Quantitative Screening of Insect Cell Transformants Stably Expressing GFP_{uv}-β1, 3-*N*-acetylglucosaminyltransferase 2 Fusion Protein

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Abstract Insect cell transformants, stably expressing human β1, 3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) as the green fluorescent protein (GFP_{uv})-fused protein, were efficiently isolated on Western blot by the quantification of the densitometric intensity of the fusion protein. From almost 150 transformants containing the fusion gene linked to three different types of signal sequence, two transformants, Tn-pXme4a and -pX28a, were successfully selected, showing 8.3 and 8.6 mU/ml β3GnT activity, respectively. ~~This is the highest, ever reported enzyme activity for β3GnT2 using a stable cell line.~~ This method requires a screening time almost one-half that required in the isolation of stably transformed cells with high expression levels, and at the same time allows the handling a large number of transformants.

Keywords: β1, 3-*N*-acetylglucosaminyltransferase 2, insect cell, green fluorescent protein, screening, Western blot

Insect cell systems can be handled easily, and are available for the expression of high-value proteins or enzymes, owing to their ability to perform post-translational modification, as observed in mammalian cells [1-3], at relatively low costs for large-scale productions. The baculovirus expression system is widely used for the production of proteins from higher eukaryotes [4]. The main advantage of this system is the high expression levels of recombinant proteins from the strong polyhedrin promoter derived from baculovirus [5]. However, this system has a constraint. As the recombinant baculovirus infection progresses, it eventually kills the host, and with this death the viral infection comes to a stop. A recombinant viral infection also causes damage to the cellular and secretory machinery, which are required for post-translational modifications [6, 7]. Overall, the expression of a protein is transient, and a large quantity of high-titer virus is required for infection.

Recently, a stably transformed insect cell system had been established using techniques to isolate transformed cell lines [8-11]. This system involves the integration of an expression cassette along with a marker antibiotic to assist in the screening of insect cell genomes. A constructed expression vector is integrated into the genomic DNA of a host. A stably transformed cell line is later isolated using the marker antibiotic gene present in the expression cassette. Although the generation of a stable cell line is simple and fast, including the screening of recombinant transformants, the selection of stable transformants, with the highest protein expression levels, which retain the enzymatic activity of the protein is very crucial. This step is time-consuming and limits the efficiency of the system; therefore, better scientific methods for the screening stable cell lines and selecting recombinant secreting proteins have to be designed.

We have used human β1, 3-*N*-acetylglucosaminyltransferase-2 (β3GnT2) fused with

GFP_{uv}, a marker protein [12], and successfully expressed the β3GnT2, in the secreted form, into a medium from which the β3GnT2 can be easily purified [13-15]. β3GnT2 plays a critical role in the synthesis of lacto-*N*-triose II, which converts into lacto-*N*-tetraose and lacto-*N*-neotetraose. The actions of β3GnT2 and its family of transferases are not completely understood, owing to lack of high-quality and a sufficient amount of enzymes retaining their native functions. We used three different types of signal peptide, prepromellitin of the honey bee (me), glycoprotein gp64 of AcNPV (gp), and cecropin B from *Hyalophora cecropia* (ce), which will further assist in the secretion of proteins. Here, we present an efficient screening method for selecting transformants expressing high levels of β3GnT2.

Tn-5B1-4 cells, from *Trichoplusia ni,* were purchased from Invitrogen (San Diego, CA, USA) and grown at 27°C in 25 cm^2 tissue culture flasks (Falcon), containing Express five SFM (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen).

The cells that stably expressed the GFP_{uv}-β3GnT2 (GGT) fusion protein were isolated using the Express Insect Vector Set Non-Viral Insect Expression system (Invitrogen, Carlsbad, CA, USA). pBlueBacHis2-GFPuv-β3GnT2 [14] was used as a template for inserting the three different signal sequences [16-18] employing the polymerase chain reaction (PCR) forward primers shown in Table 1. The PCR product was inserted into the entry vector, pENTR/D-TOPO (Invitrogen), to produce pENTR/D/me-GGT, pENTR/D/gp-GGT and pENTR/D/ce-GGT. All PCR fragments were confirmed on the basis of the dideoxynucleotide chain terminating sequence using a Thermo Sequenase Cycle Sequencing kit (USB Co., Cleveland, Ohio, USA). The expression plasmids, pXme-GGT, pXgp-GGT and pXce-GGT, were constructed with

pENTR/D/GGT and pXINSECT-DEST38 using the Gateway technology. pXINSECT-DEST38 has the cytoplasmic actin promoter from *Bombyx mori* for constitutive expression [19], a viral transcriptional enhancer, a homologous region 3 [20], a viral transactivator and BmNPV IE1 [21] from the *Bombyx mori* nuclear polyhedrolysis virus. This plasmid helps to achieve a high-level expression of the recombinant protein upon transfection. Tn-5B1-4 cells were cotransfected with either pXme- GGT, pXgp- GGT or pXce- GGT, and pBmA:neo, at a molar ratio of 10:1 using Cellfectin reagent (Invitrogen). The cells were subcultured for 3 weeks in a 24-well plate containing 700 μg/ml Geneticin (Invitrogen), and resistant polyclonal cell lines obtained.

The foci of resistant polyclonal stable cell lines for each signal sequence formed were carefully selected, maintaining the homogeneity for each transformed cell line, and then separately seeded onto 96-well plates (Falcon, NJ, USA) (Fig. 1). After 1 week of cultivation, the wells that showed confluence were seeded onto 24-well plates with a 2 cm^2 surface area (Falcon). After 4 d of cultivation in the wells, the supernatant was removed and exchanged with fresh medium. The supernatant contained the secreted protein, and was used for Western blot analysis.

The total protein of the culture supernatants was precipitated by the addition 100% trichloroacetic acid (TCA) (final concentration, 2%) and subsequently washed with 50 mM Tris-HCl (pH 7.5). The precipitated protein was dissolved in SDS-PAGE sample buffer and subjected to SDS-PAGE, using the Mini-protean II system (Bio-Rad). The purified GGT fusion protein, and its precipitate obtained using TCA, were used as the standard. After SDS-PAGE, separated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane, using a Mini Trans-Blot Electrophoretic Transfer Cell

(Bio-Rad). After blocking in 5% Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated for 1 hour in 1:10,000 diluted anti-$(His)_6$G antibody solution. The membrane was washed, and then incubated for 1 hour in 1:20,000 diluted anti-mouse IgG antibody conjugated with horseradish peroxidase. Specific bands were detected using a Fluor-S/MAX multi-imager (Bio-Rad) and analyzed using the Quantity One software (ver. 4, Bio-Rad).

Isolated transformants were seeded onto 6-well plates with a 9.6 cm^2 surface area (Falcon), and further cultivated in a 100-ml flask, with a working volume of 20 ml, at an agitation speed of 100 rpm. The β3GnT activity assay was carried out according to the previously described protocol [13].

Stable cell lines carrying the GGT fusion cDNA linked to three different signal sequences, melittin, gp64, and cecropin B, were successfully constructed using pXme-GGT, pXgp-GGT, and pXce-GGT with plasmid pBmA:neo. It took 3 weeks for the resistant cells to grow and form foci visible to the naked eye. Approximately 50 foci clones from each cell line were selected at random and seeded onto 96-well plates. These clones were allowed to grow to confluence for a polyclonal cell line for 1 week. The 50 clones seeded on wells for each stable cell line grew to form a confluent monolayer, which was easily confirmed by the naked eye. Subsequently, these stable cell lines were further seeded onto 24-well plates. After 4 d of cultivation, of the 50 seeded clones for each cell line with a signal peptide, only 19 wells were confluent for melittin, 26 for cecropin and 20 for glycoprotein signal peptides. These Geneticin-resistant cell lines, showing confluence, were maintained and their supernatants used for Western blot analyses. The supernatants from these wells were removed and total protein precipitated using 100% TCA. As a standard reference point

for the mobility, purified GGT was also precipitated with TCA, and loaded along with concentrated protein samples onto the SDS-PAGE gel, which were analyzed by Western blotting using the anti-$(His)_6$G antibodies (a-c in Fig. 2). The Western blotting of samples using the anti-$(His)_6$G antibodies showed two bands. The bands with slower mobility, similar to the standard band treated with TCA, corresponded to the GGT fusion protein [15].

To quantify the intensity of the GGT bands, their densitometric values were measured using the Quantity One software; the distribution of GGT band intensities is shown in Fig. 2d. The transformants of the Tn-pXme cell line produced a higher amount of the GGT fusion protein than those of the Tn-pXce and Tn-pXgp cell lines. Four transformants from the Tn-pXme cell line showing band intensities higher than 90% were isolated, but the cell growth of one of these was very low. Finally, Tn-pXme4a, Tn-pXme23a and Tn-pXme28a were isolated. After 6 d of cultivation in 100-ml flasks, with shaking, the β3GnT activities were confirmed, as shown in Table 2. The Tn-pXme28a cell line produced 8.6 mU/ml β3GnT activity, which was 26% higher than that of the previously isolated transformant, Tn-pXme11 [15]. The improved activity achieved by this screening method was higher than the previously reported enzyme activities. Conversely, the Tn-pXgp1a ~~and Tn-pXgp20a~~ cell line~~s~~, showing band intensities of ~~21 30~~21%, showed β3GnT activities of 1.5 ~~and 3.8~~ mU/ml~~, respectively~~. This indicates that the β3GnT activity is proportional to the band intensity of the GGT fusion protein.

Stable cell lines, expressing the GGT fusion protein, were successfully established with three different signal peptides, which assisted in secreting the protein. The extracellular secretion with the His tags on the fusion protein is helpful, and is more

rapid for protein purification. As expected, the β3GnT2 activity as well as the level of expressed protein differed between the signal peptides. Among the three cell lines, the Tn-pXme cell line showed a higher expression level of GGT, and at the same time retained a higher enzymatic activity. The Tn-pXgp and Tn-pXce cell lines showed the lowest expression levels (b and c in Fig. 2).

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The conventional screening method (15) is a series of random picking up of stable cell lines, culturing them in well-plate and flask serially for 2-3 weeks at least, detecting recombinant proteins, and then selecting high expression cell lines. In this report we skipped the cultivation of cell lines in flask and detected directly the recombinant protein using Western blotting analysis, and then chose cell lines shown a relative high intensity of GFP_{uv}. This method made shorten the time for cultivation and saved many number of well-plates and flasks. Compared with the previous study, it took us about 4 weeks and reduced the manual hours required in screening to half from the cotransfection to the selection of a stably transformed cell line retaining a high enzymatic activity, despite the simultaneous handling of many transformants. The rapid evaluation of recombinant protein productivity is a key factor in the biotechnology industry. This method required a screening time almost one-half that required in the isolation of stably transformed cells, and at the same time much more efficiently facilitated the production of high-value protein.

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Although the three different signal peptides are supposed to only assist in secretion, the rate of secretion was different for each signal peptide. This may have been due to the preference of the cell line for a particular secretory pathway, or may depend on the copy number of the cDNA randomly inserted at different sites. This method will be helpful in the screening and optimization of protein expression simultaneously using many signal

sequences and in selecting the most suitable cell line for a particular purpose as rapidly as possible.

REFERENCES

[1] Alam, M., S. Ho, D. E. Vance, and R. Lehner (2002) Heterologous expression, purification, and characterization of human triacylglycerol hydrolase. *Protein Expr. Purif.* 24: 33-42.

[2] James, D. C., R. B. Freedman, M. O. Hoare, W. Ogonah, B. C. Roony, O.A. Larionov, V.N. Dobrovolsky, O.V. Lagutin, and N. Jenkins (1995) *N*-Glycosylation recombinant human interferon-produced in different animal expression system. *Bio/Technology* 13: 592-596.

[3] E. Hericourt, S. Blanc, V. Ledeker, and I. Jupin (2000) Evidence for phosphorylation and ubiquitinylation of the turnip yellow mosaic virus RNA-dependent RNA polymerase domain expressed in a baculovirus-insect cell system. *Biochem. J.* 349: 417-425.

[4] Luckow, V. A. and M. D. Summers (1988) Trends in the development of baculovirus expression vectors. *Bio/Technology* 6: 47.

[5] Ailor, E. and M.J. Betenbaugh (1999) Modifying secretion and post-translational processing in insect cells. *Curr. Opin. Biotechnol.* 10: 142-145.

[6] Jarvis, D. L. and M. D. Summers (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol.* 9: 214-223.

[7] Morais, V. A., J. Serpa, A.S. Palma, T. Costa, L. Maranga, and J. Costa (2000) Expression and characterization of recombinant human α-3/4-fucosyltransferase

III from *Spodoptera frugiperda* (Sf-9) and *Trichoplusia ni* (Tn) cells using a baculovirus expression system. ***Biochem. J.*** 353, 719-725.

[8] Farrell, P. J., M. Lu, J. Prevost, C. Brown, L. Behie, and K. Iatrou (1988) High-level expression of secreted glycoproteins in transformed lepidopteran insect cells using a novel expression vector. ***Biotechnol. Bioeng.*** 60: 656-663.

[9] Li, E., S. L. Brown, C. S. Dolman, G. B. Brown, and G.R. Nemerow (2001) Production of functional antibodies generated in a nonlytic insect cell expression system. ***Protein Expr. Purif.*** 21: 121-128.

[10] Pfeifer, T. A., M. M. Gaurna, E. M. Kwan, G. Lesnicki, D.A. Theilmann, A. Grigliatti, and D.G. Kilburn (2001) Expression analysis of modified Factor X in stably transformed insect cell lines. ***Protein Expr. Purif.*** 23: 233-241.

[11] Wendeler, M., T. Lemm, J. Weisgerber, J. Hoernschemeyer, O. Bartelsen, U. Schepers, and K. Sandhoff (2003) Expression of recombinant human GM2-activator protein in insect cells: purification and characterization by mass spectrometry. ***Protein Expr. Purif.*** 27: 259-266.

[12] Cha, H.J., N.G. Dalal, V.N. Vakharia, and W.E. Bentley (1999) Expression and purification of human interleukin-2 simplified as a fusion with green fluorescence protein in suspended Sf-9 insect cells. ***J. Biotechnol.*** 69: 9-17.

[13] Kato, T., T. Murata, T. Usui, and E.Y. Park (2003) Improvement of GFP_{uv}-β2GnT2 fusion protein production by suppressing protease in baculovirus expression system. ***Biosci. Biotechnol. Biochem.*** 67: 2388-2395.

[14] Kato, T., T. Murata, T. Usui, and E.Y. Park (2004) Efficient production of human β1, 3-*N*-acetylglucosaminyltransferase 2 fused with green fluorescence protein in insect cell. ***Biochem. Eng. J.*** 19: 15-23.

[15] Kato, T., T. Murata, T. Usui, and E.Y. Park (2004) Comparative analysis of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 production in two insect-cell-based expression system. *Protein Expr. Purif.* 35: 54-61.

[16] Vlasak, R., C. Unger-Ullmann, G. Kreil, and A.M. Frischauf (1983) Nucleotide sequence of cloned cDNA coding for honeybee prepromelittin. *Eur. J. Biochem.* 135: 123-126.

[17] Volkman, L.K. (1986) The 64K envelope protein of budded *Autographa californica* nuclear polyhedrosis virus, *Curr. Top. Microbiol. Immunol.* 131: 103-118.

[18] van Hofsten, P., I. Faye, K. Kockum, J.Y. Lee, K.G. Xanthopoulos, I.A. Boman, H.G. Boman, A. Engstrom, D. Andreu, and R.B. Merrifield, R.B (1985) Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*, *Proc. Natl. Acad. Sci. USA.* 82: 2240-2243.

[19] Johnson, R., R.G. Meidinger, and K. Iatrou (1992) A cellular promoter-based expression cassette for generating recombinant baculoviruses directing rapid expression of passenger genes in insect cells. *Virology* 190: 815-823.

[20] Lu, M., P. J. Farrell, R. Johnson, and K. Iatrou (1997) A baculovirus (BmNPV) repeat element functions as a powerful constitutive enhancer in tranfected cells. *J. Biol. Chem.* 272: 30724-30728.

[21] Lu, M., R. Johnson, and K. Itaoru (1996) Trans-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. *Virology* 218: 103-113.

TABLE 1. Primers used in this experiment

Primers	
AcNPV-me-GGT	5’-CACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCC GGCCCGCGGGGTTCTCATCATC-3’
AcNPV-gp-GGT	5’-CACCATGCCCATGTTAAGCGCTATTGTTTTATATGTCCTTTTGGCGGCGGCGGCGCATTCTGCCTTT GCGCCCCCGCGGGGTTCTCATCATC-3’
AcNPV-ce-GGT	5’-CACCATGAATTTCTCAAGGATATTTTTCTTCGTGTTCGCTTTGGTTCTGGCTTTGTCAACAGTTTC GGCTGCACCGGAGCCGAAACCGCGGGGTTCTCAT-3’
Reverse primer	5’-CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG-3’

TABLE 2. Extracellular β3GnT activity in isolated transformants

Transformants	β3GnT activity (mU/ml)	Reference
Tn-pXme4a	8.3	This work
Tn-pXme23a	3.5	This work
Tn-pXme28a	8.6	This work
Tn-pXgp1a	1.5	This work
~~Tn-pXgp20a~~	~~3.8~~	~~This work~~
Tn-pXme11	6.8	(14)

書式変更: 右： 30 mm, 上： 35 mm, 幅： 210 mm, 高さ： 297 mm

LEGENDS FOR FIGURES

FIG.1. Schematic representation of stable cell line isolation. Cotransfection was performed using pXme-GGT, pXgp-GGT, pXce-GGT and pBmA:neo.

FIG.2. SDS-PAGE gel pictures of the GGT fusion proteins precipitated from culture supernatants of stably transformed cells using trichloroacetic acid (TCA). (a) The numbers on the lanes denote the numbers of clones containing the GGT fusion gene, with melittin as the signal peptide. (b) The numbers on the lanes denote the numbers of clones containing the GGT fusion gene, with glycoprotein 64 as the signal peptide. (c) The numbers on the lanes denote the numbers of clones containing the GGT fusion gene, with cecropin as the signal peptide. The lane marked Std is the purified GGT fusion protein used as the standard reference. The lane marked TCA is the purified GGT fusion protein, precipitated with TCA, used as the standard reference. (d) Number of transformants expressing the GGT fusion protein, with their relative intensities compared with the Std. Black, white, and slashed bars indicate number of transformant with melittin, gp64, and cecropin B signal sequences, respectively.

Fig. 1.

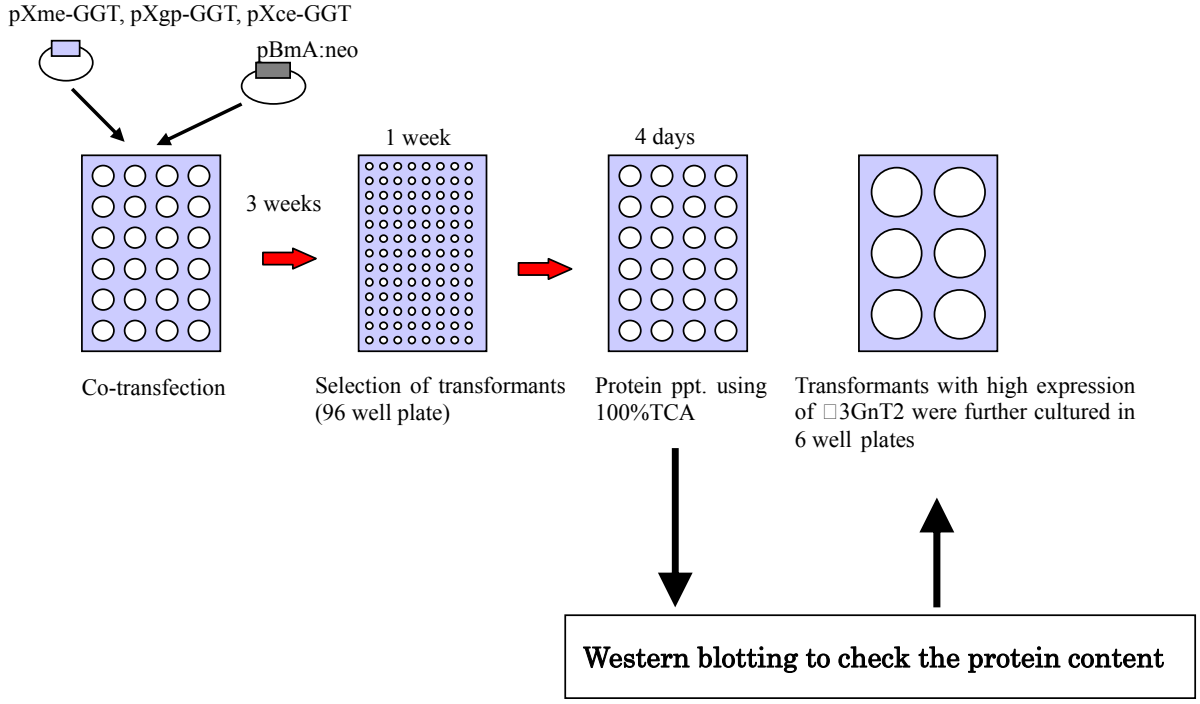
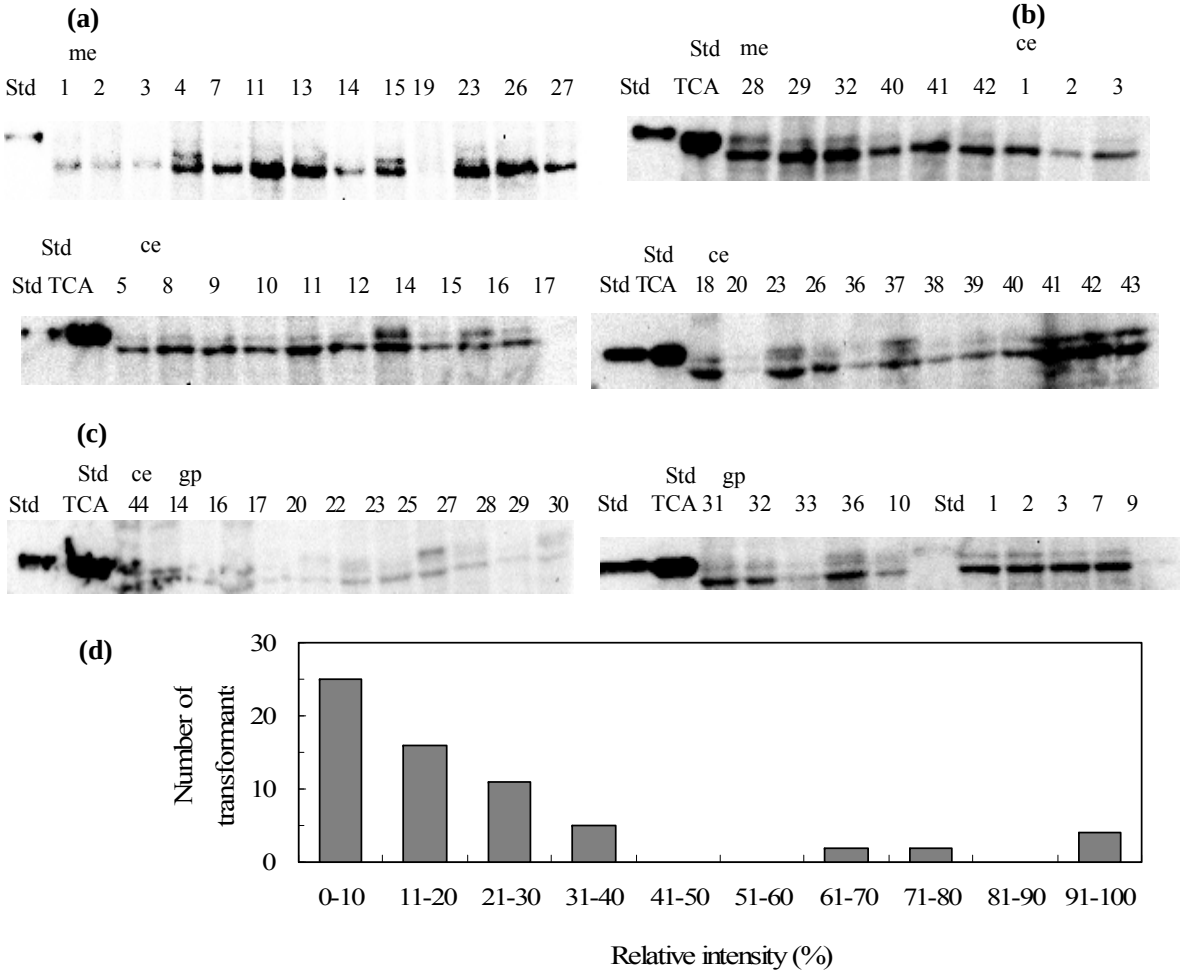
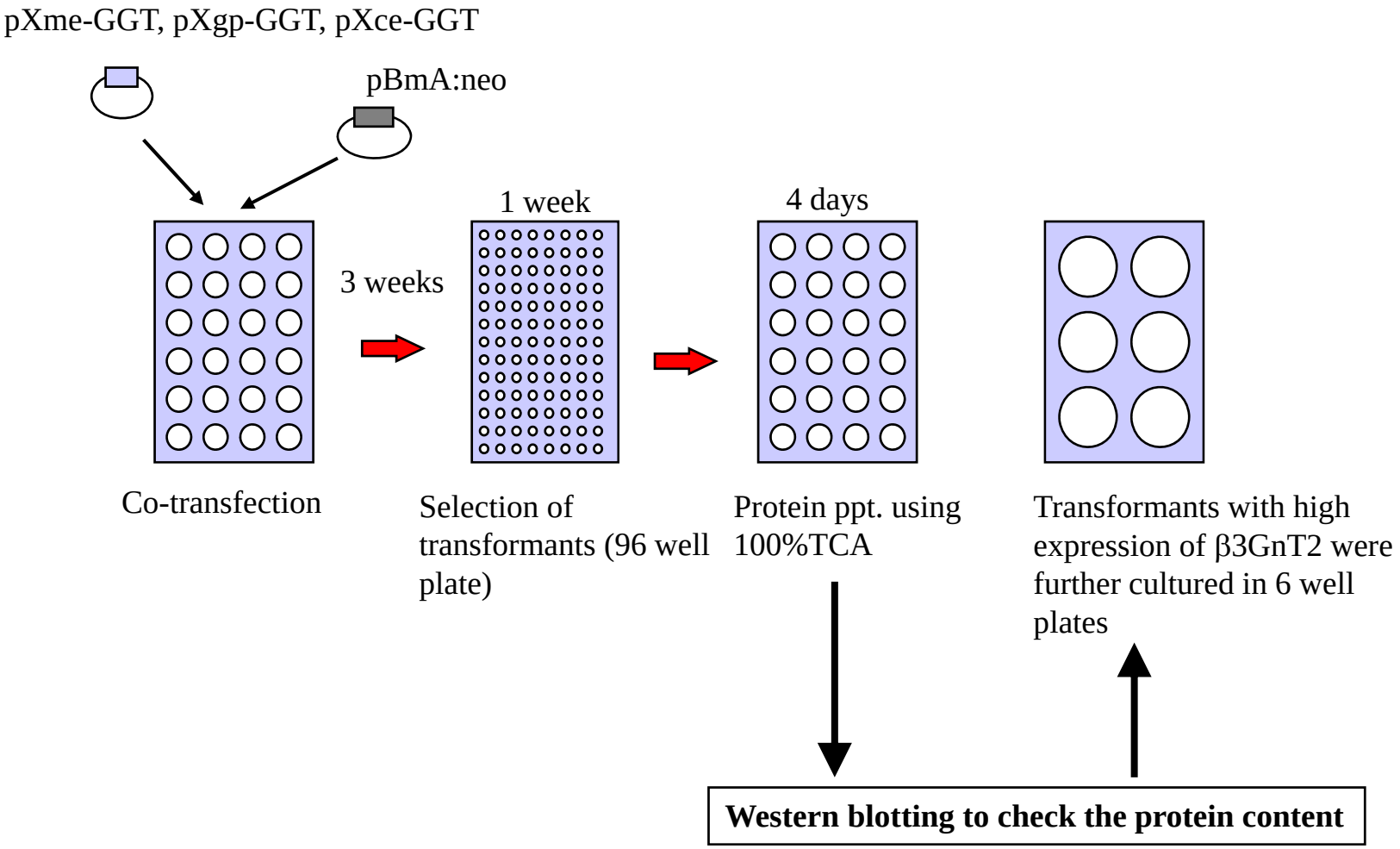

pXme-GGT, pXgp-GGT, pXce-GGT
pBmA:neo
3 weeks
1 week
4 days
Co-transfection
Selection of transformants (96 well plate)
Protein ppt. using 100%TCA
Transformants with high expression of □3GnT2 were further cultured in 6 well plates
Western blotting to check the protein content

Fig. 2.


(a)
me
Std 1 2 3 4 7 11 13 14 15 19 23 26 27
(b)
Std me
ce
Std TCA 28 29 32 40 41 42 1 2 3
Std ce
Std TCA 5 8 9 10 11 12 14 15 16 17
Std ce
Std TCA 18 20 23 26 36 37 38 39 40 41 42 43
(c)
Std ce gp
Std TCA 44 14 16 17 20 22 23 25 27 28 29 30
Std gp
Std TCA 31 32 33 36 10 Std 1 2 3 7 9
(d)
Number of transformants
30
20
10
0
0-10 11-20 21-30 31-40 41-50 51-60 61-70 71-80 81-90 91-100
Relative intensity (%)


pXme-GGT, pXgp-GGT, pXce-GGT
pBmA:neo
3 weeks
1 week
4 days
Co-transfection
Selection of transformants (96 well plate)
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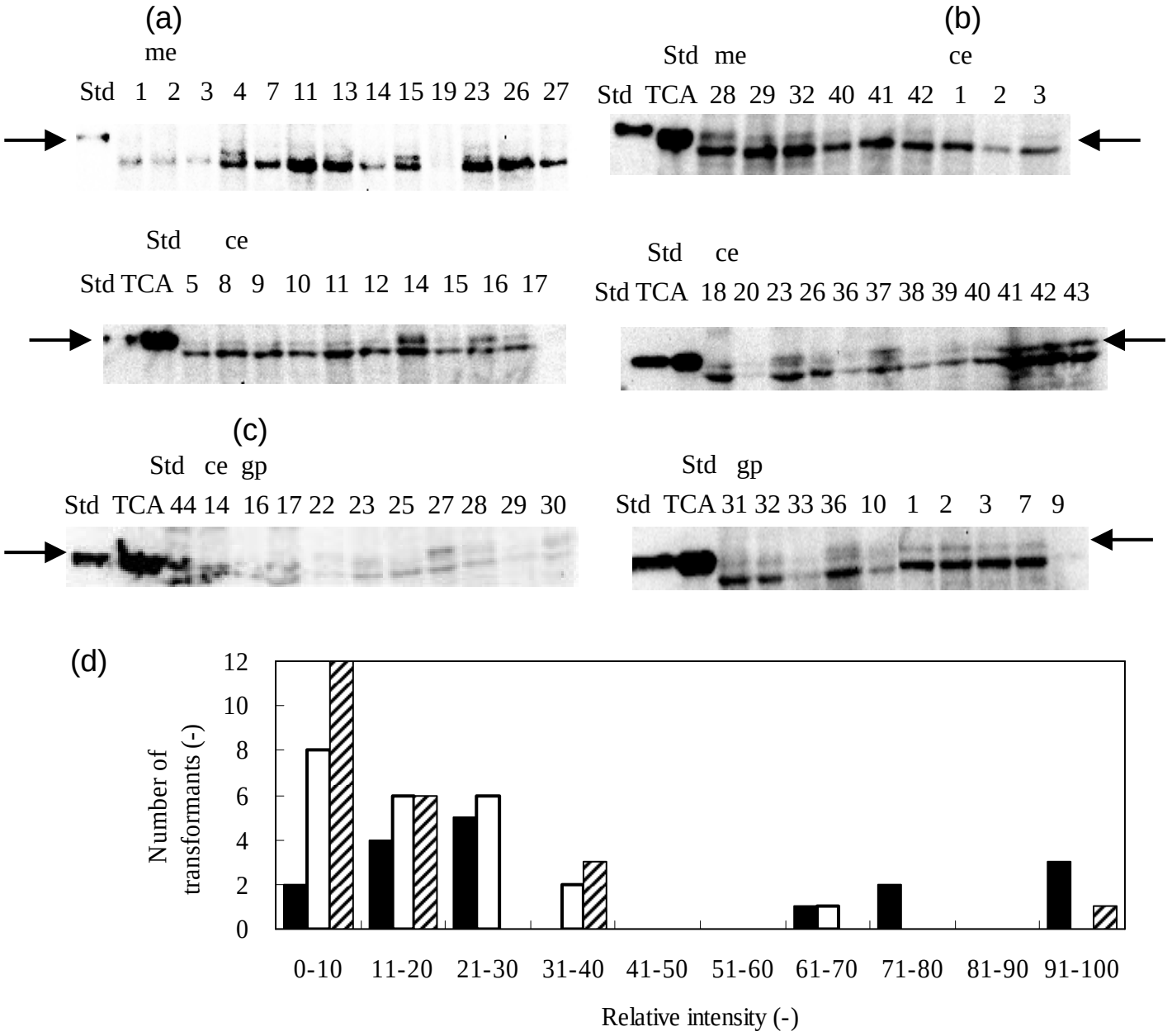
Transformants with high expression of β3GnT2 were further cultured in 6 well plates
Western blotting to check the protein content


(a)
me
Std 1 2 3 4 7 11 13 14 15 19 23 26 27
(b)
Std me
ce
Std TCA 28 29 32 40 41 42 1 2 3
Std ce
Std TCA 5 8 9 10 11 12 14 15 16 17
Std ce
Std TCA 18 20 23 26 36 37 38 39 40 41 42 43
(c)
Std ce gp
Std TCA 44 14 16 17 22 23 25 27 28 29 30
Std gp
Std TCA 31 32 33 36 10 1 2 3 7 9
(d)
Number of transformants (-)
0
2
4
6
8
10
12
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
Relative intensity (-)