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Transient expression of reteplase gene (*rPA*) under methyl jasmonat treatment in cucumber (*Cucumis sativus* L.)

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ABSTRACT INFO

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ABSTRACT

Every year, millions of people are suffering from cardiovascular diseases. Thrombolytic and fibrinolytic therapies have shown results in the treatment of cardiovascular diseases and strokes. Tissue plasminogen activator (tPA) is a pivotal thrombolytic drug that plays a crucial role in cardiovascular disorders. The recombinant version of tPA, known as alteplase (rPA), has been designated as a significant breakthrough. Reteplase, a third-generation mutant of alteplase, has also emerged as a notable variant. The objective of the present study was to design a transient expression system to analyze the gene expression of *rPA* in cucumber plants. Moreover, the effect of elicitor methyl jasmonate (MeJA) was assessed on *rPA* gene expression. To do this, the *rPA* gene was amplified and subcloned into the ZYMV vector. The recombinant vector was then inoculated in cucumber leaves, and the transfected leaves were harvested at three, five, and ten days post-inoculation (DPI). An RT-PCR reaction was performed, and the desired band was observed in the inoculated lines. Furthermore, the quantity of rPA protein expression was assessed using ELISA. The results of the study demonstrated that the presence of methyl jasmonate led to an enhancement in the production of recombinant protein in the inoculated samples. The highest level of *rPA* gene expression was observed in the samples harvested on the tenth day post-inoculation with methyl jasmonate treatment which was 450.83 ng μg^{-1} of total plant-extracted protein. For the first time, the rPA protein was successfully expressed under the influence of the methyl jasmonate elicitor in the cucumber plants.

Key words: Gene expression, Methyl jasmonate Elicitor, Pharming, Recombinant protein, Reteplase, Transient expression.

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INTRODUCTION

Cardiovascular diseases, as reported by the World Health Organization (WHO; Roth and Manwani, 2011), remain the leading cause of global mortality. Tissue plasminogen activator (tPA), plays a crucial role within the fibrinolytic system. It catalyzes the conversion of plasminogen into active plasmin, which is responsible for breaking down the fibrinogen and fibrin network present in blood clots, ultimately leading to their dissolution (Parcq *et al*., 2013). Given its mechanism of action, tPA has been extensively utilized in the management of cardiovascular diseases and ischemic stroke.

Recognizing the advantages of tPA, considerable efforts have been directed towards its production using recombinant protein technology, and tPA produced with recombinant protein is called alteplase which has received approval from the FDA and has been extensively employed for the treatment of cardiovascular diseases. Alteplase, classified as a serine protease, functions as an enzyme that cleaves peptide bonds within proteins, making it a crucial component in the dissolution of blood clots. This protein, with a molecular weight of approximately 70 kDa, is released by endothelial cells and possesses a half-life of 4 to 8 minutes. Structurally, tPA is a glycosylated protein comprising 527 amino acid residues and containing 17 disulfide bonds (Ma *et al*., 2019). The tPA has undergone significant modifications and has been developed into various variants to enhance its pharmacodynamics, pharmacokinetic properties, and overall clinical effects. These modifications have primarily focused on extending its half-life and improving its therapeutic efficacy. Reteplase, a third-generation mutated form of alteplase, exhibits a longer half-life of 14-18 minutes. It consists of 355 amino acid residues and has a molecular weight of 39.5 kDa (Nikitan *et al*., 2021).

Clinical trials have provided substantial evidence to support that reteplase exhibits enhanced thrombolytic capabilities compared to alteplase. As a result, reteplase has been identified as a suitable alternative to alteplase in thrombolysis treatment strategies (Kohnert, 1992). In this study, we employed a transient plant expression system to express the reteplase gene.

The utilization of plants as biofactories to produce vaccines, antibodies, and other beneficial biomaterials has gained significant attention. Plant-based protein expression offers a unique advantage due to the vast diversity of plant species and systems that can be utilized for production purposes. This diversity, coupled with a wide range of expression methods and protein targeting strategies, provides a suitable platform for the production of practically any desired protein product (Schillberg and Finnern, 2021).

One of the key advantages of plant expression systems is their ability to produce proteins that are not efficiently synthesized in prokaryotes and mammalian cells, primarily due to the complex structures and potential toxicity of these proteins. Additionally, offers an advantageous environment for the production of proteins derived from human pathogens that are incapable of replicating in plants, and it reduces the risk of contamination with human pathogens. (Sala *et al*., 2003; Gengenbach *et al*., 2019).

Notably, plants possess the capacity to perform multiple post-translational modifications such as glycosylation that often necessitate fulfilling the functional requirements of recombinant proteins. This makes plants advantageous hosts for producing properly functional recombinant proteins (Moustafa *et al*., 2016). Plant expression systems, characterized by low cultivation costs and the safety of resulting recombinant proteins, have emerged as a promising platform for producing essential recombinant proteins (Yamamoto *et al*., 2018). Compared to stable transformation methods, transient expression offers rapid protein production and generally yields higher quantities of the protein of interest (Xu *et al*., 2012; Egelkrout *et al*., 2012). Transient expression in plants has garnered considerable attention due to its remarkable potential, particularly in the production of valuable pharmaceutical compounds like rPA. Also because the target gene does not join the plant genome, therefore it is not inherited and causes fewer environmental concerns, (gene escape) (Gleba *et al*., 2007; Canto, 2016). Recent successful examples in the production of plant-based recombinant proteins have demonstrated the feasibility of plant-based protein production, leading to the development of plant biofactor systems (Daniell *et al*., 2009) One of these developments is using viral vectors that have shown great promise in terms of efficiency and speed of protein production because of their ability to achieve high-level expression of recombinant proteins within a short timeframe. Using the viral vectors of transient expression now are most successful expression system for the production of recombinant proteins with high yield in a short time (Egelkrout *et al*., 2012).

Viral vectors offer advantages to increasing the transient expression as a consequence of viral genome amplification and spread systematically

the target gene throughout the whole plant because of the virus's capacity to move cell-to-cell and long distance (Rodriguez-Concepcion and Daròs, 2022) and targeting recombinant proteins for accumulation on a particular subcellular compartment may affect its posttranscriptional modifications (Feng *et al*., 2022). These vectors, inserting the target gene (recombinant vectors), infect host plant cells and facilitate robust expression of the heterologous protein within the infected cells (Parcq *et al*., 2013). For the increased transcription of a target gene in a non-tissue-specific manner, constitutive promoters such as the CaMV 35S promoter are often used (Benfey and Chau, 1990) and the CaMV 35S promoter has been used mostly for increasing expression of the target gene in the shoots of dicotyledonous plants (Makhzoum *et al*., 2014).

Zucchini Yellow Mosaic Virus (ZYMV), possesses a standard single-stranded positive RNA genome. A previously constructed infectious plant virus clone, p35SZYMV2-26, that contains the full-length cDNA to the genomic RNA of a Taiwan isolate of ZYMV, driven by the cauliflower mosaic virus (CaMV) 35S promoter, was engineered as a viral vector and enabling efficient expression of the desired protein (Hsu *et al*., 2004). A *GFP* gene was originally located in the P1 and HC-Pro frame of the ZYMV vector and was used as a protein expression vector specifically in cucurbit species (Hsu *et al*., 2004). After ligation of a gene into the ZYMV vector, the recombinant vector, enters the plant cell and reproduces in the plant tissues, causing a high expression of the recombinant protein in the plant (Gleba *et al*., 2007).

The objective of this study was to utilize a transient expression system to produce pharmaceutical rPA protein in cucumber plants. To assess the potential enhancement of gene expression, methyl jasmonate was employed as an elicitor and its effect was evaluated on enhancing gene expression.

MATERIALS AND METHODS

Isolation and cloning of the *rPA* gene

The construction of the *rPA* gene was achieved by cloning it into the non-expression PMV vector, which was carried out by a gene manufacturing company (Shingene). The PMV vector, with an approximate size of 5 kb, served as a storage vector to retain the gene structure. To amplify the *rPA* gene, the PMV vectors were utilized as the template for a colony-PCR reaction using primers specifically designed to include *Sph*I and *Kpn*I restriction sites (Table 1). The resulting PCR product, which corresponded to the *rPA* gene, was then digested with *Sph*I and *Kpn*I restriction enzymes. Subsequently, purification of the digested product was performed using the Expin Como GP purification kit (GeneAll, Korea). The size of the resulting amplicon was determined to be 1165 bp. The interest construction sequence encompasses the *rPA* gene with a length of 1065 bp, along with an additional 100 bp signal peptide. The inclusion of the signal peptide aims to enhance the protein's resistance against proteases.

The ZYMV viral vector is composed of a single-stranded RNA with a genome size of 13 818 bp. Within this genome, a *GFP* gene is present, spanning a length of 735 bp.

During the ligation reaction, the *rPA* gene was successfully cloned into the ZYMV vector (Sambrook and Russell, 2001). In the ligation reaction, the *GFP* gene of ZYMV was replaced with the gene construction (Figure 1). Following the ligation reaction, the recombinant vectors were transformed into competent *E. coli* cells using the freezing and melting method (Sambrook and Russell, 2001).

Plant materials

Cucumber seeds (*Cucumis sativus L*.) of the cultivar Taft were obtained from the Faculty of Agriculture,

Table 1. Designed primers for amplifying and reverse transcription of *rPA* gene (CinaGene).

Primers	Annealing temperature (°C)	Product size (bp)	Sequences (5'-3')	GC (%)
F- K2S	67	1165	AAATTTGTCGGGCGCATGCACATGG	52
R- K2S	69	1165	TTTTCCGGTACCCATCAAGGTCTCAT	46
Primers for amplifying the rPA gene				
F- RTK2S	59	218	GGAAACAGTGACTGCTACTTTGGGAATGG	48.27
R- RTK2S	59	218	TTGATGCGAAACTGAGGCTG	50
Primers for performing Reverse Transcription PCR (RT-PCR)				

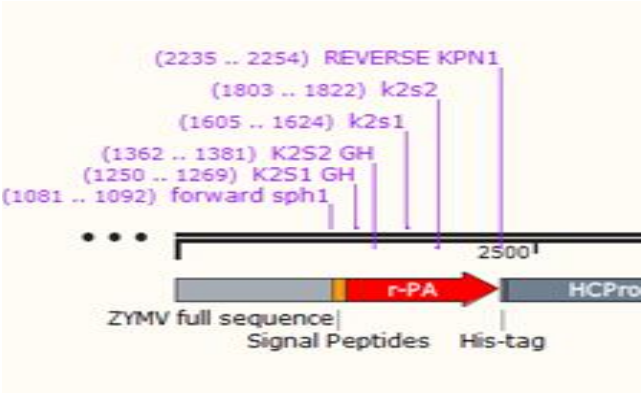

Figure 1. Construction of recombinant vector; the targeted sequence includes 1065bp *rPA* gene and 100bp signal peptide that ligated (between *Sph*I and *Kpn*I restriction enzyme sites) in ZYMV vector.

Tarbiat Modares University. Plant cultivation involved a combination of compost, vermiculite, and sterile soil. The seeds were grown in a greenhouse under controlled conditions of 25°C temperature, a 16-hour light period, and an 8-hour dark period.

Elicitation

Methyl jasmonate was employed as an elicitor to enhance the expression level of reteplase. The treatment of cucumber leaves followed the instructions outlined by Robert *et al*. (2015). Specifically, a solution containing 1 mM methyl jasmonate and 0.1% Triton 100X and without methyl jasmonate treatments was applied to the leaves 24 hours before inoculation with the recombinant vector. The control plants were grown without the inoculation of the recombinant vector and methyl jasmonate treatment.

Transfection

The transfection of cucumber leaf tissue with the viral vector containing the *rPA* gene was performed (Sablowski *et al*., 1995). Mechanical scratching of the leaf surface (plants with 3-5 leaves) was carried out using Carborundum, after which the ZYMV viral vectors were applied to the leaves. Subsequently, the leaves were gently washed with sterile water to prevent any additional damage to the plant tissue caused by excess carborundum. Each elicitated plant was inoculated with a 10 µg vector in a volume of 50 µl. The control plant was infected with the ZYMV wild-type. Subsequently, all of the samples were utilized for the analysis of reteplase gene expression. The sampling was done at 3, 5, and 10 days post-inoculation.

Investigation of expression

Evaluation of gene expression was done on RNA and protein levels.

Analyses at the RNA level

To examine gene expression at the RNA level, total RNA was extracted from the leaf tissue samples using the *RN XPLUS* kit, following the instructions provided by the manufacturer. Subsequently, cDNA was synthesized from the extracted RNA samples using a cDNA synthesis kit obtained from ParsTous Company (Iran). To confirm the authenticity of the gene expression, the synthesized cDNA served as the template for reverse transcription PCR (RT-PCR) reactions, which were conducted using specific primers designed (Table 1) for the target gene, *rPA*. The resulting amplicons from the RT-PCR reactions exhibited a 218bp band which is equal size of a small sequence of *rPA* gene that amplified in inoculated lines.

Analyses at the Protein Level

To assess the translation of the *rPA* gene and the expression of the recombinant protein in the inoculated cucumber plants, total soluble protein was extracted from the samples using the method described by Guy and Haskell (1992), following the manufacturer's instructions. The protein concentration in the extracted samples was determined (Bradford, 1976). This method involves comparing the protein concentration of the samples to a standard BSA (Bovine Serum Albumin) protein.

The quantity of expressed reteplase proteins in the plant samples was determined using an ELISA test. The quality of the recombinant protein was assessed through SDS-PAGE and western blot assays.

ELISA assay: The quantitative measurement of the expressed rPA protein in the inoculated cucumber plants was carried out using an ELISA assay, following the indirect method described by Engvall and Perlman (1971). The measurements were performed using a microplate reader (BioTek, USA). Polyclonal rabbit anti-tPA antibody as a primary antibody to rPA was used at a dilution of 1/600, while Immunoglobulin G (IgG) as the secondary antibody was diluted at 1/800.

A standard rPA curve was initially plotted using commercial rPA. Each sample was analyzed with two biological replicates and three technical replicates. Analysis of variance (ANOVA) was conducted on the data using a factorial experiment design, with a confidence level of 1%. To compare the means, Duncan's multiple-choice test was employed. A graph of the data was generated in Excel to evaluate the protein concentrations and the levels of protein expression across different treatments. Based on the results of Duncan's mean comparison, the leaf tissue samples treated with methyl jasmonate exhibited

the highest expression level of the recombinant rPA protein. Additionally, among the plants treated with methyl jasmonate, the older plants (sampled on the tenth DPI) displayed a higher expression level of the rPA protein.

SDS-PAGE: The quality of the extracted proteins from both the inoculated and non-inoculated cucumber plants was assessed using the SDS-PAGE method. Total soluble proteins were separated on a 12% polyacrylamide gel using SDS-PAGE and subsequently stained with Coomassie Brilliant Blue G-250. The proteins were loaded onto the gel, and after electrophoresis, the gel was placed in a staining solution. A decolorizing solution was then added, and decolorization continued until protein bands were visualized on the gel.

Western blot: Following the SDS-PAGE analysis, the proteins were transferred from the gel to a nitrocellulose membrane using a Trans-Blot semi-dry device (BioRad, USA) following the manufacturer's instructions. The transfer process facilitated the transfer of proteins onto the membrane for subsequent detection. For the western blot assay, a primary antibody solution specific to rPA (anti-rPA) was used at a dilution of 1/5000. Subsequently, a secondary antibody solution containing Immunoglobulin G (IgG) was applied at a dilution of 1/7000. Finally, the membrane was subjected to staining using a DAB substrate solution according to the method described by Hirano (2012).

RESULTS

Cloning of *rPA* gene into ZYMV vector

The *rPA* gene, along with a peptide signal, was amplified using PCR with specific primers (Table 1). Subsequently, the amplified construction was inserted between the downstream region of P1 and the upstream region of HCPro during the ligation process in the ZYMV expression vectors. The reteplase gene was successfully cloned into the ZYMV vector. Subsequently, the accuracy of the recombinant vectors was confirmed through DNA sequencing, colony-PCR, multiple restriction enzyme analyses, and multiple PCR interactions, before proceeding with the transformation into plants. Once the confirmation was obtained, the recombinant vector was transformed into cucumber plants. After inoculation of plants with recombinant vectors, plant samples were harvested at three, five, and ten days post-inoculation and saved for investigation.

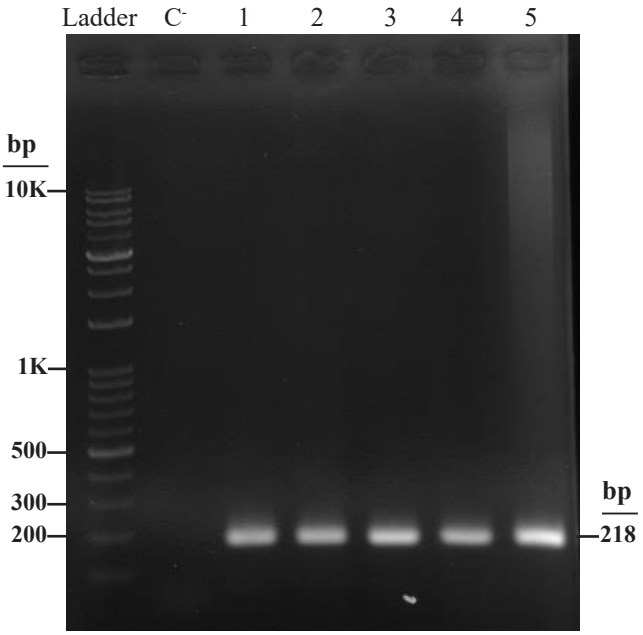

Figure 2. RT-PCR: Lane c-: the non-infected plant (control plant) and lanes 1-5: inoculated plants, observed a band of 218bp, which is related to the amplification of a short sequence using specific primers from the middle of the *rPA* gene., and size Marker is 10k (Kushanzist).

Confirming of transformation at transcription level

An RT-PCR reaction was carried out using primers (Table 1), that were specifically designed to amplify a targeted sequence of the reteplase gene. As anticipated, amplified bands were observed in the inoculated lines, confirming the successful transcription of the *rPA* gene. However, no bands were detected in the non-inoculated plant, indicating the absence of *rPA* gene transcription in that sample (Figure 2).

Confirming of transformation at translation level with ELISA and western blot assay

The quantification of expressed rPA proteins in the inoculated samples was performed using an ELISA test. This assay allowed for the measurement of the quantity of rPA protein present in the samples.

The analysis of variance for the data from the inoculated plants revealed a significant difference (p<0.01) between the factors of different sampling days post-inoculation (DPI) and the effect of methyl jasmonate treatment on the expression level of the rPA protein in the treated samples (Table 2).

Further analysis using the Least Significant Difference (LSD) method confirmed a significant difference in protein absorption between the inoculated and non-inoculated plants, as well as a significant

difference among the different sampling days post-inoculation, with increased expression observed in the presence of methyl jasmonate compared to other inoculated plants and among the plants treated with methyl jasmonate, the older plants (sampled on the tenth DPI) displayed a highest expression level of the rPA protein, that was 450.83 ng/µg of total extracted protein. In the non-inoculated samples with methyl jasmonate, the highest level of expression was 122.39 ng/µg of total extracted protein. The result demonstrated that the methyl jasmonate-treated samples produced about 3.5-fold higher expression of rPA protein than other samples (non-treated methyl jasmonate samples) (Figure 3).

SDS-PAGE analysis was performed to assess the extracted proteins from both infected and non-infected plants. A band at 39.5 kDa was observed, corresponding to the expected molecular weight of reteplase.

To assess the quality of the rPA proteins, a western blot assay was conducted using an anti-rPA antibody. The western blot test revealed a protein band at about 39.5 kDa in the inoculated plants, consistent with the molecular weight of the rPA protein, this technique confirmed rPA protein presence in the inoculated samples indicating the successful expression of the reteplase gene (Figure 4). No protein band was detected in the non-infected plant, indicating the absence of reteplase protein in the non-inoculated sample.

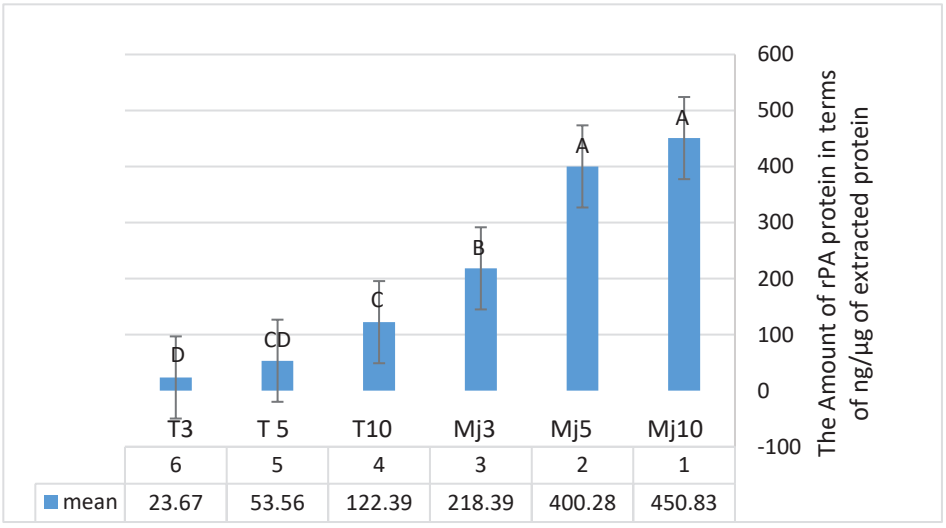

Figure 3. Comparison of the mean expression of the rPA protein by ELISA assay in inoculated plants treated with methyl jasmonate and the effect of different sampling DPI (Mj: inoculated plants that treated with methyl jasmonate. T: inoculated plants without methyl jasmonate. 3, 5 &10: DPI). The highest expression was in the inoculated plant that was treated with methyl jasmonate and harvested on the tenth DPI (Mj10) which was 450.83 ng/µg (Error amount: 5%).

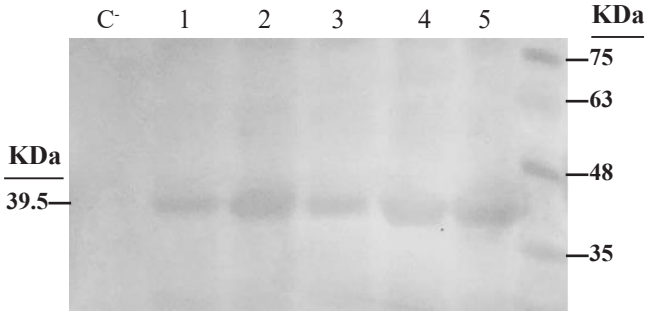

Figure 4. Western blot assay of inoculated plants showed a band of about 39.5kDa, which confirmed the desired band. Line c-: non-inoculated plant and 1-5: extracted proteins from inoculated plants, and the last line is size marker 75kDa (Zistfara company).

Table 2. Analysis of variance of reteplase protein levels in plants.

Sources of variation	df	Mean of square
DPI	2	15545.1512**
Methyl jasmonate	1	378353.3395**
DPI×Mj	5	96680.1284**
Error	14	8751.8018
Coefficient of variation (%)	-	24.80103

DPI: days post-inoculation, CV: Coefficient of Variation, **: Significancation at 1% probability.

DISCUSSION

Recombinant proteins have been extensively used in many different fields. The global protein recombinant expression market size was 1.65 billion US$ in 2017 and has been projected to increase to reach 6.47 billion US$ by 2030 (Schillberg and Spiegel, 2022). Recombinant proteins have been produced in platforms such as bacterial, mammalian, and plants. Plants have advantages over bacterial and mammalian platforms for the production of recombinant proteins (Fischer and Buyer, 2020), including the low cast and relatively high speed of protein production at a large scale (Kelada *et al*., 2021), time efficiency, high-level expression of target accumulation (Egelkrout *et al*., 2012).

In the present study, the production of reteplase protein in cucumber plants was achieved using a transient expression system facilitated by the ZYMV viral vector. Based on previous studies have shown that methyl jasmonate can effectively enhance the expression levels of foreign proteins (Robert *et al*., 2015). As expected the highest amount of rPA protein was observed in cucumber leaves harvested on the tenth day post-inoculation (DPI) in the presence of methyl jasmonate, with a production yield of 450.83 ng/µg of total extracted protein, the expression of rPA in the samples treated with methyl jasmonate was approximately 3.5 times higher than the other samples.

The *rPA* gene with the human origin and *Nicotiana benthamiana* codon usage optimization has been used in this research and by optimizing the codon usage of the *rPA* gene for expression in *Cucumis sativus*, it is expected that the production of recombinant protein can be further increased.

The selection of cucumber plants for this study was based on their advantageous characteristics, including the absence of toxic metabolites and their rapid growth, resulting in substantial biomass production within a short time (Kanamoto *et al*., 2006; Lai *et al*., 2012; Gould, 2022). Cucumber plants have the advantage of rapid and direct infection with plasmids, making them suitable for genetic engineering purposes. The use of genetic engineering techniques in cucumber plants allows for the introduction of desired traits without compromising the desirable characteristics of the plant. Additionally, the availability of the assembled draft genome sequence of cucumber has made it a valuable genetic resource for genetic modification studies (Huang *et al*., 2009). The cucumber plant has gained popularity as a highly suitable choice for biofactor applications. Moreover, cucumber plants are known to be free from toxic metabolites, making them a favorable choice for expressing genes with pharmaceutical applications. It has been extensively studied in biotechnological research, including the transgenic expression of various genes (Zhang *et al*., 2021). The potential for producing edible vaccines, antibodies, vitamins, and other biomaterials derived from cucumber is highly promising and may become a reality shortly (Wang *et al*., 2015).

This study has demonstrated the successful expression of a valuable pharmaceutical protein, rPA, in cucumber plants using the transient viral expression system. The utilization of a viral vector and methyl jasmonate as an elicitor resulted in high levels of rPA expression. It was observed that the expression level of rPA increased as the number of days post-inoculation (DPI) increased, with the highest expression detected on the tenth DPI.

However, further improvement is needed to enhance the expression level of rPA in this system.

Several factors can contribute to achieving high production of the rPA protein, including the use of a strong viral promoter, optimization of the promoter region, sequence optimization, selection of appropriate viral vectors with the ability for systematic movement, and ensuring the stability of the transgene within the vector. Increasing knowledge about protein translation, quality control, trafficking and accumulation, and degradation of expressed recombinant protein in the development of plant-based expression systems has helped to enhance the synthesis and stability of recombinant proteins in plants (Feng *et al*., 2022). However, the short half-life of rt-PA (14-18 minutes) acts as a limiting factor in achieving high expression levels (Gleba *et al*., 2007; Clark and Pazdernik, 2016). On the other hand, there are some challenges of using plants for large-scale production of recombinant proteins: first, the successful production of a few recombinant proteins on a wide scale using some developed strategies and it is not entirely clear whether they can be effective for a wide range of recombinant proteins in different sizes, structures, folding and stability. It may be necessary to integrate many of these developed methods to achieve a stronger way to produce the recombinant proteins. Many successful studies on the production of recombinant proteins on a large scale have been limited to plants' leave tissues, while many other tissues and organs such as seeds, may offer special advantages including better potential for the production of recombinant proteins (Feng *et al*., 2022).

Considering the sustainability of plant-based platforms, they hold great potential for expressing complex proteins, and plant expression systems are expected to become widely adopted expression systems in the future (Nosaki *et al*., 2021).

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