Homoplasmy stability of transplastomic tobacco plants
(\textit{Nicotiana tabacum} CV. Xhanti) containing human tissue-type
plasminogen activator (\textit{K2S} form) gene in \textit{T1} generation

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**Abstract**

The rates of recombinant protein in nuclear-transformed plants are often less than 1% of total soluble proteins. As the plant plastid is highly polyploidy, plastid transformation can lead to high-level production of recombinant protein. In addition, plastid transformation has several other advantages such as prevention of gene escape that has a high importance in molecular farming. Tissue-type plasminogen activator (\textit{tPA}) is an important protein that is used to treating clots in cardiovascular diseases. Thus, production of \textit{tPA} protein in plant system was considered. The \textit{tPA} (\textit{K2S} form) gene was transferred to tobacco chloroplast genomes. In this study, we analyzed expression and stability of \textit{tPA} gene in transplastomic tobacco plants in \textit{T1} generation. The presence of \textit{tPA} gene in transplastomic plants was confirmed with specific primer by PCR analysis. Homoplasmy, gene expression, and protein assay were confirmed with southern blot, RT-PCR, western blotting and ELISA analysis. Results showed that the \textit{tPA} gene in \textit{T1} generation of transplastomic tobacco plants is stable and is expressed. In addition, the maximum amount of \textit{tPA} protein was estimated up to 0.13% of total soluble protein.

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Introduction

The production of recombinant proteins in plants has several advantages than the other systems, based on animal cells, yeast, and bacteria, including very low production costs, very high production potential, and pathogen-free products (Ma et al., 2003). Also plants are eukaryotic organisms, so they can perform post-translational modifications (folding, glycosylation, etc) similar to animal cells on target proteins (Scotti et al., 2012). The target gene can be inserted in either nuclear or plastid genome to stable transformation, or be expressed transiently. In nuclear stable transformation, expression level generally is low, so often maximum amount of expression less than 1% of total soluble proteins (TSP) have been reported (Sheludko, 2008). However, plastid transformation can lead to high-level production of recombinant protein because the plants can be contained up to 10,000 copies (number of plastid genome) of target gene in a cell, also gene silencing and positional effects are absent in plastid transformation (Rigano et al., 2009). In addition, there are several other advantages in plastid transformation including prevention of gene escape because of maternal inheritance of plastids (Obembe et al., 2011), and increased stability of heterologous protein in stroma compared to cytoplasm (Rigano et al., 2009).

Tissue-type plasminogen activator (tPA) is a serine protease enzyme that can activate plasminogen to its active form, plasmin. Plasmin lyzes clots by breaking-down the fibrin in blood clots formed in the vessels. Therefore, tPA can be used in treating cardiovascular and cerebrovascular obstructions (Rouf et al., 1996). For plasminogen activation, tPA should be placed adjacent to plasminogen on the fibrin in clots, which means that tPA can not activate circulating plasminogen in the blood (Baruah et al., 2006).

The tPA is a single chain polypeptide that consisting of 527 amino acids whit 17 disulfide bonds between 34 cysteine residues. In addition, tPA is glycosylated and its molecular weight is approximately 70 kDa. Five domains have been identified in tPA including a fibrin-binding ‘finger’ domain, an epidermal growth factor, two disulfide looped ‘kringle’ domains, and a serine protease domain which is involved in proteolytic activity at carboxyl terminal of polypeptide chain (Rouf et al., 1996).

Reteplase is non-glycosylated truncated form that contains two domains of native tPA. It is composed of 355 amino acids with 39 kDa molecular weight. In fact, this form of tPA comprises kringle-2 and serine protease domains. Reteplase has a longer half-life (14 min) and higher thrombolytic potency compared to native form. In addition, formation of fibrin-plasminogen-tPA complex is not necessary to accurate function of reteplase (Baruah et al., 2006). The DNA sequence encoding reteplase protein so-called K2S.

The tPA protein is found in different tissues of human and animals. For natural production of tPA, tissues including melanoma, epithelial, fibroblasts, and endothelial are used through tissue culture technique and tPA is purified from these tissues. Besides, recombinant DNA technology can be used to produce tPA synthetically (Rouf et al., 1996). So far, tPA protein has been produced in mouse L cells (Browne 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 (Soleymani et al., 2006). The production of tPA in plant can be a beneficial way to commercial production of this protein.

Two reports exist about efforts to production of tPA in tobacco plant (Hahn et al., 2009; Masoumiasl et al., 2010). However, tobacco plant in these studies was nuclear-transformed and low production rates have been reported. Hence, to increase expression level of tPA gene in tobacco plant, it (K2S form of tPA) was inserted in pKCZ vector and after cloning was transferred to tobacco chloroplast using biolistic procedure (Abdoli-Nasab et al., 2013). Stability of transformation in chloroplast-transformed plants is very important, because the resulting plants are cultivated in next generation to production of
recombinant protein. If the transformation is not stable in next generation, the chloroplast-transformed plants are not suitable to cultivation and transformed chloroplasts quickly replaced with wild-type chloroplast. Therefore, chloroplast-transformed plants must be homoplastic and this homoplasmy be stable in next generation. Thus, in this study we investigated the expression and stability of K2S gene in T0 transplastomic plants that provided before (Abdoli-Nasab et al., 2013) in T1 generation.

Materials and methods

Seed culture and PCR analysis

The seeds of transplastomic tobacco (Nicotiana tabacum cv. Xhanti) plants harboring pKCZK2S construct (Fig 1a) were grown in pots contained a homogeneous mixture of perlite and peat moss (1:3 ratio).

Total genomic DNA was isolated from transgenic and wild type plant leaves using CTAB protocol (Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992). To confirm the presence of K2S gene at chloroplast genome, PCR analysis was carried out using specific primers (K2S-F 5´- GGA AAC AGT GAC TGC TAC TTT GGG AAT GG-3´ and K2S-R 5´- TCA CGG CAT GTT GTC ACG AAT CCA G -3´) designed from K2S sequence. 50 ng of genomic DNA was used as template and PCR reaction condition were as 1 cycle of 5 min at 94°C followed by 30 cycles at 94°C for 30 s, 57°C for 40 s, 72°C for 90 s and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gel.

Southern blot analysis

Homoplasy was confirmed in transplastomic plants by Southern blot analysis. At first, probe was synthesized using primers designed based on flank regions at chloroplast genome (F-F 5´- ATG TGT AAT GAT TCC CCC ATT C -3´ and F-R 5´- TTT CTC TCC CAC TTT ACA CCT C -3´). Double strand DIG-labeled DNA probe was prepared using DIG DNA-labeling Kit (Roche). Size of amplified fragment to probe was 350 bp. The total DNA was digested with Hind III enzyme; the digested DNA was electrophoresis on 1% agarose gel at 20 V voltage for 16 h. The DNA was transferred from gel on nitrocellulose membrane using a traditional wet system. The blotting procedure was carried out as according to the manufacturer’s protocol (DIG-DNase labeling and detection kit (Roche)). The expected size of restricted fragment with Hind III enzyme in non-transplastomic plant was 1 kb and fragment size in transplastomic plants and positive control (pKCZK2S vector) was 2.8 kb (fig 1b).

RT-PCR analysis

The southern blot positive plants were used to RT-PCR analysis. The total RNA was isolated from fresh tobacco leaves using RNX-plus solution according to manufacturer’s protocol (Cinnagen, Iran, Tehran). The cDNA was synthesized using a RevertAid first strand cDNA synthesis kit (Fermentas). Specific primers were designed according to K2S sequence (K2S-F 5´- GGA AAC AGT GAC TGC TAC TTT GGG AAT GG and K2S-R 5´- TTG ATG CGA AAC TGA GG). PCR condition were as 1 cycle of 5 min at 94°C followed by 30 cycle at 94°C for 30 s, 57°C for 40 s, 72°C for 40 s and a final extension at 72°C for 10 min. The RT-PCR products were analyzed by electrophoresis on 1% agarose gel.

Western blot analysis

Total soluble protein (TSP) was extracted from fresh leaves of tobacco plants (Hurkman and Tanaka, 1986). Extracted proteins were separated by electrophoresis on 12% sodium dodecyl sulphate polyacrylamide gel (Laemmli, 1970). Then, proteins were transferred to nitrocellulose membrane using semi-dry transfer system according to manufacturer’s instructions (Bio-Rad, USA). The detection of tPA protein was performed using rabbit polyclonal antibody tPA as primary antibody (Abcam, USA) at 1:1000 dilution and goat anti-rabbit IgG-HRP antibody (Santa cruz, USA) as secondary antibody at 1:1000 dilution. The bands were detected with H2O2 as substrate and 3, 3´-diaminobenzidine (DAB) detection system.

ELISA analysis
In order to estimate the amount of tPA protein in transplastomic plants, at first total soluble protein was extracted from fresh leaves of tobacco plants, then ELISA analysis was performed according to instructions as described (Abcam). Type and dilution primary and secondary antibodies were similar to western bolt analysis. The H2O2 and TMB (3, 3´, 5, 5´-tetramethylbenzidine) were used as substrate of HRP enzyme. Optical density (OD) was read at 450 nm wavelength.

Results
Amplification of K2S gene
The T1 generation plants were analyzed for presence of K2S gene by PCR analysis. In three of T1 plants and positive control (pKCZK2S vector), the expected 1,059 bp fragment was amplified. No amplification was observed in the wild type plant (Fig. 2).

Fig. 1a. pKCZK2S vector for transformation of K2S gene in tobacco plastid. The K2S gene was iserted in pKCZ vector; the shindalgarno (SD) as ribosome binding site and six histidine amino acids (6-His) as tag to aim purification of tPA protein from cell extract was fused at upstream of K2S gene. In addition, the Xa protease restriction site was inserted between his-tag and coding sequence of K2S gene to elimination his-tag after purification of tPA protein. In this cassette both tPA and aadA genes were expressed as dicistronic.

Homoplasmy assessment in transplastomic plants
The three PCR positive plants were analyzed by southern blot analysis. In all three plants, the expected (2.8 kb) transplastomic band was revealed on membrane, while wild type band (1 kb) was not observable. Hence, these transplastomic plants were homoplastic (Fig. 3).

Analysis of K2S transcripts by RT-PCR
In order to detection of K2S gene expression, RT-PCR was performed on three southern blot positive plants. The expected fragment (270 bp) was amplified in all three transplastomic plants. No amplification was observed in non-transplastomic plant and negetive control (Fig. 4). These results shown that K2S gene is expressed in transplastomic plants.

Analysis of tPA protein production in transplastomic plants
Western blot analysis was performed on transplastomic plants for detection of tPA protein production in transplastomic plants. The expected tPA protein band (39 kDa) was detected in all three transplastomic plants (Fig. 5). This showed that K2S transcripts were translated to tPA polypeptide successfully.

Fig. 2. PCR analysis of transplastomic plants with specific primers on the 1% agarose gel. M: 1kb DNA marker, C-: template-free reaction, NTP: nontransplastomic plant, C+: positive control (pKCZ
vector contain K2S gene), TP1-TP3: transplastomic plants.

Quantification of tPA protein in transplastomic plants

In order to quantification of tPA protein in transplastomic plants, ELISA analysis was performed. The highest amount of tPA protein was 0.13% of TSP (Fig. 6). The ELISA results showed that tPA protein was produced in all three transplastomic plants.

Discussion

In this study, we analyzed T1 generation of transplastomic tobacco plants in different levels (DNA, RNA, and protein). The PCR analysis using specific primers for K2S gene confirmed the presence of K2S gene in T1 generation of plants and homoplasy of three transplastomic plants was confirmed by southern blot analysis. In addition, the results showed the expression of K2S gene. Protein assay analysis confirmed the production of tPA protein in transplastomic plants. The maximum amount of 0.13% TSP for tPA protein was estimated in transplastomic plants. Different rates of recombinant protein production in transplastomic plants have been reported in various studies. In some studies rates below than 1% of TSP have been observed, for example production rates of 0.004 (Lee et al., 2006), 0.1 (Zhou et al., 2006), 0.2 (Li et al., 2006), 0.3 (Wang et al., 2008), 0.5 (Morgenfeld et al., 2009; Sim et al., 2009), 0.7 (Dreesen et al., 2010), and 0.8 (Soria-Guerra et al., 2009) of TSP have been reported (Cardi et al., 2010). However, we can use some measures to enhance the production rates of recombinant protein in plants. Several factors are affecting recombinant protein production in plant system. Enhancement of transcription, translation, and protein stability can lead to high-level accumulation of recombinant protein.

![Fig. 4. RT-PCR analysis of transplastomic plants with specific primers on the 1% Agarose. M: 1kb DNA marker, C-1: negative control (templat free), C-2: negative control (RNA as a template), TP1-TP3: transplastomic plants, NTP: nontransplastomic plant.](image)

Transcription

Use of a suitable promoter is one of the most important factors for increasing expression of transgene. In chloroplast transformation, promoters of genes with high-level expression in chloroplast organelle were commonly used. In this study, we used Prrn promoter that was widely applied in recombinant protein production in plants and high-level protein production has been reported using this promoter (Daniell et al., 2009; Michelet et al., 2011). Hence, selected promoter in our study is a suitable promoter to produce tPA protein in plastid transformation. Also, we can manipulate promoter and enhance transcription factor binding site in promoter sequence by modeling of many strong promoter to achieve a synthetic promoter (Egelkrout et al., 2011).

The other factor affecting transcription is region of transgene insertion in plastid genome. In this study, K2S gene was inserted in site between trnN and trnR genes. In some investigations on plastid transformation, transgenes were often inserted in site
between trnA and trnI genes that is located in transcriptional active site in plastid genome (Maliga, 2012) and it can be contributed to increased expression of target gene. Both above mentioned sites are in inverted repeated (IR) region in plastid genome, hence transgene can be duplicated by the phenomenon of copy correction and be inserted into the other IR in plastid genome as well (Verma and Daniell, 2007).

**Translation**

After transcription of gene to mRNA, these gene transcripts need to be translated to protein. In many cases, there is not a direct correlation between mRNA levels and rates of produced protein (Millán et al., 2003; Kim et al., 2009). Also, it should be mentioned that in chloroplast, polyadenylation do not occur on mRNA transcripts and half-life of mRNA molecules might be shorter (Rigano et al., 2009). Thus, consideration should be given to translation in production of recombinant protein in plant chloroplast.

In this study shinedelgarno sequence (SD) has been used upstream of coding region of K2S gene. This sequence is a prokaryotic ribosome-binding site (RBS). To enhance the translation conditions, use of 5’-UTRs that contain strong RBS elements can be beneficial. Also, these untranslated regions can improve mRNA stability in cell and cause mRNA transcripts to be subjected to translation by ribosome for longer time (Egelkrout et al., 2011). On the other hand, in this study rbcL terminator has been used. This terminator in many investigations has been considered as an appropriate terminator (Oey et al., 2008; Michelet et al., 2011).

**Protein stability**

produced proteins in plants require to be accumulated and purified from plant tissues. Thus, produced protein in plant cell should be preserved from degradation by proteolytic processes. In nuclear transformation, produced protein is leaded to cell organelles or apoplast by subcellular targeting of recombinant protein. The production of recombinant protein in plant chloroplast has this potential advantage but this does not mean that there is no proteolytic processes and protease in chloroplast. various proteases and proteolytic activities in chloroplast were discussed before (Adam, 2007). Hence, protein degradation can be occurred in chloroplast and prevention of protein degradation is necessary to achieve higher levels of protein accumulation.

The ratio of codons used for a given amino acid differs between organisms and is correlated to the levels of tRNAs available for that amino acid. Thus, if target gene belongs to other organisms, use of codon optimization can be favorable for its expression in plants (Oey et al., 2008; Zhou et al., 2008). Thus, it is recommended to accommodate K2S gene codons to favorite codons of tobacco chloroplast to enhance rate of tPA protein production in transplastomic tobacco plants.

**Fig. 5.** Western blot analysis on transplastomic plants contain K2S gene. M: prestained protein marker, NTP: nontransplastomic plant, TP1-TP3: transplastomic plants, C+: positive control (alteplase).

**Fig. 6.** Quantification of tPA protein in transplastomic plants. WT: nontransplastomic plant, TP1-TP3: transplastomic plants.
Half-life of truncated tPA (reteplase) is about 12-16 min (Baruah et al., 2006), and tPA protein can be degraded immediately in cell, as we believe this is one of the most important reasons for low-level accumulation of tPA in tobacco plant. Hence, we should elevate half-life of tPA protein using suitable strategies. We can use of fusion proteins (tags) in N-terminal of protein to enhance stability of recombinant proteins, for example, thermo-stable proteins can be used, because there is an strong correlation between resistance to proteolysis and thermal stability, also peptide sequences without recognition site for proteases can be used as fusion to target recombinant protein to decrease the degradation of protein (Egelkrout et al., 2011).

However, it is obvious that use of any fusion protein should be well investigated to identify its effects on target protein.

Modification of amino acid residues in recognition sites of proteases in recombinant protein polypeptide sequence can increase the stability of protein (Amin et al., 2004). Thus, protein engineering can be used as a beneficial tool to overcome post-translational degradation of recombinant protein. However, we are allowed to impose a modification that does not have an adverse impact on protein nature and function. These alterations can be performed when codon optimization is occurred on gene sequence.

As mentioned in introduction, plants have an acceptable potential as a host system to production of recombinant protein. In this study, we could approached to stable chloroplast transformed tobacco plants, this achievement is important because this plants can be cultured for years and recombinant protein to be produced. In addition, if mentioned alterations perform on our construct, high-level accumulation of tPA can be achieved in tobacco plant.

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