media of transfected cultures using either a standard colorimetric assay for AP activity or a highly sensitive bioluminescence-based AP assay (Berger, et al, 1988). The SEAP assay system is simple, cheap and sensitive. As a reporter gene in transgenic cells, SEAP can produces qualitatively and quantitatively results.

To date, there have been no reports on the application GFP as a fusion partner for monitoring foreign protein production in the plant bioprocesses. Here, We use functional SEAP-GFP fusion protein to study the utility of GFP fluorescence in bioprocess optimization and tracking of a specific foreign protein expression in the tobacco suspension cells. Although the degradation of the fusion proteins cause the different behave of the two proteins and finally contributed to the divergence of the linear

correlation at later culture time, the linear correlations exist between the fusion protein concentration and SEAP activity with the fluorescence of GFP before the growth peak in the bioreactor, indicating the utility of GFP fluorescence in monitoring other protein is feasible.

3.2. MATERIALS AND METHODS

3.2.1. Fusion protein construction

The secreted form of human alkaline phosphatase (SEAP) reporter gene was obtained from the SEAP2-Basic Vector (Clontech, Palo Alto, CA). The sequence of the SEAP gene was modified by the polymerase chain reaction (PCR) with Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA) using a forward primer 5'-GGCTACAGCTCTCCCTGGAATTCATCCCAGTTGAGGAGG-3' and a reverse primer 5'-TCATCAATGTATCTTAGAATTCCTGCTCGAAGCGGCCGG-3' to remove the SEAP signal peptide and to incorporate an *Eco*RI restriction site (underlined region) on both ends of the SEAP gene. The *Eco*RI sites were integrated such that they were in the same reading frame as the SEAP gene. The amplified fragment was digested with *Eco*RI and ligated into the same site of Bluescript SK (Stratagene, La Jolla, CA). The modifications to the sequence were verified by DNA sequencing. The binary vector pBIN-mgfp5-ER was digested with *Eco*RI which cleaves between the *Arabidopsis thaliana* basic chitinase signal sequence and the mgfp5-ER followed by ligation of the *Eco*RI-digested SEAP into the binary vector. Orientation of SEAP was verified by restriction digestion with *Xba*I. The SEAP was integrated in the same coding frame to produce a signal peptide-SEAP-mgfp5-ER fusion protein.

3.2.2. Tobacco transformation and selection

The plant expression constructs were transferred into *Agrobacterium tumefaciens* LBA4404 by electroporation. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) transformation and subsequent transformant selection were carried out essentially as described in Liu et al. (2001), which was based on the procedures reported by Horsch et al. (1985) with modifications of Fisher and Guiltinan (1995). Stem tissue from the young primary transformants was cut into small pieces and positioned on solid Murashige and Skoog medium (Murashige and Skoog., 1962) containing Gamborg's vitamins (Gamborg et al., 1968) and 2% sucrose, and supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 300 mg l^{-1} kanamycin and 500 mg l^{-1} carbencillin for callus induction and culture.

3.2.3. Suspension cell culture

Generation and maintenance of tobacco suspension cell cultures were performed as previously described (Liu et al. 2001). Media and growth conditions were identical to those reported in Liu et al. (2001) except that Murashige and Skoog medium was used instead of Gamborg's B5 medium. In shake-flask time-course experiments, to each one-liter baffled-shake flask, 300 ml of culture medium was inoculated with a 15% (v/v) inoculum from a 7-day-old cell culture. The culture was sampled periodically for the time course determination of growth and protein production parameters.

3.2.4. SEAP-GFP extraction and fluorescence determination

A three ml sample of cell suspension was collected under vacuum on a nylon mesh (10 μm pore size) fitted in a Buchner funnel, and the cells were washed three times with dH2O. The cells were resuspended in 3 ml of five different kinds of buffer (A: 50 mM Tris, 200 mM NaCl, 1 mM PMSF, pH8.0; B: 50 mM Tris, 200 mM NaCl, 1 mM PMSF, pH5.0; C: 200 mM Tris, 50 mM NaCl, 1 mM PMSF, pH8.0; D: 200 mM Tris, 50

mM NaCl, 1 mM PMSF, pH8.0; G: 10 mM Tris, 200 mM NaCl, 50 mM NaH_2PO_4, pH8.0) and lysed using an ultrasonic cell disrupter equipped with a microprobe for 5 min at its lowest output setting (Branson Model 250, Danbury, CT). The supernatant was collected after centrifugation at 3200 g for 5 min.

Off-line GFP fluorescence intensity measurements were obtained using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan) at an excitation wavelength of 473 nm and an emission wavelength of 507 nm. To minimize the interference of the inner filter effect (Srinivas and Mutharasan, 1987), samples for culture and intracellular fluorescence measurements were diluted respectively using ddH_2O and extraction buffer, when necessary, to the linear fluorescence response range, and then the fluorescence of the diluted sample was linearly extrapolated. For medium fluorescence measurements, sample dilution was unnecessary since the inner filter effect was found to be inconsequential (data not shown)

3.2.5. SEAP assay

The intracellular extraction was heated at 65°C, for 5min, then centrifuged at 7,000xg for 2 min. The supernatant was collected. A 100ul supernatant sample was taken and added into 700 ul DEA buffer (1M Diethanolamine, 0.5 mM MgCl2.6H2O, pH 9.8). The mixture was incubated it at 37°C for 10 min. Then 200 ul 54.5 mM pNPP was added into the solution continuing incubation at 37°C. 250 ul 3N NaOH was used to stop reaction in each tube. The absorbance at 405 nm was measured using a spectrophotometer (Shimadza Model UV160U, Japan).

3.2.6. Western blot analysis

Samples were mixed with an equal volume of 2× reducing sample buffer, resolved on a 10% SDS-polyacrylamide gel as described by Laemmli (1970) and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). Purified recombinant GFP (Clontech, Palo Alto, CA) was used as a standard to quantify GFP levels. Western analysis was performed as previously described (Liu et al. 2001) using an anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Quantification of the immunoreactive GFP bands on the Western blots was done using scanner (hp scanjet 5400C) at resolution 200 and the SigmaScan Pro 5.0 image-analysis software (SPSS, Chicago, IL).

3.2.7. In-gel enzyme staining and fluorescence imaging

The samples were mixed with non-reduced gel loading buffer (Bromophenol blue 1 mg/ml, 50% glycerol) without boiling and resolved on a 10% SDS-polyacrylamide gel as described by Laemmli (1970). The pictures of in-gel fluorescence imaging were taken by using blue light shining below the gel. For in-gel enzyme staining, the gel was immersed in the dye buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl2 6H2O, pH 9.5) containing 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4chloro-3-indolyl phosphate (BCIP), which is the same as that of the western blot, for at least 30 min to totally develop color. The gel was washed and kept in 7% (v/v) acetic acid solution.

3.2.8. Ammonium sulfate precipitation

A saturated salt solution (4M) was prepared and slowly added to the protein mixture to reach the salt saturated concentration 30%, 50% and 80%. The mixture was kept in the ice for 10 minutes, and then centrifuged in 4°C at 10,000xg for 10 minutes.

Pellets were re-suspended in the extraction buffer A. The microcons (Millipore) were used to desalt in the centrifuge at 2,000xg, for at least 20 minutes. For step precipitation, the supernatant from the former step precipitation was collected for next high percentage precipitation.

3.2.9. Other measurements

Cell dry weight and packed cell volume (PCV) were determined as previously described (Liu et al. 2001). Protein was determined using a protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard (Bradford, 1976). Glucose in the medium was measured using a hexokinase/glucose-6-phosphate dehydrogenase assay kit (Sigma, St. Louis, MO).

3.2.10. Bioreactor culture and online monitoring

Transgenic tobacco cell line 10-6 containing heterologous protein, SEAP/GFP fusion, selected from the transgenic calli was cultivated in a 3-liter stirred-tank bioreactor (BioFlo III fermenter, New Brunswick Scientific, Edison, NJ) with online monitoring of culture fluorescence and dissolved oxygen control. In the bioreactor culture, glucose at 20 g l^{-1} was used as the carbon source. The dissolved oxygen was controlled at 30% air saturation by automatically regulating the sparged gas composition using two mass flow controllers controlled by a PID controller. The PID algorithm was coded in LabVIEW (National Instruments, Austin, TX). The culture temperature was set at 25°C with an aeration rate of 0.5 l min^{-1} and an agitation rate of 150 rpm. Culture pH was controlled at 5.5 by adding HCl or KOH solution *via* the BioFlo reactor's pH controller. The online fluorescence monitoring system consisted of a 100W Xenon lamp light source, a 470 nm interference filter, a bifurcated fiber optic cable attached to a 12-mm diameter light rod

through a removable coupling, a monochrometer with a photomultiplier tube, and a constant amp lock-in amplifier. Except for the fiber optic cable and the light rod, which were purchased from Fiber Optic Components, Inc. (Sterling, MA), the rest of the fluorescence sensor components were obtained from Thermal Oriel (Stratford, CT). The light rod was inserted into the reactor through the head-plate. Fluorescence spectra (495-515 nm) were acquired online and analyzed using the Thermal Oriel TRACQ32 software. In this work online fluorescence intensity was defined by the area under the fluorescence peak between 495-515 nm and measured in arbitrary units. To this end, integrated intensity signal has been commonly used in culture fluorescence measurements (Li and Humphrey, 1992; Horvath and Spangler, 1992). During the bioreactor culture, fluorescence intensity of the spent medium was also measured off-line using the Hitachi F-2500 spectrofluorometer as described earlier.

3.3. RESULTS

3.3.1. Determination of the extraction conditions for the fusion protein

Four extraction buffers (A, B, C, D) and GFP extraction buffer (G) were tested for extraction of the SEAP-GFP fusion protein. All extracts were heated at 65°C for 5, 10, 20 and 30 minutes. Wild type tobacco cells and 1-13 cells that express an ER-retained GFP were used as controls, and were extracted using buffer C.

SEAP activity was measured from above samples (Fig. 3.1). Without heat treatment, the activity of endogenous alkaline phosphatase was detectable in the wild type cells and 1-13 cell line. However, the activity of endogenous alkaline phosphatase was slightly reduced in the first 5 minutes of heating and decreased a little further when exposed to additional heat treatment up to 30 minutes. Buffer pH is an important factor

to affect the enzyme activity. The enzyme in low pH buffer within 5 minutes heat treatment or without heat treatment has more activity than that in high pH. But in the continuous heat treatment longer than 5 minutes the enzyme activity in high pH increases and exceeds the activity in low pH (Fig. 3.1, 3.2). Higher NaCl concentration can help the enzyme to keep higher activity in the buffer of pH 5. Due to phosphate in the GFP extraction buffer (Bowers et al, 1966), the enzyme activity by using GFP extraction buffer is lower than by using others.

To check the pattern of fusion proteins in the assay, two kinds of antibodies, anti-GFP and anti-human placental alkaline phosphatase (PLAP), were applied in western blots. Multiple bands were observed from the Western blot membrane by using anti-GFP antibodies (Fig. 3.2). With increasing heating time, the more protein degradation was observed from the blots. One apparent band above the SEAP standard was detected on the Western blot membrane using anti-human PLAP as primary antibodies without heat treatment (Fig. 3.3). The higher position band is also found in the anti-GFP membrane indicating the bands on the top position represent the SEAP-GFP fusion protein. In addition, we notice that when using low pH buffer, fusion protein disappeared with heat treatment (Fig. 3.3B and C). Some detectable bands near SEAP standard were found, which could be the degradation of fusion protein and may contain SEAP epitope (Fig. 3.3).

3.3.2. Culture fluorescence, SEAP activity and cell growth in flask cultures

Prior to conducting the bioreactor experiments, we conducted flask experiments to evaluate the feasibility of monitoring SEAP production based on GFP fluorescence. Five flasks, each contained 300 ml of the 10-6 suspension cells, were used for time-

course studies. Growth of the suspension cells was characterized by measurements of both cell dry weight and PCV. In addition, culture, intracellular, and medium fluorescence were measured over the culture cycle. The culture fluorescence correlates with cell growth, which is similar with those reported previously (Liu et al. 2000). The medium fluorescence was detectable but with rather low intensity. The intracellular fluorescence and SEAP activity also show a similar trend over 14-day time course (Fig. 3.4).

3.3.3. Online monitoring GFP production in the batch reactor

Further studies of the 10-6 culture were conducted in the bioreactor with a fiber-optic sensor. Suspension cells of cell line 10-6 were cultivated in a stirred–tank bioreactor for 12 days. Cell growth was determined based on cell dry weight, PCV and glucose concentration (Fig. 3.5A). Both online and offline fluorescence showed correlation with the cell growth (Fig. 3.5B). The trend of SEAP activity deviated from those of cell growth and fluorescence during later stage of culture. Samples from the bioreactor culture were analyzed via Western blot using anti-GFP and anti-PLAP antibodies (Fig. 3.6). Purified GFP (Clonetech) and SEAP (Sigma) antibodies were respectively applied to each blot as a standard. The blots were scanned and the immunoreactive bands quantified by image analysis in order to examine the relationship between the fluorescence intensity and fusion protein quantity. The amounts of fusion protein determined using the two sets of antibodies and standards are quite different, although their trends are similar (Fig. 3.7). We also noted similar trends of the protein degradation over the time course using the two different antibodies (data not shown). The results from two sets of antibody and standard are not equal because the SEAP standard that was used

to determine concentration is unpurified products. So the data from anti-GFP and GFP standard could be more accurate. Analysis of western blot from the reactor samples indicated that production of fusion protein is correlated with cell growth, which it reaches the peak at the 6^{th} day. After 6^{th} day, some degradation bands appeared. Increasing degree of fusion protein degradation was noted after growth peaked on day 6 (Fig. 3.7).

The correlation between intracellular fluorescence and fusion protein concentration was linear throughout a major part of the culture (Fig. 3.8A), with a correlation coefficient for the linear regression (r^2) of 0.91. The correlation between off-line raw culture fluorescence and fusion protein concentration was also linear (Fig. 3.8B). The correlation however was not so good when considering samples from later stage cultures (i.e. samples from days 10 and 12). The correlation between the fluorescence and SEAP activity also show a similar situation (Fig. 3.8C and D).

3.3.4. Comparison of Young (7days) and Old (14 days) Cultures

To figure out the divergence between the fluorescence with SEAP activity at later culture time, specific SEAP activity from the old and young suspension cells were compared. The intracellular extractions of 7-day 10-6 culture and the 14-day culture were used to do the SDS-PAGE, enzyme stain, Western blot (based on the same protein concentration), and SEAP assay to compare the character of fusion protein (Fig. 3.9).

Using non-denaturing SDS-PAGE, the GFP fluorescence can be detected directly with blue light. The fluorescence from fusion protein was observed from both young and old samples. Some degradation bands with fluorescence, especially near GFP molecular weight, were found on the young and old samples. The old sample has more degradation bands than young sample (Fig. 3.9, A). Only one band on the fusion protein position was

found using enzyme stain directly on SDS-PAGE for all of the samples, indicating that fusion proteins have the enzyme activity (Fig. 3.9B). Meanwhile, Western blots were used to check the pattern fusion protein (Fig. 3.9C and D). There is no difference between the fusion protein parts on the blots. The degradation was found in both blots using anti-GFP and anti-SEAP, and the samples from old cells have more degraded protein fragments.

SEAP activity, western blot, and enzyme stain were also conducted among the samples with or without heat treatment to determine specific SEAP activity. Both western blot and enzyme stain are based on the same protein concentration. Only fusion proteins have the enzyme activity in enzyme stain. However, the samples after 5 minutes heat treatment have darker bands on both western blot and enzyme strain, indicating somehow after heat treatment the percentage of SEAP protein was increased. The result of SEAP assay shows that the old samples have the higher SEAP activity than the young samples. But the SEAP assay for WT did not show dramatically increasing SEAP activity compared with the young and old (data not shown). Combined with western blot analysis and the SEAP assay, the specific SEAP activity of the fusion protein calculated here is much lower than that of SEAP standard (Sigma). Meanwhile SEAP activity is somewhat different between the young and the old, heated and without heated (Fig. 3.11).

3.3.5. Salt fractionation of the fusion protein

The pellets from 30%, 50%, and 80% ammonium step precipitation were re-suspended in 1 ml extraction buffer A. The fluorescence of each sample was measured using a fluorescence spectrophotometer. The highest fluorescence was found in the sample using 50% ammonium sulfate step precipitation while there was a little

fluorescence in the sample using 80% ammonium sulfate precipitation that showed degradation GFPs as the majority on the western blot. The SEAP assay revealed the same trend. Western blot analysis showed that most fusion proteins can be obtained in the condition of 30% to 50% ammonium sulfate step precipitation and most fragment GFP will be achieved in that of 50%-80% (Fig. 3.12). The blot was scanned and the GFP concentration was calculated. Compared with former data, the fluorescence of per mole total GFP is lower than the GFP in cell line 1-13 (GFP-ER) and 7-1(sp-GFP-ER) (Fig. 3.13). It was also found that the fluorescence of the degraded GFP fragment is lower than that of the SEAP-GFP fusion protein (data not shown).

The pellets from 30%, 50%, and 80% ammonium step precipitation were re-suspended in 1 ml extraction buffer. The fluorescence was measured using a fluorescence spectrophotometer. Compared with normal sample, most part fluorescence was found in solution using 50% ammonium sulfate step precipitation and there is a little fluorescence in 80% ammonium sulfate precipitation. The SEAP assay had the same appearance. Western blot analysis showed that the most fusion proteins can be obtained in the condition of 30% to 50% ammonium sulfate step precipitation and most fragment GFP will be achieved in that of 50%-80% (Fig. 3.12). The blot was scanned and the GFP concentration was calculated. Compared with former data that was showed in chapter 2, the fluorescence of per mole total GFP, fusion form and degradation form, is lower than the GFP in cell line 1-13 (GFP-ER) and 7-1(sp-GFP-ER) (Fig. 3.13A). It was also found that the fluorescence of degradation GFP in the cell line 10-6 is also lower that the fusion part GFP (Fig. 3.12B).

3.4. DISCUSSION

The objective of this work is to study the utility of GFP fusion for monitoring foreign protein expression in a plant cell bioprocess. We already reported that the GFP fluorescence signal correlated with the increase of cell mass (Liu et al, 2000) and the utility of GFP fluorescence for on-line monitoring cell growth and protein expression (Chapter II). GFP fusion protein has often been reported in the study of the protein secretory pathway in microbial (Khalaj et al., 2001), in plant (Batoko et al., 2000) as well as animal (Kaether et al., 1995), but the studies on bioprocess applications of GFP in plant cell cultures have been rare. It has been reported that using the fusion between GFP and CAT, the fluorescence intensity of GFP correlates the activity of the downstream CAT protein in *Escherichia coli* and there is a quantitative relationship between the level of CAT protein concentration and GFP fluorescence (Albano et al, 1998). A fusion strategy to facilitate the on-line monitoring of OPH (organophosphorus hydrolase) expressed and localized in the soluble fraction of recombination *Escherichia coli* developed by the other group to improve enzyme production efficiency, enhance field use and monitoring (Wu et al, 2000). These work supported the use of GFP as an in *situ* reporter gene for scale-up and process optimization of microbial fermentation for recombinant protein production. For plant cell cultures, however, no report has been published on the kinetics and monitoring of GFP fusion protein expression.

In this study, we have successfully demonstrated the expression of SEAP-GFP fusion protein in tobacco suspension cell cultures. The fusion protein extraction condition was examined first to preserve the activity of both GFP and SEAP. We prepare four buffers with two different pHs (pH 5, 8) to check whether there is the big difference

appearance by using these buffers. Actually, we found that the high enzyme activity exist in the acid environment with short time heat treatment. It was realized later in the western blot that the high SEAP activity in the low pH extraction buffer was caused by the fusion protein degradation. The low SEAP activity found in using GFP extraction buffer is caused by phosphate that can inhibit the SEAP activity (Bowers et al, 1966).

There were the linear correlations between SEAP activity and fusion protein with GFP fluorescence in the early time of cell growth (Fig. 3.8). After cell growth reaches peak, more and more degradations of fusion protein were observed from western blots (Fig. 3.6). Since the cleavage of fusion protein exists, the relation between the fluorescence and fusion protein become complicate. It was revealed two clipped fusion fragments near the molecular weight of the wild type GFP portion using anti-GFP antibodies. This kind fusion protein degradation was also reported in the studying of GFP-OPH (Wu et al, 2000). Some GFP variants that have lost ~10 residues from their C terminus are known to retain fluorescence in the ER and cytoplasm (Gadella et al., 1999). In the experiment of comparison of young and old cultures, the fluorescence of cleaved GFP was directly observed, particularly in old cultures, by using blue light directly shining the samples in the SDS-PAGE (Fig. 3.9A). The observation on the gel was identical with the western blot for the samples from reactor (Fig. 3.6A) which showed severe GFP degradation in the last several days. Although the fluorescence from the cleaved GFP was lower than the fusion protein (Fig. 3.12B), whit its accumulation its fluorescence cannot be ignored when the majority are the degradation. The fluorescence cannot only represent fusion protein also included cleaved GFP at the later culture time. In addition, the fluorescence of per mole fusion protein is lower than the GFP

fluorescence in cell line 1-13 and 7-1 (Fig. 3.13A) although the highest GFP concentration in the fusion protein reaches 0.77 mM, compared with the highest concentration 1.1mM of GFP of cell line 1-13.

There is also a more complicate relationship between the SEAP activity and GFP fluorescence at the later culture time. Analyzed the data from western blot and SEAP assay, the specific SEAP activity in the fusion protein was much lower than that of standard (Sigma) (data not shown). Moreover, the old samples had higher enzyme activity than the young, and the heated samples had higher enzyme activity than those not heated. The old with heat treatment obtained the highest enzyme activity among those samples. The difference of the SEAP activity, especially between the heated and without heat, may be caused by the SEAP degradation from the fusion protein (Fig. 3.10A). The old sample and the heated sample had more degradation SEAP bands on the western blots. Western blots showed a SEAP fragments from the fusion protein using anti-SEAP antibodies, which position is around standard SEAP (Fig. 3.3, 3.6 and 3.9D). Although the SEAP fragment cannot be detected by the enzyme staining, by using low pH buffer and with heat treatment the fusion protein disappeared on the western blot while some SEAP degradations were detectable and the enzyme activity was still high. We suspected that the SEAP fragments have more efficient enzyme activity without GFP impeding. The failure of in-gel enzyme staining for SEAP fragments may be caused by the different degradation molecular weight, which made the fragments hard to form one band, or the SEAP fragments are more sensitive to the SDS than the fusion protein and standard SEAP. When the level of fusion protein degradation is severe, the correlation of SEAP activity with fluorescence will be weak as the GFP fragments have low fluorescent

intensity and SEAP fragments have high enzyme activity. There is less degradation at the early time course. So our data showed a perfect linear correlation between fluorescence and SEAP activity before the growth peak in bioreactor.

In conclusion, GFP-based fusion strategy is a useful tool to apply in the bioprocess study. Since the cultures will be harvested before cell growth reaches the plateau at most time, the production of foreign protein can be online monitored by using GFP as its translational partner in the bioreactor. Moreover, fusion of GFP to a foreign protein may interfere with the function of each other in transgenic plants.

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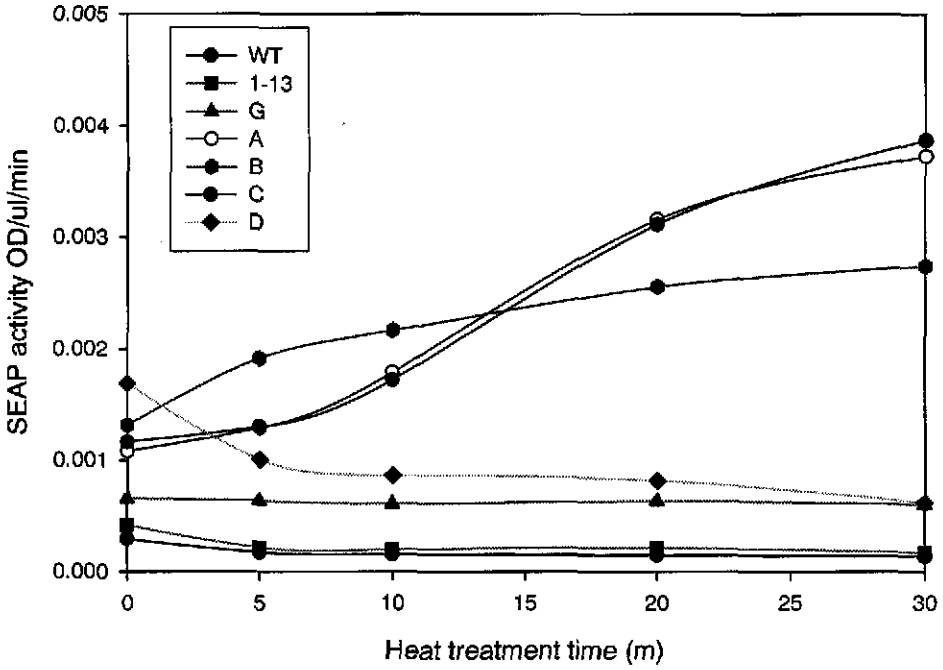

Figure 3.1: SEAP activity in different buffers. The tobacco suspension cells from 10-6 were extracted with A, B, C, D and G (GFP extraction buffer). The extraction from wild type (WT) and 1-13 cell line with buffer C is as control. The protein extract was treated with 65°C heat treatment for 5, 10, 20 and 30 min. 1-13 is the transgenic cell line that only contains GFP.

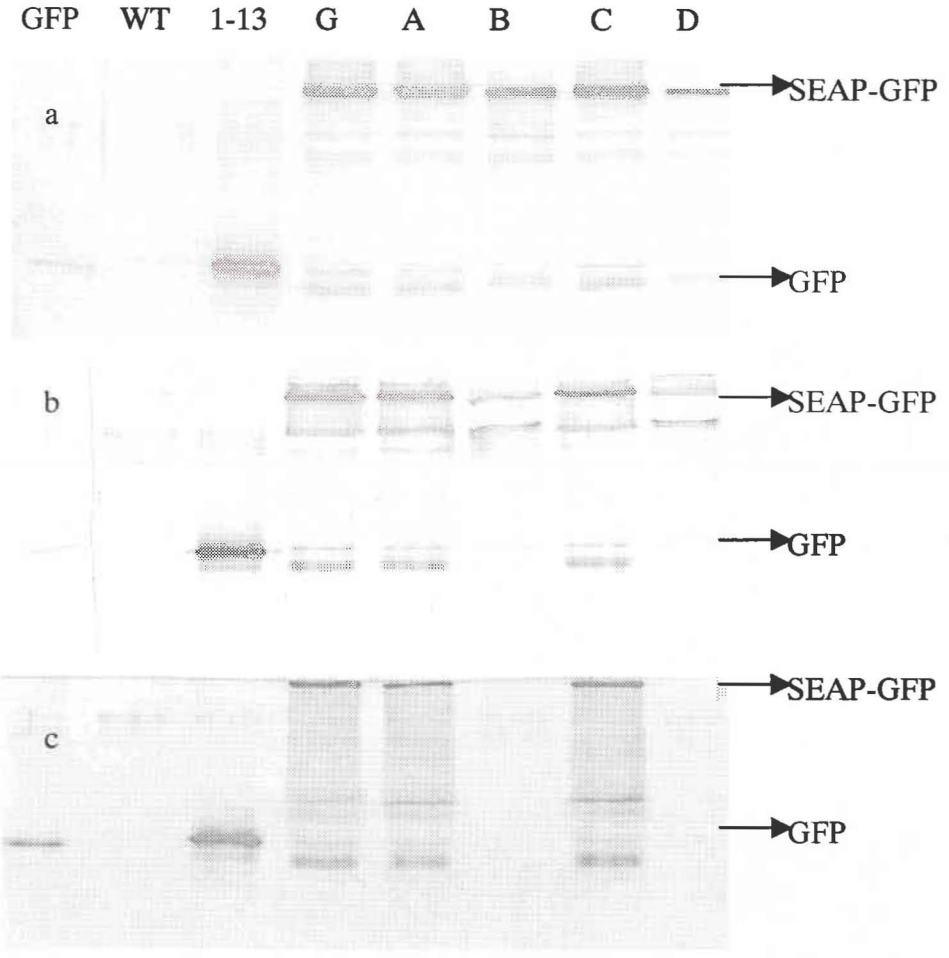

Figure 3.2: Patterns of western blot for different extraction buffers and heat treatment detected by anti-GFP as a primary antibody. The 10 ul protein samples were loaded into each lane. a. Without heat treatment. b. With 5 minutes heat treatment. c. With 30 minutes heat treatment.

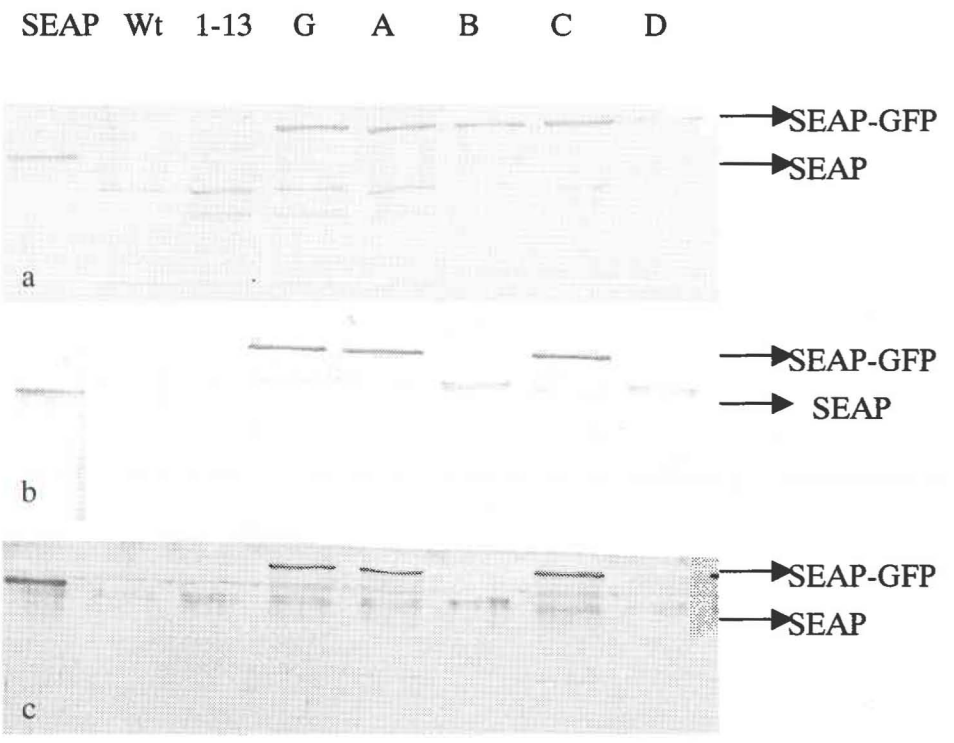

Figure 3.3: Patterns of western blot for different extraction buffers and heat treatment detected by anti-SEAP as a primary antibody. The 10ul protein samples was loaded into each lane. a. Without heat treatment. b. With 5 minutes heat treatment. c. With 30 minutes heat treatment.

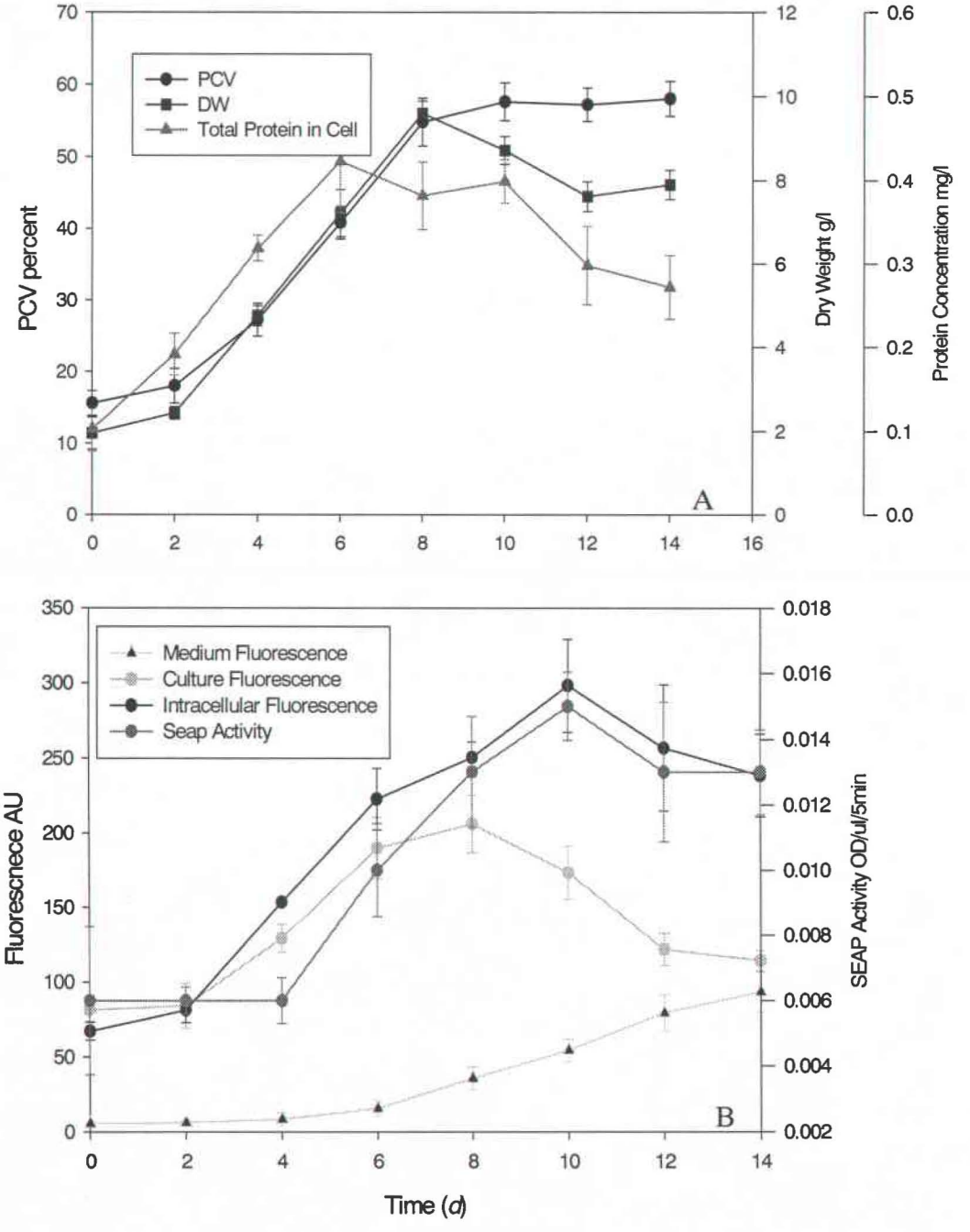

Figure 3.4: Time course for cell growth, fluorescence and SEAP activity in flasks. A. Cell growth. PCV was calculated by measuring the volume of the supernatant of 5 ml of culture sample. Cell dry weight was determined by drying cells from 5 ml culture in an oven at 50°C until a constant weight was reached. Protein was determined using a protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard (Bradford, 1976). B. Fluorescence and SEAP activity. For offline fluorescence, 1 ml sample was measured at an excitation wavelength of 473 nm and emission wavelength of 507 nm using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan)

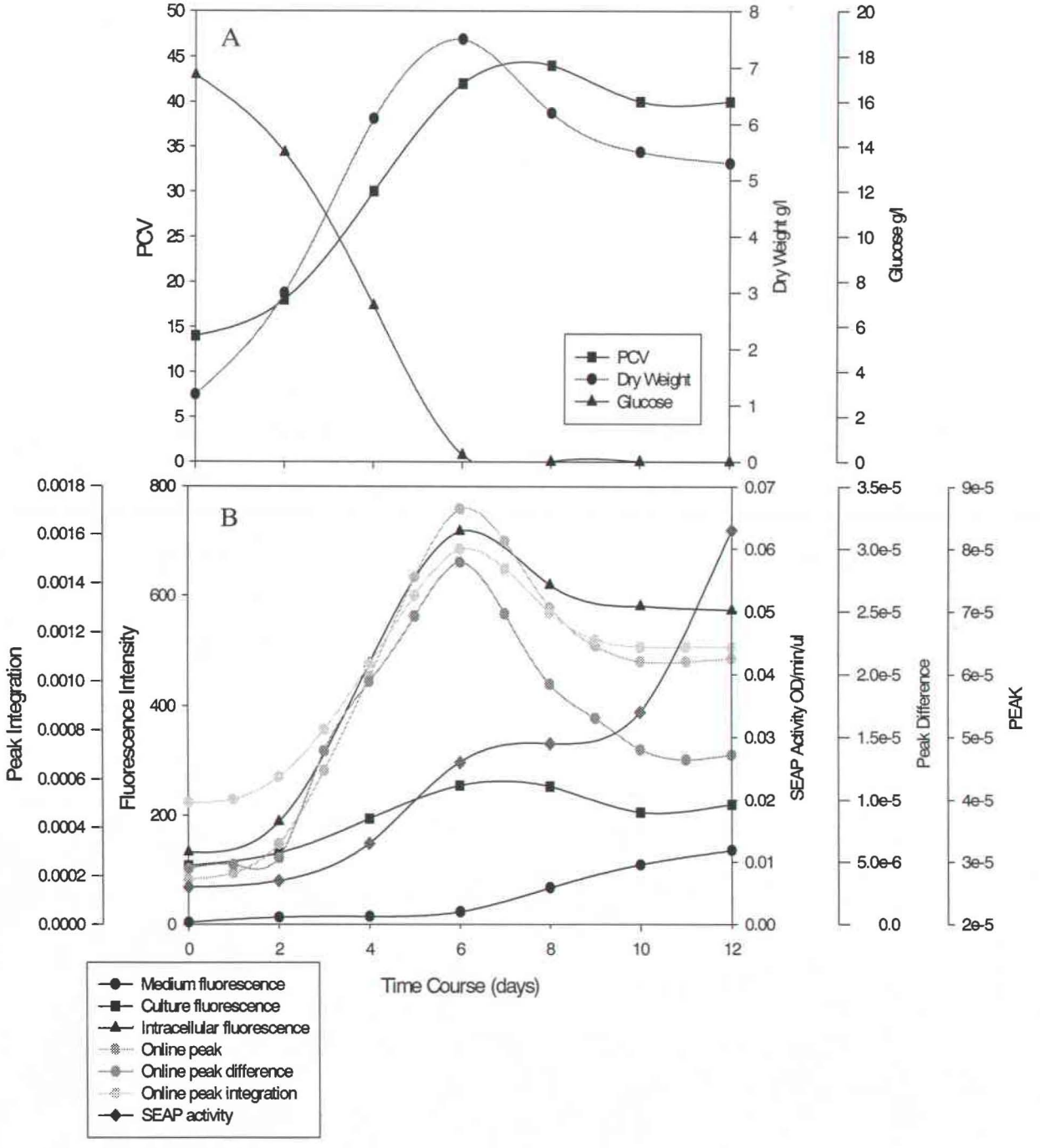

Figure 3.5: Time course of 10-6 batch bioreactor. A. Cell growth. B. Online fluorescence, offline fluorescence and SEAP activity. The method of offline fluorescence measurement is the same as that in the flasks.

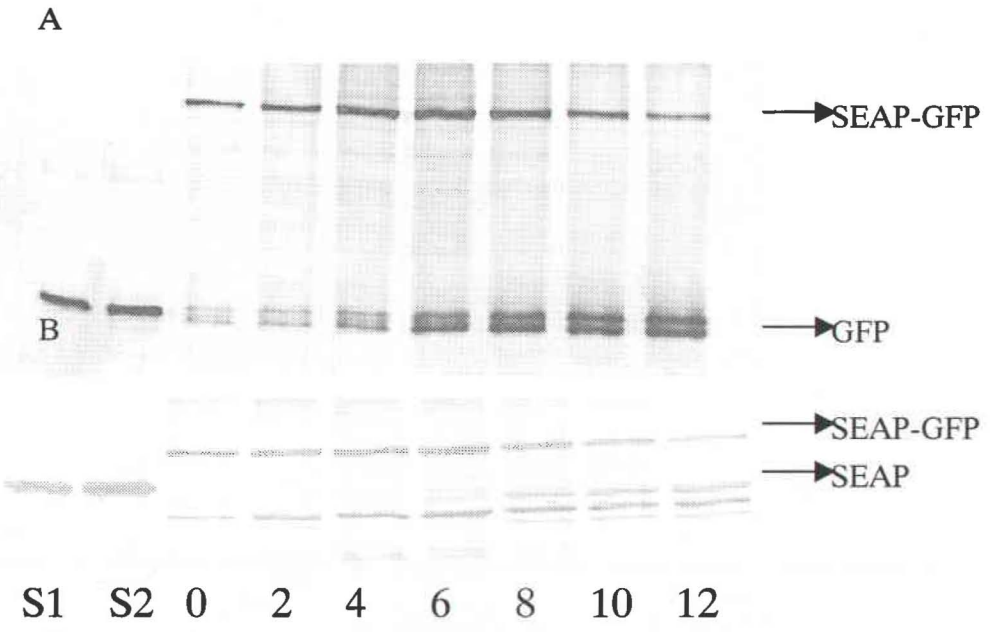

Figure 3.6: Western blot for the samples from 10-6 bioreactor. A. Anti-GFP as a primary antibody. B. Anti-SEAP as a primary antibody. Lanes 0-12 show the date of the samples taken from the reactor. 10 ul protein sample was loaded in each lame. S1 and S2 represent two different quantities of the standards. GFP standards are 20 and 50 ng. SEAP standards are 2 and 5 mg.

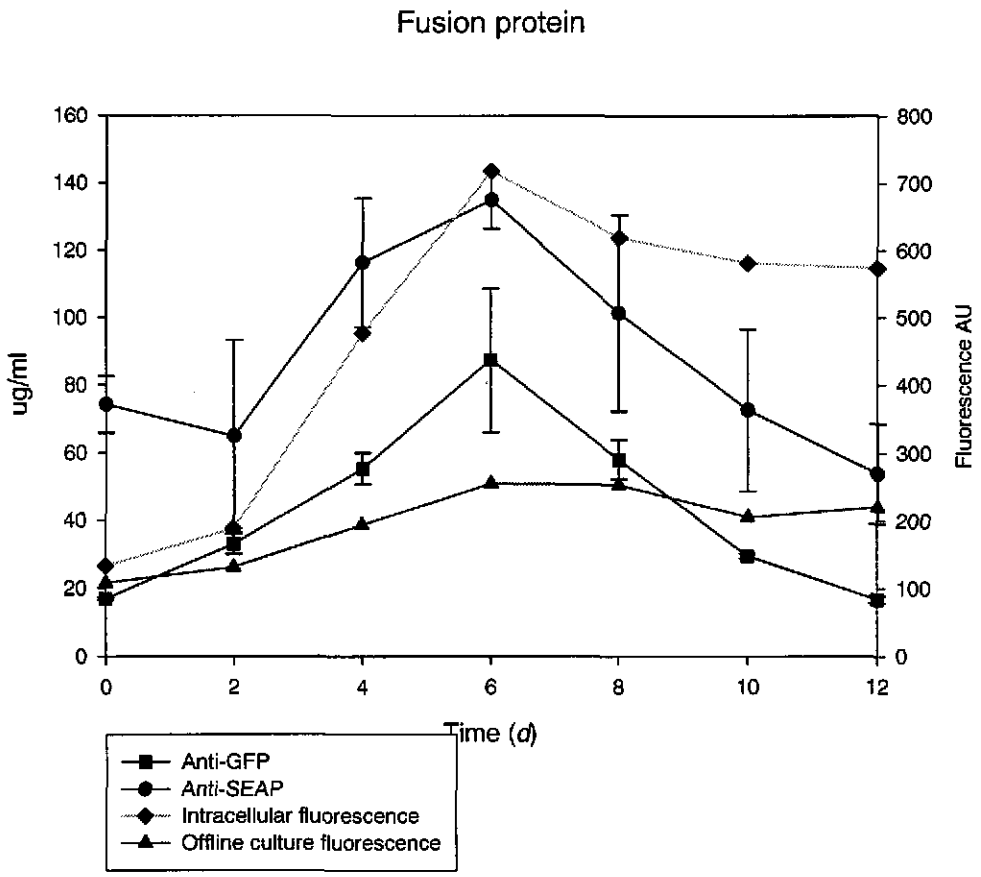

Figure 3.7: Time course of fusion protein concentration and fluorescence in the bioreactor. GFP concentration was come from the analysis of western blot intensity

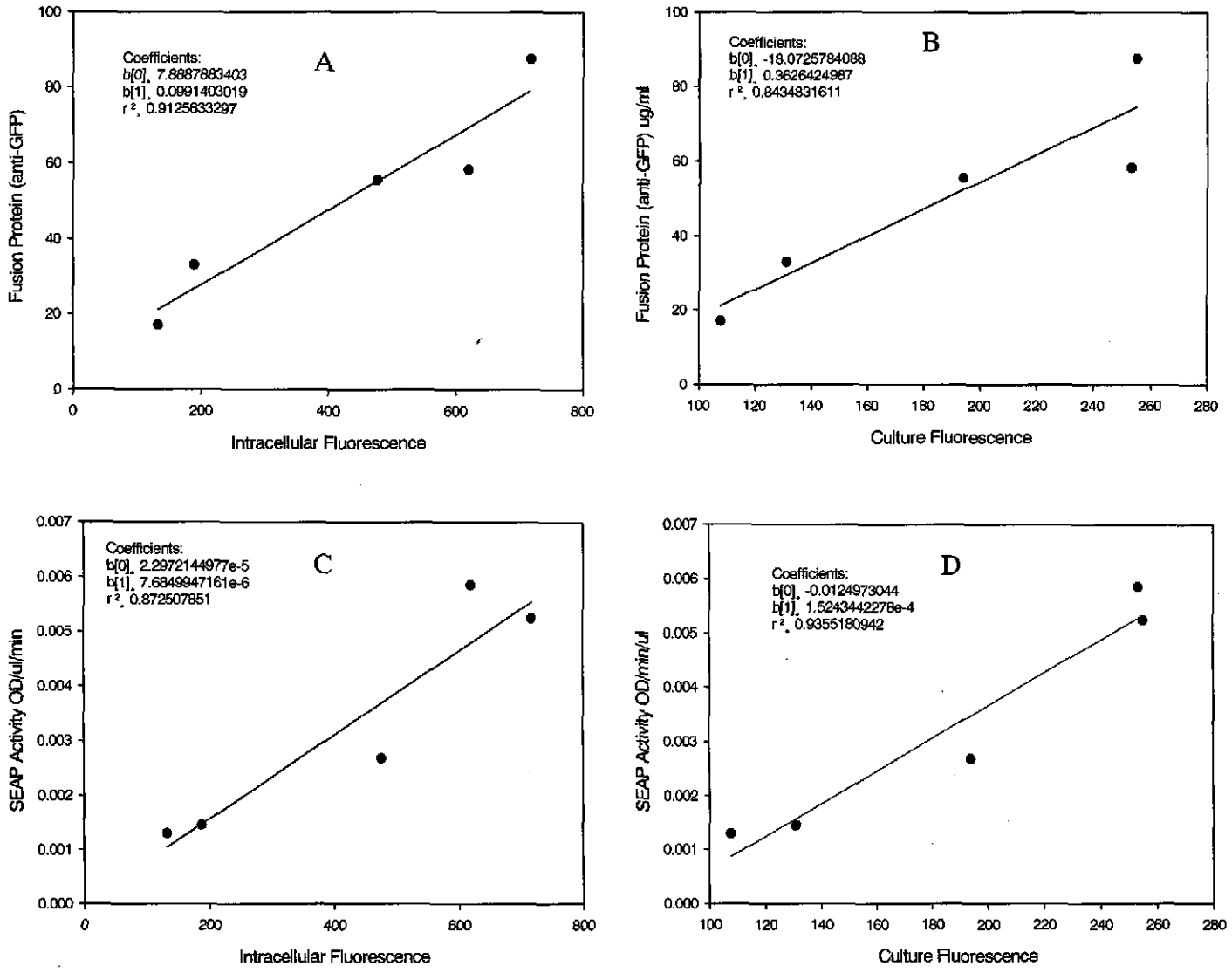

Figure 3.8: Linear correlation of fusion protein with intracellular fluorescence (A) and culture fluorescence (B) and linear correlation of SEAP activity with intracellular fluorescence (C) and culture fluorescence (D).

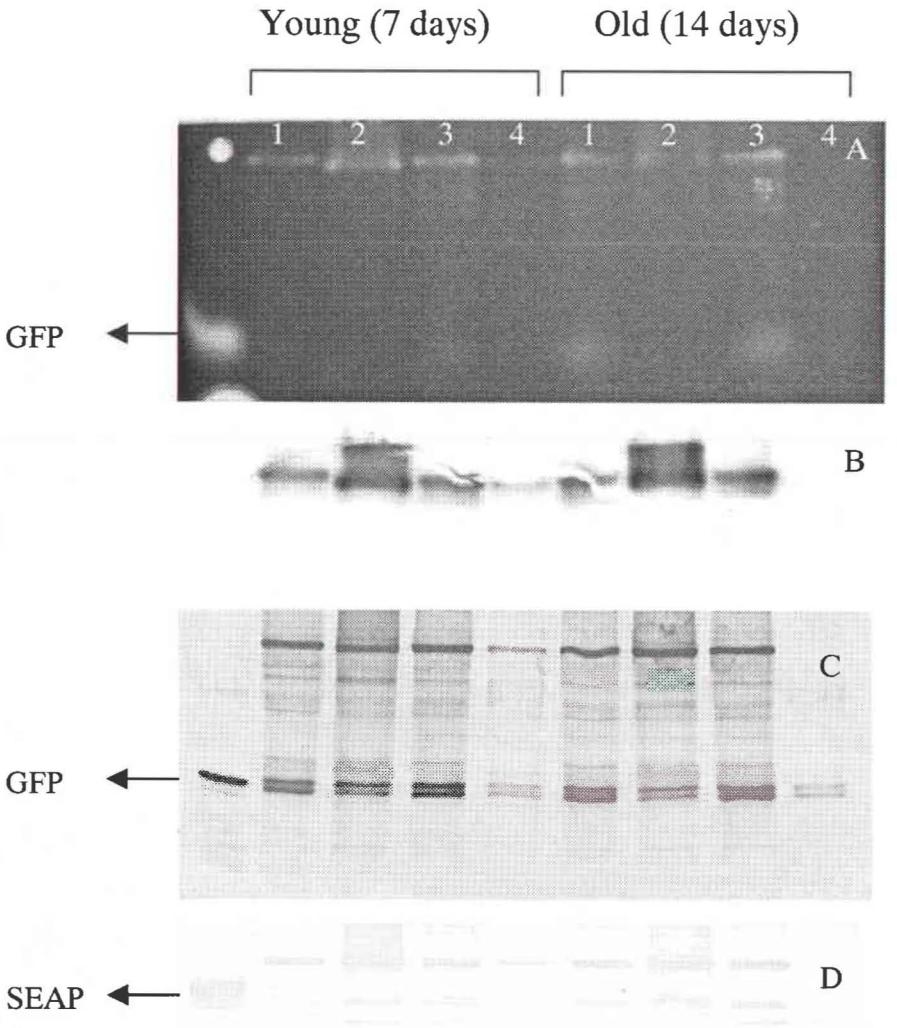

Figure 3.9: Comparison of young and old cultures. A. Fluorescence on the SDS-PAGE. The fluorescence was detected with blue light. B. Enzyme straining on the SDS-PAGE. C. Western blot using anti-GFP as primary antibodies. D. Western blot using anti-SEAP as primary antibodies. Lane 1: intracellular extract. Lane 2: 50% ammonium precipitated. Lane 3: concentrated by microcon. Lane 4: liquid from intracellular extract by using microcon. A and B are based on the same volume, 40ul. C and D were based on the same total protein.

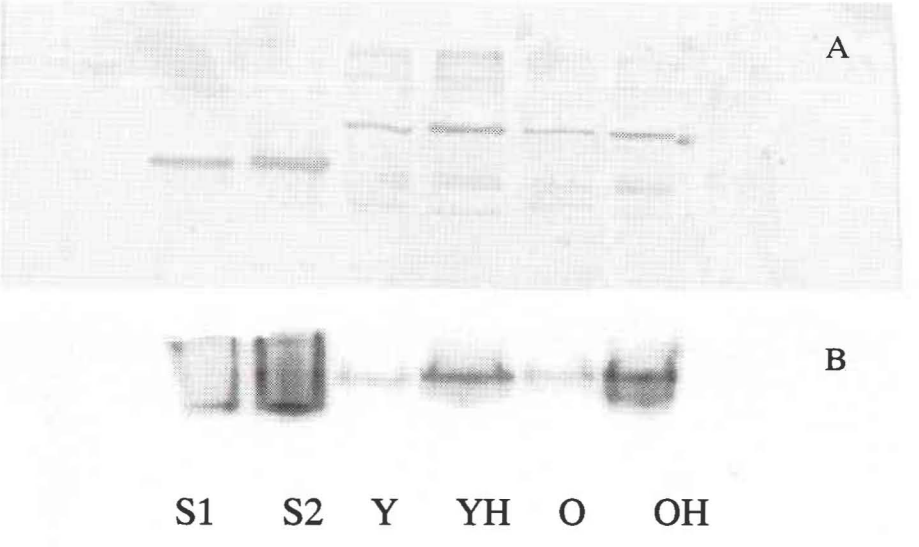

Figure 3.10: SEAP activity in the fusion protein with/without heat treatment. A. Western blot. B. Enzyme stain on the SDS PAGE. All the samples are based on the same protein concentration. S1 and S2 are the SEAP standard from Sigma, containing total protein 2 mg and 5mg. Y represents young sample. O is old sample. H means heat treatment. Polyclonal anti-SEAP was used for detection.

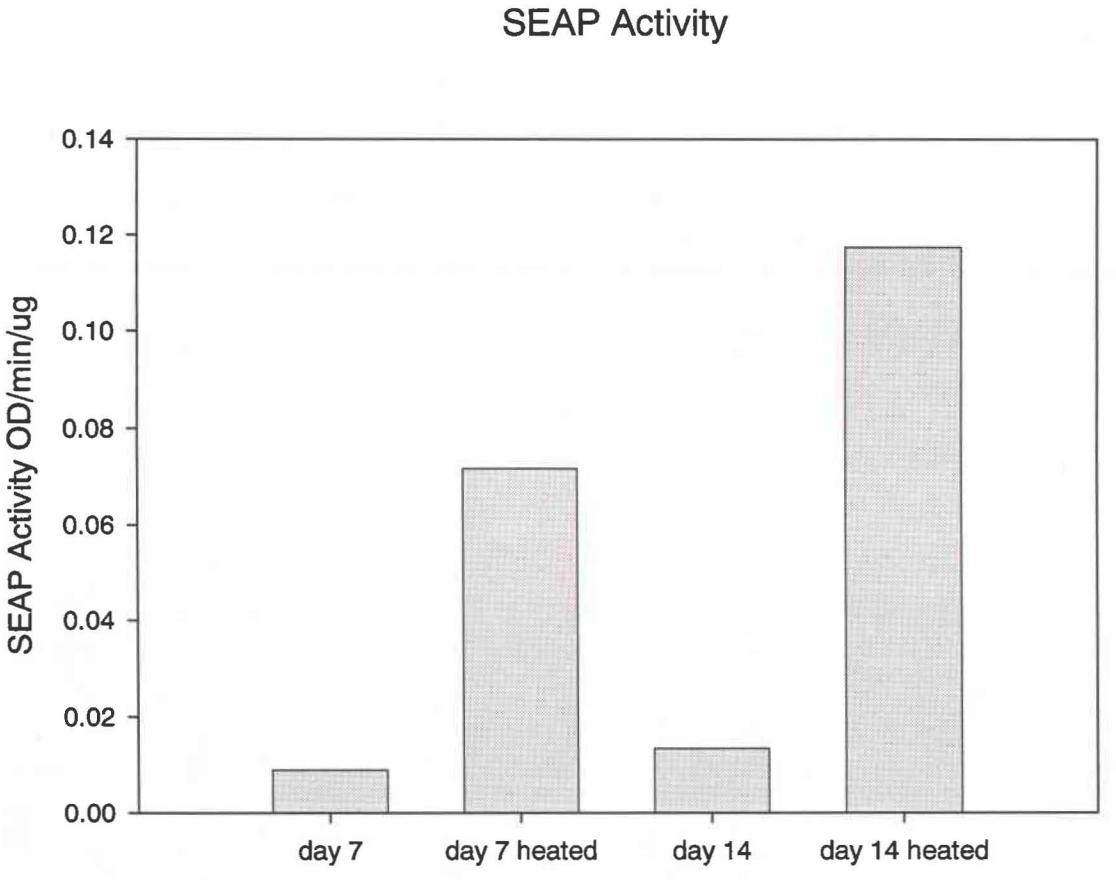

Figure 3.11: Comparison of SEAP activity for young and old, heat and without heat samples. SEAP activity was determined using SEAP assay. SEAP concentration was determined using western blot analysis.

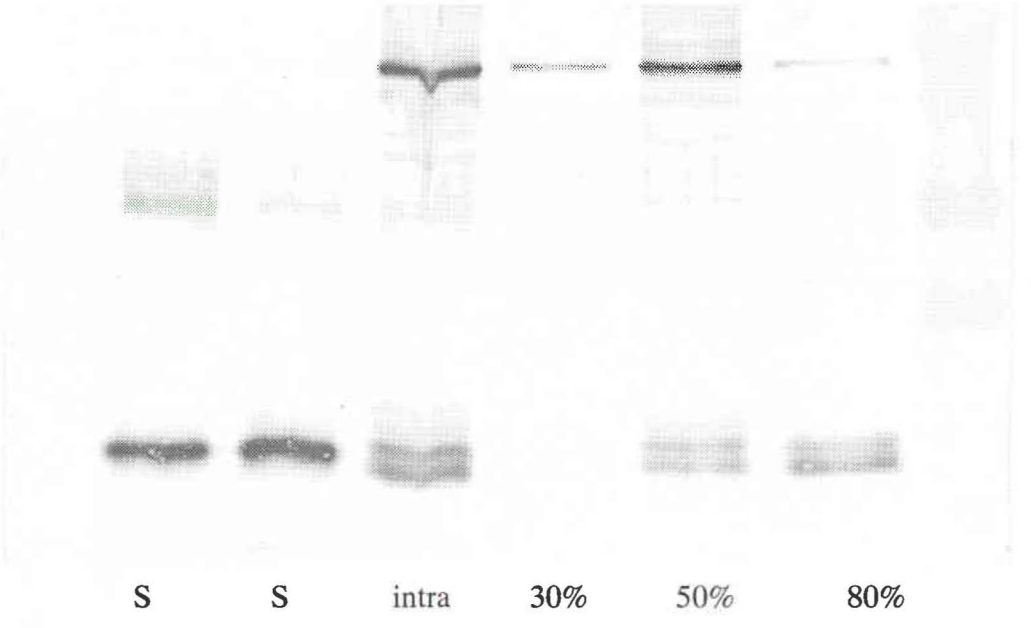

Figure 3.12: Western blot for ammonium precipitated samples. 10 ul protein sample was loaded into each. 1 ml extract of 10-6 cells was used to step ammonium precipitation. All the pellets from 30%, 50% and 80% ammonium precipitation were resuspended into 1 ml extraction buffer A. S is 50 ng GFP standard.

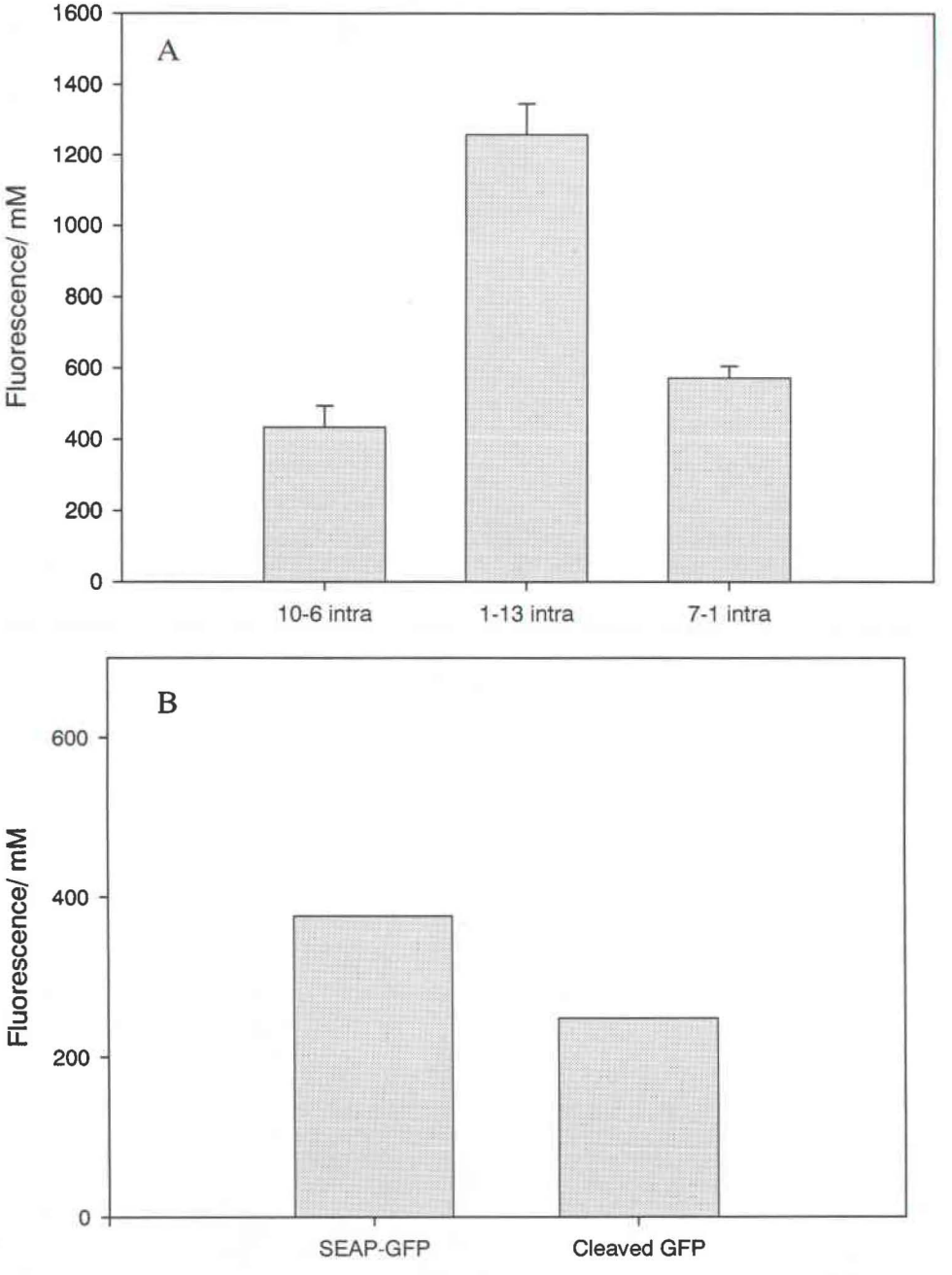

Figure 3.12: Comparison of GFP fluorescence intensity in three cell lines (A) and between fusion protein and cleaved GFP (B).

CHAPTER 4

PRELIMINARY STUDY ON INDUCIBLE GFP EXPRESSION IN TOBACCO SUSPENSION CULTURES

ABSTRACT

Expression of green fluorescent protein in transgenic tobacco cell suspension cultures using the ethanol-inducible alc promoter system was achieved. Here we report the first experiments to characterize the expression. Specifically, we have determined the dosage response and best timing of induction with the optimal ethanol dosage. The optimal dosage is found to be between 0.1% and 1% (v/v) for the suspension cells. When induced with an ethanol concentration at 0.1%, the GFP fluorescence is detectable after 1 day, with maximal fluorescent intensity occurring after 4 days. There is no significant fluorescence in the absence of ethanol. These preliminary data support the use of the alc system in tobacco cell suspension cultures for tightly controlled expression of foreign proteins.

4.1. INTRODUCTION

The ability to exogenously regulate expression of transgene in higher plants has considerable advantages for both basic biosciences and biotechnology (Gatz, 1997). They avoid problems associated with constitutive over-expression, and can uncover primary molecular events resulting from activation of a particular gene. An additional feature of chemical induction is that by varying dosage it may be possible to titrate the activity of a target gene (Salter et al., 1998). In bioprocess, transformed plant cells with an inducible

promoter are suitable for the production of useful products since the growth phase and the production phase can be separated.

Several different methods of chemically regulating transgene expression in plants have been reported (Gatz, 1997). Of those systems previously reported, the alc regulon from *Aspergillus nidulans* (Felenbok et al., 1988; Pateman et al., 1983; Sealy-Lewis and Lockington, 1984) provide a promising chemically regulated gene expression cassette for high plants with the potential to be accepted for routine use in the field. The alc promoter system for plants consists of two components. The alcR transcriptional regulator is expressed from the cauliflower mosaic virus (CaMV) 35S promoter such that in the presence of ethanol, alcR induces expression of any gene fused to a modified alcA promoter (Caddick et al., 1998; Salter et al., 1998). The induction of gene expression using the alc system in whole plants has been studied upon uptake of ethanol via roots or after foliar sprays (Salter et al., 1998). More recently, ethanol vapor has been reported as an inducer of β-glucuronidase (GUS), Luciferase (LUC) and green fluorescent protein (GFP) in *Arabidopsis* (Roslan et al., 2001).

GFP was isolated from the jellyfish *Aequorea victoria* and it has been commonly used as a reporter protein or biomarker for studying subcellular localization of proteins (Akashi et al. 1998; Yamaguchi et al. 1999) and protein trafficking (Imlau et al. 1999; Oparka et al. 1999). The development of various mutants of GFP, also offer new opportunities for developing innovative bioprocess sensing techniques based on this versatile reporter. For bioprocess applications, GFP has been utilized in several studies to monitor bacterial fermentations (Randers-Eichhorn et al. 1997; Poppenborg et al.

1997; DeLisa et al. 1999). GFP has also been used as a reporter to study inducible promoter systems in plant cells (Granger et al, 2001; Hughes et al, 2000).

Here, we demonstrate in transgenic tobacco suspension cells that GFP expression driven by the alc gene switch is efficiently induced by low concentrations of ethanol. Preliminary results from the determination of the inducer dose-response and optimal timing of induction are presented. The results support the use of the alc system for tightly controlled foreign gene expression in cultured plant cells with bioprocess applications. Inducible expression is particularly useful for expressing harmful proteins.

4.2. MATERIALS AND METHODS

4.2.1. Plant expression vector

The pBIN-mgfp5-ER vector was generously provided by Jim Haseloff (Siemering et al. 1996; Haseloff et al. 1997). The mgfp5-ER insert was obtained by digestion of pBIN-mgfp5-ER with *Bam*HI-*Sac*I and ligated into the respective sites of pGEM-11Zf (Promega, Madison, WI). The sequence of the mgfp5-ER was modified by the polymerase chain reaction (PCR) with Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA) using a forward primer 5'-GTCGACAAGGAGATATAACAATGAAG-3' and a reverse primer 5'-GTCGACTTAAAGCTCATCATGTTTGTATAG-3' to incorporate a *Sal*I restriction site (underlined region) on both ends of the cDNA. The plasmids pACN and binSRNACatN for expression of genes under the control of an ethanol-inducible promoter were generously provided by Andrew Greenland at Zeneca Agrochemicals (Caddick et al. 1998). Plasmid pACN was digested with *Sal*I to remove the CAT gene and the *Sal*I digested mgfp5-ER was ligated into the plasmid. Orientation of the mgfp5-ER with respect to the ethanol-inducible promoter was verified by

restriction digestion with *Eco*RI. The expression cassette containing the ethanol-inducible promoter, mgfp5-ER and the *nos* terminator was excised from pACN-mgfp5-ER with *Hin*dIII and ligated into the same site in the binary vector binSRNACatN (*Hin*dIII digestion removes the CAT expression cassette).

4.2.2. Suspension cell culture

Generation and maintenance of tobacco suspension cell cultures were performed as previously described (Liu et al. 2001). Media and growth conditions were identical to those reported in Liu et al. (2001) except that Murashige and Skoog medium was used instead of Gamborg's B5 medium. In shake-flask time-course experiments, to each one-liter baffled-shake flask, 300 ml of culture medium was inoculated with a 15% (v/v) inoculum from a 7-day-old cell culture. The culture was sampled periodically for the time course determination of growth and protein production parameters.

4.2.3. Induction

The suspension cells were prepared in 250 ml flasks containing 50 ml culture. Cultures were induced by adding ethanol at preset concentrations. After the appropriate induction time, the suspension cells were collected for GFP extraction, protein assay, and fluorescence measurement.

4.2.4. GFP extraction and fluorescence determination

Extraction of cell suspension samples was performed as previously described (Liu et al 2001). The supernatant following centrifugation was filtered through a 0.2 μm filter and used for determining intracellular GFP. For extracellular GFP determination, a sample of cell suspension was centrifuged at 3200 g for 5 min and the supernatant filtered through a 0.2μm filter.

Off-line GFP fluorescence intensity measurements were obtained using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan) at an excitation wavelength of 473 nm and an emission wavelength of 507 nm. To minimize the interference of the inner filter effect (Srinivas and Mutharasan, 1987), samples for culture and intracellular fluorescence measurements were diluted, when necessary, to the linear fluorescence response range, and then the fluorescence of the diluted sample was linearly extrapolated. For medium fluorescence measurements, sample dilution was unnecessary since the inner filter effect was found to be inconsequential (data not shown) and the extracellular GFP concentrations in all samples were within the linear response range.

4.2.5. Protein assay

Protein was determined using a protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard (Bradford, 1976).

4.2.6. Western blot analysis

Samples were mixed with an equal volume of 2× reducing sample buffer, resolved on a 12% SDS-polyacrylamide gel as described by Laemmli (1970) and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). Purified recombinant GFP (Clontech, Palo Alto, CA) was used to quantify GFP levels. Western analysis was performed as previously described (Liu et al. 2001) using an anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR).

4.3. RESULTS

4.3.1. Characterization of inducible GFP expression in transgenic tobacco cell culture

0.1% ethanol (v/v) was added into a 8-day-old tobacco suspension culture of cell line 9-5 and 9-12. Two days post induction, cell extracts were analyzed for GFP fluorescence. Cell line 9-5 showed a much higher fluorescence based on the same total soluble protein. Compared with induced cells, the extremely low fluorescence was found in the cells without induction, and the value is closed to wild type, which can be considered as negligible. Western blot analysis was performed to investigate GFP expression and pattern. Immunoreactive bands with molecular weight similar to that of standard GFP portion were observed for both cell lines 9-5 and 9-12. Their patterns were similar as that of cell line 1-13, which showed multiple bands near GFP position (Fig. 1). This is expected since the same ER signal peptide and ER-retention sequence were used in all three cell lines. The induced suspension cells were also checked under the fluorescent microscope. Most part of cells showed fluorescence, but not all (Fig. 2).

4.3.2. Characterization of the dose-response of induction in transgenic tobacco suspension cells

To determine the optimal ethanol concentration for induction, 8-day-old tobacco suspension cells from cell line 9-5 were induced with a range of ethanol concentrations. After addition of ethanol, the suspension cells were incubated for 16 h before measuring GFP fluorescence. The expression was monitored over 7 days. The comparison of GFP fluorescence intensity is based on the same total protein (Fig. 3). All the suspension cells, except 0.01% ethanol induction, showed a continuous rise in GFP fluorescence with ethanol concentrations from 0.05 to 1% after 16 h. The peak of GFP fluorescent intensity was observed with 0.1% to 1% ethanol (v/v) 3-4 days later. After that the fluorescence

decreased. The fluorescence intensity of the suspension cell induced with 0.01% ethanol is very low, and the reading is similar with wild type.

4.3.3. Characterization of the effect of timing of induction in transgenic tobacco suspension cells

The optimal ethanol concentration of 0.1% was subsequently used for further characterization of the time course of induction in 9-5 tobacco suspension cells. 0.1% ethanol (v/v) was added into different ages of suspension cells, 4, 6, 8 and 10-day old. GFP fluorescence was measured after incubation for 4 days. Fluorescence per mg total protein was monitored over a 6-day time course, as indicated in figure 3. The highest level of GFP fluorescence per mg protein is obtained using 8-day old tobacco cells. Compared with other inductions, the level of GFP fluorescence is rather low at 10-day induction. In addition, extended induction time to 6 days or 8 days, fluorescence intensity decreased.

4.4. DISCUSSION

These experiments demonstrated that the alc system is sensitive to low concentration ethanol in tobacco suspension cells. In earlier work (Caddick et al., 1998; Salter et al., 1998; Roslan et al., 2001), alc system was applied in whole plants or part of plant. The optimum ethanol concentration for CAT induction is 0.1% in transgenic tobacco plants. At ethanol concentrations higher than that, plants were visibly stressed (Salter et al., 1998).Here, we introduced using transgenic tobacco suspension cell as material to study alc system mediating GFP expression. Suspension cells are more homogeneous than the whole plant system. It has been found that whole-plant induction by ethanol would only result in alc-regulated gene expression in the tissue or organ

expressing alcR (Sweetman, 2002). Meanwhile, ethanol is not readily translocated within the plant (MacDonald and Rimmerer, 1993). However, some cells without fluorescence were observed under the fluorescent microscope in our experiment (Fig. 2). It could be caused by the cell senility, more complicate situation related to gene expression, or uneven uptake of ethanol by the plant cells.

On application of the inducer in tobacco cells, alc-mediated GFP expression was not as rapid and sensitive as that of GUS expression in *Arabidopsis* plants reported by Roslan et al. (2001), which a dramatic increase in GUS expression was detected after 4 h induction. Significant GFP fluorescence was only detectable after 1 day. However, like GUS expression, GFP fluorescent levels showed a slow and stable increase, reach a peak at 4-5 days. GUS (Roslan et al., 2001) and CAT (Salter et al., 1998) activities were found after induction with 0.01% ethanol whereas no significant fluorescence was detectable after induction with 0.01% ethanol in our cell suspension culture system. In addition, in the study by Roslan et al. (2001), the induced expression levels achieved was similar to the constitutive expression mediated by the CaMV35S promoter in *Arabidopsis thaliana*. However, the fluorescence of cell line 9-5 after 4 days of GFP accumulation is not comparable with cell line 1-13 which could be reach 1000 AU per 1 ml intracellular extraction at 8-day-old age of suspension cells using CaMV35s promoter.

In conclusion, alc system is a powerful tool for regulating protein production in plant cells. Transgenic tobacco suspension cells expressing GFP regulated by alc system is effective and reversible, which can be used as a model system to study regulation of gene expression in the bioreactor.

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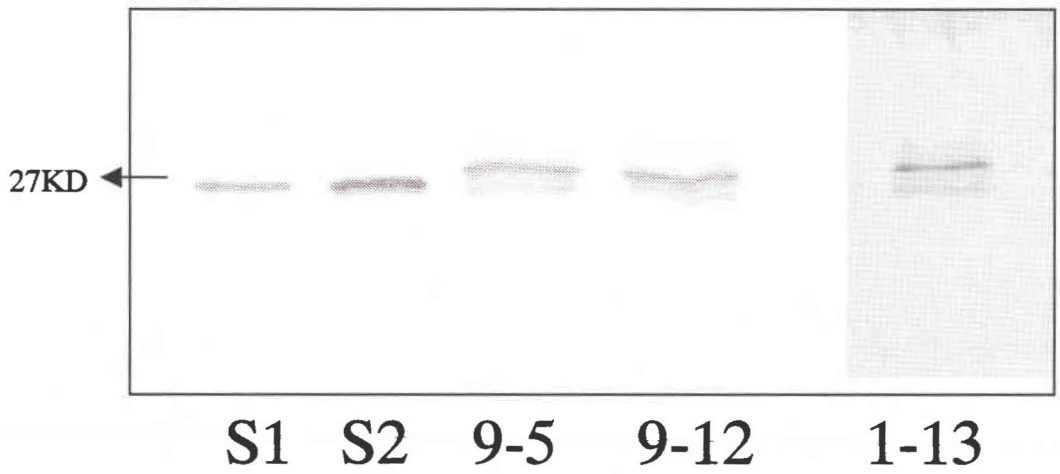

Figure 4. 1: Western blot for 9-5,12. 10 ul protein extraction was loaded in each lane. S1 and S2 represent 20 and 50ng GFP. The same volume of cell culture was used to extract protein. The cell density in 9-12 was higher than in 9-15.

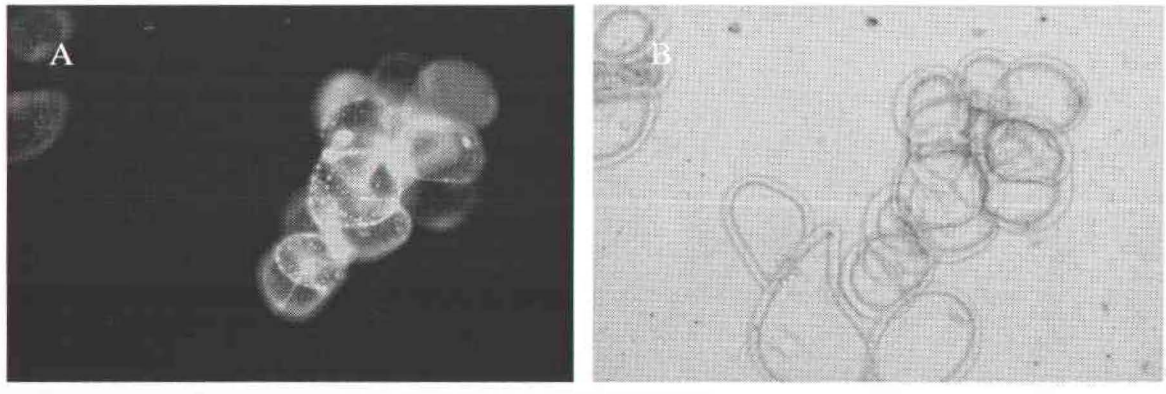

Figure 4.2: Visualization of fluorescent distribution in tobacco suspension cells by using fluorescent microscope. The cells was shined with blue light (A), or with normal light (B)

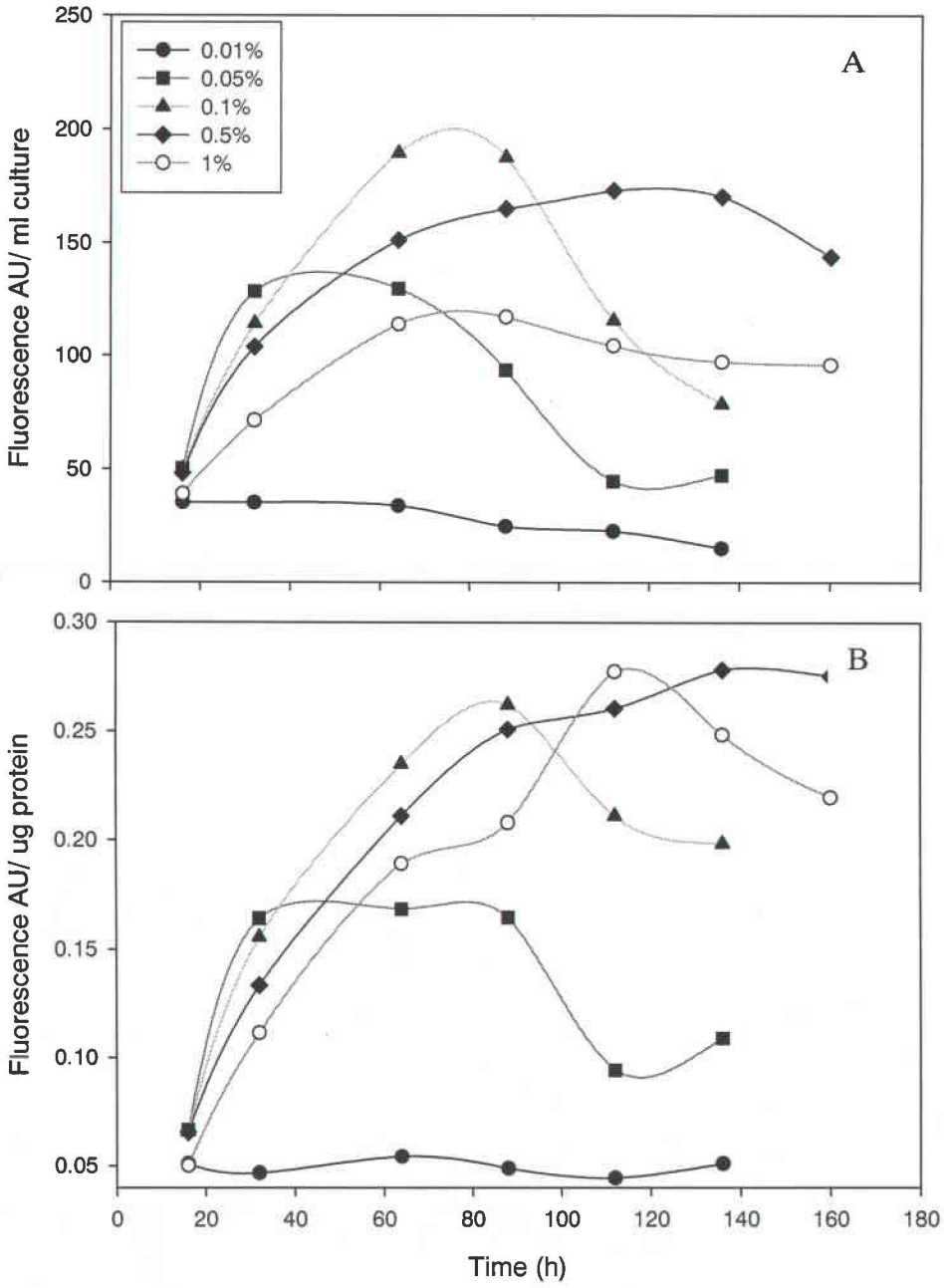

Figure 4.3: Ethanol dose-response in cell line 9-5. A. Based on 1 ml culture. B. Based on per µg protein. 8-day-old tobacco suspension cells were monitored for GFP fluorescence after addition of ethanol. A range of ethanol concentrations was applied. The values represent GFP fluorescent intensity per mg protein.

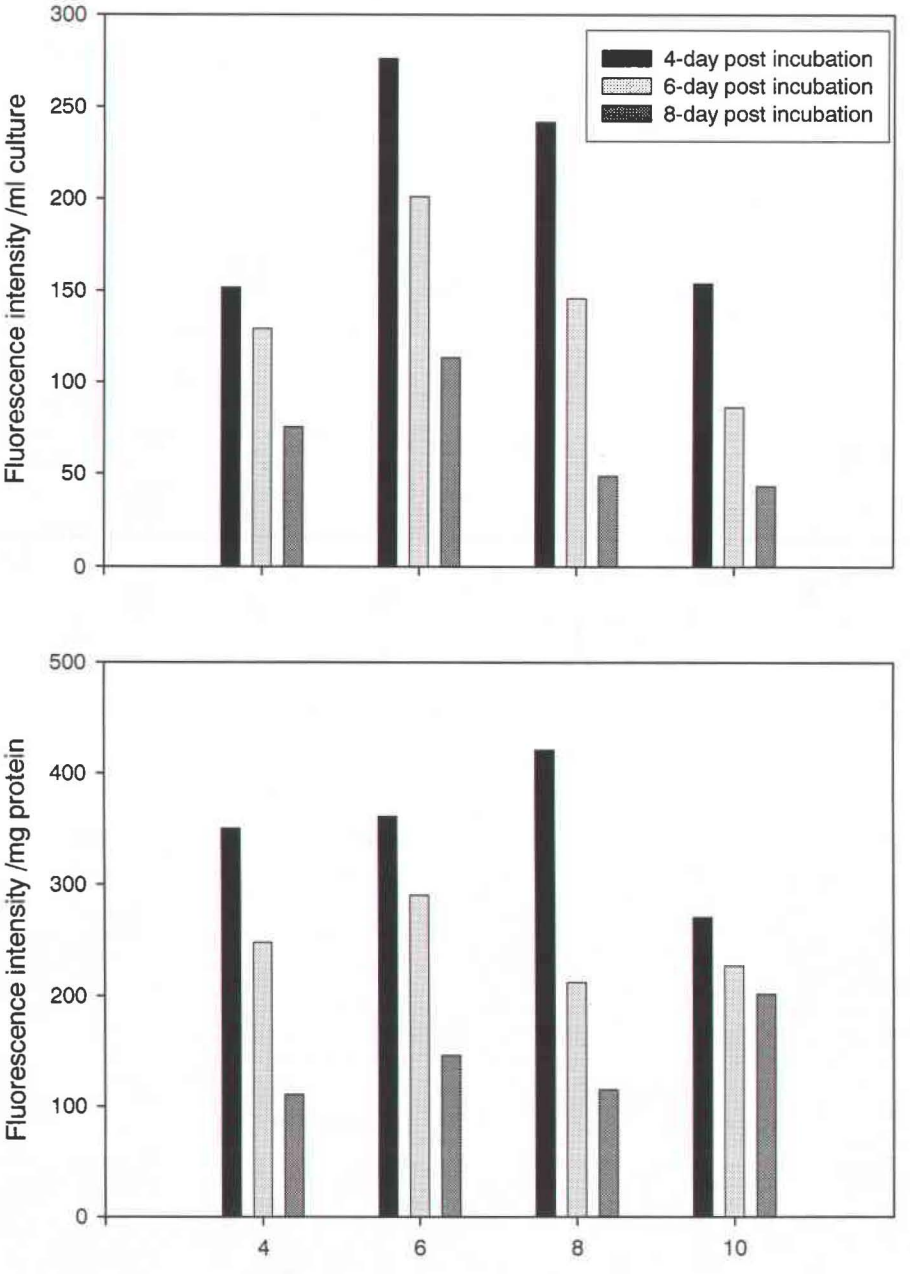

Figure 4.4: Time course of induction. A. Based on 1 ml culture. B. Based on per mg protein. 0.1% ethanol was added into different age tobacco suspension cells at 4, 6, 8, 10 days. Fluorescence was measured after induction at 4, 6 and 8 days.

APPENDIX I

COMPARISON OF CELL LINES 1-13, 6-15 AND 7-1

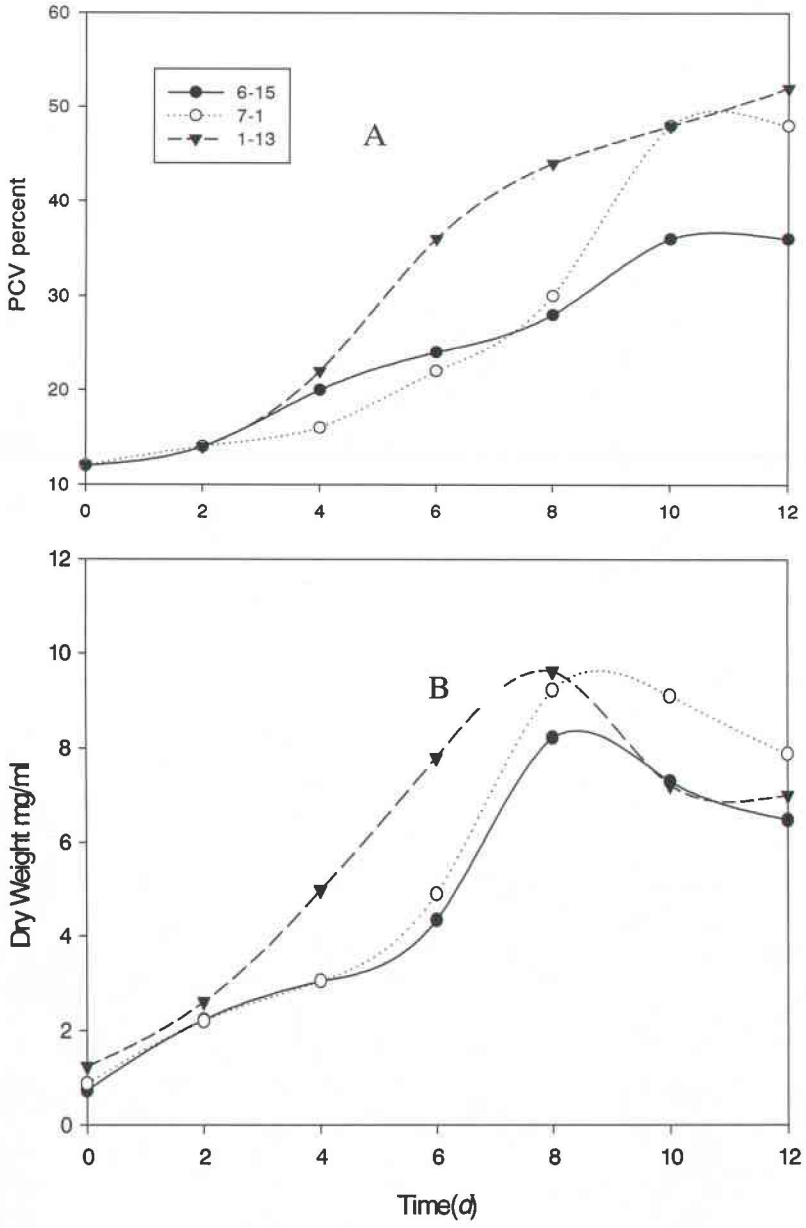

Figure I-1: Comparison of three cell lines' cell growth. Cell dry weight was determined by using cells from 5 ml culture in an oven at 50°C until a constant weight was reached.

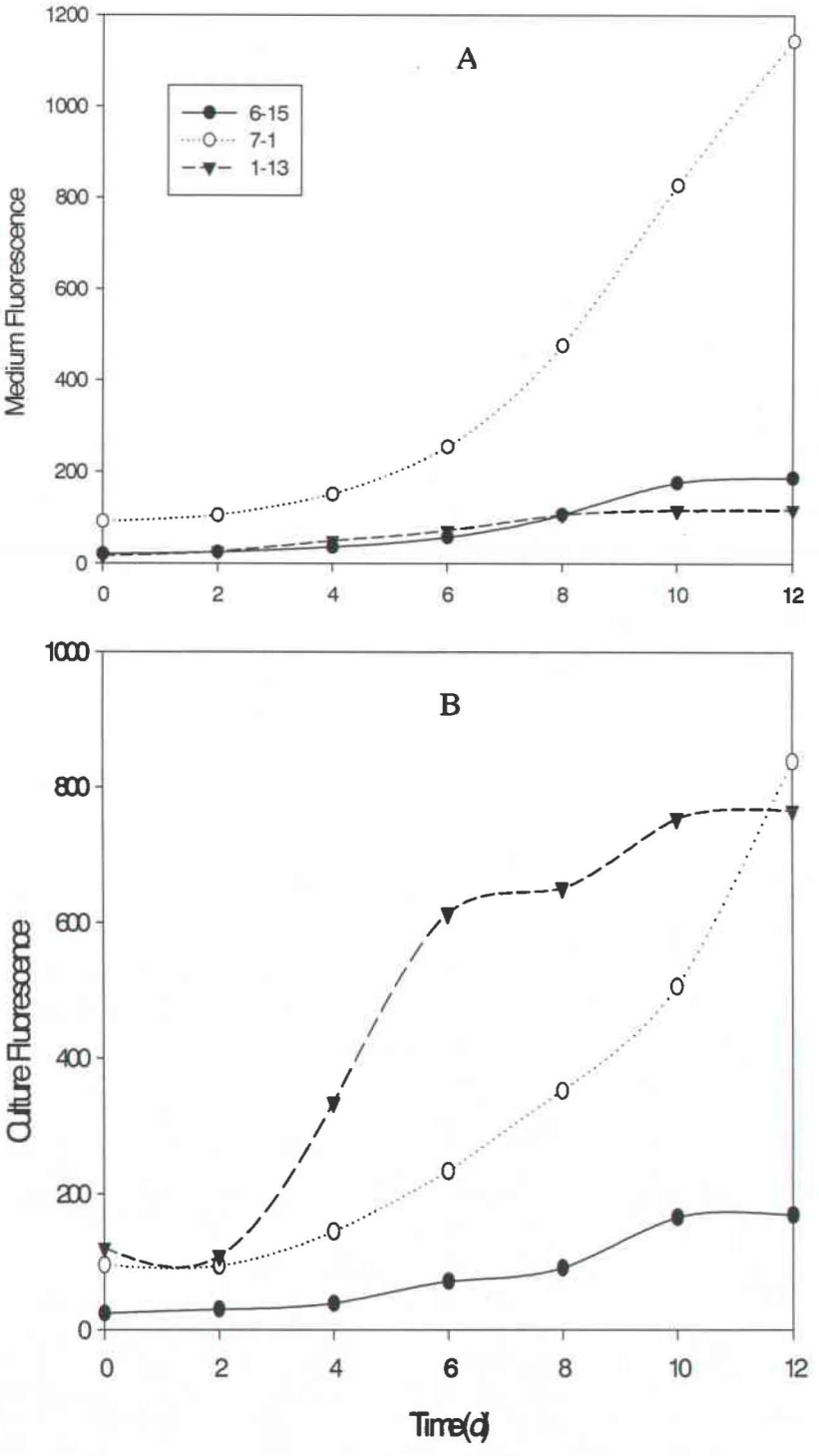

Figure I-2: Fluorescent intensity in the three cell lines. Fluorescence of 1 ml sample was measured at an excitation wavelength of 473 nm and emission wavelength of 507 nm using a fluorescence spectrophotometer (Hitachi Model F-2500, Tokyo, Japan)

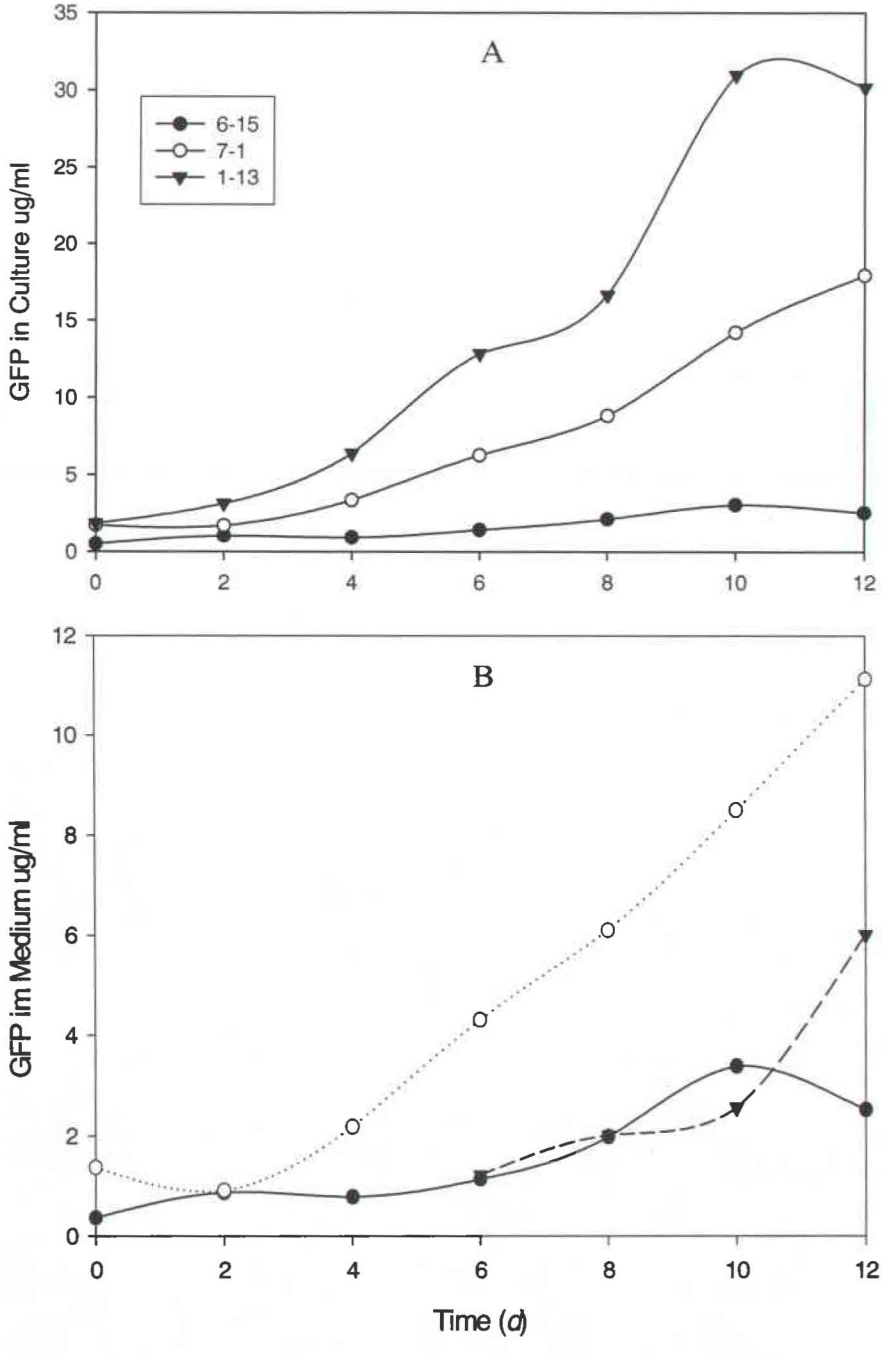

Figure I-3: The comparison of GFP concentration in three cell lines. GFP concentration was determined using western blot analysis. Total GFP concentration in the culture was calculated with formula $GFP_{total} = GFP_{cell} + (1\text{-}PCV) \cdot GFP_{medium}$.

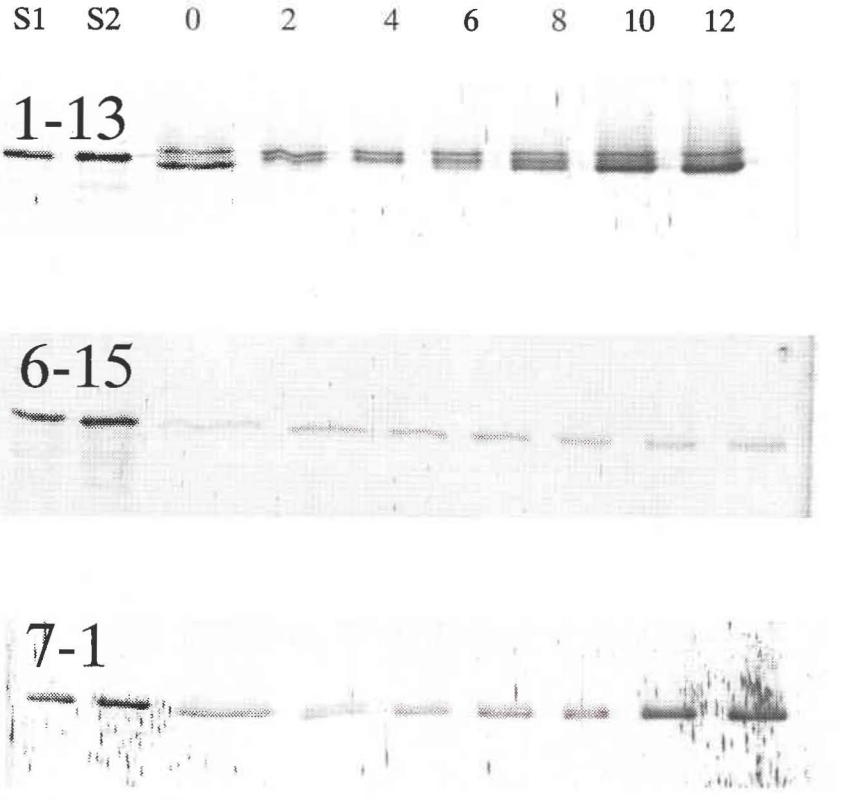

Figure I-4: Western blot for intracellular Samples from three cell lines. The same total protein was loaded into 12% SDS-PAGE from lane 0-12, and polyclonal anti-GFP antibody was used for detection. S1 and S2 represent GFP Standard 20ng and 50ng. 0-12 represents the time course of the experiment.

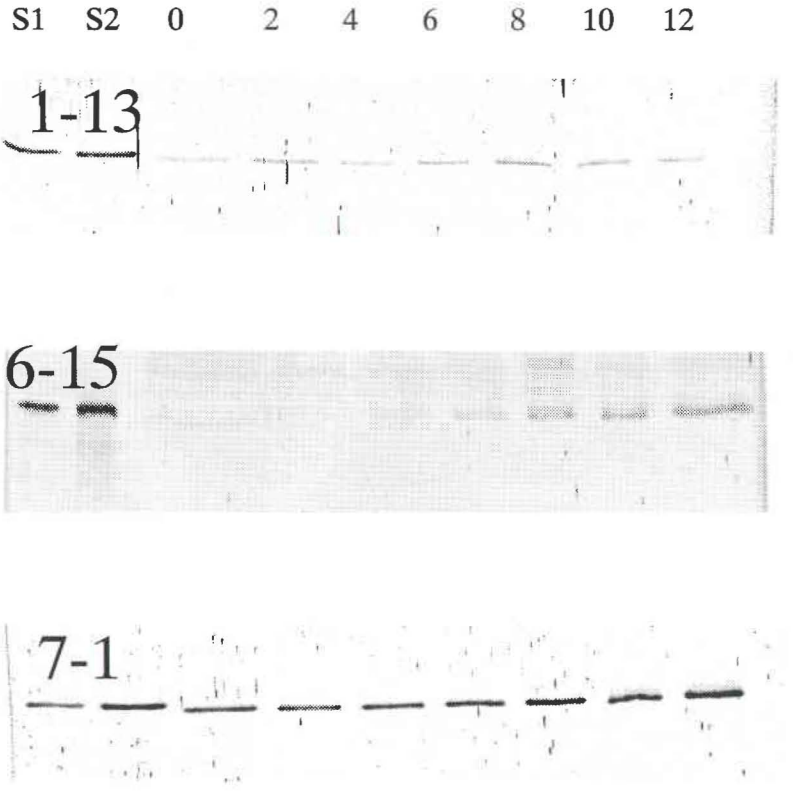

Figure I-5: Western blot for extracellular samples from three cell lines. The same total protein was loaded into 12% SDS-PAGE from lane 0-12, and polyclonal anti-GFP antibody was used for detection. S1 and S2 represent GFP Standard 20ng and 50ng. 0-12 represents the time course of the experiment.

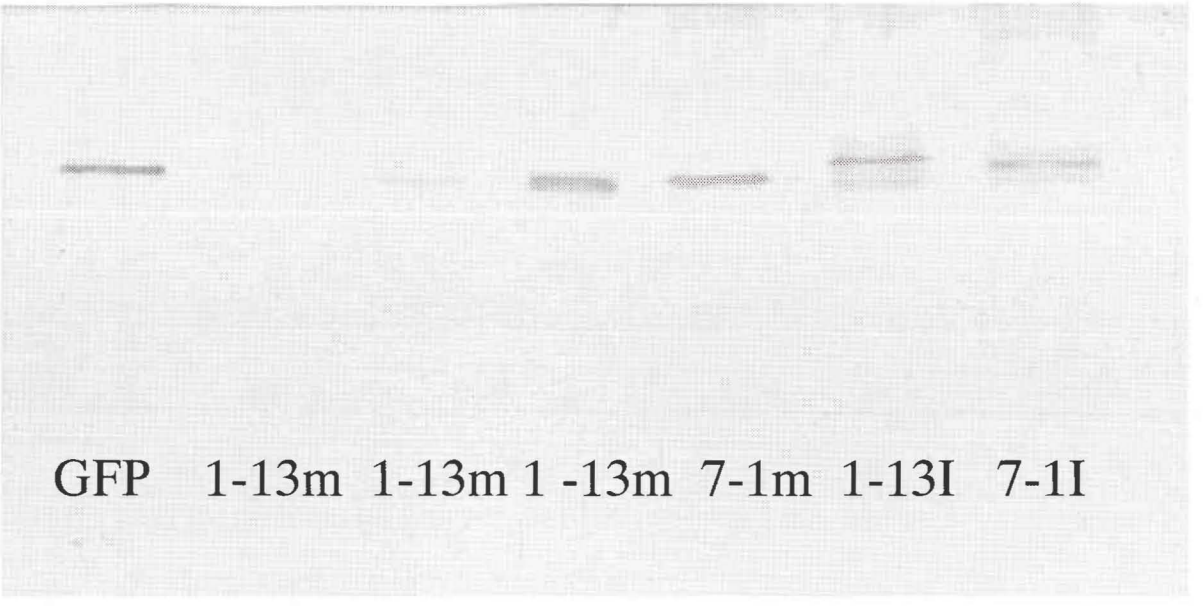

Figure I-6: Western blot for 1-13 and 7-1 intra- and extra-cellular samples using 15% SDS-PAGE. 5μl protein sample was loaded in each lane except GFP standard 10μl. M is the sample from medium. I represents intracellular sample.

APPENDIX II

PERFUSION BIOREACTOR FOR CELL LINE 7-1

Method

Transgenic tobacco cell line 7-1 was cultivated in a perfusion bioreactor with online monitoring of pH and dissolved oxygen. B5 medium was used with 30g/l glucose as the carbon source. The dissolved oxygen was controlled at 40% air saturation. The aeration rate is $0.51 min^{-1}$. The agitation rate at the beginning is 70 rpm. Depending on the cell density, agitation rate can be raised to 200 rpm at the end. Culture pH was set at 6.0 and controlled by adding HCl or NaOH 1N solution via the pH controller of BioFlo reactor. 600ml culture was prepared for initiation. Perfusion began at the 8^{th} day with the perfusion rate 0.56 ml/min. In order to keep the certain cell density, PCV around 45%, the culture was pumped out from the bioreactor. The date and volume are showed as below:

Times	Date	Volume (ml)
1	8^{th}	710
2	10^{th}	450
3	10^{th}	100
4	11^{th}	180
5	12^{th}	160
6	13^{th}	160
7	14^{th}	170
8	15^{th}	240
9	17^{th}	240
10	18^{th}	120
11	19^{th}	120

13mg/ml antifoam, totally 150ml, was used for 3.2 L culture medium during the 24 days to prevent the foam in the bioreactor. Ammonium in the medium was measured using ammonia cyanurate reagent poweder pillows (Hach, 26531-99) and ammonia salicylate reagent powder pillows (Hach, 26532-99). Other methods are the same as those described in chapter 2.

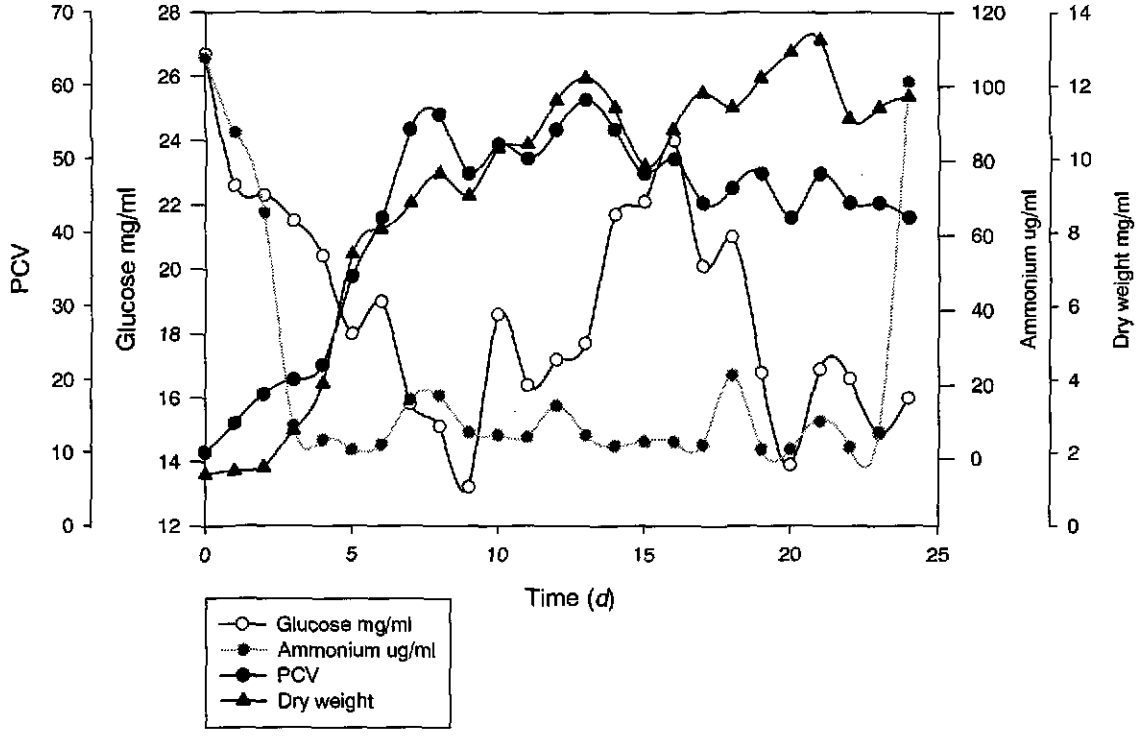

Figure II-1: Cell growth in the perfusion bioreactor.

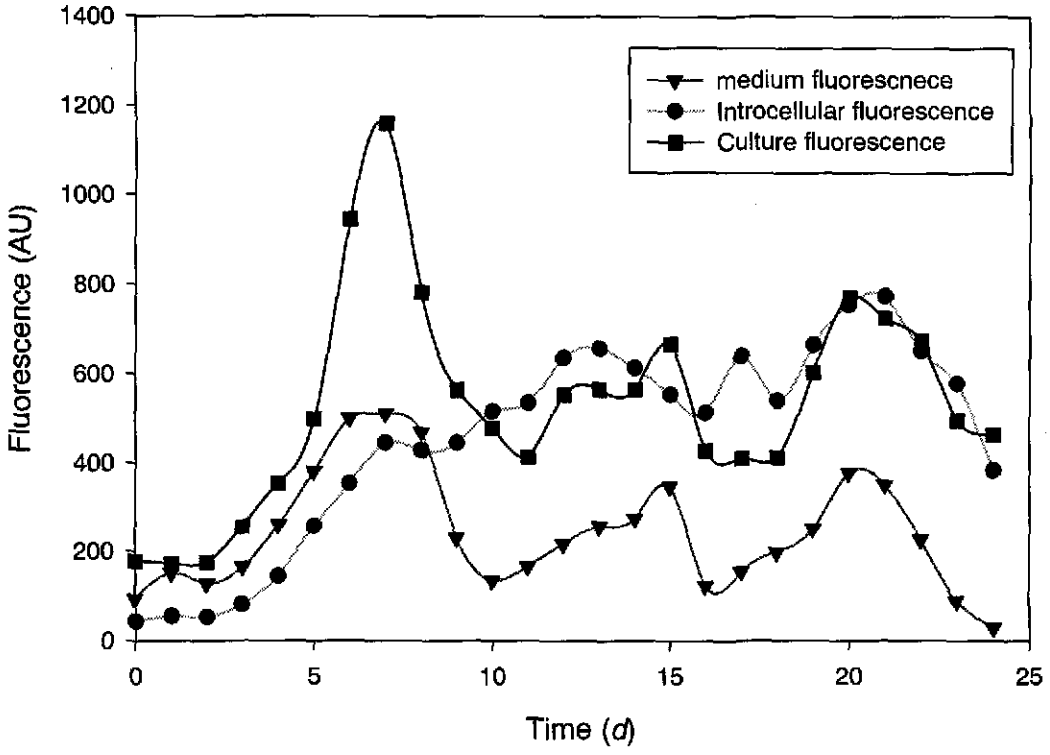

Figure II-2: Offline fluorescence in the perfusion reactor.

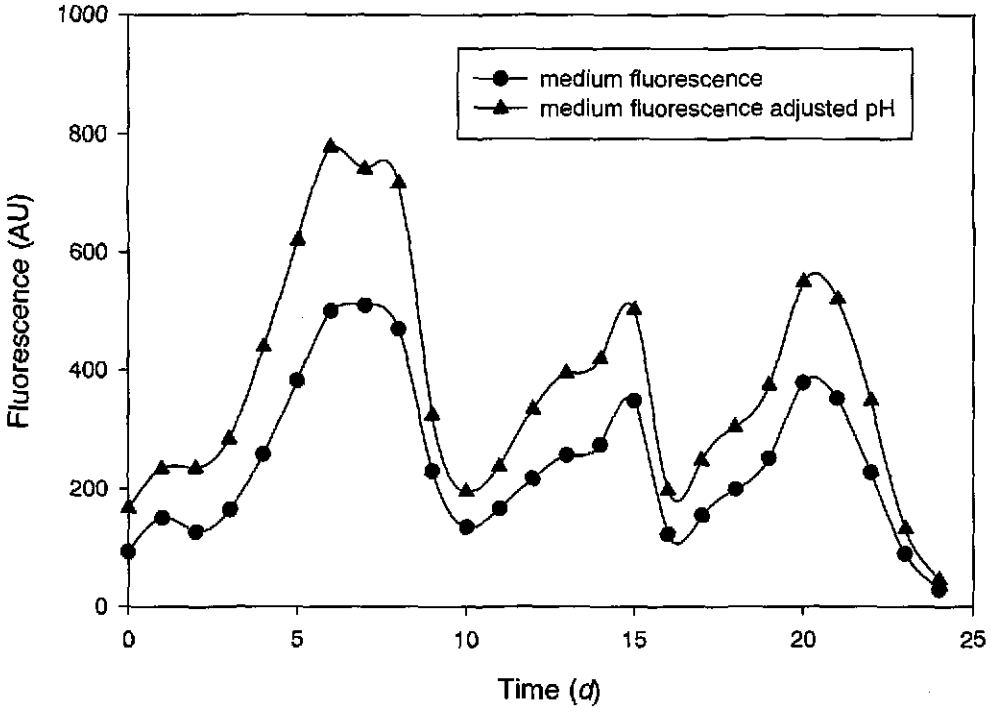

Figure II-3: Comparison of medium fluorescence with or without adjusted pH.

APPENDIX III

PRELIMINARY STUDY ON GUS-GFP FUSION PROTEIN EXPRESSING IN TOBACCO CELLS

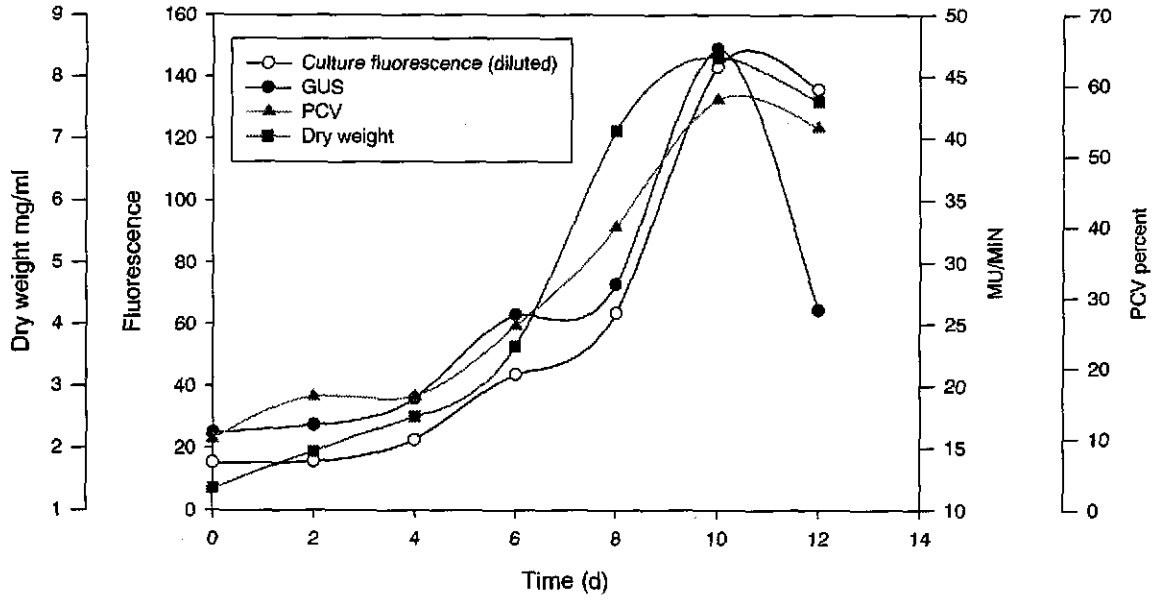

Figure III-1: Time course of cell growth, fluorescence and GUS activity in flasks.

Note: Cell line 8-3 was used in the experiment. For initiation, 60 ml suspension cells were added into 250 ml MS medium. The measurements for cell growth and fluorescence are the same as those in chapter 2 and chapter 3. For GUS activity assay, described as blow.

GUS assay: Fluorometic assay for GUS activity using 4-methylumbelliferyl β-D-glucuronide (MUG) (Sigma) was conducted by a modified protocol based on the method of Jefferson et al. (1987). The fluorescenct product of 7-hydroxy-4-methylcoumarin (MU) is produced through hydrolysis of MUG by GUS. The tobacco cells are collected, washed with water and resuspended in GUS extraction buffer containing 50 mM NaPO4(pH 7.0), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100. The cells lysed using an ultrasonic cell disrupter for 5 min at the lowest output setting (Branson Model 250, Danbury, CT). The supenatant was collected after centrifugation at 14,000 rpm 10 min at 4°C. 2 µl aliquots were added into 1ml volume of GUS assay buffer containing 1 mM of MUG in GUS extraction buffer and incubated at 37C. 200 ul sample were taken after 0, 5, 10 and 20 min and reaction was

stopped by adding the sample into 800 ul of a buffer containing 0.2 M NaCO3. The fluorescence was measured with Hitachi F2500 fluorescent spectrophotometer. The wavelength of the spectophotometer was set to 365 nm for excitation and 455 nm for emission.

Reference:

Quaedvlieg NEM, Schlaman HRM, Adimiraal PC, Wijting SE, Stougaard J and Spaink HP (1998) Fusions between green fluorescent protein and β-glucuronidase as sensitive and vital bifunctional reporters in plants. Plant Molecular Biology 38: 861-873.