PRELIMINARY ASSESSMENT OF THE EFFECT OF PLANT PROTEASE INHIBITORS ON THE EXPRESSION OF HETEROLOGOUS GENES IN PLANT CELLS

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ABSTRACT

Despite the successful development of pant-based expression systems for the production of a wide range of recombinant proteins in recent years, there remain serious limitations in the final yield accumulation of the foreign protein in the plant cells used. Plant protease activity represents a significant barrier to efficient and hence economical recombinant protein production. Inadequate information is available on specific plant proteases and its role in foreign protein degradation. Some strategies have been reported recently to overcome endogenous plant protease effects on foreign protein integrity and activity in plants including the co-expression of recombinant proteases to reduce the endogenous protease activity. The lack of information in this area suggests that more studies should be carried out to assess different plant protease inhibitors for the improvement of the stability of foreign protein production in particular host plants. The aim of this research was to evaluate the effect of recombinant plant protease inhibitors on the expression of a cloned heterologous ScFv antibody gene in plant cells. The recombinant tomato Cathepsin D Inhibitor (CDI) gene fused to a KDEL sequence was ligated to a previously construcuted recombinant antibody (scFv) gene developed against toxoplasmosis epitopes (pCTOXO-CDI-KDEL) and then transferred successfully into *Nicotiana tabacum* cv. BY-2 cell suspension using *Agrobacterium*-mediated transformation method. In addition, the scFv gene was also ligated to a construct containing only the endoplasmic reticulum retention signal, KDEL, (pCTOXO-KDEL) and this was also transferred into tobacco cells as a comparison against CDI-KDEL containing cells. Both constructs contained GUS marker gene. To assess the effect of the CDI gene in putative transformed tobacco cells GUS fluorometric assay was performed. Total soluble protein of the tobacco cells was also analyzed by Bradford protein assay. The tobacco cells carrying the CDI protease inhibitor gene showed higher TSP levels in comparison with plant cells lacking this

gene. This finding suggests the positive effect of co-expression of the CDI gene on the expression of scFv anti-toxoplasmosis in tobacco cells. However, quantitative GUS enzymatic activity expressed in pmol 4-MU/mg protein/min did not show any significant differences between two constructs in paired t-test ($p>0.05$). Overall results suggest that this strategy could be viable for increasing the production of ScFv antibodies in plant cells.

ABSTRAK

Walaupun perkembangan sistem ekspresi berasaskan tumbuhan dalam penghasilan protin rekombinan beberapa tahun kebelakangan ini telah berjaya,tetapi masih terdapat halangan untuk memastikan pengumpulan produk akhir yang maksima di dalam sel tumbuhan yang digunakan. Di antara faktor penghalang yang utama dalam penghasilan protein rekombinan yang cekap dan produktif adalah aktiviti enzim protease. Walaupun fungsi sesetengah protein protease dalam degradasi protein asing telah diketahui, namun maklumat yang ada tidak mencukupi. Beberapa strategi dilaporkan telah digunakan untuk mengatasi masalah ini termasuklah ekspresi bersama(co-expression) protease rekombinan untuk mengurangkan aktiviti protease dalaman (endogenous) tumbuhan. Kurangnya maklumat dalam bidang ini menunjukkan lebih banyak kajian perlu dijalankan untuk menilai potensi perencat protease tumbuhan (plant protease inhibitor) yang berbeza bagi meningkatkan pengeluaran/penghasilan protein asing dalam tumbuhan. Kajian ini bertujuan untuk menilai kesan perencat (inhibitor) rekombinan protease tumbuhan ke atas pengekspresan klon gen 'heterologous ScFV' antibodi dalam sel tumbuhan. Gabungan antara gen 'Cathepsin D Inhibitor' (CDI) daripada tomato rekombinan dan jujukan KDEL telah dicantumkan ke dalam gen rekombinan antibodi yang dapat mengecam 'toxoplasmosis epitope' (pCTOXO-CDI-KDEL). Fragmen DNA ini (pCTOXO-CDI-KDEL) seterusnya dipindahkan ke dalam cell suspension *Nicotiana tabacum* cv. BY-2 melalui kaedah transformasi berasaskan *Agrobacterium*. Di samping itu, gen scFv turut digabungkan ke dalam konstruk (fragmen DNA) yang hanya mengandungi isyarat pengekalan reticulum endoplasmik, KDEL, (pCTOXO-KDEL) dan seterusnya dipindahkan ke dalam sel tembakau untuk dijadikan perbandingan dengan sel yang mengandungi CDI-KDEL. Kedua-dua fragmen DNA ini mempunyai gen penanda GUS. Ujian fluorometrik GUS dilakukan untuk menganalisa/menilai kesan gen CDI dalam sel tembakau tersebut. Jumlah protein larut dalam sel tembakau turut

dianalisa menerusi ujian protin Bradford. Sel tembakau yang mengandungi gen 'CDI protese inhibitor' menunjukkan jumlah protein larut yang lebih tinggi berbanding sel tumbuhan yang tidak mempunyai gen ini. Penemuan ini menunjukkan kemungkinan wujudnya kesan positif ekspresi bersama gen CDI ke atas pengekspresan scFv anti-toxoplasmosis dalam sel tembakau. Namun begitu, aktiviti kuantitatif pmol enzim GUS yang dikira berdasarkan t-berpasangan ($p>0.05$) dalam unit pmol 4-MU/mg protein/min di antara kedua-dua DNA tidak menunjukkan perbezaan yang ketara. Secara keseluruhannya, data daripada eksperimen yang dijalankan menunjukkan petanda positif keberkesanan kaedah ini dalam meningkatkan pengeluaran antibodi scFv dalam sel tumbuhan.

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ABBREVIATION

A: Absorbance

Agrob: *Agrobacterium*

anti-toxo: anti-toxoplasmosis

BBTI: Bowman–Birk trypsin inhibitor

bp: base pair

BSA: Bovine serum albumin

BY-2: Bright Yellow–2

CaCl2: Calcium chloride

CDI: Cathepsin D Inhibitor

cDNA: Complementary deoxyribonucleic acid

conc: Concentration

cv: Caltivar

DNA: deoxyribonucleic acid

E.coli: *Escherichia coli*

EDTA: Ethylenediaminetetracetic acid

ER: Endoplasmic reticulum

ETOH: Ethanol

Fab: Fragment antigen binding

g: Gram

GAB: GUS assay buffer

GEB: GUS extraction buffer

HDEL: His-Asp-Glu-Leu

HIV: human immunodeficiency virus

Kb: Kilobase

KCl: Potassium chloride

KDEL: Lys-ASP-Glu-Leu

l: Liter

LB: Luria-Bertani

M: Molar

M2: Media 2

mAB: Monoclonal antibody

mg: milligram

min: minute

ml: milliliter

mM: Millimolar

MnCl2: Manganese chloride

MOPS: 3-(N-morpholino)propanesulfonic acid

MS: Murashige and Skoog media

MSO: Murashige and Skooge basal medium

MUG: 4-methylumbelliferyl-ß-D-glucuronide

MW: Molecular weight

Na2CO3: Sodium carbonate

NaOH: Sodium hydroxide

nM: Nanomolar

OCPI: Oryzacystatin protease inhibitor I

OD: Optical density

PCR: polymerase chain reaction

pCTOXO-BBTI: pCAMBIA 1301-scFv anti –Toxo-BBTI

pCTOXO-CDI-KDEL: pCAMBIA 1304-scFv anti –Toxo-CDI-KDEL

pCTOXO-CDI: pCAMBIA 1301-scFv anti –Toxo-CDI

pCTOXO-KDEL: pCAMBIA 1304-scFv anti –Toxo-KDEL

pCTOXO-OCPI: pCAMBIA 1301-scFv anti –Toxo-OCPI

pCTOXO-PI: pCAMBIA 1301-scFv anti –Toxo-Protease inhibitor

pH: Potential hydrogen

PI: Protease inhibitor

pmol: picomole

pUC-PI: pUC57-Protease inhibitor

RE: Restriction endonuclease

RNA: ribonucleic acid

RNAse A: Ribonuclease A

rpm: Revolutions per minute

scFv: Single chain variable fragment

sdH2O: Sterile distilled water

SDS: Sodium dodecyl sulphate

sec: Second

TBE: Tris borate

Ti: Tumor inducing

TOXO: Toxoplasmosis

Tris-Cl: Trisbase-Hydrochloric acid

TSP: Total soluble protein

u: Unit

UV: Ultraviolet

v: Volume

Vir: Virulence

vs: Versus

w: Weight

w/v: Weight over volume

X: Times

2,4-D: 2,4-Dichlorophenoxyacetic acid

4-MU: 4-methylumbelliferone

µ µl: Micro liter

g: Micro gram

µM: Micro molar

˚C: Degree Celsius

%: Percentage