Production of HIV-1 p24 Protein in Transgenic Tobacco Plants

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Abstract

The production of antigens for vaccines in plants has the potential as a safe and cost-effective alternative to traditional production systems. Toward the development of a plant-based expression system for the production of human immunodeficiency virus type I (HIV-1) p24 capsid protein, the p24 gene was introduced into the genome of tobacco plants using *Agrobacterium tumefaciens*-mediated gene transfer. Southern blot analyses confirmed the presence of the p24 coding sequence within the genome of transgenic lines. Western blot analysis of protein extracts from transgenic plants identified plant-expressed p24 protein that cross-reacted with a p24-specific monoclonal antibody, thus confirming the maintenance of antigenicity. Quantification of the p24 protein using enzyme-linked immunosorbent assay (ELISA) estimated yields of approx 3.5 mg per g of soluble leaf protein. Similar accumulation levels of p24 were also detected in T1 plants, confirming that the p24 gene is transmitted stably. Our results indicate that plant-based transgenic expression represents a viable means of producing p24 for the development of HIV vaccine and for use in HIV diagnostic procedures.

Index Entries: Human immunodeficiency virus type I (HIV-1); p24 capsid protein (p24); plant-based expression, plant transformation; protein expression; vaccine antigen.

1. Introduction

With recent technical advances in molecular engineering, plants have been investigated increasingly as biological factories for the production of commercially valuable and clinically important recombinant proteins, particularly antigens for vaccines ***(1–3)***. Plant-based production systems have the potential to be safe and cost-effective alternatives to the traditional production systems ***(4)***. Following the pioneering work on the expression of the hepatitis B virus surface antigen in tobacco plants ***(5)***, transgenic approaches have been used to successfully produce several different protein antigens in plants including Norwalk virus capsid protein, heat-labile enterotoxin B, cholera B subunit oligomers, and gastroenteritis coronavirus glycoproteins ***(3)***. When the purified proteins or crude leaf extracts were introduced into animals, they were able to elicit immunological responses ***(6,7)***, thus supporting the use of plant-based antigens for the development of vaccines. More interestingly, recent studies have shown that antigens produced in plants are able to induce immune responses in animals that are directly fed antigen-containing plant tissues, thereby supporting the feasibility of edible vaccine production ***(8–10)***. In addition to stable expression in transgenic plants, several RNA plant viruses have been used successfully to transiently express vaccine antigens in plants ***(3,11–13)***.

As acquired immunodeficiency syndrome (AIDS) is progressively threatening human lives worldwide, the development of vaccines against the causative agent of this disease, human immunodeficiency virus (HIV), is of great importance to human health. The HIV-1 p24 capsid protein is

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an important early marker of HIV infection ***(14–16)*** and has been shown to induce cellular and humoral immune responses ***(17,18)***. Therefore, p24 represents a promising candidate antigen for the development of HIV vaccines, where it would be used to induce protection against HIV infection in combination with glycoproteins encoded by the envelope gene ***(17,18)***. In fact, p24 protein is included in the vaccine cocktails being tested in several current therapeutic HIV vaccine trials ***(19);*** for current HIV/AIDS vaccine clinical trials, visit the web page of the National Institute of Allergy and Infectious Disease Division of AIDS at http://www.niaid.nih.gov. In addition, p24 is routinely used in blood screening, clinical diagnoses and HIV-related research ***(20,21)***.

Although several studies have documented the expression of epitopes from HIV-1 gp120 and gp41 in plants using viral vectors ***(3,13)***, there is only one report on the production of a full-length HIV protein, HIV-1 p24, using tomato bushy stunt virus ***(21)***. However, in this system the inserted p24 open reading frame (ORF) was not stably maintained in the viral vector. Toward the development of a stable plant-based production scheme for p24 protein, we have introduced a p24 gene cassette into the genome of tobacco plants using *Agrobacterium*-mediated gene transfer. Our data confirm successful and stable *in planta* production of p24 and the results support the use of this plant-based expression system for the production of this important antigen of HIV.

2. Materials and Methods

2.1. Construction of a Binary Vector

To clone the p24 ORF into a binary system, the HIV-1 p24 coding region was amplified by PCR using the construct p98-5 (provided by Aventis Pasteur Ltd., Toronto, Canada) as template. The PCR reaction was carried out using a forward primer PG3 that included an *Nco*I site (underlined) at its 5' end (PG3: 5'-GCGGCCGCCATGGT-ATCAGGTGGTCCTATAGTGCAGAACATC) and a reverse primer PG4 containing an *Xba*I site (underlined) (PG4: 5'-GGCCCCCTCGAGTCT-AGAGGATCACCCAACTCTTGCCTTATG-GCCGGGTCC).

Following amplification with Vent DNA Polymerase (New England Biolabs) the PCR product was digested with *Nco*I and *Xba*I, and ligated into the expression cassette present in an intermediate plasmid pRTL2. The entire p24 expression cassette, containing cauliflower mosaic virus (CaMV) 35S promoter with duplicated enhancer (P_{35S}), the tobacco etch virus leader (TL) from pTL-7SN, the p24 coding sequence, and the 35S terminator (T_{35S}), was released using *Hind*III and ligated into the *Hind*III site of a binary plasmid pGA482, thereby generating pGA482-p24 (**Fig. 1**). A tri-parental mating system was used to introduce pGA482-p24 into an *Agrobacterium tumefaciens* strain C58C1, which harbors a disarmed Ti-plasmid (pGV3850).

2.2. Tobacco Leaf Disc Infection and Subsequent Regeneration of Transgenic Plants

Tobacco (*Nicotiana tobacum* var. Burley 21) were cultured on MS medium ***(22)*** in Magenta boxes in a growth chamber with 16 h of light (25°C) and 8 h of dark (20°C). Fully expanded leaves were removed from plants, cut into 1 to 2 cm^2 pieces and used for *Agrobacterium*-mediated transformation ***(23)***. Shoots were generated through organogenesis. Following root induction, plantlets were removed from the culture medium and transferred to soil to grow to maturity in a greenhouse.

2.3. Southern Blot Analysis of Genomic DNA from Transgenic Plants

Genomic DNA was isolated from tobacco plants according to Fedoroff et al. ***(24)***. Ten micrograms of genomic DNA was digested with *Eco*RI, separated in a 0.8% Agarose gel, and transferred onto nylon membrane (Hybond-N, Amersham). Randomly labeled probe corresponding to the p24 ORF was generated using Klenow polymerase from the NEBlot kit (New England Biolabs) and was used in Southern hybridizations.

2.4. Immunological Detection of p24 Protein in Extracts from Transgenic Plants

Total soluble protein was extracted from 30–40 d old tobacco leaves according to Chong et al. ***(25)***. Briefly, fully expanded leaves were excised from

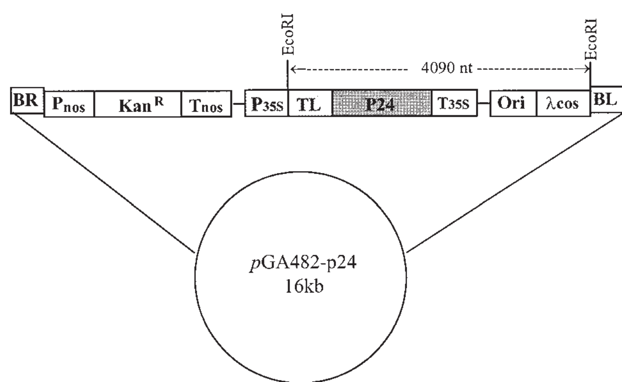

Fig. 1. A schematic representation of the transfer DNA (T-DNA) region of the binary vector pGA482-p24. The p24 expression cassette contains the cauliflower mosaic 35S promoter with duplicated enhancer (P_{35S}), the tobacco etch virus leader (TL) lacking the first 12 nt from pTL-7SN, the p24 ORF followed by the 35S terminator (T_{35S}). The Tn5 neomycin phosphotransferase gene (KanR) driven by the pTiT37 nopaline synthase promotor (P_{NOS}) provides a marker for selecting stable transformants. The origin of replication (Ori) from pBR322 and the cos site from λ were also included in the T-DNA region and are flanked by the right (BR) and left (BL) border. The 4 kb segment flanking by EcoRI sites was subsequently used to identify the presence of p24 in transgenic tobacco lines.

plants. After removal of major veins, leaf materials were immersed in liquid nitrogen in a mortar and ground to fine powder with a pestle. The resultant powder was resuspended (3 mL per g leaf material) in extraction buffer (50 m*M* Tris-HCl [pH 7.5], 2 m*M* EDTA, 0.5 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride [PMSF], and 1% Triton-X 100) and further homogenized with the pestle. The homogenate was centrifuged at 40,000*g* for 15 min and the supernatant was precipitated with 10% trichloric acid (TCA). The TCA precipitate was solubilized using resuspension buffer (25 mM Tris-HCl [pH 7.5] and 2% SDS). Protein content was estimated according to Lowry et al. ***(26)***.

For Western blot analysis, soluble protein (20 μg) was separated by 12% SDS-PAGE and then transferred onto Immobilon-P membrane (Millipore). The membrane was incubated with anti-p24 mouse monoclonal antibody followed by goat anti-mouse Ig (H+L) conjugated to horseradish peroxidase (HRP) as the secondary antibody.

p24 protein was quantified by enzyme-linked immunosorbent assay (ELISA) using AVP HIV-1 p24 Antigen Capture Assay Kit (SAIC Frederick). Microtiter plates precoated with mouse monoclonal antibody to HIV-1 p24 and blocked with 0.5% casein (SAIC Frederick) were used essentially as previously described by the manufacturer ***(27)***. Soluble plant protein extracts were diluted in PBS containing 0.1% BSA and 0.2% Tween-20 (BDH, Poole, U.K.), added to the microtiter plates, and incubated at room temperature for 2 h. Washes were performed in PBS containing 0.1% Tween-20 and 0.1% BSA. For p24 detection, wells were incubated for 1 h with rabbit anti-HIV-1 p24 serum (SAIC Frederick; Lot No. SP451) followed by goat anti-rabbit Ig (H+L) conjugated to HRP (SAIC Frederick; SP524D) as the secondary antibody. 3,3',5, 5'-Tetramethylbenzidine (TMB) substrate was used for color development. The assay standards were both retrovirus-like particle samples that had been assayed using commercially available p24 assay kit (Coulter, Hialeah, FL) as described by Persson et al. ***(28)*** and Triton X-100 lysed HIV-1 provided by the manufacturer (SAIC Frederick; Lot No. SP436F; 10.2 ng/mL).

3. Results and Discussion

Eighty-one plants were recovered in the presence of the selectable marker kanamycin following infection of tobacco leaf discs with the *Agrobacterium* strain carrying the p24 expression cassette. Total DNA was extracted from leaves of regenerants and analyzed using PCR. The screening of eighty-one regenerants using primers flanking the p24 ORF indicated that seventy-nine plants contained the p24 ORF (data not shown).

Southern blot analysis of *Eco*RI-digested genomic DNA was used to confirm that the p24 gene was integrated into the genome of p24-expressing tobacco plants. A ^{32}P-labeled probe specific for the p24 sequence detected p24-containing fragments of the predicted size in 18 transgenic lines tested **(Fig. 2)**, confirming the integration of the p24 gene.

To determine whether p24 protein accumulated in the transgenic plants, total soluble protein was extracted from the leaves of lines 14 and 15, representing lines with high and low copies of p24 transgene. Western blot analysis revealed a protein that cross-reacted with a p24-specific monoclonal antibody **(Fig. 3)**. Compared to the standard HIV p24 protein from virus-like particles (VLP) of mammalian origin, which is free of glyco residues, the plant-derived product displayed a lower migration rate, which corresponded to approx 32 kDa. Analysis of the amino acid sequence of p24 protein using a glycosylation-site-predicting program ***(29)*** showed that the 49th amino acid serine and the 124th amino acid threonine of p24 are potential sites for *O*-glycosylation. Therefore, the reduced migration observed may have resulted from the presence of glyco-residues. Despite possible posttranslational modifications, the plant-based p24 protein was fully reactive with the p24 specific antibody.

ELISA was used to quantify the yield of p24 protein. The average yield was approx 3.5 mg per g leaf soluble protein **(Table 1)**. This yield is higher than that observed for hepatitis B virus surface antigen (0.1 mg per g leaf soluble protein), but comparable to that of Norwalk virus capsid protein (2.5 mg per g leaf soluble protein), both of which were also produced in transgenic tobacco ***(5,7)***. No difference in p24 protein accumulation between lines 14 and 15 although Southern analysis indicated significant difference in copy number of p24 transgene **(Fig. 2)**.

To assess the stability of p24 expression in T1 plants, lines 14 and 15 were self-pollinated and the seeds generated were collected and planted. Soluble proteins were extracted from the leaves of T1 plants for both lines. Western blot analysis confirmed the accumulation of p24 protein (data not shown). ELISA analysis showed similar level of p24 protein to those observed for T0 **(Table 1)**, thus demonstrating that p24 gene is expressed stably in these transgenic lines.

In summary, *Agrobacterium*-mediated gene transfer was used to successfully introduce an HIV-1 p24 gene into the genome of tobacco plants and to stably express *in planta* an antigenically-active form of this viral protein. To our knowledge, the only other *in planta* expression of a full-length HIV-1 protein is also of p24 using a tomato bushy stunt virus-based viral vector ***(21)***. However, in that case the p24 ORF was not stably maintained in the vector and was deleted within the initially-infected plant. The p24 transgenic system described here offers the important advan-

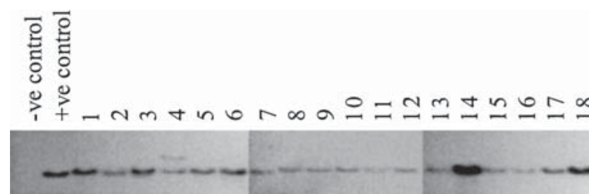

Fig. 2. Southern blot analysis. Genomic DNA was isolated from leaves of transgenic tobacco lines, digested with *Eco*RI, separated in a 0.8% agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled probe specific for the p24 ORF. The plant line number is indicated at the top. "+ve control" represents a negative control where Southern blotting was carried out on DNA isolated from untransformed tobacco plants. "+ve control" represents a positive control in which 1 pg *Eco*RI-digested pGA482-p24 was added to DNA isolated from untransformed tobacco plants.

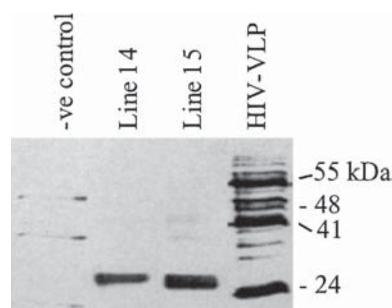

Fig. 3. Immunoanalysis of leaf soluble protein. Total soluble protein was extracted from leaves of 30- to 40-d-old plants, separated by 12% SDS-PAGE, transferred to nylon membrane, and hybridized with mouse anti-HIV-1 p24 monoclonal antibody. The plant line number is indicated at the top, and the position of the p24 protein (labeled 24 kDa) is shown on the right. The HIV-VLP lane represents total HIV-1 virus-like particles, which includes the p24 protein (in addition to other proteins). The "–ve control" lane contained protein extracts from nontransformed tobacco plants.

Table 1
p24 Content (mg g^{-1} Soluble Leaf Protein) of T0 and T1 Transgenic Tobacco Lines

	Control	line 14	line 15
T0	0.09±0.10*	3.55±0.18	3.70±0.81
T1	0.09±0.12	3.60±0.16	3.65±0.44

*mean+SD (*n*=3)

tage of stable expression, in addition to excellent yield. These promising results support further development of the transgenic plant-based system for effective production of p24 protein for use in HIV vaccines and diagnostic procedures.

Acknowledgments

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