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Expression of Tissue Plasminogen Activator in Transgenic Tobacco Plants by Signal Peptides Targeting for Delivery to Apoplast, Endoplasmic Reticulum and Cytosol Spaces

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***Abstract*—**Tissue plasminogen activator (tPA) as a serine protease plays an important role in the fibrinolytic system and the dissolution of fibrin clots in human body. The production of this drug in plants such as tobacco could reduce its production costs. In this study, expression of tPA gene and protein targeting to different plant cell compartments, using various signal peptides has been investigated. For high level of expression, Kozak sequence was used after CaMV35S in the beginning of the gene. In order to design the final construction, Extensin, KDEL (amino acid sequence including Lys-Asp-Glu-Leu) and SP (γ-zein signal peptide coding sequence) were used as leader signals to conduct this protein into apoplast, endoplasmic reticulum and cytosol spaces, respectively. Cloned human tPA gene under the CaMV (Cauliflower mosaic virus) 35S promoter and NOS (Nopaline Synthase) terminator into pBI121 plasmid was transferred into tobacco explants by *Agrobacterium tumefaciens* strain LBA_{4404}. The presence and copy number of genes in transgenic tobacco was proved by Southern blotting. Enzymatic activity of the rt-PA protein in transgenic plants compared to non-transgenic plants was confirmed by Zymography assay. The presence and amount of rt-PA recombinant protein in plants was estimated by ELISA analysis on crude protein extract of transgenic tobacco using a specific antibody. The yield of recombinant tPA in transgenic tobacco for SP, KDEL, Extensin signals were counted 0.50, 0.68, 0.69 microgram per milligram of total soluble proteins.

***Keywords*—**Recombinant tissue plasminogen activator, plant cell comportment, leader signals, transgenic tobacco.

I. Introduction

PLASMINOGEN activators are thrombolytic factors that digest fibrin to soluble products by converting the proenzyme, plasminogen, into plasmin that is an important enzyme present in blood and its deficiency may lead to thrombosis. Two plasminogen activators have been known: the tissue-type activator (t-PA) and the urokinase-type plasminogen activator (u-PA) [1]. tPA protein includes four domains (a serine protease domain a finger domain, Kringle 1 and Kringle 2 domains, a growth factor domain) [2]. tPA has been expressed in different kind of systems. Firstly, bowes melanoma cell line was used as a source for production rtPA [3]. For clinical purpose, tPA was produced in *Escherichia coli* as recombinant tPA [2], [3]-[5]. Mammalian cell culture system such as CHO cells has been used for commercial production of tPA to date [6], [7]. Recently, plants have been introduced as cost-effective, safe and large-scale systems for production number of biopharmaceutical components like as anti-cancers [8]-[12]. The low amount of expression of recombinant protein in plants might be occurred, due to proteolytic activities in plant cells. This fact has been demonstrated in some studies including production of enzyme inhibitors and some antibodies in transgenic plants [13]-[18]. To overcome these problems, some solutions including organelle-specific targeting and tissue-specific expression have been suggested [10]. In this investigation, use of some targeting signals for delivery recombinant tPA to different cell compartments, was a possible solution to decrease of protein lysis. Three signal peptides such as KDEL, Extensin and Sp (the first part of Zera signal) were used for protein delivery to ER, Apoplast and cytosolic spaces respectively [18].

This is the first report of the production recombinant tPA in different cell compartments of tobacco that they were active in terms of enzymatically active.

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II. Materials and Methods

A. Construction of Plasmids for t-PA Expression in Different Plant Compartments

To create, pBI-SP construct a fragment with *Xba*I/*Sac*I contain Kozak sequence, encoding SP signal, CPP, thrombin site and His-tag was cloned into plant binary vector. The fragment encoding tPA gene (1,668 bp) was cloned in mid-construct pBI-SP and finally complete construct (pBI-SP-tPA) was formed. A fragment with *Xba* I / *Sac*I containing Kozak sequence, encoding extensin signal (GeneBank accession no. X02873), CPP, thrombin site and His-tag were cloned into binary vector pBI121 and mid-construct (pBI-Ext) was formed. After that a fragment encoding tPA with *Bam H* I / *Xho* I was cloned into mid-construct (pBI-Ext) and second construct (pBI-Ext-tPA) contain tPA gene was formed. The third construct was made as well as two previous constructs. A fragment contains thrombin site and His-tag and KDEL signal was cloned into binary vector pBI121and mid-construct (pBI-KDEL) was formed. After that, a fragment encoding tPA and kozak with *Bam H* I / *Xho* I was cloned into mid-construct, (pBI-KDEL) and the third construct (pBI-KDEL-tPA) was formed. Binary vector pBI121 is driven by cauliflower mosaic virus 35S (CaMV 35) promoter and a 35S terminator.

B. Plant Transformation

Leaf pieces of tobacco plant (*Nicotiana tabacum* cv. Xathi) were used for agrobacterium-mediated transformation by A. tumefaciens strain LBA_{4404}. Agrobacterium-mediated transformation method was used for transformation tobacco plants by pBI-Ext-tPA, pBI-SP-tPA, pBI-KDEL-tPA cassettes. Transformed leaves of tobacco regenerated by MS-based medium containing 2- naphthaleneacetic acid (NAA) (0.1 mgL^{-1}), 6-benzylaminopurine (BAP) (3 mgL-1), cefotaxim (200 mgL^{-1}), and Kanamycin (100 mgL^{-1}) contain selective medium including kanamycin (100 mgL^{-1}) and cefotaxime (200 mgL^{-1}) for selection of the transformed cells. However, no plant regenerated in control cases (infected by agrobacterium without pBI121).

C. PCR Analysis

Presence of tPA gene was confirmed by PCR analysis of tobacco plants that were transformed by (pBI-tPA-KDEL), (pBI-SP-tPA), (pBI-Ext-tPA) constructs. Two primers were used for amplification of tPA gene. Forward primer that was selected of the CaMV35S promoter (Forward 5´ CTT CAA AgC AAg Tgg ATT gAT gTg ATA TCT CC 3 ´) and reverse primer was selected of the end of the tPA gene (Reverce 5´ TATCTCGAGCGG TCG CAT GTT GTC ACG 3 ´) under the following conditions PCR was performed in a thermo-cycler: 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1.30 min for a total of 35 cycles. The PCR products were separated on a 1% agarose gel and photographed.

D. Southern Blotting Analysis

For the Southern Blot analysis, about 40 µg of genomic DNA was digested with Hind III and separated by electrophoresis in a 0.9% agarose gel. The DNA was transferred to a positively charged nylon membrane (Roche). Hybridization was carried out following the standard procedures, the tPA gene was used as probe. Hybridization and detection were carried out using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche) according to the manufacturer's instruction.

E. Enzyme-Linked Immunosorbent Assay

The amount of tPA protein in transgenic carrot root was determined by using an ELISA (enzyme linked immunosorbent assay). Polystyrene microtiter plates were coated with 100 µl/well TSP (total soluble protein) samples from transgenic carrot and wildtype carrot and with known concentrations of tPA protein quantified using the Coomassie dye-binding assay (Bio-Rad, Hercules, CA, USA) with BSA (bovine serum albumin) as a standard [9-12], and the plates were incubated overnight. They were then blocked with 5% (w/v) powdered milk and incubated with tPA rabbit polyclonal antiserum (diluted 1:1500) and then treated with goat anti-rabbit HRP-conjugated antibodies (diluted 1:2000). When the reactions were complete, the absorbance at 450 nm was recorded by using an automated plate reader. To determine the expression level for each transgenic tobacco, three leaves explants were analyzed for tPA expression, and an average expression level was obtained.

F. Zymography

Gelatin zymography is like SDS-PAGE but in non-reducing condition and different loading buffer. After loading 300 µg of TSP in each well of 12% SDS-PAGE gel that containing 1% gelatin and 100µg/µl plasminogen, Gel was run in 4 °C for 5 h and after that washed with Triton X-100 (2.5%) for 1 h at room temperature afterward incubation in the Glycine (0.1 M) for 3 h at 37 ºC. The gel was stained in Coomassie Brilliant Blue overnight and destined by destining solution (Acaetic Acid: Methanol) (overnight). Enzymatically activity of rtPA was determined by white band in blue background of gel [22].

III. Result

A. Construction of Plant Binary Vector

In order to delivery of rtPA to different compartments of tobacco cell plants in each construct was located different signals. In pBI-SP-tPA construct after of 35S promoter, Kozak sequence, LTB peptide, tPA, CPP, thrombin cleavage site, and His-tag was placed respectively. For pBI-Ext-tPA construct Kozak, Extensin signal, CPP, thrombin cleavage site and His-tag were placed and for pBI-tPA-KDEL construct Kozak, tPA, thrombin site, His-tag and KDEL signal, respectively. The t-PA gene and other peptides were expressed and stabilized under the 35S promoter and 35S terminator, respectively. In order to increase the expression level a Kozak leader sequence was inserted downstream of the 35S promoter. A schematic representation of constructs is presented in Fig. 1.

B. Molecular Analysis of Transient Tobacco and Alfalfa Leaves

PCR

By PCR analysis confirmed presence of the tPA gene. Two primers that were used for amplified fragments belong to *CaMV35S* promoter as forward primer and reverse primer that belongs to end of the tPA gene. In brief, for tobacco plants that were transformed by pBI-SP-tPA construct amplified fragment was 2250 bp (tPA 1700bp- LTB 350bp- SP signal 120bp- *CaMV35S* promoter amplified 130bp), for tobacco plants that transformed by pBI -tPA-KDEL construct amplified fragment was 1830 bp (tPA 1700bp- *CaMV35S* promoter amplified 130bp), for tobacco plants that were transformed by pBI-Ext-tPA construct amplified fragment was 1950 bp (tPA 1700bp- Extensin signal 120bp- *CaMV35S* promoter amplified 130bp) (Fig. 2).

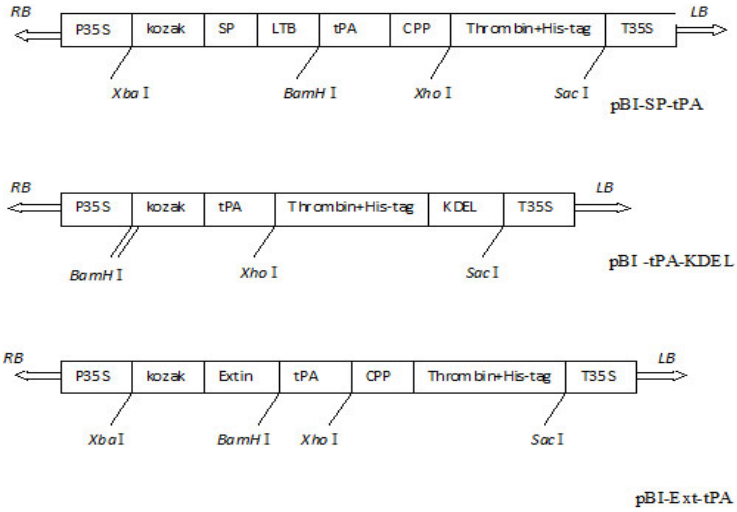

Fig. 1 pBI121 as binary vector that was driven by the cauliflower mosaic virus 35S promoter (P35S) and polyadenylation signal of cauliflower mosaic virus 35S T35S for end processing of the transcript was used. The structure of T-DNA region of binary vector pBI-SP-tPA, pBI-tPA-KDEL, pBI-Ext-tPA was constructed for expression of t-PA and other fusion peptides. Expression cassettes for t-PA and other fusions are located between the left and right T-DNA borders (LB, RB). Kozak an expression enhancer sequence used after promoter for three constructions. pBI-SP-tPA respectively containing: Kozak sequence, sp signal, LTB peptide, tPA gene, cpp peptide and thrombin, His-tag. pBI-Ext-tPA respectively containing: Kozak sequence, extension signal, tPA gene, cpp peptide and thrombin, His-tag. pBI -tPA-KDEL respectively containing: Kozak sequence, tPA gene, thrombin, His-tag and KDEL signal. TAA end codon was used before *sac* I site in each construction

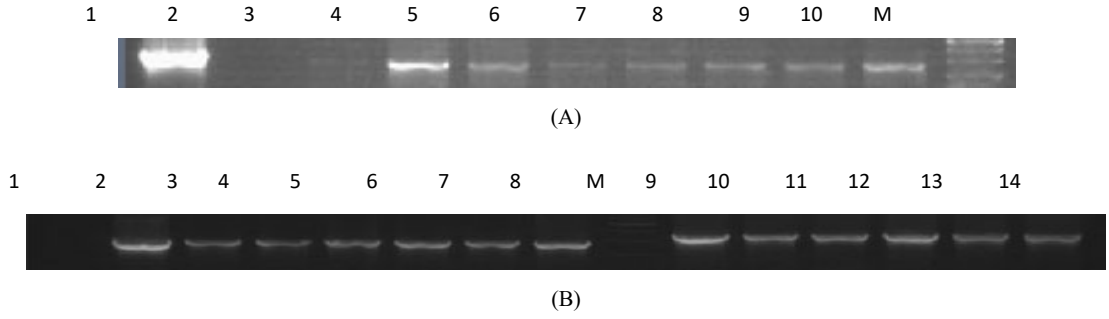

Fig. 2 A PCR products of transgenic tobacco plants that transformed with pBI-tPA-KDEL construct, amplified fragments with two primers (F-pBI and R-tPA) were 1800 bp. Lane 1 positive control, lane 2 wild type control, lanes 3-10 tobacco plants, lane M molecular ladder. B PCR products of transgenic tobacco plants that transformed with pBI-Ext -tPA and pBI-SP -tPA constructs. Amplified fragments for plants that transformed with pBI-Ext -tPA were 1900 bp, lane 1 wild type, lane 2 positive control, lanes 3-8 transgenic plants with pBI-Ext -tPA construct. M molecular ladder. Amplified fragments of the transgenic plants were 2200 bp. Lane 9 positive control, lanes 10-14 were transgenic plants

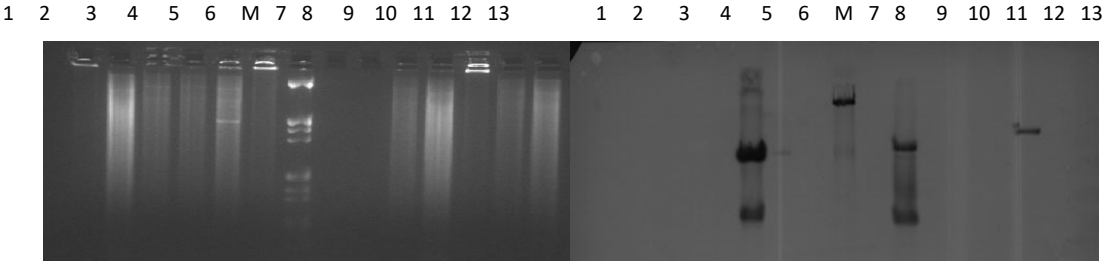

Fig. 3 Copy number of tPAs that were transformed to each transgenic plant by pBI-SP-tPA, pBI-tPA-KDEL, pBI-Ext-tPA constructs was determined. A digestion of genomic DNA wild type and tobacco plants with *Hind* III, lane 1 wild type plant, lanes 2-6 plants that were transformed with pBI-tPA-KDEL construct, M molecular ladder, lane 7 plasmid as a positive control, lane 8 water as negative control, lanes 9-11 tobacco that were transformed with pBI-Ext-tPA construct, lanes 12, 13 tobacco that were transformed with construct pBI-SP-tPA. B southern blotting of transgenic plants and copy number of transformed to each tobacco plant

C. Southern Blotting

Southern blotting confirmed that some of the transgenic tobacco plants have more than one copy number of tPA gene that integrated into genomic DNA. Ten tobacco plants which by different constructs were transformed were investigated by southern blotting and demonstrated that lane 5, 6 tobacco plants transformed by pBI-tPA-KDEL construct have three and one copy number respectively. Lane 9 tobacco plant transformed by pBI-Ext-tPA construct has two copy numbers. And lane 12 tobacco plant that transformed by pBI-SP-tPA construct has one copy number. As positive control, pBI-Ext-tPA by Hind III was digested and used. Wild type of tobacco plant as negative control does not have band (Fig. 3).

D. ELISA

Using ELISA immune-activity of recombinant tPA from tobacco plants was analyzed. Normal immune-activity of rt-PA was observed. Amount of recombinant protein rtPA for tobacco plants was demonstrated by ELISA analysis. The yield of recombinant tPA in tobacco for SP, KDEL, Extensin signals respectively 0.50, 0.68, 0.69 (µg/mg) of total soluble protein was obtained. Ten tobacco plants that were transformed by different signals analyzed by ELISA and amount of the rt-PA of each plant was counted (Figs. 4 and 5).

E. Gelatin Zymography of rt-PA

Enzymatic activity of rtPA to convert of deactivate pro enzyme plasminogen to plasmin was determined using gelatin zymograpy and it was positive for separated proteins were produced in each of three cell compartments. This assay has been shown that enzymatic activity was conserved even after insertion various peptides to target of main protein to different compartments, although they had different activities. There was no activity in the control plants. In addition, Altplase was used as positive control (Fig. 6).

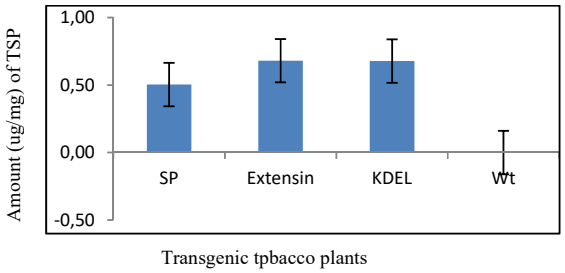

Fig. 4 ELISA assay. Three hundred micrograms of total soluble protein were used for ELISA. Transgenic tobacco plants were transformed with different construct. Wilde type of tobacco was used for negative control

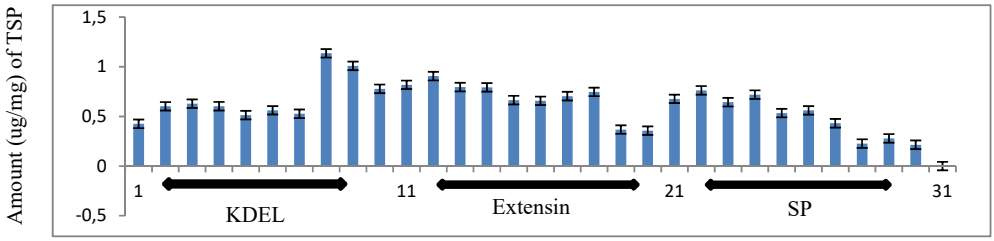

Fig. 5 ELISA assay, ten plants were transformed with pBI -SP-tPA, pBI-tPA-KDEL, pBI-Ext-tPA constructs was used. 300 µg of TSP used. 1-10 tobacco plants were transformed with pBI-tPA-KDEL, 11-20 tobacco plants were transformed with pBI-Ext-tPA, 21-30 tobacco plants were transformed with pBI -SP-tPA. 31 wild type tobacco plant

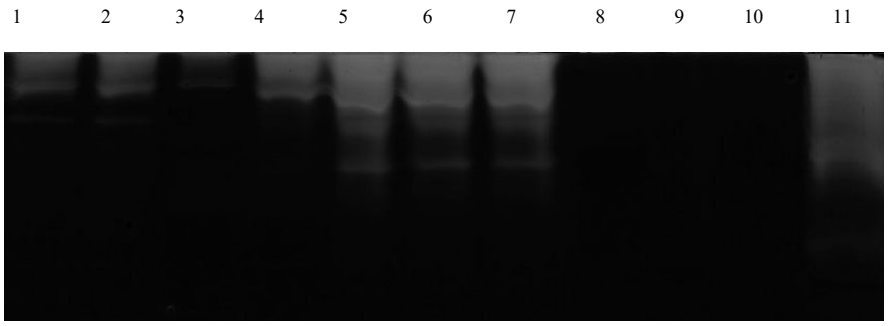

Fig. 6 Gelatin zymography for wild type and the transformed tobacco plants by three constructs were done. Produced rtPA by transgenic tobacco that was transformed by different construct was different. Lane 1, 2 tobacco plants that were transformed by pBI-tPA-KDEL and measure of the rtPA was 66 kDa. Lane 3, 4, tobacco plants were transformed by pBI-SP-tPA Lane and measure of the rtPA was 85 kDa 5, 6, 7 tobacco plants that were transformed by pBI-Ext-tPA and measure of the rtPA was 67 kDa. Lanes 8, 9, 10 are wild type of tobacco. Lane 11 positive control with altplase and measure was 63 kDa

IV. Discussion

To obtain high level of accumulation of proteins in plant cell, it is necessary to target heterologous proteins to different organelles to escape from hydrolytic activity of protease. Here, the reported active tPA proteins can be expressed in different cell compartments of transgenic tobacco plants using agrobacterium-mediated transformation. In some studies, targeting recombinant protein by signal peptides to the apoplast, the mitochondrion, vacuolar compartments, the plastid, and the nucleus have also been reported [19], [20]. Apoplast (inter-cellular space beneath the cell wall), endoplasmic reticulum and cytoplasm can be targeted in order expression recombinant proteins [12]. Using appropriate signal peptide, such as a transit peptide, can transfer recombinant proteins from cytoplasm where they are degraded by proteases to subcellular organelles such as apoplast [5], [7]. By targeting recombinant proteins to the apoplast or ER where they are protected from degradation by cytoplasmic proteases, the amount of recombinant proteins may be further enhanced. For the ER lumen in plant cells and mammalian, KDEL is known as a retention signal [22]. The most post-translational modification of therapeutic occurs in ER and Golgi compartments. A greatly enhanced accumulation of the recombinant protein was being produced by presence of the ER-targeting signal. The recombinant protein is then destined for secretion to the apoplast, where it significantly accumulates to higher levels compared to the cytosol [12], [21]. The range 0.5–2% TSP was expected for the standard expression levels of recombinant proteins for stably transformed plants in the apoplast and ER are usually (Conrad and Fiedler 1998). In this study, the size of recombinant tPA was different for constructs, 85kDa for pBI-SP-tPA, 66kDa for pBI-tPA-KDEL, and 67kDa for pBI- Ext- tPA and 63 kDa for commercial tPA (Altplase) as positive control. Expressed-rtPA in different compartments showed enzymatically activity and was more active in apoplast (150 µg of TSP for tobacco used) (Fig. 6). The CPP peptide in N-terminal of the rt-PA had the role to transport protein and can used for faster delivery during in vivo condition. In this report, we suggested using this peptide in molecular farming for the first time. In addition, his-tag and thrombin cleavage site can use for easier extract of rt-PA from other plant materials. Pure rt-PA was obtained by cleavage thrombin site and removed His-tag. Results showed that the expression of the rt-PA in different compartments had enough quality and activity.

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