Transgenic Res
DOI 10.1007/s11248-009-9358-9

BRIEF COMMUNICATION

Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant

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Received: 11 June 2009 / Accepted: 26 December 2009

Abstract HIV-1 Tat plays a major role in viral replication and is essential for AIDS development making it an ideal vaccine target providing that both humoral and cellular immune responses are induced. Plant-based antigen production, due to its cheaper cost, appears ideal for vaccine production. In this study, we created a plant-optimized *tat* and mutant (Cys30Ala/Lys41Ala) *tat* (*mtat*) gene and ligated each into a pBI121 expression vector with a stop codon and a *gusA* gene positioned immediately downstream. The vector construct was bombarded into tomato leaf calli and allowed to develop. We thus generated recombinant tomato plants preferentially expressing a Tat-GUS fusion protein over a Tat-only protein. In addition, plants bombarded with either *tat* or *mtat* genes showed no phenotypic difference and produced 2–4 μg Tat-GUS fusion protein per milligram soluble plant protein. Furthermore, tomato extracts intradermally inoculated into mice were found to induce a humoral and, most importantly, cellular immunity.

Keywords AIDS · Antibody response · Cellular immune response · HIV-1 · Tat · Transgenic tomato

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Introduction

HIV-1 has already claimed millions of victims worldwide and despite billions of dollars spent on HIV-1/AIDS research annually (Walker and Burton 2008; Watkins et al. 2008), no promising candidate HIV-1 vaccine has been made to date due to: (a) specific viral characteristics including extreme genetic variability among various isolates collected worldwide and even within the infected individuals; (b) a high mutation rate allowing rapid escape of variants from immune responses; and (c) biological properties of HIV-1 regulatory proteins, such as Nef and Tat, which avoid immune responses (Walker and Burton 2008; Watkins et al. 2008; WHO 2008; Potts et al. 2008). As widely believed, these characteristics pose a major obstacle towards controlling AIDS (Gaschen et al. 2002; Moore et al. 2008).

An ideal strategy against HIV-1 is one that stimulates passive protection or neutralizing immunity by

Published online: 14 January 2010

producing both antibodies and cytotoxic T lymphocytes or CTLs (Walker and Burton 2008; Watkins et al. 2008; Addo et al. 2001). Earlier works have shown that CTLs can control HIV-1 replication in the absence of antibodies (Borrow et al. 1994) prompting several attempts to stimulate anti-viral CTL responses using a combination of varying HIV-1 proteins and their epitopes (Betts et al. 2005; Matano et al. 2004; Mwau et al. 2004). The Tat protein has been one of the well studied HIV-1 proteins (Barboric and Peterlin 2005; Emerman and Malim 1998; Goldstein et al. 2001; Okamoto and Wong-Staal 1986; Ramirez et al. 2007). It is a small regulatory protein composed of either 86 or 101 amino acid residues (14 or 18 kDa, respectively) encoded by two exons (Okamoto 1995). Among the HIV-1 proteins already studied, Tat shows great potential for CTL induction covering a wide variety of HIV-1 clones besides from little variability among distinct viral subtypes and is highly conserved in both inter- and intra-patient variants (Addo et al. 2001; Goldstein et al. 2001).

Over 4 million people become infected with HIV-1 each year (WHO 2008; Fox 2007) in third-world countries in particular (Flexner 2008). Cheap and affordable production of pharmaceutical products for third-world consumption has prompted the development of plant-made pharmaceuticals for often neglected diseases (Zahn et al. 2008), including HIV-1 (Ramirez et al. 2007; Flexner 2008; Shchelkunov et al. 2006; Webster et al. 2005). Previous attempts to utilize the tomato plant for HIV-1 Tat vaccine development in the form of an edible-vaccine was only successful in inducing antibodies or humoral immune response (Ramirez et al. 2007; Shchelkunov et al. 2006). At present, no report has been made with regards to induction of CTLs or cellular immune responses using Tat protein (Addo et al. 2001), more so, using a plant-expressed Tat protein.

In this study, we demonstrate the evidence of preferential expression of a Tat-GUS fusion protein over the Tat-only protein in tomato plant and is expressed much higher than previously reported (Ramirez et al. 2007). In addition, we were able to induce both humoral immune response and, surprisingly, cellular immune response using Balb/c mice when tomato extracts were intradermally introduced. To our knowledge, this is the first report of cellular immune induction using Tat expressed in a plant system.

Materials and methods

Vector construction and tomato transformation

The *tat* gene from the HXB2 strain of HIV-1 and *mtat* were synthesized following a specific codon-usage table based on tomato was used (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4081). The plant-optimized M2 epitope directly fused to either *tat* or inactive *mtat* (Imai et al. 2005) genes with a stop codon, were individually ligated into a pBI121 expression vector (Clontech) upstream of a *gusA* gene (Fig. 1). Transformation was performed using a particle gun

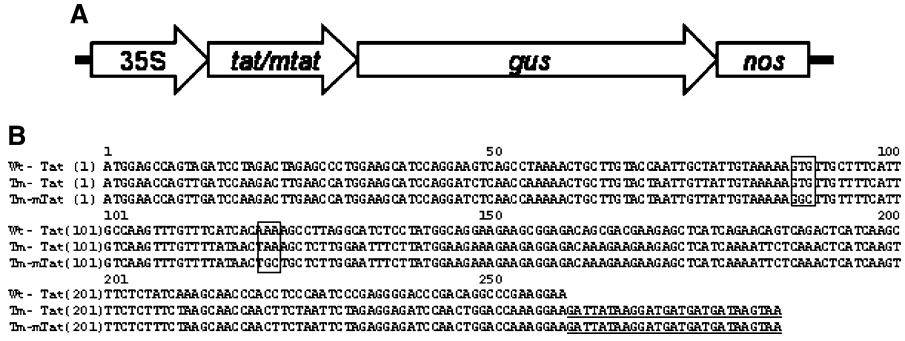

Fig. 1 Construction of the pBI121 plant expression vector containing either *tat* or *mtat* indirectly fused to *gusA* gene. **a** Expression was driven by 35S CaMV promoter and terminated with NOS termination signal located downstream of *gusA* gene. The inserted *tat*/*mtat* gene is located upstream of the *gusA* gene containing the termination codon (TAA) in between. **b** Codon-optimized *tat* and *mtat* were synthesized following the codon usage of tomato. The *boxed regions* represent point mutations at Cys30Ala and Lys41Ala found in *mtat* [23]. The *underlined segment* represents M2 epitope added to serve as an expression tag. Wt-Tat represents the Tat sequence from the HXB2 strain. Tm-Tat and Tm-mTat represents codon-optimized tomato Tat and mTat, respectively

(Tanaka Co., Ltd, Tokyo, Japan) and the tomato var. *Improved Pope* as previously published (Bhatia and Ashwath 2004; Sheeja et al. 2004). Briefly, the sterilized seeds were grown in MS medium and allowed to grow for 7–10 days. Callus induction of the explant material was performed in a MS medium containing 1.0 ppm zeatin for 7 days. Bombardment was carried out in tomato calli grown in MS medium containing 0.5 ppm zeatin, 1.0 ppm indole-butyric acid and 1.0 ppm giberillic acid. All reagents used for tomato transformation were purchased from Sigma.

Reverse transcription-PCR

Triplicates of bombarded tomato calli, regenerated leaf and shoot tissues were freshly obtained for mRNA extraction. The MicroFastTrack^{TM} 2.0 mRNA Isolation Kit (Invitrogen) was used to isolate mRNA according to manufacturer's instructions. The Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) was used to synthesize cDNA according to manufacturer's instructions. The forward primer, TAT-F (5′-ATG GAA CCA GTT GAT CC-3′), used was based on the tomato codon-based HIV-1 *tat*, whereas, the reverse primer, GUS-R (5′-CGG TAT AAA GAC TTC GCG CTG-3′) was based on *gusA*. Both primers were synthesized by Invitrogen. The TOUCHDOWN PCR condition was performed using the TaKaRa *Taq*^{TM} Hot Start Version (Takara Bio Inc., Japan) with an initial denaturation temperature of 95°C for 5 min proceeded by 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. This was followed by another 5 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min. The last set of cycles consists of 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. A final 10 min extension at 72°C was also performed. Reverse transcription-PCR (RT-PCR) products were resolved on 1% agarose gel.

Protein extraction

Protein extracts were obtained from 2-weeks old transgenic tomato plants. Protein extraction was done on all transgenic samples using the P-PER Plant Protein Extraction Kit (Thermo Scientific) according to manufacturer's recommendation. Tomato protein extracts acquired ($\sim$100 µl) were divided for use in Western blot assay and Immunogenicity testing.

Western blot assay

Western blot using both antibodies against Tat and M2 (Sigma) was performed and amount of Tat expressed in bombarded tomato plants were estimated using the Bio-Dot Mircofiltration Apparatus (BIO-RAD) as previously published (Ota et al. 2005). The Tat protein standard used was a recombinant Tat (ImmunoDiagnostics, Inc.) with various dilutions.

Immunogenicity testing

Balb/c mice were intradermally immunized with the recombinant tomato protein extracts mixed in an incomplete Freund's adjuvant (IFA). The peptides used in this study were the Tat CTL epitope (Morris et al. 2001) and B cell epitope (Goldstein et al. 2001). Tat-specific antibody responses were measured by ELISA. Briefly, synthetic peptides for Tat and mutant Tat (mTat) diluted in PBS were coated in multiwell plates overnight at 4°C followed by 30 min of blocking with non-fat milk. Test samples were then added and incubated at room temperature for 1 h. After washing, the reacted antibodies were detected using the HRPO-labeled goat anti-mouse IgG (H + L) and ABTS substrate (Roche Diagnostics). The OD_{405} was recorded and used as a relative measure of antibody titer.

The number of Tat-specific IFN-γ secreting cells indicating specific CTL activity was determined by ELISPOT assay (Takamura et al. 2005). Briefly, a 96-well nitrocellulose plate (Millipore Corporation) was coated with anti-mouse IFN-γ mAb R4-6A2 (Phermingen) and incubated at 4°C overnight. After washing with PBS, complete medium with 10% fetal calf serum was added and incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen of the immunized mice were plated in two-fold dilutions from 5×10^5 to 6.25×10^4 cells/well [29], added with Tat CTL peptide and incubated for 24 h at 37°C in 5% CO_2. After washing with PBS-T, biotinylated anti-mouse IFN-γ mAb XMG1.2 (Phermingen) was added and incubated overnight at 4°C. Plates washed with PBS-T were added with streptavidin-conjugated alkaline phosphatase (AP) (Mabtech AB) and visualized using AP color development buffer

(BIO-RAD) and counted by KS ELISPOT (Carl Zeiss, Inc.).

Results and discussion

Preferential expression of mTat/Tat-GUS fusion protein in tomato plant

Comparison between the control and transgenic tomato lines (Fig. 2a) showed that the transgenic lines were stunted in growth compared to the control tomato lines consistent with Tat expression (Ramirez et al. 2007; Karasev et al. 2005). Among 82 total calli bombarded (with either Tat or mTat), 55 (67.1%) survived with 14 (25.5%) confirmed plant regeneration. To confirm transformation, tomato extracts were used for Western blot analysis using anti-Tat and anti-M2 antibodies. A ~40 kDa protein, representing the mTat/Tat-GUS fusion protein (mTat/Tat is 14 kDa in size and GUS is 26 kDa in size), was detected instead of the expected 14 kDa size representing an mTat/Tat-only protein (Fig. 2b, c).

The indirect fusion between *mtat/tat* and *gusA* genes was designed to allow the tomato plant to selectively express either a fusion protein, a single protein or both. As seen in Fig. 2b, Tat and mTat were successfully expressed in all tomato extracts but only as a fusion protein and regardless of the stop codon found downstream of the *tat* gene which would have allowed a Tat-only protein to be expressed. Figure 2c illustrates the expected (Tat-only) and actual (Tat-fusion) proteins detected in the transgenic tomato lines. Expression of a fusion protein is suggestive of a codon read-though event (Tork et al. 2004) implying preferential expression of the fusion protein over the Tat-only protein in tomato plants. To resolve the transgenic nature of the Tat- and mTat-bombarded tomato calli, RT-PCR was performed. Figure 2d confirms the active transcription and presence of *mtat/tat* mRNA in all three sets of the transgenic tomato callus, shoot and leaf. In addition, since we were able to amplify a PCR product using a forward

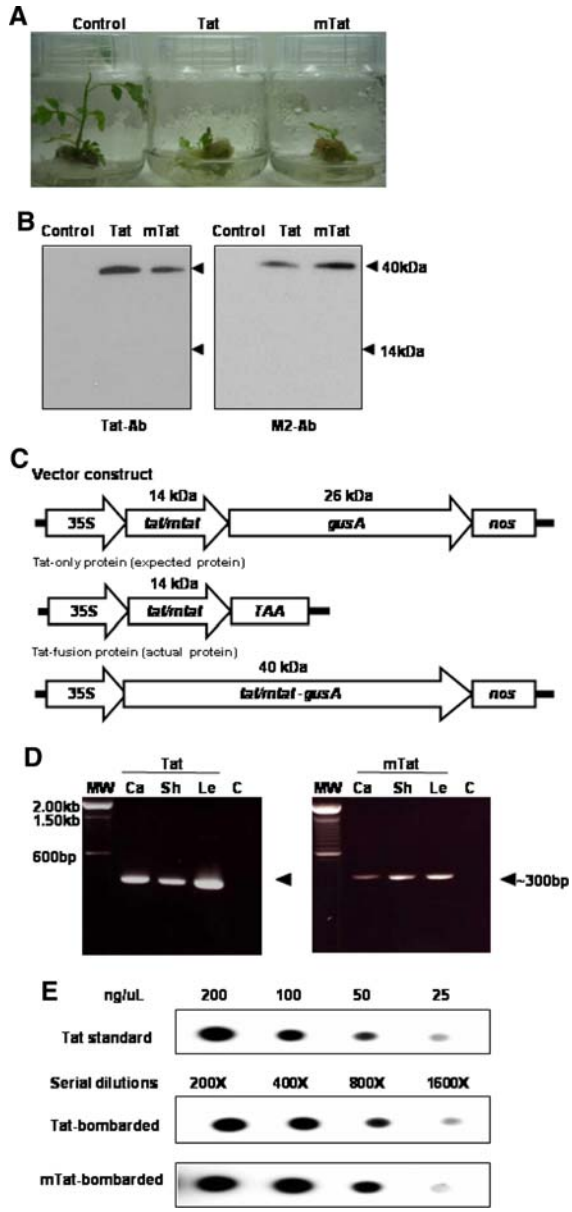

Fig. 2 Expression of both Tat and mTat proteins in tomato plant using particle-gun bombardment. **a** All tomato calli bombarded followed the same bombardment conditions and grown for 3 weeks. Control samples were bombarded with pBI121 vector only. Tat and mTat samples were bombarded with Tat- and mTat-pBI121 vectors, respectively. **b** Western blot assay using tomato extracts from the bombarded samples and detected with anti-Tat and anti-M2 antibodies as indicated in the *bottom*. Only a 40 kDa protein was clearly detected from either Tat- and mTat-pBI121 bombarded tomatoes representing a mTat/Tat-GUS fusion proteins. No 14 kDa protein was detected. **c** Schematic illustration of expected (Tat-only) and actual (Tat-fusion) protein transiently expressed. **d** Reverse transcription-PCR was performed using cDNA obtained from both Tat- and mTat-bombarded callus (*Ca*), shoot (*Sh*) and leaf (*Le*) tissues. Likewise, cDNA obtained from pBI121-bombarded tomato were used as controls. **e** Dot-blot assay providing estimated amounts of Tat expressed in both Tat- and mTat-bombarded tomato plants

primer based on the *tat* gene and a reverse primer based on the *gusA* gene, we show that a *tat-gusA* mRNA is transcribed further confirming production of Tat-GUS fusion protein. Furthermore, tomato extracts were found to contain ~2–4 μg mTat/Tat-GUS fusion protein per milligram plant protein (Fig. 2e) much higher than previous attempts (Ramirez et al. 2007; Karasev et al. 2005). This would imply that in tomato, Tat-GUS fusion protein is the protein form preferentially expressed allowing for a higher amount of protein production. Though the reason that drives the tomato plant to preferentially express the fusion protein is unclear, the significance of both the fusion protein and the amount produced in tomato plant was tested for its immunogenicity by injecting the tomato extracts into Balb/c mice.

Induction of antibody and CTL in Balb/c mice using recombinant tomato extracts

To test the immunogenicity of the fusion protein, Balb/c mice were intradermally injected with the recombinant tomato protein extracts and checked for immunogenic responses. Considering, previous attempts using a Tat-only protein have, thus far, successfully induced a humoral immune response (Ramirez et al. 2007; Karasev et al. 2005), we first established the consistency of humoral immune induction using Tat, in fusion form, in Balb/c mice. Humoral IgG immune responses were detected in the range of 1:10–1:160 titers before leveling-off at 1:320 for both mTat- and Tat-bombarded extracts (Fig. 3). The tomato extracts were found to induce a humoral immune response, regardless of the nominal amount used, and showed that Tat in fusion form could still induce an antibody response consistently with previous works using Tat-only protein (Ramirez et al. 2007; Karasev et al. 2005).

Interestingly, a cellular immune response, though minimal, was also detected. Cellular immune responses as detected by IFN-γ production were modestly induced at 22–24 cells/1 $\times$ 10^6 splenocytes using the recombinant tomato protein extracts (Fig. 4). It is noteworthy that Balb/c mice are normally used to test Th2 immune responses which are known to inhibit macrophage activation and instead stimulate antibody production (Mills et al. 2000), explaining the relatively low IFN-γ produced using our tomato extracts. Nevertheless, of greater importance is the ability of Tat protein, in fusion form, to induce a CTL response. The vaccine potential of utilizing HIV-1 Tat relies heavily in its ability to induce both humoral and cellular immunity in the host. Given that both IFN-γ and IgG were detected from the same induced Balb/c mice, it was

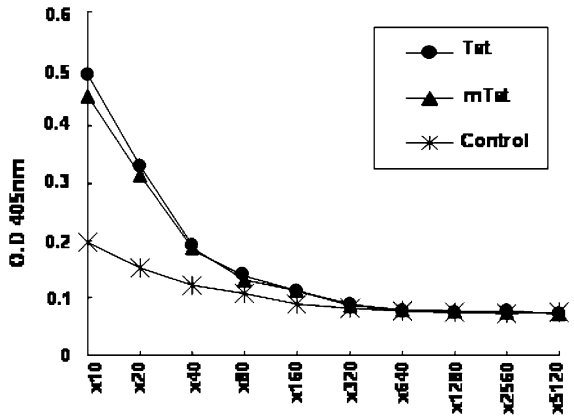

Fig. 3 Induction of IgG humoral immune response in Balb/c mice by recombinant tomato extracts. Plant extracts containing 5 μg Tat were inoculated in five mice intradermally. After 2 weeks, these mice were bled and the anti-Tat IgG antibody titer was measured by ELISA. The figures represent the mean and standard deviation of anti-Tat antibody response but the standard deviations could not be seen because of very small differences. Normal tomato extracts were used as control

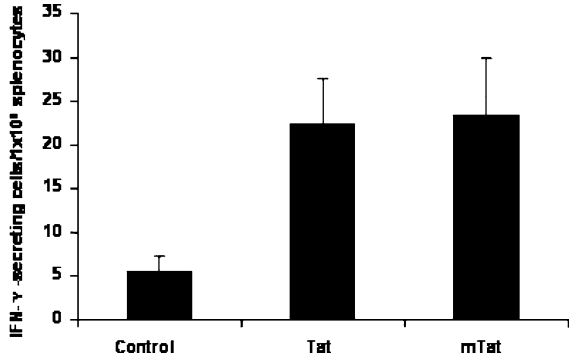

Fig. 4 Induction of cellular immune response in Balb/c mice by recombinant tomato extracts. IFN-γ secreting cells were determined by ELISPOT assay. $CD8^+$ cells were prepared from the spleen of each mouse and approximately 5–6.25 $\times$ 10^4 cells per well were incubated with the synthetic Tat peptide (Tat17-25) for 24 h, plates were washed by PBS-T, further incubated overnight at 4°C in the presence of 2 μg/ml of biotinylated anti-mouse IFN-γ monoclonal antibody, and the number of IFN-γ secreting $CD8^+$ T cells were visualized by adding streptavidin conjugated alkaline phosphatases. Data represent the mean and standard deviation of three independent experiments

clearly demonstrated that a Tat-GUS fusion protein was preferentially expressed over the Tat-only protein in tomato plants. Furthermore, Tat in fusion form would seem to be ideal in inducing both humoral and cellular immune responses which coincidently are the requirements of a model HIV-1 vaccine (Walker and Burton 2008; Gaschen et al. 2002; Borrow et al. 1994). To our knowledge, this is the first report of induction of anti-Tat cellular immunity in Balb/c mice, using Tat protein in fusion form expressed in tomato plant.

No significant difference between wild-type and mutant Tat expressed in tomato plant

It is worth mentioning that tomato plants bombarded with either *mtat* or *tat* gene are both stunted in growth (Fig. 2a) and found to have no significant difference (Fig. 2b, c) implying that the mutations found in mTat are insufficient to distinguish it from Tat when expressed in tomato. With regards to immunogenicity, although previous findings (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002) suggested that wild-type Tat might inhibit immune responses by downregulating the function of antigen presenting cells, the extent of immune responses elicited by either Tat or mTat did not apparently show any difference in mice. This is perhaps because murine Cyclin T1 does not bind to HIV-1 Tat (Bieniasz et al. 1998). Thus, a similar study using primates is warranted since, in human and primate cells, we expect a lower immunogenicity for Tat compared to mTat because of the recruitment of Cyclin T1 that is required for the action of class II transactivator expressing class II MHC molecules (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002).

Acknowledgments We thank Professor Masahiro Sugiura for helpful comments and encouragement throughout the study. This work was supported by grants-in-aid from Ministry of Health, Labor and Welfare, Japan, and Ministry of Culture, Science and Technology, Japan. The anti-Tat reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Tat Monoclonal (7D5.1) from Dr. Jonathan Karn.

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