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Full Length Research Paper

Cloning and expression of tissue plasminogen activator (t-pa) gene in tobacco plants

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Plants offer a promising alternative to microbial fermentation and animal cell cultures for production of recombinant proteins, the major advantages are safety, low cost, post-translation modifications and high volume of production. t-PA (tissue Plasminogen activator) is a trypsin-like serine proteinase and a superior thrombolytic agent. In this report, recombinant cDNA of tissue Plasminogen activator was transformed to tobacco plants. t-PA recombinant protein was expressed under the control of *CaMV35S* promoter and *NOS* terminator. A high-expression sequence (Kozak sequence) and KDEL signal (for endoplasmic reticulum retention of recombinant protein) were linked to amino and carboxy-termini of t-PA *gene*, respectively. The vector containing t-PA (pBIt-PA) was transferred to *Agrobacterium tumefaciens* and the t-PA *gene* was inserted into the plant genome by *agrobacterium*-mediated transformation. Transgenic plants were selected on kanamycin (100 mgL^{-1}), maintained in perlite and then the soil, subsequent generations were obtained. The presence and expression of the transgene was confirmed in the transformants by PCR, SDS-PAGE, RT-PCR, Zymography and Western blotting. This report examines the transformation and expression of t-PA *gene* in tobacco plants.

Key words: Recombinant protein, tissue plasminogen activator, tobacco, zymography.

INTRODUCTION

Plants have been used as recombinant protein production systems over the past decades because of low cost of production in comparison to microorganisms and especially animal cell systems (Streatfield, 2007). Plants provide an inexpensive and convenient system for the large-scale production of valuable recombinant proteins (Twyman et al., 2003). The numbers of biopharmaceuticals and therapeutic plantibodies that have been expressed in plants via nuclear transformation have grown considerably (Ding et al., 2006).

Blood coagulation is an enzymatic event initiated by substances from injured tissues and culminating in the formation clot-forming fibrin monomers. The fibrin clot is degraded by fibrinolytic enzyme system (Mosher, 1990). Plasminogen activators play an important role in the fibrinolytic system. These enzymes are capable of converting plasminogen to its catalytically active form, plasmin, which degrades the fibrin network formed within blood clots. There are two major types of plasminogen activators, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) (Brown et al., 1985).

Human t-PA is synthesized as a single-chain polypeptide. This protein is a 63-68KDa serine protease composed of 527 amino acid residues (Pennica et al., 1983), 17 intrachain disulfide bonds and one unpaired Cys residue. Furthermore, the human tissue-type plasminogen activator is N-glycosylated at three positions, while one of them is utilized approximately to 50% of the molecules (Waldenstrom et al., 1991).

Recombinant DNA technology provides sufficient quantities of this protein, to examine its clinical effect on pulmonary embolism, deep vein thrombosis and heart attacks and so on. The strong thrombolytic effect of t-PA, which acts locally on the thrombus (in contrast to streptokinase and u-PA), was shown in various animal

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models. To date, the t-PA used for commercial purposes has been produced using a mammalian cell culture system such as CHO cells (Cartwright, 1992). However, the increasing demand for this protein in the treatment of thrombolytic disease requires a safe, cost-effective and large-scale production system. Plant expression system will be beneficial for lower cost and large-scale t-PA production. Here, we report transformation and expression of t-PA and cDNA into tobacco. This is the first report of expression of recombinant t-PA in tobacco plants.

MATERIALS AND METHODS

Materials

Some of biochemical materials purchased from Sinagen, Fermentas, Chromogenix, abcam and Dako companies dedicated some vectors, bacteria and tobacco plants from some Iranian institutes such as Pasteur institute and NIGEB.

Construction binary vector for plant transformation (pBIt-PA)

DNA fragments, encoding t-PA (GeneBank accession No. I01047) were arranged into a pBI121 binary vector as follows. The t-PA was amplified by primers containing *Hinc*II and *Xba*I sites 5´-gagtctagataaacatggatgcaatgaagagaaccc-3´ containing the Kozak sequence before the start codon as the forward primer (FA) and 5´-atagtcaactcatagctcatctttcggtcgcatgttg-3´ containing KDEL sequence before stop codon as the reverse primer (RA) and inserted under the control of the cauliflower mosaic virus 35S (*CaMV35S*) promoter and *NOS* terminator into the plant binary vector pBI121 (dedicated from NIGEB). The middle of this *gene* was amplified by two primers (FB: 5'-ttgatgcgaaactgaggctg-3' and RB: 5-'cttctcagatttcgtgtgcc-3'), the final cassette was named as BIt-PA. The 1.7 kb PCR product was ligated into the *CaMV35S* upstream of *NOS* terminator (from *npt*II *gene*) and downstream of the pBI121 vector using *Xba* I and *BamH* I restriction sites (during subcloning from pCR2.1 to pBI121). Cloning of t-PA *gene* in pBI121 was confirmed by colony PCR, double digestion and sequencing.

Plant transformation

The resulting cassette, pBIt-PA was transformed to *Escherichia coli* and *Agrobacterium tumefaciens*. *Agrobacterium* containing pBIt-PA was grown on Luria-Bertani (LB) media containing streptomycin (50 mgL^{-1}) and kanamycin (50 mgL^{-1}). The colony PCR confirmed the existence of the fragment in agrobacterium. Tobacco (*Nicotiana tabacum* cv. Xathi (NC)) leaf pieces were used for *agrobacterium* mediated transformation (*A. tumefaciens* strain LBA_{4404}). The pBIt-PA cassette was transformed into tobacco plants by the *agrobacterium*-mediated transformation method. After transformation, leaf pieces were transferred to Murashige and Skoog-based medium containing 6-benzylaminopurine (BAP) (1.5 mgL^{-1}), 2-naphthaleneacetic acid (NAA) (0.1 mgL^{-1}), cefotaxim (200 mgL^{-1}), and Kanamycin (100 mgL^{-1}).

The tobacco leaves inoculated by agrobacterium showed organogenesis on selective medium containing kanamycin (100 mgL^{-1}) and cefotaxime (200 mgL^{-1}). However, no plant regenerated in control cases (infected by *agrobacterium* without pBI121). The regenerated plants were transferred to vermiculite and then were planted in soil. After regeneration, the total of 36 kanamycin-resistant plants with independent origin were obtained, 30 normal plants were used for molecular analysis. After 4 - 5 weeks, established transgenic tobacco lines were later transferred to soil for subsequent generations by self-fertilization in a greenhouse conditions. In this research, we analyzed only T_0 plants.

PCR analysis

For examining the presence of t-PA, polymerase chain reaction (PCR) on genomic DNA from tobacco was preformed, using primers described above. The amplification consisted of 30 cycles as follows: denaturation, 95ºC/60 s; annealing, 60ºC/60 s; extension, 72ºC/ 120 s and finally the reaction mixture were placed for 15 min at 65ºC. The plants with t-PA *gene* were determined.

Preparation of total soluble protein from plant leaves

For the extraction of total soluble protein (TSP), 20 mg of tobacco leaves were used. The young tobacco leaves were ground in liquid nitrogen to a fine powder with a mortar and pestle. Soluble protein was extracted by using 200 µl of extraction buffer (E.B.: 200 mM Tris-HCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 0.1 mM 2-Mercapthoethanole (2-ME)) per gram of leaf material. Cell debris were removed by two rounds of centrifugation (in 24000 g, 21 min, at 4ºC), and the supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE of proteins was performed using 12% acrylamide gel followed by staining with Coomassie brilliant blue (Laemmli, 1970).

In this research, we were not used to 2-mercapthoehthanole in total protein extraction buffer for Zymography analysis, but were used to total protein extraction buffer for SDS-PAGE.

RT-PCR

For the extraction of RNA, 10 mg of tobacco leaves was used. The young tobacco leaves were first frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Total RNA was extracted by using 1 ml of RNX (-Plus) buffer (E.B: guanidinium thiocyanate, phenol). 300 µl of chloroform was added and it was centrifuged at 24000 g for 15 min at 4ºC. Then the aqueous phase was collected and it was re-extracted with an equal volume of isopropanol and then stored 20 - 30 min at -20ºC. The samples were centrifuged again at 24000 g for 15 min at 4ºC. RNA precipitate formed a white-yellow pellet at the bottom of the tube. The supernatant was removed and RNA pellet was washed once with 1 ml 75% ethanol and it was subsequently centrifuged at 9500 g for 8 min in 4ºC, at last, it was dried and resuspended in 30 µl of DEPC- treated sterile water.

cDNA synthesis was preformed by a First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was done on cDNA by specific Primers (FA, RA, FB and RB primers).

Zymography

Tobacco leaves were homogenized in a protein extraction buffer (200 mMTris-HCl, 5 mM Ethylene Diamine Tetra Acetic acid (EDTA)) and then, 100 µg of each extract was mixed with the equal volume of loading buffer and loaded in the wells of 12% acrylamide gel that have gelatin (1%) and plasminogen (chromogenix)(75 µg/100 µl), the gel was run in 10 mA and then washed with TritonX-100 (2.5%) for 1 h at room temperature followed by incubation in the Glycine (0.1 M) for 3 h at 37ºC. The gel was stained in Coomassie Brilliant Blue for 0.5 h and destained by destaining solution (Acaetic Acid: Methanol) (overnight). t-PA

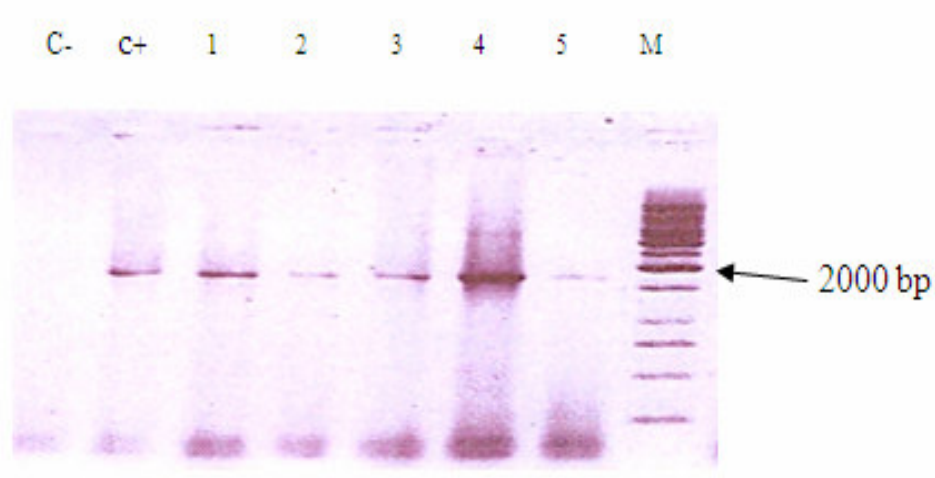

Figure 1. Electrophoresis of the PCR products on transgenic tobacco plants. C-: Wild-type plant. C+: Positive control. Lanes 1-5: Transformed tobacco plants. M: 1 kb marker.

activity was identified by white band of enzyme in blue background (Hu et al., 2006).

Western blotting

Tobacco leaves were homogenized in a protein extraction buffer and then 100 µg of each extract was mixed with the equal volume of loading buffer, then it was loaded in the wells of 12% acrylamide gel and then transferred protein bands to nitrocellulose membrane by semi-dry transfer cell for 30 min in voltage of 12. Then, the membrane was stained by ponceau-s dye, membrane was blocked by BSA (1.5%) overnight, washed by PBS buffer and triton X-100 three times and then, polyclonal t-PA antibody (abcam) was added and was shaken for 3 h and then, wash three times and second antibody (Dako) was added and again it was shaken for 1.5 h and then, it was washed three times, finally Dab (3,3'-diaminobenzidine) substrate was added (2 - 5 min) and bands appeared (Sambook and Russel, 2001).

RESULTS

PCR analysis

PCR analysis using specific primers on extracted genomic DNA of 30 normal regenerated plants showed 1.7 kb bands in transformants. No band was observed in the wild-type plant used as control; this shows that, the transgenic plants have received at least one copy of the t-PA *gene*. The PCR results of 5 plants by specific primers (FA and RA primers) (transgenic plants T_1, T_2, T_3, T_4, T_5) are shown in Figure 1.

RT-PCR analysis

RT-PCR analysis using specific primers (FA, RA, FB and RB primers) on cDNA of four transgenic plants showed 635 bp and 1.7 kb bands in transformants. However, no bond was observed in the wild-type plant used as negative control. The RT-PCR results of four plants by main and second specific primers are shown in Figure 2.

SDS-PAGE

SDS-page analysis on the proteins extracted from transgenic plants (for example vent No. 4) showed a clear band with 65 KDa weight, but this band not shown in non-transgenic plants. The weight of this protein was equal to t-PA protein weight (Figure 3).

Zymography

We evaluated t-PA activity in each of tobacco plants by gelatin zymography analysis. This analysis detected gelatinolytic enzyme activity related to the proteins of molecular weight corresponding to 63 - 68 KDa, which is indicative of the presence of t-PA and its activity. The transparent areas were observed on the gel indicating serine-protease digestion of plasminogen, were only observed in transgenic plants (events No. 4 and 5 in Figure 4).

Western blot analysis

A protein with estimated 63 - 68 KDa weight was detected in western blots of samples prepared from the transgenic plants (event No. 4). No protein band was observed in protein samples from non-transgenic plants (Figure 5).

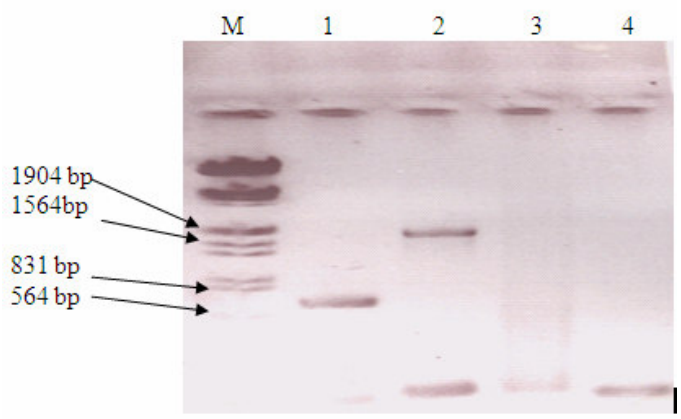

Figure 2. RT-PCR on cDNA of transformed plant with pBIt-PA (for event No.4). M: 100bp marker. Lane 1: RT-PCR with specific primers (FB and RB). Lane 2: RT-PCR with specific primers (FA and RA). Lane 3: Negative control. Lane 4: wild-type plant.

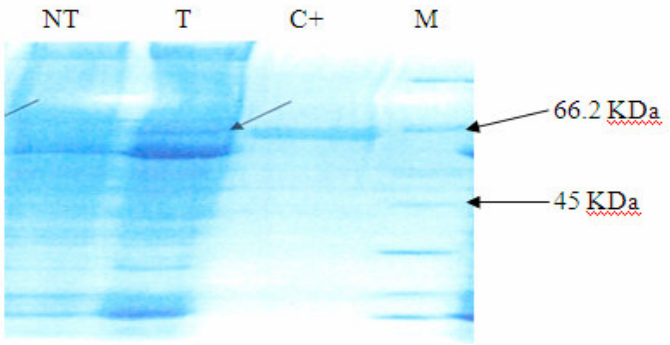

Figure 3. SDS-PAGE analysis on transgenic and nontransgenic plants. Lane C+: Positive control (Actylase), lanes T : transgenic plant (event No.4), lane NT : non-transgenic plants (negative control). Lane M: Protein marker.

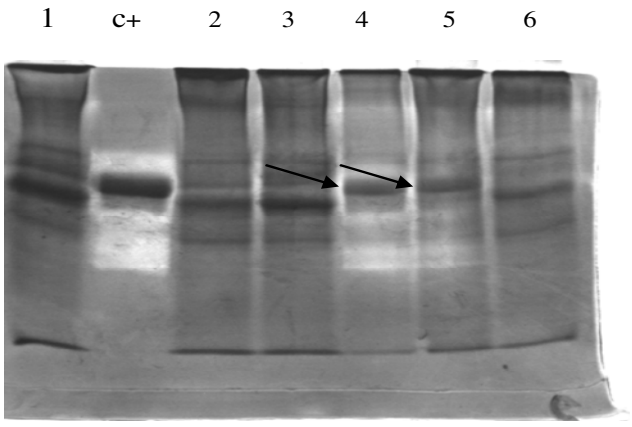

Figure 4. Gelatin zymography was done on protein extract of transgenic and non-transgenic plants. C+: Positive control (Actylase). Lane 1: non-transgenic plant. Lanes 2, 3, 4, 5 and 6 transgenic plants (events No. 4 and 5 are positive).

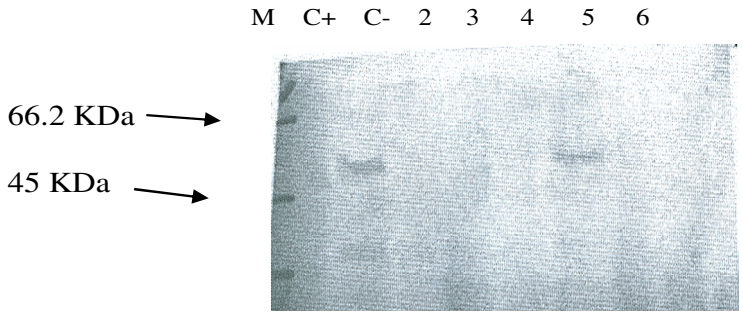

Figure 5. Detection of t-PA in protein extracts from the leaves of transgenic plants using western blot analysis. M: Protein Marker, C+: t-PA drug, C-: non-transgenic plant, Lanes 2-6: transgenic plants (only event No. 4 is positive).

DISCUSSION

In this report, expression of recombinant active t-PA protein in transgenic tobacco plants was confirmed. The integration and expression of t-PA *gene* and activity of t-PA protein in transgenic tobacco plants were confirmed by PCR, RT-PCR, Western blot and Zymography.

Overall, 39% recombinant proteins are made by *E. coli*, 35% by CHO cells, 15% by yeast, 10% by other mammalian systems and 1% by other bacteria and other systems (Rader, 2008). Recently, using plants for the synthesis of recombinant proteins in comparison to traditional microbial and mammalian production systemshas received a great deal of attention because of advantages in economy, scalability and safety (Hellwig et al., 2004). Plant expression systems are believed to be even better than microbes in terms of cost, protein complexity, storage and distribution. Some added advantage of plant systems are glycosylation and targeting, compartmentalization and natural storage stability in certain organs (Hood, 2002).

Up to three million people had developed stroke in the USA in 1999 and ~10% of them would have benefited from thrombolytic therapy. Because of several reasons only 1 - 6% was treated with t-PA (Benchenane et al., 2004). t-PA has been expressed in mouse L cells (Brown et al., 1985), Bowes melanoma cell line (Dodd et al., 1986), mammalian cell lines (Jalanko et al., 1990), *E. coli* (Obukowicz et al., 1990), Chinese Hamster Ovary (CHO) Cells (Fann et al., 2000) and Leishmania (Soleimani et al., 2006), but there is no report of t-PA production in plants. Based on previous studies, none of the traditional expression systems would be able to fulfill the demand for recombinant protein needed for full-scale treatment at an inexpensive cost. Hence, the plant expression system is suitable for this aim.

In this study, we used *CaMV35S* promoter because it has showed a high level induction of transcription, about 18 - 60 fold more than *Ubi* in the same plants (Kwon et al., 2003). *NOS* terminator in this construct is another important factor inducing high level expression of protein (Fischer and Schillberg, 2004). In addition, Kozak sequence influences the transcription too, resulting in an increased expression of target *gene* (Kozak, 1992). Also, KDEL signal was added at the C-terminal of *gene*. When this tetra peptide is translocated into the secretary pathway, it is a signal for retaining proteins in the endoplasmic reticulum (ER); KDEL has been found to function as a retention signal for the ER lumen in mammalian and plant cells (Okamoto et al., 1994).

Most modifications of therapeutic proteins (such as coagulation factor IX) occur in ER and Golgi compartments. These proteins are co-translationally inserted in the lumen of the ER and then transported to the lysosomal compartment, the extra cellular matrix or the blood stream via Golgi system. When a protein KDEL retrieval signal at the C-termini is expressed in tobacco, the addition of immunogenic glycans to Plant Made Proteins is inhibited. KDEL induces the storage of the therapeutic protein within the ER by this way (Gomord and Faye, 2004).

The combination of *CaMV35S* with *NOS* terminator or other terminators, without Kozak sequence, has resulted in lower level of expression, while the combination of *CaMV35S* with *NOS* terminator and Kozak sequence was suitable for expression in tobacco (Rajabi-memari et al., 2006). It appears that, the presence of two functional signal sequences, in tandem at the amino terminus of the signal peptide/protein/KDEL construct interferes with the synthesis and/or processing of the protein in plant cells, but the combination of protein and KDEL is better than the above mentioned combination (Huang et al., 2001). In this study, Due to post-translation modifications of t-PA protein, we combined signal peptide with KDEL and the results showed that these modifications to this protein have been done correctly. The designed construct was transferred to plant cells using the agrobacterium-mediated transformation method (Chen et al., 2003). Tobacco plants are very susceptible to infection by strains of *A. tumefaciens* (such as LBA_{4404}) (Fischer and Schillberg, 2004).

Zymography analysis for evaluation of the

serine-protease activity of t-PA was used. The activity of the produced t-PA protein in tobacco plants can be determined by using the simple, sensitive and specific gelatin-zymography method (Kim et al., 2007). Zymography is the same as western blotting method which allow us to analysis proteinase in crude extracts and purified states (Pagano, 1999). Western blot will identify not just the presence or absence of a protein that reacts with the antigen, but also the size and an estimate of its relative levels of expression (Dale and Von Schantz, 2002). Western blot is extremely useful for the identification and quantitation of specific proteins in complex mixtures of proteins that are not radio labeled (Sambrook and Russel, 2001). In some of transformed plants, t-PA *gene* was detected by PCR analysis, but the protein was not expressed. This may be due to a low level expression of recombinant protein or altered structure of t-PA protein (Prasad et al., 2003).

In conclusion, our results suggested that plants is a suitable system for the expression of t-PA and may aid basic study for the high production of t-PA in plants. More studies are needed to optimize protein production in plants (by nucleuos or chloroplast transformation) and extraction/purification methods which have a substantial influence on final yields. The novel and challenging task in molecular farming is the combination of genetic engineering, protein extraction, and development of adequate manufacturing and processing technology (Daniell, 2003).

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