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Anti-VEGFR2 protein accumulation under the influence of methyl jasmonate treatment in *Brassica napus* and *Nicotiana benthamiana* plants

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ABSTRACT

This study was performed to investigate the effect of methyl jasmonate (MeJA) elicitor on green fluorescent protein (GFP) and Anti vascular endothelial growth factor (Anti-VEGFR2) proteins accumulation. A factorial experiment based on completely randomized design (CRD) was performed primarily to evaluate the effect of MeJA and days after inoculation on GFP expression in *Brassica napus* and *Nicotiana benthamiana*. RT-PCR and qRt-PCR assay was performed for GFP gene transcription confirmation at mRNA level. ELISA was done to investigate effect of the elicitor on GFP and Anti-VEGFR2 accumulation. The optimum treatment combination was then used to check Anti-VEGFR2 accumulation. GFP expression was significantly enhanced using M1D10 combination (the best treatment combination) in the two plants. This increase was greater in *Brassica napus* than in *Nicotiana benthamiana*. GFP and Anti-VEGFR2 transcript levels showed no significant differences between the optimized and control treatments in the qRT-PCR experiment, but the quantity of Anti-VEGFR2 protein accumulated in optimized treatments was higher than in control. A simple explanation for the improved accumulation of GFP and Anti-VEGFR2 would be a low 'sink pressure' on amino acid pools towards RuBisCO biosynthesis in RuBisCO-depleted leaves, and a resulting increased availability of metabolite and cellular resources for the production of less abundant (e.g., recombinant) proteins. This research showed that MeJA elicitor increased the accumulation of both proteins.

***Key words*:** Elicitor, GFP, Molecular farming, Recombinant protein, Transient expression.

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INTRODUCTION

The use of plant bioreactors for recombinant (pharmaceutical and industrial) proteins production is defined as molecular farming (Mirzaee *et al.*, 2022; Wani and Aftab, 2022; Long *et al.*, 2022). The advantages of using the plant bioreactors for molecular farming are high scalability, rapid production, low cost of production, ability to perform post-translational modifications, and biosafety (no risk of contamination with animal or human pathogens). So far, a wide number of recombinant proteins such as proinsulin (Yarbakht *et al.,* 2015), tissue plasminogen activator (Javaran *et al.,* 2017; Izadi *et al.*, 2021), gamma interferon (Razmi *et al.,* 2019), Anti-VEGF (Soleimanizadeh *et al.,* 2019), and etc (Park and Wi, 2016; Schillberg and Finnern, 2021) have been successfully produced using molecular farming.

Over the last few years, transient expression of proteins using plant viral vectors have attracted the attention of manufacturing companies and have progressed to the commercialization of target proteins. The viral vectors offer the numerous advantages including the tiny genome of the virus, simple manipulation, high replication speed, protein production in high quantities, and the short period of protein production (Hefferon, 2017; Kopertekh and Schiemann, 2017; Mahmood *et al.*, 2023).

The transient expression of recombinant proteins in plant systems can be performed with the use of full-virus or deconstructed expression vectors full-virus expression vectors are functional viruses, mostly derived from positive-sense RNA viruses such tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), and potato virus X (PVX) proteins. Full-length viral expression vectors based on these viruses have been successfully applied for the expression of various recombinant proteins. Deconstructed viral vectors, in which non-essential elements of the viral genome for systemic movement of the virus are deleted, include MagnICON system which is used to express different types of pharmaceutical proteins at high levels, Bean yellow dwarf virus (BeYDV)-based virus vector which is applied for the production of monoclonal antibodies (MAbs) and virus-like particles, and the reported deconstructed CPMV-based viral vector for the production of human gastric lipase (Kopertekh and Schiemann, 2017).

Insufficient accumulation level of recombinant protein is the major challenges of molecular farming that must be solved. To achieve a high accumulation of recombinant protein, various strategies have been applied by researchers (Desai *et al.,* 2010; Gerasimova *et al.,* 2016; Saberianfar and Menassa, 2018; Karimzadegan *et al.,* 2019; Heidari-Japelaghi *et al.*, 2020; Soleimanizadeh *et al.*, 2022). One of these strategies is to use abiotic and biotic elicitors (Karimzadegan *et al.*, 2019). It has been demonstrated that the production of some elicitors such as MeJA increases during wounding and as a result, it causes a significant amount of energy to be allocated to plant defense mechanisms. MeJA elicitor could induce downregulation of photosynthesis-related genes such as RuBisCO (ribulose bisphosphate carboxylase, oxygenase) (Bilgin *et al.,* 2010; Zubo *et al.,* 2011; Duceppe *et al.,* 2012; Robert *et al.,* 2015). Therefore, additional cellular and metabolite resources such as free amino acids can be used for the production of low-frequency proteins (for example, target recombinant proteins) (Robert *et al.,* 2015; Karimzadegan *et al.,* 2019).

One of the main causes of death in the worldwide is cancer (Bray *et al.,* 2018). Angiogenesis is a process that is required for the growth, incursion, and spread of cancer tumors (Behdani *et al.,* 2012). One of the strategies used to treat cancer tumors is the inhibition of the angiogenesis process (Folkman, 2007). VEGFR2 (Vascular endothelial growth factor receptor2) is a critical tumor-related receptor that plays an important role in the process. Hence, blocking of VEGFR2 signaling using an Anti-VEGFR2 nanobody can prevent neovascularization and metastasis of cancer tumors (Behdani *et al.,* 2012; Karami *et al.*, 2022).

Nanobody is a unique type of antibody functional fragment that is found in Camelidae serum (Muyldermans *et al.,* 2009; Jovčevska and Muyldermans, 2020). They are useful tools for therapeutic applications because of their unique features including very little immunogenicity, tiny size, high affinity and specificity, high stability, and solubility (Buelens *et al.,* 2010; Behdani *et al.,* 2012; Soleimanizadeh *et al.,* 2019).

The Anti-VEGFR2 nanobody was isolated from the camel in 2012 using a phage display technique (Behdani *et al.,* 2012). Before, we successfully expressed this nanobody in tobacco and lettuce plants using (Turnip mosaic virus) TuMV vector (Mirzaee *et al.,* 2018; Modarresi *et al.,* 2018). This study aimed to evaluate the influence of the MeJA elicitor as an elicitor on GFP and Anti-VEGFR2 nanobody genes expression using quantitative reverse-transcriptase (RT) PCR and ELISA analysis.

MATERIALS AND METHODS

Plant material

Seeds of *Nicotiana benthamiana* and *Brassica napus* plants were cultivated in pots containing sterilized soil (50%), perlite (25%), and peat moss (25%) and after weathering they were kept in a growth room at 25±2 °C and 16 h light8/ h dark cycles.

Recombinant viral vector construction

p35STuMVGFPH a (pTGFP) viral vector (Figure 1) was previously provided by Prof. Shui-Dong Yeh (Department of Plant Pathology, National Cheng Hsing University, Taichung city, Taiwan) which was used in this research. The pTGFP vector includes the 35S promoter, the coding sequences of GFP protein, native genes of TuMV virus, and 6x his tag at the C-terminal. Anti-VEGFR2 (3VGR19) nanobody gene was placed between nuclear inclusion protein (NIb) and the capsid protein (CP) genes in the pTGFP vector by replacing the GFP gene (Mirzaee *et al*., 2018). The pTuMV-3VGR19 recombinant viral vector and pTGFP vector were used for the expression of 3VGR19 nanobody and GFP proteins in target *(Nicotiana benthamiana* and *Brassica napus)* plants, respectively.

Elicitor (MeJA) treatment and plant inoculation

Three concentrations of MeJA treatment (0, 1 mM and 2 mM) were used for the Spraying of plants with 4 leaves. Spraying of each plant was carried out using 50 mL of MeJA concentration dissolved in water including 0.1% ($v.v^{-1}$) Triton X-100 (Sigma-Aldrich). Inoculation of plant cotyledon leaves was performed with 10 µl of pTGFP viral vector using mechanical rubbing and dusting with carborundum after 24 h after MeJA treatment (Mirzaee *et al*., 2018). Plant leaf samples were collected 5, 10, and 15 days after inoculation and then kept in -80 °C until the extraction of RNA or protein. The best treatment combination (M1D10) was selected and used to investigate its effect on 3VGR19 recombinant protein expression after plants inoculation with a pTuMV-3VGR19 recombinant viral vector.

RNA extraction and RTPCR

Total RNA extraction from 100 mg *Nicotiana benthamiana* and *Brassica napus* leaf samples was carried out using the Qiagen kit (RNeasy Plant Mini Kit) according to the recommended method. RNA was treated with RNase-free DNaseI (Fermentas) and complementary DNA (*cDNA*) synthesis was carried out using HyperscriptTM Reverse Transcriptase (GeneAll Biotechnology, Korea). Anti-VEGFR2 and GFP-specific primers (Table 1) were used to perform the RT-PCR procedure in this study.

Quantitative reverse-transcriptase (RT) PCR

Quantitative RT-PCR (qRT-PCR) was performed for the quantification of GFP and 3VGR19 transcription levels using the BioRad system and Eva Green (Amplicon, Denmark) fluorescent dye method. The qRT-PCR amplification consisted of the following condition: 95 °C, 30 s, 35 cycles at 94 °C, 30 s, 58 °C, 20 s, and 72 °C, 15 s. Three technical replicates were used for each sample in this experiment. GAPDH gene

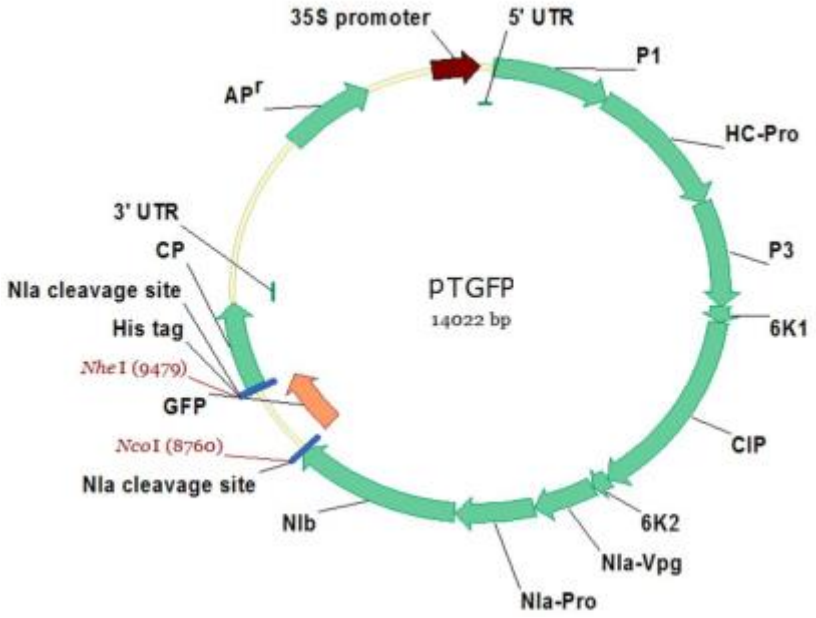

Figure 1. The schematic representation of the pTGFP viral vector used in this study: Anti-VEGFR2 recombinant protein encoding gene is integrated between NIb and CP genes using *Nco*I and *Nhe*I restriction enzymes.

Table 1. Specific primers for the amplification and detection of the GFP, Anti-VEGFR2, and GAPDH genes.

Primer's name	Primer's sequence (5′→3′)	Primer length (bp)	Annealing temperature (°C)
GFP-F GFP-R	ACGACGGCAACTACAAGACC TTGTACTCCAGCTTGTGCCC	160	60.5
NF NR	TCATCACCATCACCATCACTCT TCCAGGAGCTTGTCTAAACCAT	196	58
GAPDH-F GAPDH-R	AAGCCAGCATCCTATGATCAGATT CGTAACCCAGAATACCCTTGAGTT	132	60

was also selected as an internal reference for studying the target gene expression. Finally, the $2^{-\Delta\Delta Ct}$ method (reference) was used for qRT-PCR data analysis.

Protein extraction and quantification using ELISA

Total soluble protein (TSP) was extracted from the plant leaf samples (200 mg) according to the Guy *et al*. (1992) method. TSP concentration was determined using Bradford's (1976) assay. Bovine serum albumin (BSA) was also used as a protein standard in this assay. ELISA was performed to investigate the effect of elicitor (MeJA) on GFP and 3VGR19 proteins accumulation. The extracted proteins (50 ng) were mixed with 100 mM carbonate, bicarbonate buffer (pH 9.6) and coated in ELISA plate wells in four replicates. Plate incubation was then performed at 4 °C overnight. After washing the wells with phosphate-buffered saline (PBS-T), the plate was blocked using %1 BSA for 1h at 37 °C. ELISA Plate washing was repeated and then the wells were incubated with Mouse anti-His tag antibody (Biolegend, 1 µg/ml) at 37 °C (1h) for the detection of GFP protein. The wells were washed again and incubated with secondary antibodies (Goat anti-mouse IgG-HRP, Sigma, 1µg/ml). Afterwards, detection was performed using 1% TMB **s**ubstrate solution at RT (room temperature) for 15-20 min. The enzymatic reaction was stopped with H_2SO_4 (1M) and a microplate reader was used to read the absorbance at 450 nm. ELISA method was also carried out for quantification of 3VGR19 nanobody expression as described above. Polyclonal rabbit anti camel IgGs and Goat anti-rabbit IgG- HRP (Sigma) were used as primary and secondary antibodies, respectively. 3VGR19 nanobody quantification was calculated using a standard curve created by serial dilution of bacterial expressed (3VGR19) nanobody.

Statistical analysis

Statistical analysis (a factorial experiment in a completely randomized design) was carried out with two factors including 0, 1 and 2mM concentrations of MeJA and sampling days post-inoculation (5, 10, and 15) using SAS software version (v) 9.1. This software was used for ELISA data analysis of GFP expression. Interaction effects of MeJA (M) treatment and days post-inoculation (D) were highly significant at (1%) probability level in *Brassica napus* (Table 2) and *Nicotiana benthamiana* (Table 2) plants. Therefore, the mean comparisons were performed for M×D interaction effects using the least significant differences (LSD) method.

Table 2. The analysis of variance of factorial experiment in a CRD (comletely randomized design) based on the effects of two factors of MeJA and days on the expression of GFP protein in the inoculated *Brassica napus and Nicotiana benthamiana* plants, using ELISA assay results.

Sources of variation	df	Mean of square	
		Brassica napus	*Nicotiana benthamiana*
M	2	0.01850337**	0.00024026**
D	2	0.09276104**	0.02074404**
M×D	4	0.00629359**	0.00264643**
Error	18	0.00000596	0.00002826
Total	26		

RESULTS

GFP recombinant protein expression

Brassica napus and *Nicotiana benthamiana* plants were transiently inoculated with p35STuMVGFPHis (pTGFP) viral vector containing the GFP reporter gene (Figure 1). RT-PCR analysis using GFP-specific primers was applied for the confirmation of its expression in the inoculated plants. An expected fragment of 160 bp was amplified in the inoculated *Brassica napus* and *Nicotiana benthamiana* plants; while no bands were observed in the control (inoculated with the wild-type TuMV) plant (Figure 2). This analysis also confirmed that the pTGFP viral vector can inoculate the target plants and expressed its genes in these plants. Furthermore, an ELISA was performed to study the

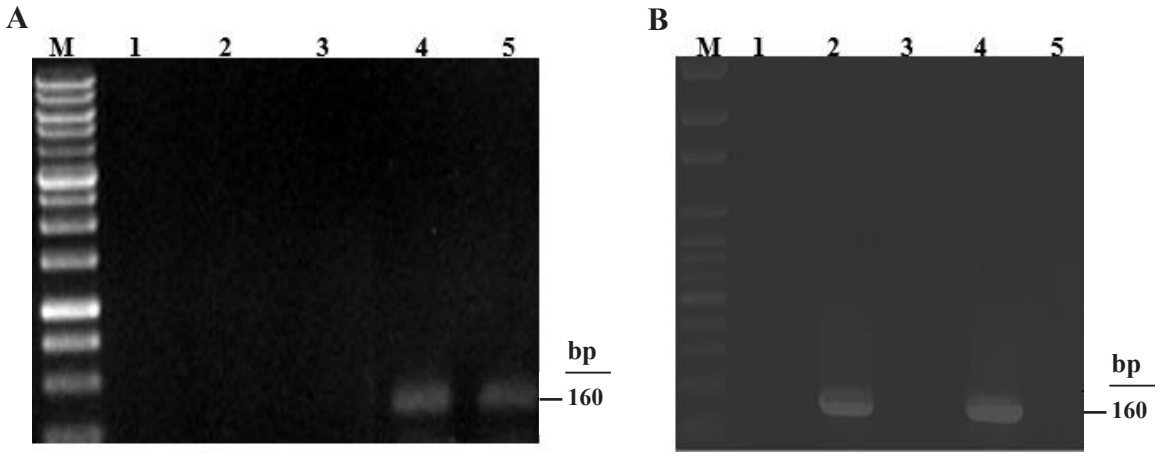

Figure 2. RT-PCR analyses of GFP expression in **A:** *Nicotiana benthamiana* and **B:** *Brassica napus*. **A:** M. molecular size markers (1 kb), lanes 1 negative control, 2 PCR amplification of total RNA as a negative control, 3 PCR amplification related to control (inoculated with the wild-type TuMV) plant, 4 PCR amplification related to M0D10 (0 MeJA and harvested after 10 days) treatment, 5 PCR amplification related to M1D10 (1 mM of MeJA and harvested after 10 days) treatment. **B:** M. molecular size markers (1 kb), 1 negative control, 2 PCR amplification related to M0D10 treatment, 3 PCR amplification of total RNA as negative control, 4 PCR amplification related to M1D10 treatment, 5 PCR amplification related to control (inoculated with the wild-type TuMV) plants.

effect of the MeJA elicitor on the GFP recombinant protein expression in *Brassica napus*, 15 days after the inoculation (Figure 3A) of *Nicotiana benthamiana* (Figure 3B) inoculated plants. As expected, there was a significant difference between MeJA-treatment and control plants regarding GFP production. This result indicates that the utilization of MeJA leads to a higher expression level of GFP protein. The highest level of recombinant protein accumulation was observed in M1D10 (1mM MeJA and sampling on the tenth day after inoculation) treatment for both (*Brassica napus* and *Nicotiana benthamiana*) plants (Figure 3A, B). For M1D10 treatment, the expression of GFP protein in *Brassica napus* was 2 fold higher than its expression in *Nicotiana benthamiana* plants (Figure 3A, B). Therefore, the best treatment combination (M1D10) was selected to evaluate the effect of MeJA treatment on target (anti-VEGFR2) recombinant protein expression in the *Brassica napus* plant.

Investigation of Anti-VEGFR2 expression in *Brassica napus* plants

The coding sequence of the Anti-VEGFR2 gene was integrated between NIb and CP genes and downstream of the 35S promoter into the pTGFP viral vector (Mirzaee *et al.*, 2018). Then, *Brassica napus* plants were inoculated using the new (pTuMV-Anti-VEGFR2) recombinant viral vector. After total RNA extraction and cDNA synthesis, the detection of Anti-VEGFR2 gene transcripts in inoculated plants was performed using RT-PCR analysis and the target gene-specific primers. The results of RT-PCR analysis showed the presence of an expected 196 bp band in the inoculated plants (Figure 4). However, the control plants did not show a similar band.

Quantitative RT-PCR analysis

To investigate the effect of MeJA on transcription, the relative transcript levels of GFP and anti-VEGFR2 nanobody genes were measured using quantitative RT-PCR analysis in MeJA-treated (M1D10) and untreated (M0D10) plants. The results of quantitative RT-PCR analysis for GFP and anti-VEGFR2 genes indicated that there is no significant difference between plants treated with the best treatment combination (M1D10) and untreated plants (Figure 5). Although GFP and anti-VEGFR2 proteins accumulation were enhanced with the use of MeJA treatment, there was no significant change in the transcription level of these genes. Several reports have demonstrated that MeJA reduces the levels of transcription of photosynthetic genes, including RuBisCO (Robert *et al.*, 2015; Karimzadegan *et al.*, 2019). In the other words, the suppression of RuBisCO production leads to an increased access to free amino acids inside the cell, which ultimately proceeds to an increase in GFP and anti-VEGFR2 recombinant proteins expression.

ELISA analysis

Anti-VEGFR2 nanobody expression in MeJA-treated (M1D10, 1 mM of MeJA and harvested after 10 days) and control (M0D10, 0 MeJA and harvested after 10 days) plants was measured with ELISA using an anti-camel antibody. The quantity of the recombinant

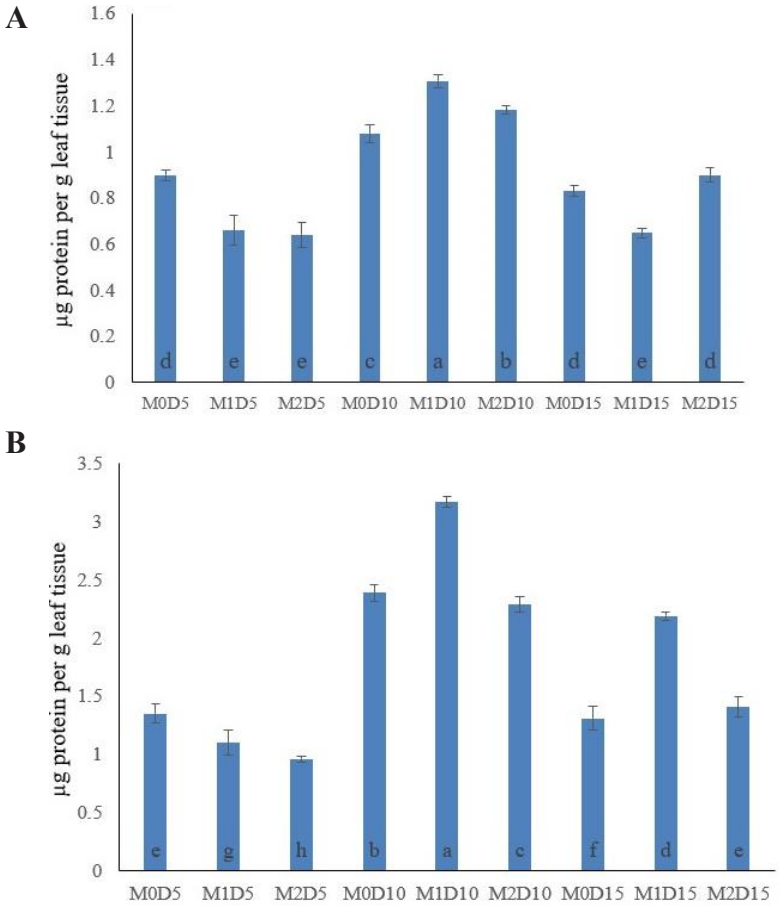

Figure 3. The influence of different treatments of MeJA on expression of *GFP* gene using ELISA assay in **A:** inoculated *Brassica napus* plants and **B:** *Nicotiana benthamiana* plants with the pTGFP viral vector. M, MeJA, and D, Day after inoculation, a significant difference between various treatments was indicated with small letters on each column. Also, there is no significant difference between treatments with similar letters. Each bar representes the mean of 3 independent replicates.

nanobody (Anti-VEGFR2) was evaluated by a standard curve created by determinate concentrations of bacterial expressed (3VGR19) nanobody. ELISA results demonstrated that the maximum accumulation of the recombinant nanobody in M1D10-treated plants was higher than in M0D10-treated plants (Figure 6). Therefore, MeJA can increase the recombinant nanobody expression. The possible reason for the increase is the inhibitory effect of MeJA on the expression of photosynthetic proteins as have been proven by various reports. Furthermore, increased availability of released amino acids leads to the high expression of recombinant (Robert *et al.*, 2015; Karimzadegan *et al.*, 2019).

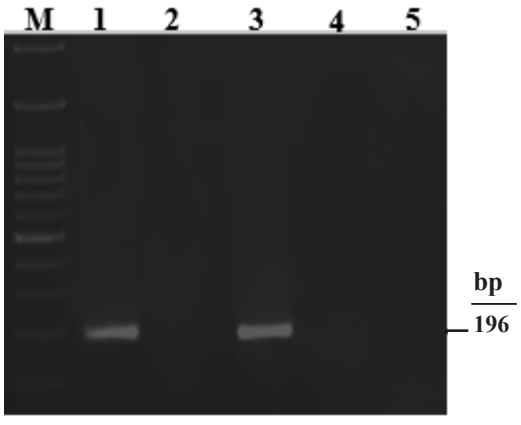

Figure 4. RT-PCR analysis of Anti-VEGFR2 expression in *Brassica napus*. M. molecular size markers (1 kb), 1 PCR amplification related to M0D10 treatment, 2 negative control, 3 PCR amplification related to M1D10 treatment. 4 PCR amplification of total RNA as a negative control, 5 PCR amplification related to control (inoculated with the wild-type TuMV) plants.

DISCUSSION

The need to create inexpensive and effective ways for cancer therapy is essential due to the increasing growth of the cancer (Cortes *et al.*, 2020; Mao, *et al.*, 2022; Pulumati *et al.*, 2023).

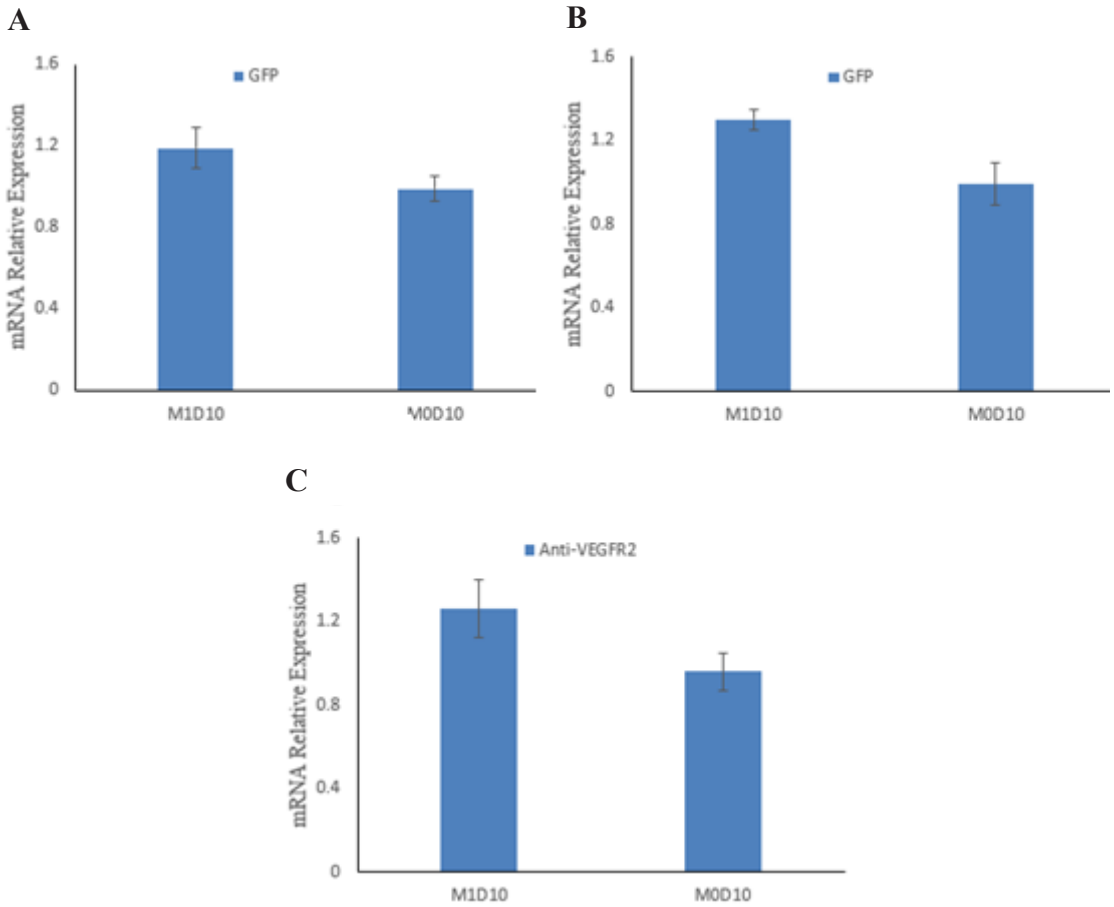

Figure 5. Frequency of transcription of *GFP* and *Anti-VEGFR2* genes for M1D10 and M0D10 treatments. Relative expression of *GFP* gene in **A:** the inoculated *Nicotiana benthamiana* plants and **B:** *Brassica napus* plants with pTGFP vector, relative expression of *Anti-VEGFR2* nanobody gene in **C:** the inoculated *Brassica napus* plants with pTuMV-Anti-VEGFR2.

One of the most effective strategies for cancer treatment is the use of neutralizing nanobodies against VEGF (vascular endothelial growth factor) (Soleimanizadeh *et al.*, 2022) or its receptors (Jovčevska and Muyldermans, 2020). Our research team successfully produced an Anti-VEGFR recombinant nanobody in lettuce plants using chloroplast transformation (Mirzaee *et al.,* 2018) and viral vectors (Modarresi *et al.,* 2018). The use of a plant viral vector is a simple and quick strategy for transient expression of important recombinant proteins. In the present study, we successfully expressed GFP and Anti-VEGFR2 recombinant proteins in *Nicotiana benthamiana* and *Brassica napus* using pTGFP and pTuMV- Anti-VEGFR2 viral vectors. The effect of MeJA elicitor treatment was also evaluated on GFP and Anti-VEGFR2 recombinant proteins expression. When plants are exposed to wounding and herbivores attack, MeJA increases, resulting in the allocation of significant part of energy to defense mechanisms rather than developmental processes. A conserved

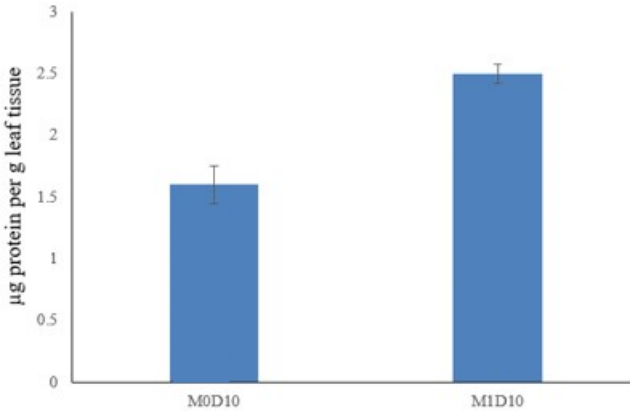

Figure 6. The quantity of Anti-VEGFR2 protein accumulated in the inoculated *Brassica napus plants.* M0D10 untreated plants collected on the tenth day after inoculation, M1D10, MeJA-treated plants collected on the tenth day after inoculation, each bar representes the mean of 3 independent replicates.

trait of plant reactions to a large number of stress conditions is the downregulation of photosynthesis-related genes. Previous studies have demonstrated that the production of proteins associated with stress and recombinant proteins can be up-regulated using the MeJA elicitor. This elicitor can also reduce the expression of photosynthesis-related proteins such as RuBisCO. These data demonstrate the effectiveness of MeJA as a potent inducer in *Nicotiana benthamiana* and *Brassica napus* leaves. The goal of this study was to use the potential of MeJA to increase recombinant proteins (GFP and anti-VEGFR2 nanobody) production in *Nicotiana benthamiana* and *Brassica napus* plants.

The results of Real-Time PCR showed no significant differences between the transcription of recombinant protein-coding genes between the best combination of MeJA and control. MeJA caused a significant increase in the expression of recombinant protein. A simple explanation for the improved accumulation of GFP and anti-VEGFR2 nanobody would be a low 'sink pressure' on amino acid pools towards RuBisCO biosynthesis in RuBisCO-depleted leaves resulting in an increased availability of metabolites and cellular resources for the production of less abundant (e.g., recombinant) proteins. Our research results, in this case, were consistent with the findings of studies by other researchers (Robert *et al.,* 2015; Karimzadegan *et al.,* 2019).

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