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Challenge towards plant recombinant protein expression: instability in nuclear and chloroplast transformation

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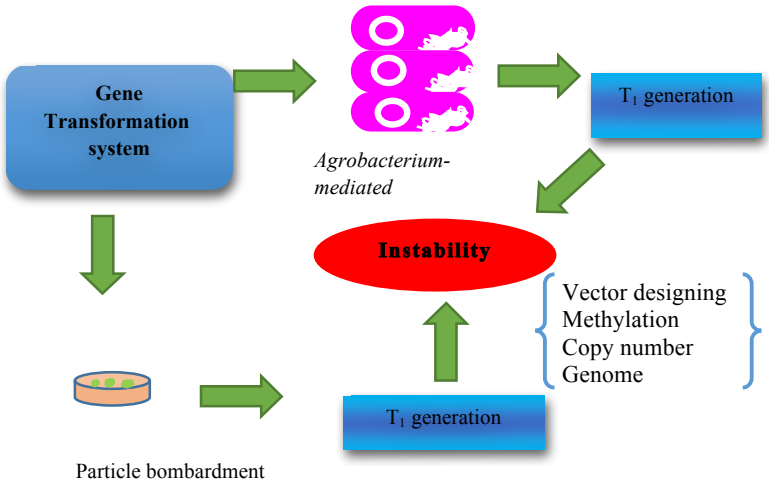
Grafical Abstract



Abstract: It is crucial to maintain the stability of transgene and its expression level. It seems the transformation method and the target organ can influence this instability. To this aim, two transformation systems, *Agrobacterium*-mediated and particle bombardment systems which have been applied to introduce tissue plasminogen activator (tPA) into nuclear and chloroplast respectively, have been compared to determine transformation efficiency, tPA expression, and stability. The presence of the tPA gene in transformants has been confirmed by PCR analysis. The gene expression in nuclear transformants and homoplasmy in transplastomic plants have been assayed by ELISA and southern blot analyses, respectively. Some of the *Agrobacterium*-derived transformants have shown the heritability and stability of the integrated T-DNA harboring the transgene, which encodes the tissue plasminogen activator and instability of its expression in the T1 generation. Using Southern blot analysis of bombardment-mediated transformants has surprisingly led to detecting the inheritability of tPA. There are several factors lead to the silencing of in transgenic plants, which should be considered. Possible reasons for these silencing are likely vector designing, methylation, copy number, and genome rearrangement.

Keywords: Recombinant Protein, Tissue plasminogen activator, *Agrobacterum*, Particle Bombardment, Instability

Introduction

The pharmaceutical industry has needed the production of recombinant proteins in sufficient quantity in order to keep up with demands [1], [2]. There are other factors, besides the quantity, which can be obtained by recombinant technology such as increased quality, enhanced safety, and decreased cost [3]. Some living cells have been engineered as heterologous expression platforms to produce recombinant protein, including bacteria, animals, and plants [4]. Higher

plants offer some advantages [4] which emphasize the practicality of utilizing them as green factories to express useful recombinant protein [5]: lowering costs of production, no human pathogens, synthesizing protein with correct folding, and post-translational modification like glycosylation [6].

Considering these advantages, up to date, several biopharmaceutical proteins have been produced in the plants [7], so, plant molecular farming has impacted positively on the important pharmaceuticals [8]. Plant molecular farming has enrolled genetically engineered plants as vehicles for expression of recombinant protein and provided an attractive perspective to produce these important proteins in a large scale at low costs [9].

This plant engineering refers to the introduction and integration of "interested" DNA in plant cells, which can lead to transient or stable expression of interested DNA [10]. Through either nuclear or plastid genomes, stable-transformed plants can be obtained [11], although, chloroplast transformation offers several advantages in comparison with nuclear transformation such as minimizing or avoiding the gene escape, eliminating the position effect, and higher expression level [12]. As a consequence of stable transformation, the interested DNA is integrated into the host DNA and eligibly predicted to be passed on to the next generation [10]. After about two decades, in which molecular farming is coming of age [7], the concentration has moved away from technical and principal studies towards a serious attention of necessity for sustainable production of recombinant protein [9].

In fact, it can be remarked that the stability is as important as the expression level of transgene for the large-scale commercialization of transformants [13], it is clear that the expression and stability are not guaranteed over generations [14]. Transgene instability can be defined as the loss of a transgene or its expression in genetically engineered organisms[15]; methylation, genome rearrangement, and the site of insertion found to be responsible for the phenomenon [16]. The instability has been reported in both biolistic bombardment and *Agrobacterium* mediation [17].

Tissue plasminogen activator (tPA), a serine protease, hydrolyses the plasminogen to convert it to plasmin [18]. tPA and plasminogen bind to the fibrinogen and fibrin to modulate proteolytic activity, enable the dissolution of blood clots. It has been found that it is useful to treat myocardial Infarction, thrombosis, and stroke [19]. It should be noted that there is a single-chain nonglycosylated form of tPA called reteplase, with a longer plasma half-life, better diffusion, and higher fibrinolytic activity [20]. As utilizing tPA one hour after heart attack can lead to increasing the survival chance, there has been an interest to produce tPA in large scale [21]. Attempts have been made to produce the protein in several expression systems like *Saccharomyces cerevisiae* [22], *Aspergillus nidulans* [23], *Escherichia coli* [24], Chinese hamster ovary (CHO) cells [25], *Leishmania* [26], Bowes melanoma cell line [27], mammalian cell lines [28], [29],and insect cells [30], however the aforementioned disadvantages lead to recent interests in plants. It has been expressed in tobacco [21], [31], [32] and oriental melon [33].

In this study, we compared the stability and inheritance of two forms of transgene in transformants transformed by different methods (*Agrobacterium*-mediated transformation and particle bombardment technique) in different organs (nuclear and chloroplast, respectively). In fact, transgenic plants were investigated to determine the transgene and expression stability over generations because the probability of instability/ transgene silencing, through generations, increases [34]. Our attempts were made to find the stable transgenic plants and discuss the stability and expression level of transgene.

Methods and materials

Seed culture

Two groups of transgenic plants were considered to assay. *Nicotiana tabacum* CV. Xhanti were transformed by the *Agrobacterium*-mediated method and particle bombardment technique.

Nuclear primary transformants (T0) created through *Agrobacterium*-mediated transformation harboring pBIt-PA construct containing the Kozak sequence before the start codon and a KDEL sequence before the stop codon, nptII gene was controlled by 35S promoter of the cauliflower mosaic virus (CaMV) and NOS terminator of the *Agrobacterium* tumefaciens [32]. The seeds of T0 were cultivated to obtain T1 seeds. pKCZK2S carrying *K2S* and *aadA* gene controlled by Prrn as the rRNA operon promoter and rbcl3′chl of the Chlamydomonas rbcl gene as a terminator was applied to make chloroplast transgenic plants. After several selection rounds and four rounds of regeneration, plants were allowed to produce T1 seeds [21]. All seeds were grown in the pots containing a homogeneous mixture of perlite and peat moss (1:3 ratio of perlite:peat moss).

PCR analysis

Genomic DNA was extracted from fresh leaves of transgenic and non-transgenic plants utilizing the CTAB method [35] and used as the template in the PCR assays. PCR was conducted to confirm the presence of both *tPA* and *K2S* genes (a truncated form of tPA) in the nuclear and chloroplast genome, respectively. Two primer sets were used on each DNA template: (1) one to amplify a 1.7 kb tPA (forward: 5′-GAGTCTAGATAAACAATGGATGCAATGAAGAGAGG G-3′; reverse: 5′-ATAGTCAACTCATAGCTCATCTTTCGGTCGCATGTT G-3′) and (2) another to amplify a 1.2kb *K2S* (forward: 5′-GGAAACAGTGACTGCTACTTTGGGAATGG-3′ and revers 5′- TCACGGTCGCATGTTGTCACGAATCCAG-3′).

Southern blot

To confirm the homoplasmy, a Southern Blot analysis was conducted on chloroplast transgenic plants. High-quality genomic DNA was isolated from the fresh leaves of T_1 PCR-positive plants by the CTAB method [36]. High quality genomic DNA of transgenic and non-transgenic plants were digested by HindIII, fractionated at 20V through a 1% agarose gel for 16 h, and transferred onto nitrocellulose membranes (BioRad, USA) utilizing a traditional wet system. Fragments designed based on a flank region in the chloroplast genome, specifically amplified using the DIG-DNA Labeling and Detection kit (Roche, Germany) and the primer set (P-Forward 5′-ATGTGTAATGATTCCCCCATTC-3′ and P-Reverse 5′-CTTCTCTCCCACTTCACACCTC-3′), were utilized as hybridization probes, producing a 1kb size fragment in non-transgenic(non-transplastomic) plants and a 2.8 kb in transgenic plants (transplastomic) along with a positive control(pKCZK2S vector). Based on the manufacturer's protocol, the probe was hybridized and resolved on the membrane.

ELISA

The level of specific tPA in leaves of T1 PCR-positive transgenic plants was estimated by enzyme-linked immunosorbent assay (ELISA) on protein extracted just before the assay. tPA was assayed by an indirect ELISA procedure as previously described [21].

Result

Analysis of transformed plants by PCR

PCR was carried out with two primers sets to confirm the presence of recombinant tPA gene in transformants. The presence of 1.7 kb in some nuclear transformed plants, about 20 plants out of 200 plants, has showed the inherited integration of the transgene in these plants (Fig.1). DNA from some chloroplast transformed plants has represented the expected size of amplified product to be 1.2 kb with *K2S*-gene-specific primers and some of them have shown no band.(Fig.2). Untransformed plants (negative control) have shown no PCR product.

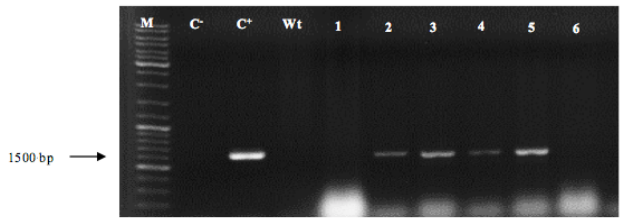


Figure 1 PCR amplification of a 1.7 kb fragment. M: 1 kb molecular weight marker (Fermentas), C⁻: negative control, C⁺: positive control (pBIt-PA vector contain *tPA* gene), Wt: non-transformed plant, 1-6: transformed plants.

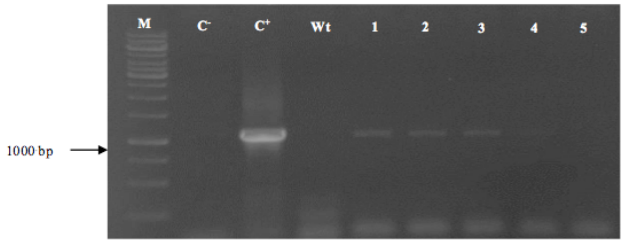


Figure 2 PCR amplification of a 1.2 kb fragment. M: 1 kb molecular weight marker (Fermentas), C⁻: negative control, C⁺: positive control (pKCZK2S vector contain K2S gene), Wt: non-transplastomic plant, 1-5: transplastomic plants.

Southern blot analysis

After confirming the presence of interested gene *K2S* in the chloroplast transformed plants by PCR, it was subjected to the southern blot analysis. As it was expected to observe just a 2.8 kb band on the membrane revealing homoplastic plants and a 1kb size band on the me

mbrane indicating a heteroplasmic plant, we just observed the 1kb size band in all of our T1 PCR-positive plants (Fig.3). Our analyses which represented the heteroplasmic plants were not suitable for more analysis like ELISA.

ELISA

An ELISA was conducted to quantify protein by comparing the absorbance readings of the three replicates of T1 PCR-positive plants of nuclear transformation with known quantities of the commercial tPA protein (Alteplase). After estimating, it was found there was not any difference between transgenic and non-transgenic plants in tPA protein content (Data was not shown).

Discussion

As it can be observed in the Fig.1 and Fig.2 the positive-PCR transgenic plants have been obtained in both nuclear and chloroplast transformations, about 10% plants in nuclear and, surprisingly, some chloroplast transformants. Considering the advantages of chloroplast genetic engineering over nuclear ones, which was mentioned above; due to the lack of gene silencing [37]; it is assumed to see all chloroplast transformants represent the expected 1.2 kb band. So, by this experiment, we can observe the genomic instability of chloroplast transgenic plants causing the loss of the transgene.

To assess the homoplasty, the Southern blot was conducted on the positive-PCR transgenic plants transformed by particle bombardment. Plants show only the expected size for the wild-type plastom band while no integrated transgene bands can be observed. It seems this lack of the 2.8 kb transgene band is precise and needs to be considered.

It can be inferred this phenomenon is the consequence of the intramolecular recombination event resulting in keeping the wild-type plastome copies during the chloroplast segregation procedure [38]. To find out more about this phenomenon, a detailed study has been made on the cloning procedure and

the vector structure. pKCZK2S vector was used to create transgenic plants producing tPA. The problem faced in plants transformed by pKCZ has been the same in their next generation [38–40]; plants have never met the genetically uniform lines, and comes back to the intramolecular recombination via the repeated sequence element, especially regulatory elements controlling foreign gene expression. As it is visible in the Zou (2003) study, there are three copies of plastid promoter prrn governing both selection markers (aadA) and reporter gene(uidA), two prrn copies are oriented as the direct repeats; specific to transplastomic aadA and endogenous rrn16 (16S rRNA) genes. The result of intramolecular recombination of these direct repeats of prrn leads to the removal of ~10kb including all rRNA (5S, 4.5S, 23S and 16S), the whole uidA cassette, and several tRNA genes and keeps the aadA cassette resulting in aberrant transplastomes which are spectinomycin resistant.

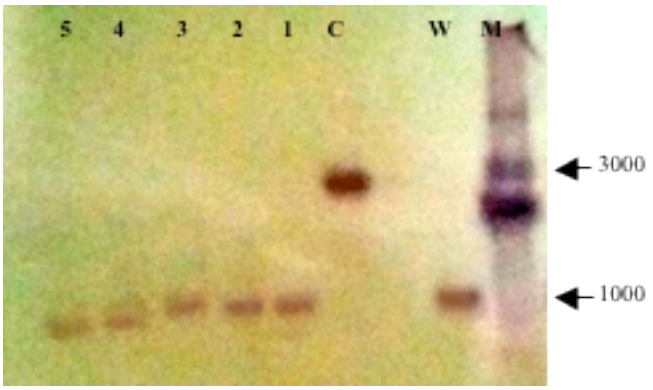


Figure 3 Sothern blot analysis of transplastomic T1 Plants with Hind III enzyme. M: 1 kb molecular weight marker (Fermentas), Wt: non-transplastomic plant, C^+: positive control (pKCZ vector contain K2S gene), 1-5: transplastomic plants.

Abdoli-Nasab *et al* (2013) decided to add the tPA cassette before aadA one to overcome this problem, so, in their opinion, transplastomes could express both tPA and aadA simultaneously in spectinomycin resistant plants. They remained unaware that the removal of all rRNA and several tRNA genes were lethal for transplastomes, however aadA was expressed resulting in the resistance to the spectinomycin. In their analysis, on the one hand, they did not address the issue as they analyzed transformants in the early stages of growth in T_0 generation. The average of transgene loss was estimated about 5%, but it was a continuous process and did not stop until all intramolecular recombination via the direct repeats happened, resulting in all rRNA and several tRNA genes to be removed [41]. On the other hand, the intramolecular recombination procedure under selection stress seemed to be a slow process, but accelerated in the absence of antibiotics like growing in the soil, so, it seemed to be a plausible interpretation as to why we met the loss of transgene in the T_1.

Intramolecular recombination via direct repeats is not always a potentially lethal adverse phenomenon in transformation; this strategy has been helpfully used to create antibiotic marker-free transplastomic plants, [41] while one of the direct repeats has been left in the chloroplast genome after the recombination.

To applicate transformation technologies commercially, it is extremely important that transformants express the recombinant protein over generations [42]. The evaluation of genetic instability seems to be obvious via a loss or sudden change in the phenotype of agronomically engineered plants [43] while more studies need to be considered in non-agronomically engineered ones. In our study, as shown in Fig.1, we have found 20 plants out of 200 plants have shown positive in PCR. These PCR-positive plants have not represented any difference among transgenic and non-transgenic plants in tPA protein content.

The transgene instability can increase over generations especially when it advances to stabilize plants in the homozygous level [44].The instability of transgene inheritance, in the form of transgene lost or rearrangement, has been reported in the PEG-mediated direct gene transfer, bilolistic bombardment or *Agrobacterium* mediation [17]. Although *Agrobacterium*-mediated transformation shows less rearrangement, co-suppression and instability in subsequent generations in comparison with bombardment transformation [44]. There are several reasons which should be considered as the answer for this event; several factors are generally accepted to be responsible for transgene expression instability such as methylation, copy number, genome rearrangement, integration site in genome, and endogenous gene homology to the transgene [16].

It is crucial to produce single-copy number inserted transformants, because it is known that the loss of transgene expression and the expected phenotype can also result from multiple transgene copy numbers [45], [46]. In addition, the tandem copies of T-DNA are often transferred at a single locus in the *Agrobacterium*-mediated transformation and it is found that the T-DNA repeats which stand inverted and head-to-head around the right border are often associated with transgene silencing [47].

Epigenetic interactions as another potential source of transgene instability have been known as gene silencing system in plants [48], resulting from the interaction among multiple transgene copy number or an endogenous gene homology to the transgene leads to the homology dependent gene silencing (HDGS). The mechanism has been unclear, although some theoretical hypothesis have put forward that HDGS may be observed either through the transcription repression, called transcriptional gene silencing (TGS), or through mRNA degradation, called posttranscriptional gene silencing (PTGS) [49]. TGS is characterized as heavy methylation to be the mechanism of the silenced promoter and is meiotically heritable [50], it can be interpreted that the chromatin environment of the transgene is epigenetically modified in a way that can be heritable, most likely before or during gametogenesis. In fact, promoter regions in the repeated transgenes are often methylated, these repeated transgenes cause the methylation of homologous regions placed in trans; also transcription of aberrant promoters shows promoter methylation. Heavy methylation can be seen

when prokaryotic vector sequences integrate into the plant genome, the excess vector sequences cannot be well tolerated by the eukaryotic genome and are often spontaneously resulted in heavy methylation that can extend into neighboring transgenes 49 it is possible these sequences have unusual compositions to bind the eukaryotic nuclear proteins, which subsequently are susceptible to plant methyltransferases 49 and to a conversion to different epigenetic states 50. PTGS is not meiotically inheritable and should be reinstituted in each gametogenesis. It leads to methylation of an increased amount of DNA within the protein-coding region, resulting in the appearance of specific low molecular weight fragments of RNA [49].

Studies reported that the heritable instability could occur in the T_2 to T_4 generations, although Travella *et al* (2005) reported that there was some evidence in which transgene instability occasionally could be appeared in the T_1 population which contained the single-copy number of interested gene, as mentioned above the extra integrated vector sequence led to gene silencing, however it was recently reported that this extra vector sequence might influence transgene expression [51]. Sometimes the meiotic instability results in the loss of T-DNA partially or completely, this instability frequently has been reported to be low for *Agrobacterium*-mediated transformants with single-copy inserts [17].

Considering the aftermentioned reasons for the problem which we have met in the *Agrobacterium*-mediated transformants in the T1 generation, according to Scott *et al* (1998), it seems it is early to discuss the definite answer for this event now and more experiments require to be assayed. Using scaffold attachment regions (SAR) can be useful in nuclear stable transformation, as SARs can be attached to the proteins of the nuclear scaffold and can lead to the organization of the chromatin into loop exposure to transcription machinery. So, flanking the transgenes with SAR elements have been shown to contribute to the reduction of position effects and silencing of transgene expression [52–54].

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