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Transformation of HIV-1 Envelope Glycoprotein120 in Tobacco Plant

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ABSTRACT

HIV-1 infection which causes AIDS is still be health, social and economical problems in Thailand. Early diagnosis of HIV-1 infected individuals is one of the strategies to confine the spreading of the virus. Every year, commercial kits for HIV-1 infection diagnosis were imported to Thailand at several hundred millions Baht. The development of protein production system for using as antigens in diagnostic kit is very important for self-depending and decreasing an import of recombinant proteins and commercial kits to Thailand. In this study, tobacco plant was transformed with the entire 1515-bp of HIV-1 glycoprotein 120 (gp120) coding region by A*grobacterium*-mediated gene transformation. We could demonstrate the presence of integrated gp120 coding region in the genomic DNA of transformed tobacco plants by nested-PCR, which was confirmed by sequencing. However, neither gp120 mRNA nor protein was detected. The reason that HIV-1 gp120 could not be expressed may due to an inappropriate system used in our study. Naturally, HIV-1 infects only human and use human's advance machinery to produce all viral proteins. Furthermore, gp-120 is expressed as gp160 precursor, which then cleaved into gp120 and gp41 during viral maturation. The research and development of transgenic plant or other organisms to produce such a special protein likes HIV-1 gp120 would be desired.

Key words: HIV-1, gp120, transformation, tobacco

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS), is classified into at least 9 subtypes and 12 circulating recombinant form (CRF), which have been distributing at different prevalence in each region of the world (UNAIDS. 2004). Information about the HIV-1 and pathogenesis of AIDS, including vaccine and diagnostic method, are mostly come from the studies on HIV-1 subtype B, which is epidemic in North America and Europe (Kanki *et al*., 1999). In Thailand, HIV-1 CRF01_AE spans over 80% of infected individuals and the rest is HIV-1 subtype B (Gao *et al*., 1996). An estimated number of new HIV-1 infected cases in Thailand is 20,000 persons per year (AIDS Division, Bureau of AIDS, TB and STDs, Department of Diseases Control, Ministry of Public Health,

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www.aidsthai.org). Whereas AIDS is still being the incurable disease, early diagnosis of HIV-1 infected individuals is one of the strategies to confine the number of new infected cases.

Current methods to diagnose HIV-1 infection are based on the detection of specific anti-HIV-1 antibody. Every year, Thailand imports commercial kits to diagnose HIV infection for several hundred millions Baht. There are only two commercial kits produced in Thailand, but using imported HIV-1 antigens (personal communication). Traditional recombinant protein production systems using microbial fermentation, yeast, and insect and mammalian cell culture have drawbacks in term of cost and scalability. To overcome these drawbacks, simple and inexpensive alternative approaches, which allow the large scale of production, would be highly desired.

The antigens used in HIV-1 diagnosis assays are derived from viral structural proteins. The most important antigens are envelope glycoprotein gp120 and gp41 because these antigens induce antibody response earlier than other HIV-antigens. Moreover, anti-gp120 and anti-gp41 antibodies are persisting last long throughout the infection (Allian *et al*., 1986). The sources of antigens for HIV diagnostic assays included whole viral lysate or purified viral proteins from cell culture, recombinant proteins, and synthetic peptides. However, recombinant proteins and synthetic peptides have been increasingly used because of low risk of exposure to the hazardous virus in the production process. In addition, using the selective HIV antigen(s) that have been proved not to cross-react with other pathogens can increase specificity of the test. However, there is a limitation in using synthetic peptide as antigen since it represents only linear antigenic epitope but not conformational epitope. Principally, recombinant protein is supposed to be the most appropriate representative for antigen in diagnostic assays.

The developments of genetic transformation technology for plant has facilitated the study of plant gene expression for enhanced production traits, and then were consequently applied as a novel manufacturing system to produce foreign proteins for medical sciences. Genetically engineered plants for the production of immunogenic proteins also provide a new approach for the delivery of a plant-based subunit vaccine, including Hepatitis B surface antigen, Rabies virus glycoprotein, Cholera toxin B subunit (Ma *et al.*, 2003).

For HIV, there was a reported of the production of p24 capsid protein in transgenic tobacco using A*grobacterium*-mediated gene transfer (Zhagg *et al.*, 2002). Estimated yield of p24 protein was 3.5 mg/g of soluble leaf protein. Kim and colleagues reported the expression of fusion protein between cholera toxin B subunit and a fragment of HIV-1 gp120 (103 amino acids) (Kim *et al.*, 2004). So far, there was no report of the expression of the whole HIV-1 gp120 (505 amino acids) or gp41 (353 amino acids) in transgenic plant. Therefore, this study aimed to determine the possibility to produce the whole HIV-1 gp120 by transgenic tobacco**.**

MATERIALS AND METHODS

Construction of plasmid for plant transformation

Coding region of HIV-1 gp120 (1515 bp) was amplified from a plasmid clone of HIV-1 CRF01_AE whole-genomic (95TNIH022; GenBank Acuession number AB032740) (Auwanit *et al.*, 2001), with primers 6G_NcoI_gp120F: 5'-GGGGGGCCATGGTAGATCTGACTAGTATGAGAGTGAAGGAGACACAG-3' and 6G_NheI_gp120R: 5'-GGGGGGCTAGCTCTTTTT-TCTCTCTCCACCAC-3' using a proof reading DNA polymerase (*Pfu* DNA polymerase, Promega, Madison, USA). The thermal profile for amplification was as follow:

95°C for 5 min; 25 cycles of 95°C for 30 sec, 55° C for 15 sec, 72°C for 2 min; and then 72°C for 5 min and soak at 4°C. The PCR product (1539 bp) was cut with *Nco*I and *Nhe*I, and ligated into pCAMBIA1303 (GenBank Accession number AF234299), which was also cut with *Nco*I and *Nhe*I. The HIV gp120 coding region now was under the control of cauliflower mosaic virus (CaMV) 35S promoter and *Nos* poly-A. This expression plasmid (named pCAMBIA1303_HIV-1 gp120) was propagated in *E.coli* (JM109, Promega, Madison, USA), and the purified plasmid was checked for the entire insert sequences and the joining area by BigDye Terminator v3.1 cycle sequencing kit and automate DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, CA., USA). The pCAMBIA1303_HIV-1 gp120 was electroporated into *Agrobacterium tumifaciens* strain AGL1 under the selection with 100 mg/L of Kanamycin. After confirming for the presence of pCAMBIA1303_ HIV-1 gp120 by cutting the purified plasmid from the transformed *Agrobacterium tumifaciens* with *Nco*I and *Nhe*I, the plasmid was subsequently transformed into tobacco plant (*Nicotiana tabacum* cv. Xanthi) by leaf disc transformation (Horsch *et al.*, 1985). Transformed calli were selected on Murashige and Skoog medium (MS Basal Medium, Sigma, MO., USA) containing 50 mg/L of hygromycin.

Analysis of transgenic tobacco

Genomic DNA was extracted from the leaves of transformed and untransformed tobacco plants (MasterPure^{TM} Plant Leaf DNA Purification kit, Epicentre, Madison, USA), and used as templates for amplification by nested-PCR with outer primers; pCAMBIA-Sq1: 5'-CGTCTTC AAAGCAAGTGG-3' and pCAMBIA-Sq2: 5'-AACATAGATG-ACACCGCG-3', and inner specific primers for HIV-1 gp120 with thermal profiles as described above. The PCR products were analysed under 1% agarose gel electrophoresis compared with DNA size marker (Hyper Ladder I, Bioline, MA., USA). The PCR products that had an expected size of 1.5 Kb were confirmed for the HIV-1 specific sequence by sequencing.

Analysis of gene expression

Total RNA were extracted from tobacco leaves (TRIzol Reagent, Invitrogen, CA, USA) and used as templates in RT-PCR (Superscript III One-step RT-PCR System with Patinum Taq DNA Polymerase, Invitrogen, CA., USA) to verify the HIV-1 gp120 transcription. The presence of Ubiquitin mRNA was used as a housekeeping gene expression control (Brunner *et al.*, 2004).

Tobacco leaves (approximately 300 mg) were crushed in 200 µl of extraction buffer (50 mM Tris HCl, pH 9.0, 150 mM NaCl, 1 mM EDTA, 20 mM DTT and 1% PVP). Total soluble proteins were determined by Bradford dye-binding procedure (Coomassie Plus Protein Assay Reagent Kit, Pierce Biotechnology, IL, USA) by using Bovine serum albumin as a standard. Dot blot and Western blot were performed to determine the immunogenic property of HIV-1 gp120. Extracted proteins were directly blotted (dot blot), and also transferred from SDS-PAGE (Western blot) onto a membrane (Immun-Blot PVDV membrane, Bio-Rad , CA., USA), which then incubated with serum from HIV-1 infected patient. The immune complex, if presented, would be bind to anti-human immunoglobulin conjugated with horseradish peroxidase (HIV Blot 2.2; Genelabs Diagnostics, Singapore) which digest the substrate into purple color.

RESULTS AND DISCUSSION

The construction of plant expression vector; pCAMBIA1303_HIV-1 gp120, is shown in Figure1. We did sequencing of the whole fragment of HIV-1 gp120 coding region (1515 bp) and the joining area at both ends. The entire sequence results did not contain any unexpected

base change, which ensured correct sequences of HIV-1 gp120 coding region and the translation initiation (Kozak) for further steps of transcription and translation. We designed for expression the whole gp120 because we want to have all the possible antigenic epitopes and there was the database showed antibody map along the entire molecule (HIV molecular immunology, HIV sequence database; http:// hiv-web.lanl.gov).

From 150 tobacco leaf-discs, 4 calli were propagated under a selective of hygromycin resistance. The low percentage of transformation may be due to the integration position of HIV-1 gp120 or the expression of gp120 (if any) disturbing the development mechanism of tobacco callus. The genomic DNA extracted from these transformed tobacco leaves showed the presence of the transgene after nested-PCR with specific primers of HIV-1 gp120 coding region, whereas untransformed tobacco leaves did not showed this specific band (Figure 2). This result suggested that the HIV-1gp120 coding region had been transformed successfully into tobacco plants.

RT-PCR was performed to check the transcription of HIV-1 gp120, and none of the 4 transformed lines gave positive results. All of these 4 lines gave positive for RT-PCR of ubiquitin housekeeping gene. For protein expression analysis, the total soluble proteins per 300 mg fresh leaf of transformed tobacco line 1 to 4 (1080, 1845, 1922, and 1023 μg/μl; average 1467.5 μg/μl) were not different ($p > 0.05$) from untransformed tobacco (1905 μg/μl). And all of these 4 lines showed negative results by dot blot and Western blot analysis. These results suggested that there were no HIV-1 gp120 mRNA expression and

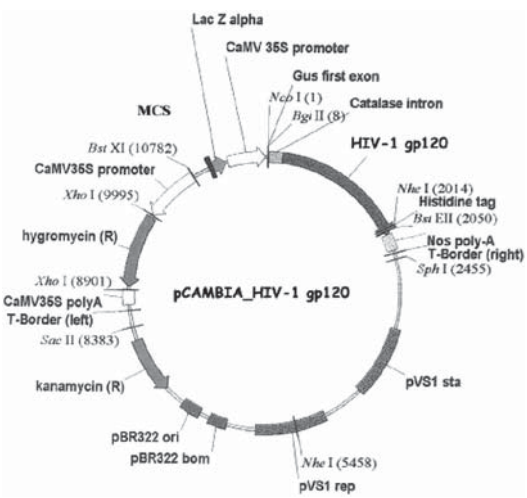

Figure 1 The construction of plant expression vector pCAMBIA1303_HIV-1 gp120. HIV-1 gp120 coding region was inserted between *NcoI* and *NheI* site of pCAMBIA1303 under the control of CaMV 35S promoter and *Nos* poly-A.

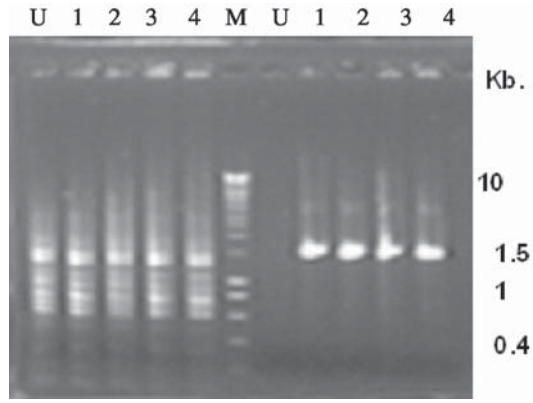

Figure 2 1% Agarose gel electrophoresis of PCR products. U: untransformed tobacco control; 1-4: transformed tobacco line 1-4. M: DNA size marker of 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, and 10 Kb, respectively. The outer PCR products from pCAMBIA-Sq1/ pCAMBIA-Sq2 primers are shown in the left side of M. All samples presented smear and nonspecific bands without the expected specific band of 1954-bp. The expected inner PCR products of1515-bp from HIV-1 specific primers in all 4 transformed lines, but not untransformed control, are shown in the right side of M.

protein translation. Naturally, replication of HIV-1 occurs inside the specific cells of human ($CD4^+$ T-lymphocyte). Envelope proteins of HIV-1 are synthesized as a single co-translational glycosylated polyprotein precursor (gp-160; 858 amino acids). Individual gp160 precursor molecules initiate folding, presumably in the presence of ER-associated chaperonins and assemble to form an oligomeric structure (Hunter and Swanstrom, 1990). These polyprotein precursors are cleaved later by the host enzyme; furin-like Golgi proteinase, into gp120 (505 amino acids) and gp41 (353 amino acids) during viral maturation (Hallenberger *et al*., 1997). This study used single protein (gp120) expression, not co-expression, which may cause the failure of expression. Furthermore, protein production system in plant has some differences from animal system that might not suitable for proteins having complicated modification like HIV-1 gp120. Therefore, it is possible that HIV-1 gp120 mRNA in tobacco plant may not stable enough for passing through out the whole process of transcription and translation.

CONCLUSION

In this study, we demonstrated the transformation of the entire HIV-1 gp120 coding region into tobacco plant. However, the transgenic tobacco did not express HIV-1 gp120 since we could not detect the specific mRNA and gp120. Research and development of other strategies or using other organisms to produce such a special protein likes HIV-1 gp120 would be desired.

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