IMPROVING HETEROLOGOUS PROTEIN EXPRESSION IN PLANT LEAVES – COMPARISON OF PEAQ-HT VECTORS WITH CONVENTIONAL BINARY PLANT VECTORS

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Tiivistelmä — Referat — Abstract

Transientti ekspressio on suosittu tapa tuottaa proteiineja verrattuna stabiiliin ekspressioon, koska se on sopiva konstruktien nopeaan tarkasteluun. pEAQ-HT-vektorit ovat helppoja ja nopeita vektoreita ilman tarvetta virusten replikaatioon, ja ne on kehitetty transienttiin ekspressioon. Ne edistävät korkeampia ekspressiotasoja ja sallivat usean ekspressiokasetin sisällyttämisen yhteen plasmidiin. Agroinfiltraatio on laajasti käytetty metodi transientin ekspression indusoimiseen kasveissa, sillä se on tehokas ja suoraviivainen keino.

Tämän tutkielman tavoitteena oli parantaa heterologista proteiinieksпressiota käyttämällä Gateway-yhteensopivia pEAQ-HT-DEST-vektoreita. Geenit G2PS1, 2 ja 3 alikloonaattiin näihin sekä pK2GW7-vektoreihin, ja infiltroitiin tupakan (*Nicotiana benthamiana*) lehtiin. pK2GW7-vektoreita käytettiin vertailuun tuloksia tarkastellessa western blot -analyysillä, ELISA:lla sekä HPLC:llä. Tupakan pitäisi tuottaa 6-metyyli-4-hydroksi-2-pyronia sekä 4,7-dihydroksi-5-metyylikumariinia, kun jokin näistä geeneistä on ilmentynyt, mutta molempia ei ole vielä havaittu samanaikaisesti. Korkeammat ekspressiotasot voisivat auttaa näkemään puuttuvat yhdisteet. Se olisi tärkeää tulevia tutkimuksia varten, koska jos synteesi saataisiin alkamaan infiltraation aikana, kotransformaatio reduktaasien kanssa voisi johtaa gerberiinin, parasorbosiinin ja 4-hydroksi-5-metyylikumariinin biosynteesiin, täten todistaen geenien toiminnan.

Western blot -analyysi oli onnistunut ja signaali oli vahvempi kaikilla pEAQ-HT-vektoreilla verrattuna pK2GW7-vektoreihin. Seitsemän päivää infiltraation jälkeen kerätyt näytteet osoittivat vahvemman signaalin kuin kaksi päivää infiltraation jälkeen kerätyt. Vaikka kvantitatiivisia tuloksia ei saatu ELISA:sta, tuloksista kuitenkin selvästi näki pEAQ-HT-vektoreiden aikaansaavan korkeamman ekspressiotason. HPLC näytti vain 6-metyyli-4-hydroksi-2-pyronin, muttei 4,7-dihydroksi-5-metyylikumariinia. Aiemmat tutkimukset ovat osoittaneet korkeampia ekspressiotasoja pEAQ-HT-vektoreita käyttämällä, joten voidaan todeta niiden parantavan transienttia ekspressiota verrattuna tavanomaisiin binaarivektoreihin. Vaikka pEAQ-HT-vektoreiden on todistettu toimivan tehokkaasti, on kuitenkin jo useita tutkimuksia, joissa niitä on entisestään parannettu.

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Agroinfiltraatio, heterologinen proteiini, pEAQ-HT, transientti ekspressio

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Tiivistelmä — Referat — Abstract

Transient expression is a preferred way to produce proteins compared to stable transformation because it is suitable for rapid screening of constructs. pEAQ-HT vectors are easy and quick vectors without the need for viral replication, and they are developed for transient expression. They promote high expression levels and allow the insertion of multiple expression cassettes in a single plasmid. Agroinfiltration is a widely used method to induce transient gene expression in plants because it is efficient and straightforward.

This study aimed to improve heterologous protein expression using Gateway compatible pEAQ-HT-DEST vectors. The genes G2PS1, 2 and 3 were subcloned to these and pK2GW7 derived expression vectors and infiltrated into tobacco (*Nicotiana benthamiana*) leaves. pK2GW7 derived expression vectors were used as a reference when samples were analyzed by western blotting, ELISA and HPLC. Tobacco should produce 6-methyl-4-hydroxy-2-pyrone and 4,7-dihydroxy-5-methylcoumarin when one of these genes is expressed, but they both have not yet been observed at the same time. Higher expression levels could allow us to see that. That would be important for further studies because if the synthesis could get started under agroinfiltration, co-transformation with reductases can lead to gerberin, parasorboside and 4-hydroxy-5-methylcoumarin biosynthesis, thus proving function.

Western blotting was successful and showed a stronger signal with all pEAQ-HT derived expression vectors compared to pK2GW7 derived expression vectors. Samples collected seven days after agroinfiltration showed a stronger signal than samples collected two days after infiltration. Results from ELISA also showed more protein from pEAQ-HT vectors even though quantitative data was not obtained. HPLC showed only 6-methyl-4-hydroxy-2-pyrone but not 4,7-dihydroxy-5-methylcoumarin. Previous studies have also shown increased expression using pEAQ-HT vectors. Hence, using pEAQ-HT vectors does increase transient gene expression compared to conventional binary vectors. Even though pEAQ-HT vectors are proved to work efficiently, there are already studies about improving them.

Avainsanat — Nyckelord — Keywords
Agroinfiltration, Heterologous protein, pEAQ-HT, Transient expression

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ABBREVIATIONS

2PS	2-pyrone synthase
AS	Acetosyringone
CPMV	Cowpea mosaic virus
DHMC	4,7-dihydroxy-5-methylcoumarin
DMSO	Dimethyl sulfoxide
ds	Double-stranded
EAQ-HT	Easy and quick-hypertranslatable
ELISA	Enzyme-linked immunosorbent assay
His	Histidine
HMC	4-hydroxy-5-methylcoumarin
HPLC	High-performance liquid chromatography
HT	Hypertranslatable
nos	Nopaline synthase
NPT	Neomycin phosphotransferase
PKR	Polyketide reductase
PKS	Polyketide synthase
PTGS	Posttranscriptional gene silencing
RISC	RNA-induced silencing complex
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
siRNA	Small interfering RNA
ss	Single-stranded
T-DNA	Transferred DNA
TAL	6-methyl-4-hydroxy-2-pyrone, triacetolactone
Ti-plasmid	Tumor-inducing plasmid
UTR	Untranslated region
vir	Virulence
VLP	Virus-like particle

1 INTRODUCTION

Heterologous proteins are proteins that are produced outside their natural host system (Desai et al. 2010). Protein production in plants is a good alternative for animal, bacterial or yeast production systems because plants are cost-effective, and contamination risks are lower because plant pathogens do not infect humans. Certain plants like tobacco can also produce a lot of biomass in a very short time (Peyret & Lomonossoff 2013). Other advantages are that recombinant proteins accumulate at high levels and posttranslational modifications like glycosylation are possible (Kusnadi et al. 1997).

The research about plant-produced proteins is in the spotlight, and the development has been significant. Transient expression and utilization of *Agrobacterium* have promoted gene delivery technology. There is an increasing interest in pharmaceutical proteins produced in plants. The main categories are antibodies, therapeutic enzymes, subunit vaccines and virus-like particles (VLPs) (Lomonossoff & D´Aoust 2016). VLPs are like viruses but without the infection ability. However, they are still able to elicit an immune response making them perfect vaccine candidates (Marsian & Lomonossoff 2016). Taliglucerase alfa was the first plant-produced biopharmaceutical approved by The US Food and Drug Administration in May 2012. It is an enzyme for treatment of Gaucher disease, and it is produced in genetically engineered carrot cells (Fox 2012). Makatsa et al. (2021) demonstrated in their research the use of plant-derived recombinant proteins in the fight against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). They developed a serological assay and showed that recombinant SARS-CoV-2 proteins enable a robust detection of SARS-CoV-2-spesific antibodies. Antibody responses to SARS-CoV-2 can be detected by serological assays and it is crucial when studying immunity to the virus.

The research was conducted from October 2018 to May 2019 at the Gerbera laboratory of the University of Helsinki. The HPLC experiment was done in May 2021. The topic of the thesis was given directly by prof. Teemu Teeri as it was going to be investigated and well suited for a master’s student.

2 LITERATURE REVIEW

2.1 Stable and transient gene expression

There are two ways to produce heterologous proteins in plants: stable transformation and transient expression (Peyret & Lomonossoff 2013). Stable transformation involves the integration of the gene of interest into plant nuclear DNA (Magori & Citovsky 2011). Although it is a time-consuming approach and not suitable for the rapid screening of constructs, it has the advantage of potential large-scale production. Transient expression is a quick way to produce high amounts of recombinant proteins because it does not involve the integration process. It can be performed using modified plant viruses or agroinfiltration using *Agrobacterium tumefaciens* (Peyret & Lomonossoff 2013). Other methods to produce transient gene expression are particle bombardment, protoplast transformation and microinjection, but they are also time-consuming, so agroinfiltration is more widely used (Bashandy et al. 2015). Transient gene expression has many advantages over stable transformation. In addition to it reducing the time needed to produce proteins, it is also simple and easy to perform, and the gene expression can be measured directly after agroinfiltration (Wydro et al. 2006). Because the genes are not heritable by transient expression, increasing the production means more infiltration, so maximizing the expression is essential (Lomonossoff & D´Aoust 2016).

RNA plant viruses are the base for many vectors used for transient expression. They include *Cowpea mosaic virus* (CPMV), which is a bipartite comovirus (Sainsbury & Lomonossoff 2008). RNA viruses are used because they can replicate fast and spread throughout the plant. However, using replicating RNA viruses has disadvantages like restrictions on the size of the insert and complexity of the expressed proteins. Because the vectors can move throughout the plant, there are also concerns about genetic drifting and problems with biocontainment (Peyret & Lomonossoff 2013). To overcome these problems, Sainsbury and Lomonossoff (2008) developed a method based on a non-replicating version of CPMV RNA-2. It became the base of the pEAQ vector series (Sainsbury et al. 2009).

2.1.1 RNA silencing and the silencing suppressor P19

Transient expression of genes may be significantly limited by RNA silencing (Johansen & Carrington 2001). It is a highly conserved defense system among most eucaryotic organisms. In plants, RNA silencing is known as posttranscriptional gene silencing (PTGS) (Lakatos et al. 2004). In higher plants, PTGS has a role in protecting the whole plant. It can be triggered by a viral infection or transgene expression (Silhavy et al. 2002). The PTGS pathway can be divided into three steps. First, double-stranded (ds) RNA accumulation induces RNA silencing, and viral dsRNAs are converted into small interfering RNAs (siRNAs) by DICER-LIKE proteins. Second, siRNAs are amplified, and finally, antiviral RNA-induced silencing complex (RISC) is assembled and targeted viral RNAs (Burgyán & Havelda 2011).

By using RNA silencing suppressors, the limitations caused by RNA silencing can be overcome (Johansen & Carrington 2001). One of many RNA silencing suppressors and lots of studied is the 19 kDa protein (P19) of tombusviruses (Csorba et al. 2015). P19 functions by specifically binding 21-25 nucleotide (nt) long dsRNA molecules generated in PTGS and synthetic 21 nt ds siRNAs with 2 nt 3’ overhanging ends. Hence, P19 inhibits the movement of mobile silencing signal and prevents the forming of RISC (Silhavy et al. 2002).

There are many studies about the ability of P19 to enhance the transient expression levels of heterologous protein. In Lombardi et al. (2009) study, the goal was to enhance the production of HIV-1 Nef protein. They agroinfiltrated tobacco plants, and by using the combination P19 with Nef, they obtained 4.4-fold increase of Nef yield compared with Nef alone. Boivin et al. (2010) attempted to improve the yield of IgG1 protein. Their study demonstrated 2-fold increase in IgG1 production when P19 was infiltrated together with the expression vector compared to the expression vector alone. Mohammadzadeh et al. (2015) showed in their experiment that co-infiltration with P19 resulted in significantly higher expression levels of the HCVpc-HBsAg fusion protein than without P19.

2.2 pEAQ vectors

pEAQ vectors are easy and quick expression vectors made for transient expression developed by Sainsbury, Thuenemann and Lomonossoff (2009). pEAQ vectors were designed to be modular, which allows the insertion of multiple expression cassettes, including the silencing suppressor P19, into a single plasmid. pEAQ vectors are small binary vectors utilizing the backbone of pBINPLUS vector which was originally constructed by van Engelen et al. (1995). Sainsbury et al. (2009) determined that three regions of pBINPLUS were essential for a functioning binary vector: the transfer DNA (T-DNA) region, the origin of replication OriV, and a segment containing the neomycin phosphotransferase (NPT) II gene, the ColEI replication origin providing higher copy number in *Escherichia coli*, and a replication-essential locus TrfA for promoting replication. The vector size was reduced by removing more than 7 kb of non-essential sequence from the backbone.

CPMV RNA-2 was termed CPMV-hypertranslatable (HT) because high expression levels were result of enhanced translation instead of viral replication. It involves inserting the desired gene between the modified 5'-untranslated region (UTR) with the U162C mutation, and the 3'-UTR from CPMV-RNA-2. It was shown that modified 5'-UTR provided at least 10-fold increase in GFP levels than obtained with unmodified 5'-UTR (Sainsbury & Lomonossoff 2008). After cloning the wanted gene into the CPMV-HT system, it had to be transferred into the binary vector with Cauliflower mosaic virus 35S promoter and nopaline synthase (nos) terminator. It was a quite time-consuming procedure, so it was improved (Sainsbury et al. 2009).

pEAQ-HT vectors are expression vectors already containing the CPMV-HT cassette, and they are developed by Sainsbury et al. (2009). pEAQ-HT vectors were created to remove a two-step cloning procedure needed with pEAQ vectors. pEAQ-HT vectors include a construct containing a polylinker between a modified CPMV RNA-2 5'-untranslated region (UTR) and the 3'-UTR (Figure 1). The polylinker allows direct insertion of a gene of interest into the CMPV-HT cassette and the fusion of an N- or C-terminal Histidine (His) tag. The modified 5'-UTR also contains an additional mutation (A115G) which promotes higher expression levels than the modified 5'-UTR with only the U162C mutation.

Traditional restriction enzyme-based cloning might cause a bottleneck for high performance expression systems, so Sainsbury et al. (2009) created three Gateway-compatible pEAQ-HT destination vectors to ease this problem. The double-mutated (A115G/U162C) 5'UTR is included to these vectors for maximizing the expression. The expression is allowed for an unfused protein, a protein with N- or C-terminal His-tag by pEAQ-HT-DEST1, pEAQ-HT-DEST2 or pEAQ-HT-DEST3, respectively (Figure 1).

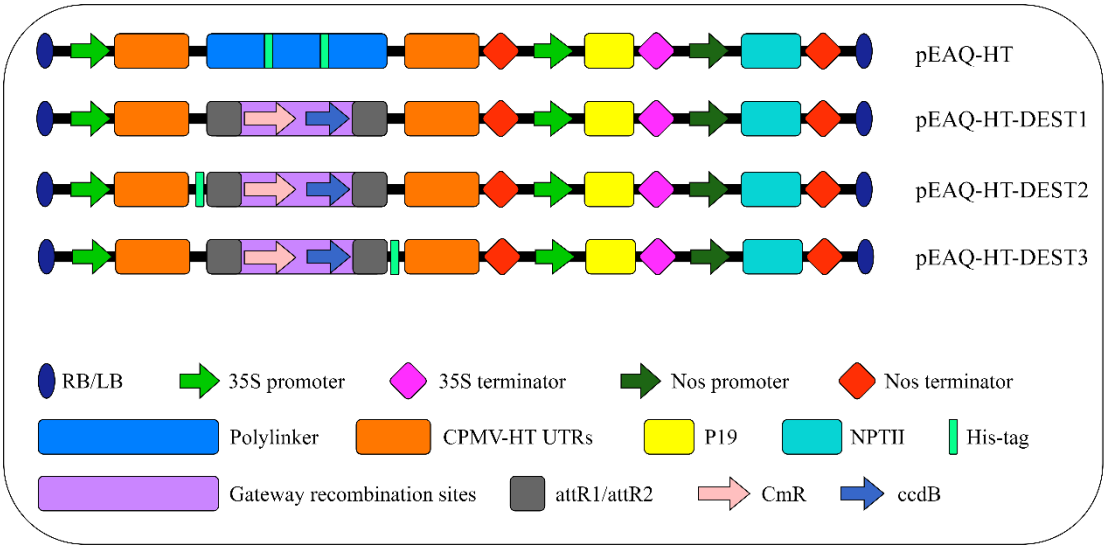

Figure 1. Diagrammatic representation of pEAQ-HT, pEAQ-HT-DEST1, pEAQ-HT-DEST2 and pEAQ-HT-DEST3 vectors and HT expression cassettes. The backbones are the same for all the vectors. CmR is chloramphenicol resistance gene, ccdB is *E. coli* lethal gene. Adapted from Sainsbury et al. (2009) and Peyret & Lomonossoff (2013).

2.3 *Agrobacterium*-mediated gene transfer

Agrobacterium is a gram-negative soil bacterium genus that includes many species, of which most are pathogens. *A. tumefaciens* is the most used species for gene transfer, and it causes crown gall disease in nature. If the plant is infected, its water and nutrient flow is interfered by the formation of galls on the plant stem. *A. tumefaciens* can infect most of the dicotyledonous plants in nature and many monocotyledonous species under laboratory conditions (Tzfira & Citovsky 2000).

Agrobacterium is the only known organism that can naturally transfer its DNA to the host plants (Tzfira & Citovsky 2000). Bacterial tumor-inducing (Ti) plasmid is an essential part required for gene transfer between *Agrobacterium* and a plant cell. Ti-plasmid has multiple regions with specialized tasks (Gelvin 2003). Especially two regions are required for plant genetic transformation: T-DNA and virulence (vir) region (Tzfira & Citovsky 2000). T-DNA sequences are defined by their right and left borders (Gelvin 2003). Vir genes located in vir region encode proteins that make T-DNA move (de la Riva et al. 1998).

Gene transfer from *Agrobacterium* to a plant cell is a complicated series of events. It begins with signal perception when *Agrobacterium* notices chemical signals from the activation of the host plant's defense mechanism. The main phenolic compound emitted by the host plant and regulating the virulence is acetosyringone (AS). *Agrobacterium* responds by changing the activation of its virulence system, and vir genes become transcriptionally activated (Lacroix & Citovsky 2013). Signal perception also induces the attachment of *Agrobacterium* to the host cell, which is a requirement for gene transfer (Citovsky et al. 2007). Activation of vir region produces a single-strand (ss) copy of T-DNA. ssT-DNA is produced by cutting it from its border sequences, and it is called T-strand. *Agrobacterium* transports T-strand through a type 4 secretion system to the host cytoplasm, where it is actively transported to the nucleus with the help from both bacterial effector proteins and plant proteins (Gelvin 2010). Nuclear pores have multiple physical and molecular barriers that T-strand must conquer before entering the nucleus. *Agrobacterium* uses the interaction between nuclear localization proteins from vir genes and importing α protein, which leads T-strand to proceed through the nuclear pore (Dafny-Yelin et al. 2008). For stable transformation, the second strand of T-DNA must be synthesized before integration can happen (Lacroix & Citovsky 2013). For transient expression, the non-integrated T-DNA remains transiently present and can be transcribed (Kapila et al. 1997).

2.3.1 Agroinfiltration

Agroinfiltration is a technique initially developed by Kapila et al. (1997). It is a simple and efficient method to induce transient expression on plant cells (Wydro et al. 2006). This can be done with vacuum or syringe infiltration. Vacuum infiltration was developed for species that cannot achieve sufficient results with syringe infiltration. Before vacuum infiltration, leaves are submerged into *Agrobacterium* suspension containing the gene of interest. When the plant is exposed to the vacuum, the air is drawn out from the interstitial space of the leaves. After that, the vacuum is released, and *Agrobacterium* suspension can enter the leaves (Chen et al. 2013).

Syringe infiltration is done with a syringe without a needle. First, a small slit is made with a sharp blade on the abaxial side of the leaf. Then Agrobacterium suspension containing the target gene is injected into the leaf with a syringe (Figure 2). The expression can be detected only a couple of days after infiltration (Chen et al. 2013). AS is a crucial element in the infiltration suspension buffer because it induces vir gene expression (Kanneganti et al. 2006).

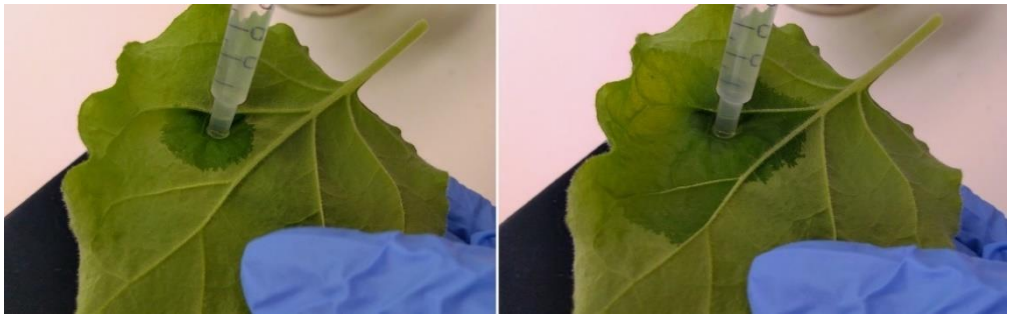

Figure 2. Agroinfiltration using a needless syringe. *Agrobacterium* suspension spreading inside the leaf is easily detectable.

2.4 G2PS1, 2 and 3 in polyketide synthases in *Gerbera hybrida*

Gerbera (*Gerbera hybrida*) belongs to the Asteraceae family, and besides being an important ornamental species, it is also a model plant for secondary metabolism and flower development (Teeri et al. 2006). Plant secondary metabolites are compounds that are not essential in life functions like growth and reproduction. Instead, they have a significant role in the plant's adaptation to the environment (Bourgaud et al. 2001).

Type III polyketide synthase (PKS) superfamily includes enzymes mainly found in plants but also in bacteria. Type III PKSs catalyze synthesis of several secondary metabolites in plants including chalcones, pyrones and stilbenes (Austin & Noel 2003). Gerberin, parasorboside and 4-hydroxy-5-methylcoumarin (HMC) are polyketide derivates produced in gerbera and they have a role in defending a plant against phytopathogenes and herbivores. Three PKSs specifically expressed in gerbera are involved in synthesis of these compounds: gerbera 2-pyrone synthase 1 (G2PS1), G2PS2 and G2PS3 (Pietiäinen et al. 2016).

G2PS1 is accountable for the biosynthesis of 6-methyl-4-hydroxy-2-pyrone (triacetolactone, TAL) which is a precursor of gerberin and parasorboside aglycones (Figure 3, part A). G2PS1 performs two condensation reactions between acetyl-CoA and malonyl-CoA (Eckermann et al. 1998). G2PS2 and G2PS3 also produce TAL from the same starter substrates. In addition, they are responsible for the biosynthesis of HMC and 4,7-dihydroxy-5-methylcoumarin (DHMC) (Figure 3, part B). DHMC is an unreduced precursor of HMC formed in tobacco (Pietiäinen et al. 2016).

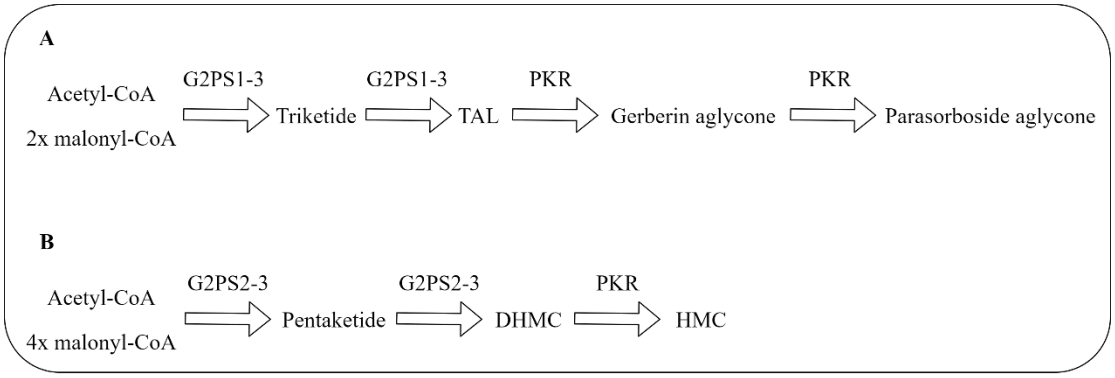

Figure 3. A. A pathway of G2PS1-3 producing TAL. Polyketide reductases (PKR) are needed to generate gerberin and parasorboside aglycones. B. Biosynthesis of DHMC and its reduction to HMC by PKR. Adapted from Pietiäinen et al. (2016).

3 RESEARCH OBJECTIVES

Research objectives are to subclone the genes of interest (G2PS1, G2PS2 and G2PS3) into pEAQ-HT-DEST1 and pEAQ-HT-DEST2. Next, by analyzing with western blot, we aim to see if the expression levels are higher with these vectors compared with pK2GW7 derived expression vectors. With ELISA assay, the aim is to quantify the amount of protein we can obtain with these vectors. Finally, we aim to observe TAL and DHMC in tobacco leaves by HPLC.

Previously used pK2GW7 derived expression vectors designed for stable transformation work reasonably well, but pEAQ-HT vectors provide high yields of recombinant protein through high translational efficiency. Tobacco and petunia should produce TAL and DHCM when G2PS1, 2 or 3 is expressed, but both have not yet been observed at the same time. Improved expression levels could reveal the missing compounds. This is important because if the synthesis could get started under agroinfiltration, co-transformation with reductases can lead to gerberin, parasorboside and HMC biosynthesis, hence proving function.

4 MATERIALS AND METHODS

4.1 Plant material

Tobacco (*Nicotiana benthamiana*) plants were used for infiltration. Plants were germinated from seeds in a soil containing peat and vermiculite in ratio 1:1. Plants were grown in a growth room at the temperature of 24 °C, the photoperiod was 16 hours, and the humidity was 65 %. Plants were watered twice a week with soluble fertilizer SUBSTRAL VitaPlus containing 6 % N, 1.3 % P and 5 % K. The infiltration was done when plants were 6-weeks old and at a 10-leaf stage.

4.2 Plasmid minipreps

To get the entry plasmid DNA, plasmid minipreps from entry vectors pHTT832, pHTT839 and pHTT836 were made using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Saint Louis, Missouri, USA) and following Experienced User Protocol from User Guide. Optional 500 µl Optional Wash Solution was included, and Elution Solution was reduced to 80 µl. After that, the concentrations from 1.5 µl purified plasmid DNA were measured using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Then plasmids were tested on 0.8 % agarose gel (appendix 1) electrophoresis with 0.5x TBE buffer (appendix 1) (1 h, 200 V). 2 liters of 1x TBE buffer with 80 µl EtBr (2.5 mg/ml) was added to the electrophoresis machine so that the gel was completely covered. Plasmids were digested in 1x FastDigest Green Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), MQ water and restriction enzymes, loaded into wells and added λ-PstI into own well as a size standard.

4.3 Gateway LR cloning to pEAQ-HT-DEST1 and 2

Both the entry vectors made in pDONR221 and the pEAQ-HT-DESTX destination vectors have kanamycin resistance as the bacterial selectable gene, and that is why the entry vector must be first linearized. pK2GW7 derived expression vectors were used as references and they already had all genes cloned into them by prof. Teemu Teeri. The names of entry vectors and expression vectors with corresponding genes are shown in Table 1. pEAQ-HT-DESTX derived expression vectors are produced in Gateway LR reaction described in 4.3.3.

Table 1. Entry vectors and expression vectors with corresponding genes.

Gene	Entry vector in pDONR221	pK2GW7 derived expression vector	pEAQ-HT-DEST1 derived expression vector	pEAQ-HT-DEST2 derived expression vector
G2PS1	pHTT832	pHTT842	pATU1	pATU11
G2PS2	pHTT839	pHTT847	pATU2	pATU12
G2PS3	pHTT836	pHTT846	pATU3	pATU13

4.3.1 Linearization of the entry plasmid

Enzyme for linearization was selected to cut the backbone of the entry plasmid but not the gene of interest between the attL sites. The total volume of 30 µl was made for each construct, including 3 µl of 10x Fast buffer (clear) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1000 ng of entry vector according to concentration, MQ water according to DNA volume and 0.6 µl of PvuII enzyme. The components were added together and incubated for 30 minutes at 37 °C. Next, the mixtures were incubated for 10 minutes at 65 °C to inactivate the enzyme. Plasmids were tested on 0.8 % agarose gel electrophoresis (described in 4.2) to check that enzyme does not cut the insert.

Linearization of pHTT832 was done twice. On the first time, the total volume was 15 µl, the plasmid was used 500 ng and the enzyme was Pvu1. Gateway LR reaction to pEAQ-HT-DEST1 (described in 4.3.3) was done with this first plasmid. Second linearized pHTT832 was extracted from the gel (described in 4.3.2), and it was used in LR reaction with pEAQ-HT-DEST2 (described in 4.3.3).

4.3.2 Isolation of the linear plasmid from gel

Linear plasmids were isolated from the gel by using GelJet™ Gel Extraction Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purification protocol A: DNA extraction from the gel using centrifuge was followed. Optional steps were not used, and elution was made into 25 µl of TE (appendix 1) instead of Elution buffer. Concentrations were measured with NanoDrop™ Lite Spectrophotometer.

4.3.3 Gateway LR reaction

For each reaction, 150 ng of one entry plasmid and 150 ng of one destination vector were needed according to Table 1. Gateway™ LR Clonase™ Enzyme Mix (Invitrogen Corporation, Carlsbad, California, USA) was used for LR reaction. The total volume of 10 µl was made for each construct, including 2.5 µl of pEAQ-HT-DEST1/2 in TE, 5.5 µl of entry plasmid in TE and 2 µl of LR clonase. The exception was pHTT832 entry vector with pEAQ-HT-DEST1 destination vector. Volumes for that reaction were the same except entry plasmid in TE was 3 µl and TE was 2.5 µl. The components were added together and incubated for 3 hours at room temperature. Then 1 µl of Proteinase K was added to each reaction, and they were incubated for 10 minutes at 37 °C.

Competent DH5α *E. coli* cells were used for kanamycin resistance selection. DH5α cells were recovered from -80 °C and thawed on ice for 15 minutes before use. 5 µl of DNA products produced in LR reaction were added to each DH5α cell tube, mixed and incubated on ice for 30 minutes. A heat shock at 42 °C for 30 seconds was given to the tubes, and after that, the tubes were returned to the ice. 900 µl of SOB (appendix 1) (650 µl of SOB to pHTT832 in pEAQ-HT-DEST1) was added into the tubes and incubated for 30 minutes at room temperature. Next, the tubes were spun in a microcentrifuge for 5 minutes at 2500 xg, the supernatant was discarded, and the remaining drop in each tube was resuspended. The cells were plated on Petri dishes containing LB medium (appendix 1) with 50 µg/ml kanamycin and grown overnight at 37 °C.

Pure cultures were made on LB plates with 50 µg/ml kanamycin. The plates were divided into six sections, and four big and two small colonies from each construct were transferred to the new plate. The plates were incubated overnight at 37 °C, and the clones were tested with restriction enzymes using STET protocol.

4.3.4 STET protocol

Boiling prep for plasmids from *E. coli*, called STET protocol, was used for checking the clones. 50 µl of STET buffer (appendix 1) was pipetted in Eppendorf tubes, and the tubes were placed on ice. Avoiding any agar, a fair amount of bacteria was scraped

from the plate and resuspended in STET buffer by vortexing. Single colonies were left on the plates so that further propagation of the clones was possible. Next, 4 µl of lysozyme (10 mg/ml in water) was added to each tube, and the tubes were boiled in a water bath for 50 seconds. After that, the tubes were spun for 10 minutes at 17000 xg. The sticky pellet was removed, 50 µl of cold isopropanol was added to the tubes and incubated for 10 minutes at -20 °C. Then the tubes were spun for 10 minutes in a cold room, the supernatant was removed, 500 µl of cold 70 % ethanol was added to each tube, and the tubes were spun for 1 minute. Ethanol was removed, and the pellets were dried in a vacuum for 5 minutes. After that, the pellets were resuspended in 20 µl of TE for a minimum of 20 minutes.

4 µl of suspension was used for testing the preps in the gel (described in 4.2) with RNase in 5x TD (10 µl of 10 mg/ml stock into 1 ml of 5x TD). The proper enzymes were used for digestions. Two promising clones from each plasmid were selected, new pure cultures were made on LB plates with 50 µg/ml kanamycin, and the plates were incubated overnight at 37 °C. Minipreps were prepared, DNA concentrations were measured, and plasmids were checked in the gel (described in 4.2) with more restriction enzymes. After that, one was discarded, and the other was selected as an accepted plasmid. They were given names (Table 1), and the strains were stored in glycerol at -80 °C.

4.4 Transformation of expression plasmids to *Agrobacterium*

Expression vectors produced in Gateway LR reaction were transformed into *Agrobacterium tumefaciens* strain C58C1(pGV2260). Before the transformation, competent *A. tumefaciens* cells were prepared by prof. Teemu Teeri using the following procedure. Fresh pure culture of C58C1(pGV2260) was made on an LB plate containing 100 µg/ml rifampicin and 100 µg/ml carbenicillin and incubated for 3 days at 28 °C. A single colony was inoculated with 1 ml of LB medium and grown overnight at 28 °C with shaking. Then, 1 ml of the overnight culture was inoculated with 50 ml of LB medium and shaken at 28 °C 5-6 hours until OD_{600} reached 0.500. The cells were cooled on ice and pelleted for 10 minutes at 1600 xg at 4 °C. After that, the cells were resuspended in 15 ml of ice-cold 20 mM $CaCl_2$, pelleted again, and resuspended in 750

µl of ice cold 20 mM $CaCl_2$. The suspension was kept on ice overnight, 250 µl of cold 87 % glycerol was added, mixed well, divided into 100 µl aliquots, frozen in liquid N_2, and stored at -80 °C.

4.4.1 DNA transformation into *Agrobacterium*

Competent *Agrobacterium* cells were recovered from -80 °C and thawed on ice for 15 minutes. 100 ng of expression plasmids were added to the tubes, mixed, and kept on ice for 5 minutes. The tubes were frozen in liquid N_2, thawed in a 37 °C water bath for 5 minutes, and taken on the ice. 1 ml of LB medium was added to the tubes, and they were kept on a bench for 30 minutes to express the selectable markers. Then, the cells were centrifuged for 5 minutes at 4900 xg, the supernatant was discarded, and the cells were resuspended in the remaining ca. 100 µl. The suspensions were plated on selection plates containing LB with 100 µg/ml rifampicin, 100 µg/ml carbenicillin and 100 µg/ml kanamycin. The plates were incubated for 3 days at 28 °C. From each plate, two colonies were picked, made new pure cultures, and incubated for 3 days at 28 °C. One colony from each plate was inoculated with 5 ml of LB medium, and new pure cultures were made from the same colonies. Inoculations were grown overnight at 28 °C, and pure cultures were grown 3 days at 28 °C. Plasmid minipreps were made from inoculations and checked on the gel as described in 4.2. The names of accepted constructs are shown in Table 2. pK2GW7 derived expression vectors in *Agrobacterium* were already made by prof. Teemu Teeri.

Table 2. DNA constructs for agroinfiltration.

Gene	pK2GW7 derived expression vector in *Agrobacterium*	pEAQ-HT-DEST1 derived expression vector in *Agrobacterium*	pEAQ-HT-DEST2 derived expression vector in *Agrobacterium*
G2PS1	TAT842	TATU1	TATU11
G2PS2	TAT847	TATU2	TATU12
G2PS3	TAT846	TATU3	TATU13

4.5 Agroinfiltration

pEAQ-HT-DEST vectors already contain the silencing suppressor 35S-P19 in the plasmid, but pK2GW7 derived vectors do not. Thus, they must be coinfiltrated with *Agrobacterium* strain P19 = C58C1(pGV2260, pBin61-P19).

Pure cultures were made from all expression vectors in *Agrobacterium* and P19 and grown 3 days at 28 °C. From fresh pure cultures, a single colony was inoculated with 5 ml of LB. Inoculations were grown overnight at 28 °C with shaking. 2 ml Eppendorf tubes were filled with inoculations, the tubes were spun for 10 minutes at 4900 xg, the supernatant was discarded, and the pellets were resuspended in 800 µl of Mg-MES-AS buffer (appendix 1). After that, 150 µl of suspension was diluted to 2.85 ml of Mg-MES-AS buffer, OD_{600} was measured and adjusted to 0.500. Then the suspensions were kept at room temperature for 3 hours.

TAT842, TAT847 and TAT846 were mixed with P19 in ratio 1:1. Infiltration was done on the abaxial side of the two uppermost *N. benthamiana* leaves. A small cut was made on the leaf using a sharp blade, and the suspension was infiltrated with a 1 ml plastic syringe without a needle. The plants were kept in the growth room, and samples were collected two days and seven days after infiltration. The samples were taken using a cork bore which was punched through the leaf. The leaf disk was 5.5 mm in diameter, and one sample contained two leaf disks. Control samples were taken similarly from uninfiltrated leaves. The samples were placed into Eppendorf tubes and stored at -20 °C until analyzed. The infiltrated and sampled leaves were also cut off and stored at -20 °C until analyzed with HPLC.

4.6 Western blotting

4.6.1 Preparation of plant protein extracts

The leaf samples were taken from the freezer and placed in a box with dry ice. 100 µl of Extraction buffer Exp7.5 (appendix 1) was added to each tube, the samples were ground with a small pestle, and the tubes were placed on ice. Next, the tubes were spun for 5 minutes at 17000 xg at 4 °C. Protein concentration was measured from the

supernatant using Bradford assay. 195 µl of MQ water was pipetted into wells of the 96-well flat bottom plate, and 5 µl of the sample was added. 5 µl of Extraction buffer was used for blank samples, and 200 µl of MQ water only was used in the last sample. 50 µl of Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Hercules, California, USA) was added to the wells and mixed well with a pipette. Absorbance was measured at 595 nm against an Exp7.5 blank using iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories Inc., Hercules, California, USA). Next, protein concentration was calculated by assuming 1 A_{595} = 8 µg protein, so 8 * A_{595} = amount of protein in 5 µl of the sample. Then was calculated how much sample corresponds to 5 µg of protein using formula 5 / (µg of protein in 5 µl) * 5. The resulted number was corrected for 4xSB (appendix 1) multiplying by 4/3, and that was the volume of the gel sample (5 µg of soluble protein).

4.6.2 Running the gel

Bio-Rad's ready-made gel (Bio-Rad Laboratories Inc., Hercules, California, USA) was used for running the gel. The comb and the tape from the bottom were carefully removed, and the gel was assembled in the gel gasket. Running buffer (appendix 1) was used to fill the gasket, rinse the wells using a syringe, and fill the lower container. 1/3 volume of 4xSB was added to each sample, they were heated at 98 °C for 5 minutes, and the debris was spun down. The samples were loaded in the gel along with Bio-Rad's Precision Plus Protein Standard (Bio-Rad Laboratories Inc., Hercules, California, USA), and the gel was run for 30 minutes at 200 V. After that, the gel cassette was cracked open, and the gel was placed on a box.

4.6.3 Transfer of the proteins to the membrane

The gel was shaken for 10 minutes in Transfer buffer (appendix 1). Blotting was done on Amersham™ Hybond-ECL membrane (GE Healthcare, Chicago, Illinois, USA). A sheet of nitrocellulose membrane was cut to size to fit the gel. Eight slightly smaller pieces of filter paper were cut, of which four were wet with Transfer buffer. They were placed on a lower plate of Bio-Rad Trans-Blot Turbo Tray and checked for bubbles

with a roller pin. The membrane was wet in Transfer buffer, placed on the filter paper stack, checked for bubbles, and then the gel was placed on the membrane. Another stack of four filter papers was wet in Transfer buffer and placed on the gel, checked for bubbles, and placed the tray in the Trans-Blot Turbo machine (Bio-Rad Laboratories Inc., Hercules, California, USA). Blotting was run for 30 minutes at 25 V, 1.0 A. After the blotting, the membrane was moved into the box with tap water.

4.6.4 Ponceau S staining of the membrane

Ponceau S staining was optional, but it was used for checking both equal loading and even transfer. Water was poured off, ca. 100 ml of Ponceau S (appendix 1) was added into the box, shaken for 10 minutes at room temperature, and then the stain was poured back to the bottle. Excess stain was rinsed away with tap water, ca. 75 ml of water was added into the box and shaken for 15 seconds. The above step was repeated to remove the background, and then excess water was removed from the membrane with a paper towel. The membrane was placed on a glass plate, quickly photographed, and placed back into the box with tTBS (appendix 1).

4.6.5 Blocking of the membrane and antibody incubations

The membrane was washed with tTBS for 5 minutes at room temperature. tTBS was poured off, 25 ml of Blocking buffer (appendix 1) was added and shaken for 1 hour. After that, the membrane was washed with 100 ml of tTBS for 10 minutes. Primary antibody was prepared by diluting 4 µl of Rabbit anti-2PS antibody in 10 ml of Blocking buffer. The membrane was carefully rolled and placed in a 50 ml Falcon tube with a protein side facing the center of the tube. Primary antibody was added, and the tube was placed in the roller overnight at 4 °C.

The membrane was transferred back to the box, rinsed with tTBS and washed 3 x 10 minutes with 100 ml of tTBS. Secondary antibody was prepared by diluting 1.25 µl of HRP-linked Anti-rabbit IgG in 10 ml of Blocking buffer. The membrane was incubated with it for 1 hour at room temperature. Then the membrane was washed 4 x 10 minutes

with 100 ml of tTBS. A double-layered plastic sheet was cut to size clearly larger than the membrane, and it was placed in the film cassette. ECL™ western Blotting Detection Reagent (GE Healthcare, Chicago, Illinois, USA) was used, and 200 µl of ECLA solution was mixed with 200 µl of ECLB solution. The membrane was taken from tTBS, dried between paper towels, and placed on a glass plate. 400 µl of reagent was pipetted on the membrane and spread evenly using a roller pin. The surface of the membrane was dried, and the membrane was placed between the plastic sheets in the film cassette. The cassette was closed and taken to the film developing room with a box of Amersham Hyperfilm ECL (GE Healthcare, Chicago, Illinois, USA). Two exposures were made: 2 seconds and 2 minutes.

4.7 Sandwich ELISA

Sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify the antigen levels. The first antibody was prepared by diluting 20 µl of α-2PS IgG C3 (4.8 mg/ml) in 10 ml of Coating buffer (appendix 1). An ELISA plate was pre-coated with 100 µl of Coating buffer with the first antibody to each well and incubated for 3 hours at 37 °C. Next, the plant protein extracts were prepared. The leaf samples were taken from the freezer and placed on ice. 200 µl of PVP-PBST buffer (appendix 1) was added to the tubes, and the samples were ground with a small pestle. The tubes were spun for 5 minutes at 17000 xg at 4 °C. Protein concentration was measured from the supernatant using Bradford assay as described in 4.6.1. The absorbance values were converted to µg protein by multiplying with 8.

After 3 hours, the coated ELISA plate was washed with PBST (appendix 1) 3 x 3 minutes. Next, a dilution series of the samples with ¼ intervals was made. 100 µl of PVP-PBST was pipetted to the dilution wells 2, 3 and 4. 100 µl of the undiluted sample was pipetted to the dilution well 1. 33 µl of the undiluted sample was pipetted to the dilution well 2 and mixed well with a pipette. 33 µl of the diluted sample from well 2 was pipetted to the dilution well 3 and mixed well with a pipette. 33 µl of the diluted sample from well 3 was pipetted to the dilution well 4, mixed well with a pipette and removed 33 µl from well 4. The plate was incubated overnight at 37 °C.

The plate was washed with PBST 3 x 3 minutes. The second antibody was prepared by diluting 16 µl of α-2PS IgG-Prx 2 (450 µg/ml) in 10 ml of Antibody buffer (appendix 1). 100 µl of Antibody buffer with the second antibody was pipetted to each well, and the plate was incubated for 3 hours at 37 °C. After that, the plate was washed with PBST 3 x 3 minutes. 100 µl of the peroxidase substrate TMB-ELISA was pipetted to each well, and the plate was incubated for 30 minutes at room temperature. Absorbance at 655 nm was measured with Bio-Rad's iMark Microplate Reader (Bio-Rad Laboratories Inc., Hercules, California, USA) operated through the computer. Next, 100 µl of 2 M H_2SO_4 was pipetted to each well, and absorbance at 450 was measured with the plate reader.

4.8 HPLC

High-performance liquid chromatography (HPLC) was used to analyze secondary metabolites. The leaves were taken from the -20 °C freezer, placed the tubes on a liquid nitrogen and crushed the tissue with a glass rod. 5 times of volumes of methanol was added and the tubes were sonicated in a dish sonicator for 15 minutes. After that, the tubes were centrifuged for 10 minutes at 3220 xg, 1 ml of sample was moved to an Eppendorf tube and centrifuged for 10 minutes at 17000 xg. 700 µl of the supernatant was taken in HPLC ampules and HPLC was run with appropriate compounds using Agilent 1100 machine (Agilent technologies, Santa Clara, California, USA). Detection was done at 210 nm and 280 nm.

5 RESULTS

5.1 Gateway LR reaction

Constructs were made by Gateway LR reaction. The constructs were digested with appropriate enzymes shown in Figure 4, part A and tested in 0.8 % agarose gel electrophoresis. Gene fragments from the gel picture were the same as the expected fragments (Figure 4, part B), which shows that the constructs were correct and Gateway LR reactions were successful. DNA concentrations of accepted plasmids are shown in Table 3.

Table 3. Names and DNA concentrations of accepted pEAQ-HT-DEST1/2 derived expression vectors.

Expression plasmid	Concentration, ng/µl
pATU1	100
pATU2	205
pATU3	148
pATU11	177
pATU12	140
pATU13	102

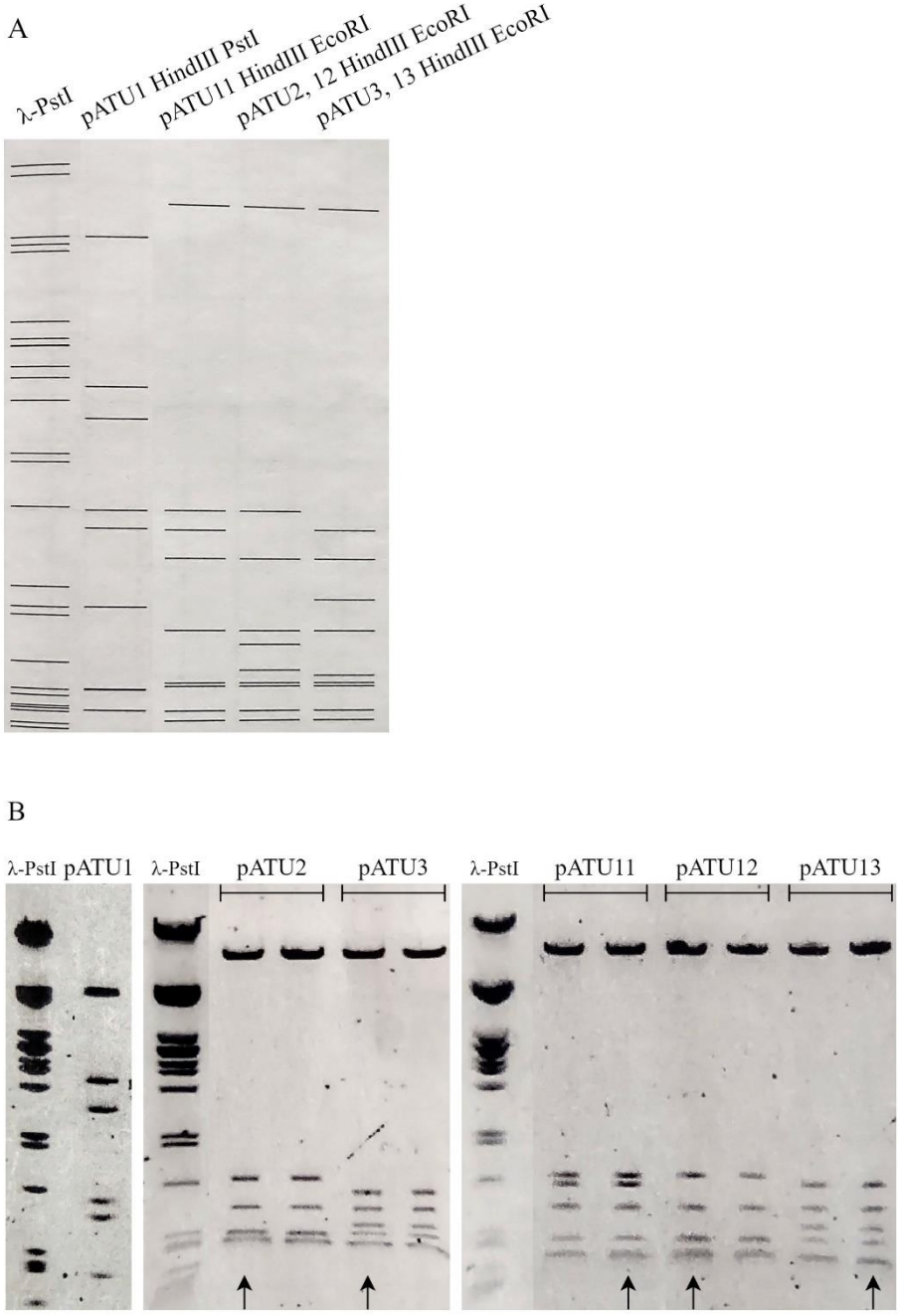

Figure 4. A. Expected fragments and enzymes used for digestion. B. Gel picture of enzyme digestions of constructs. Arrows show the accepted plasmids of each construct.

5.2 Western blotting

Western blot analysis was made with 2 seconds and 2 minutes exposures. The signal was already strong enough at 2 seconds exposure to get the results. TATU constructs showed stronger signal from each sample compared to TAT constructs (Figure 5). Bands are shown very clearly, and they are visible for each sample. Samples collected on day 7 showed higher expression levels than samples collected on day 2. Constructs made with pEAQ-HT-DEST1 and 2 showed relatively similar results between each other.

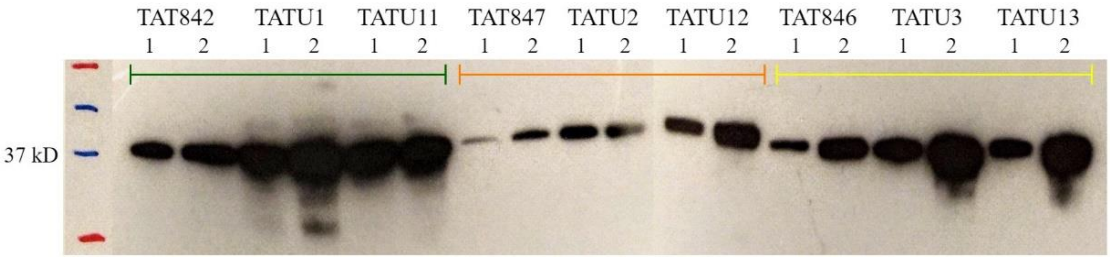

Figure 5. Western blot analysis, 2 seconds exposure. Samples collected at day 2 are marked as 1 and samples collected at day 7 are marked as 2. Correct sized bands are shown at above 37 kD from all the samples.

5.3 ELISA

Quantitative results were not obtained from ELISA. The signal was saturated, so the only thing we could get from those was that pEAQ-HT vectors provided a lot more protein than comparative vectors.

5.4 HPLC

TAL was visible in all samples and results presented in Figures 6, 7 and 8 are from detection at 210 nm. Uninfiltrated and empty agro were used as a comparison and the samples were also compared with reference TAL peak at the retention time 4.047 minutes (Figure 9). The samples were between 4.571- 4.607 minutes, so they can be

confirmed as TAL. G2PS2 and 3 should also produce DHMC, and the expected peak should have been at 9.405 minutes, but it was not seen in any of the samples.

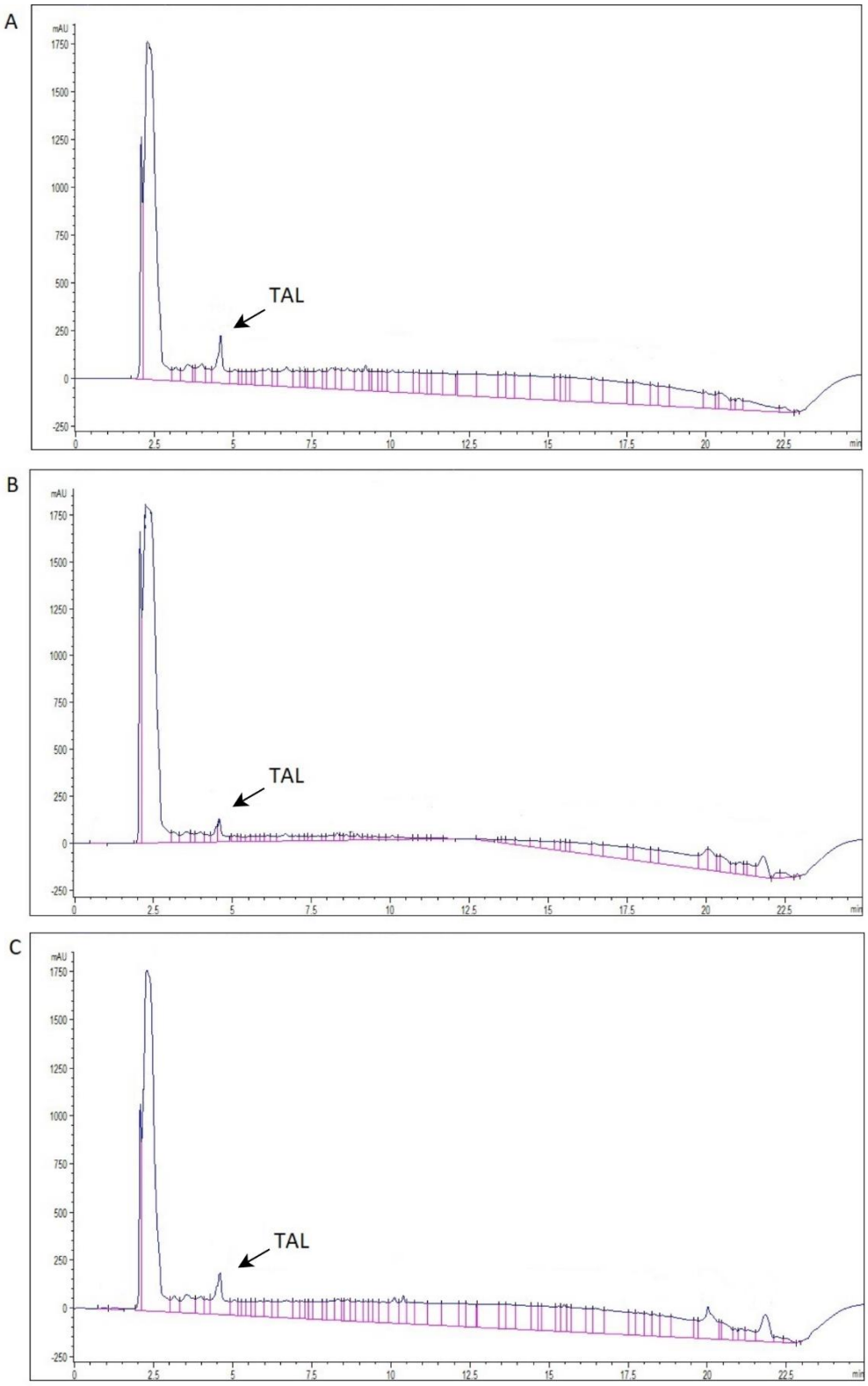

Figure 6. G2PS1 constructs show TAL, detection at 210 nm. A. TAT842 4.594 min, B. TATU1 4.582 min and C. TATU11 4.588 min.

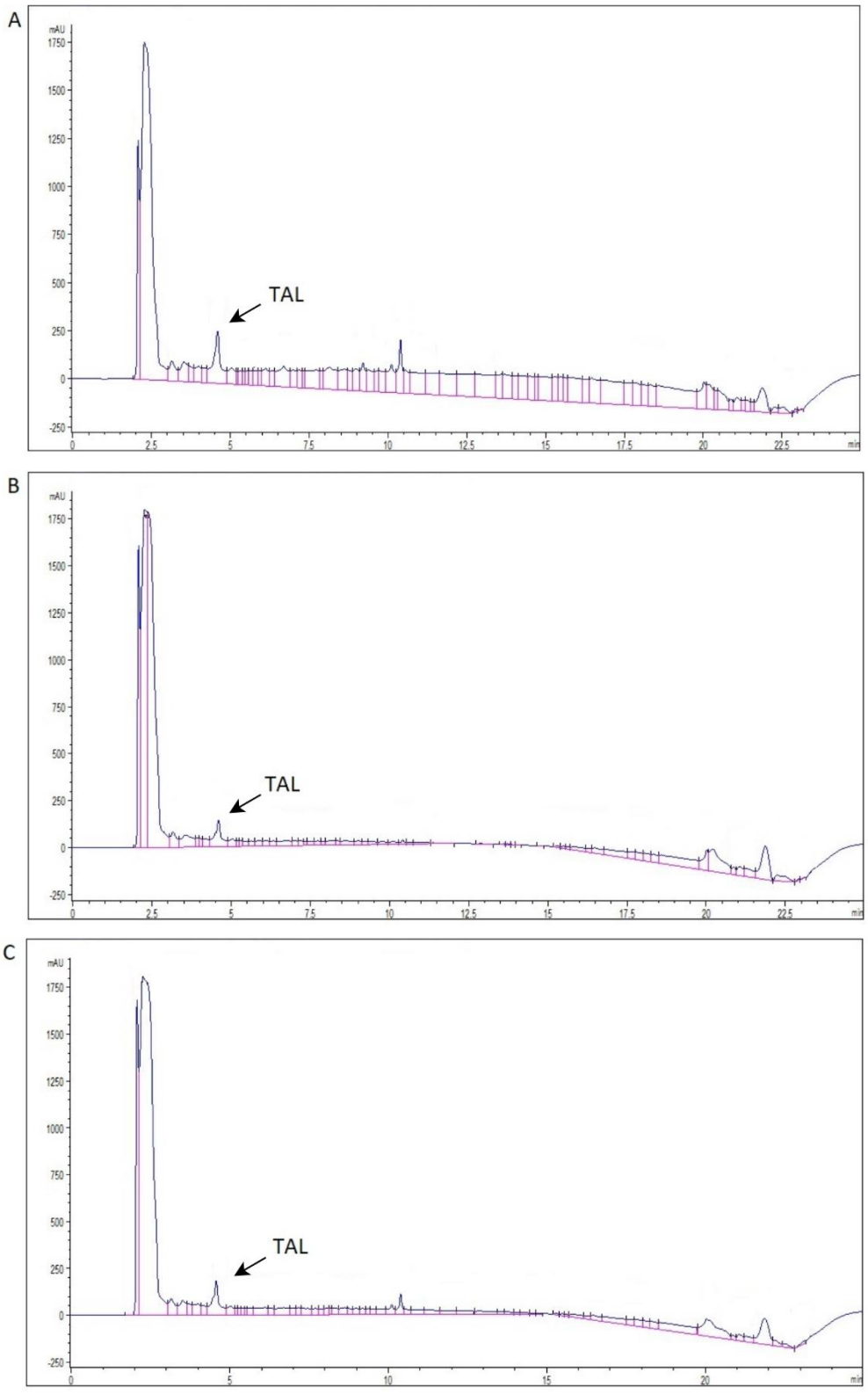

Figure 7. G2PS2 constructs show TAL, detection at 210 nm. A. TAT847 4.594 min, B. TATU2 4.595 min, C. TATU12 4.571 min.

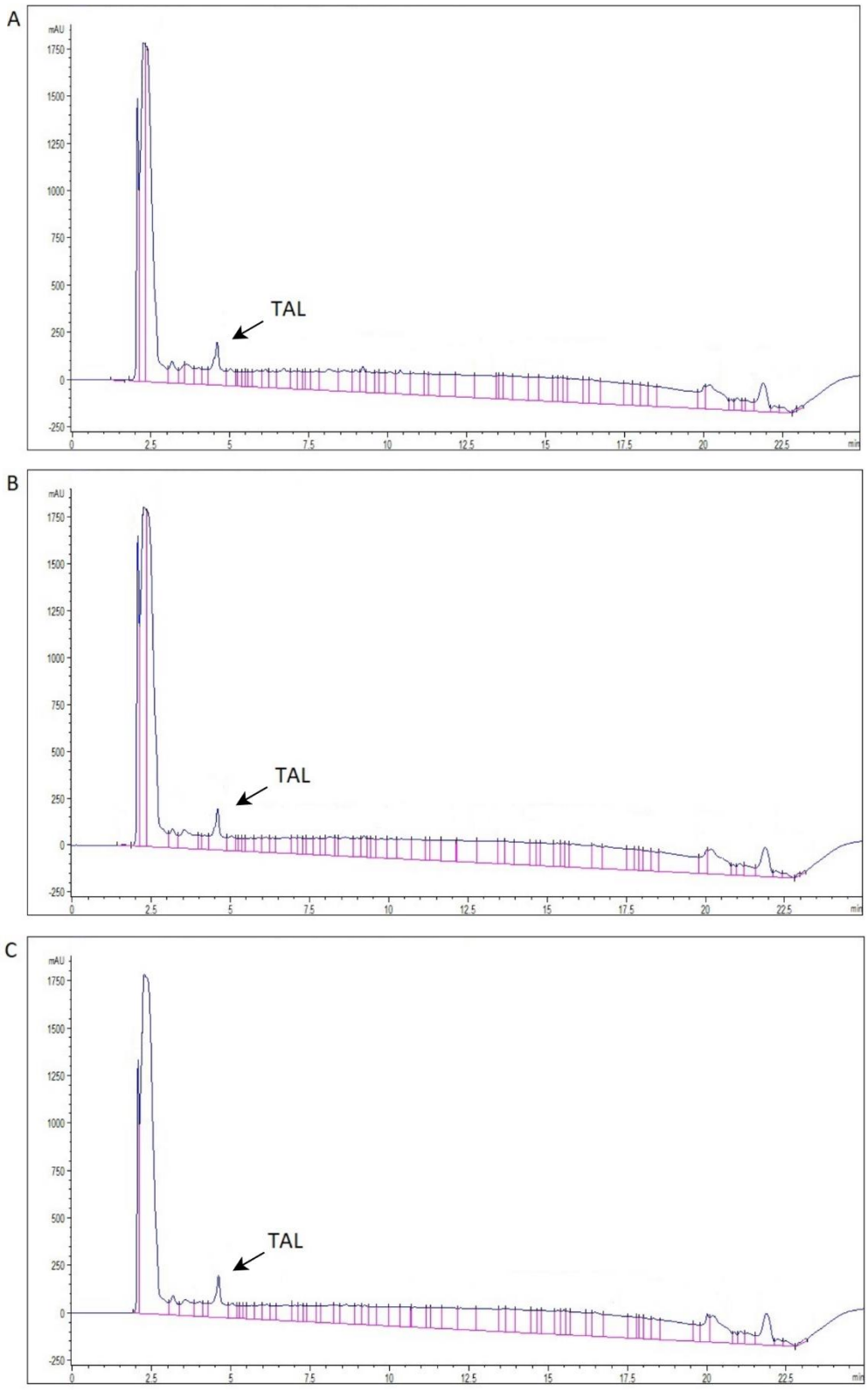

Figure 8. G2PS3 constructs show TAL, detection at 210 nm. A. TAT846 4.595 min, B. TATU3 4.591 min, C. TATU13 4.607 min.

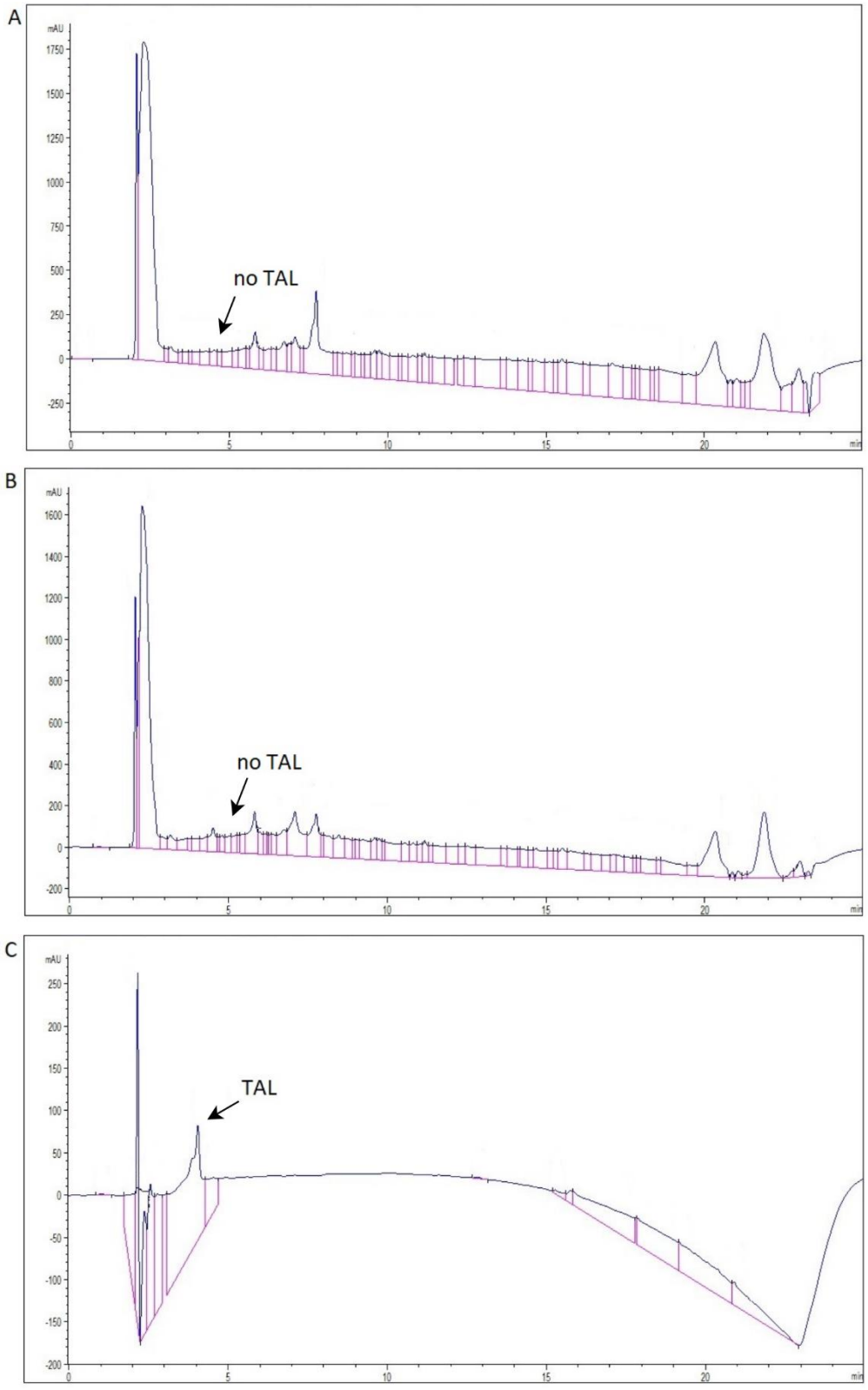

Figure 9. A. Uninfiltrated control, detection at 210 nm (Mamunur Rashid), B. Empty agro, detection at 210 nm (Mamunur Rashid), C. TAL 10 µg/ml 4.047 min, detection at 210 nm.

6 DISCUSSION

Western blotting showed results as expected, and results can be considered quite reliable as it was done twice, and results did not change between them. All the pEAQ-HT derived constructs showed higher expression levels than comparative pK2GW7 derived constructs. Obviously more testing would be required for more demanding purposes, but twice was enough for this work. ELISA was also done twice, and even though we did not get quantitative data as it was supposed to, it did provide information about pEAQ-HT vectors' better performance over comparative vectors. The second time two extra dilutions were done for TATU1 and TATU11, but it was not enough. More dilutions would have been required for finding the correct range of values for the signal, and only after that it would have been possible to analyze data for quantitative results. DHMC was not shown in this experiment, and the samples had been in the freezer for two years, so the results from HPLC might not be reliable. Even though all the aims were not met, western blotting and ELISA clearly showed improved expression with pEAQ-HT vectors, thus confirming earlier studies' results about pEAQ-HT vectors providing higher expression than comparative binary vectors.

Love et al. (2012) reported the first successful Bovine papillomavirus vaccine candidate produced in plants. They used a pEAQ-HT based system, and by transient expression, they obtained 4-fold higher yields than the basic level needed for economic production. Sun et al. (2011) demonstrated in their study that pEAQ-HT vectors also work for stable high-level expression of proteins. By producing recombinant human serum albumin in transgenic tobacco cells, they obtained 20-40 times higher yields than from other recombinant proteins produced with the same cell line. That indicates the potentially important role of pEAQ-HT vectors in improved protein production and could make it possible to produce pharmaceutical proteins in this system.

Shah et al. (2013) compared transient expression vectors in their study. Their results showed that pEAQ-HT vectors were among two other vectors showing the highest expression levels. pEAQ expression system has also been used to produce enzymes. Kanagarajan et al. (2012), Miaymoto et al. (2012) and Vardakou et al. (2012) all reported the production of high titres of enzyme by using pEAQ-HT vectors.

6.1 Improvements

Even though pEAQ-HT vectors have proven to improve recombinant protein production, there is always room for improvement. Berthold et al. (2019) studied these vectors and created 15 new pEAQ-HT-DEST1-based plasmids which utilize Gateway technology. They were categorized into three subsets: A, B and C. Subsets A and C were designed for high expression of recombinant proteins. The A and B vectors allow the subcellular localization of proteins, providing knowledge about their function. C vectors were designed to allow post-translational modifications of proteins, like glycosylation, which are produced through the secretory pathway. After testing the vectors in multiple ways, Berthold et al. conclude that all the new vectors were found functional.

Another improvement was shown by Norkunas et al. (2018), who aimed to improve expression levels delivered by pEAQ-HT vectors. They presented in their study a new dual vector delivery system using pEAQ-HT deconstructed vector system together with GUS (β-glucuronidase) reporter. After optimizing and combining the most effective components for transient expression by agroinfiltration, they showed 3.5-fold higher levels of absolute GUS protein using a new dual vector system compared to pEAQ-HT vector.

Peyret et al. (2019) presented a rational design of new synthetic 5’ and 3’UTRs. These novel UTRs can be used as expression modulators and it was shown that synthetic 5’UTRs outperform the modified 5’UTR of the CPMV-HT system. They also created novel expression vectors named pHRE and pHREAC based on their results. pHRE gave similar expression levels as pEAQ-HT and it is ideal for rapidly testing new combinations of UTRs. pHREAC was created to give maximum yield of recombinant protein. Peyret et al. (2019) conclude that in the future, these new vectors might facilitate the development of even better plant transient expression vectors.

7 CONCLUSIONS

Transient expression is a popular way to produce proteins. Because there is no need to produce new lines like with stable transformation, transient expression is a rapid method. Agroinfiltration is a preferred method to induce transient expression, and syringe infiltration can be easily conducted.

pEAQ-HT vectors have provided a valuable tool for both research and industrial views. Compared to earlier binary vectors, the smaller size does not compromise transient expression, and pEAQ-HT vectors are easy to use since there is no need for a two-step cloning process. Western blot was successful experiment and results clearly showed stronger signals from pEAQ-HT vector constructs. The aim with ELISA was not met, but it was still clearly visible that there was much more protein produced with pEAQ-HT vectors than with pK2GW7 derived constructs. HPLC showed only TAL, and DHMC was not visible. Even though all the research objectives were not completely met, this study showed that pEAQ-HT would be a better choice over conventional binary plant vectors.

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APPENDIX 1: MEDIA AND BUFFERS

0.8 % agarose gel (300 ml)

300 ml 1x TBE

2.4 g agarose

Heat in microwave until agarose is dissolved, allow to cool to 65 °C in a water bath and add 12 µl EtBr (2.5 mg/ml). Pour the mixture into gel tray with well comb and allow to solidify.

10 x TBE

108 g Tris base

55 g Boric acid

9 g 0.5 M EDTA, pH 8

1000 ml RO water. Use as 0.5x TBE.

TE

10 ml 1 M Tris-Cl, pH 8

1 ml 0.5 M EDTA, pH 8

1000 ml RO water.

SOB

0.186 g KCl

2.4 g $MgSO_4$

0.5 g NaCl

20 g Tryptone

5 g Yeast extract

1000 ml RO water. Autoclave 20 min 120 °C.

LB medium

10 g Tryptone

5 g Yeast extract

10 g NaCl

1000 ml RO water. For plates, add 15 g agar. Autoclave 20 min 120 °C.

STET buffer

8 %	sucrose	40 ml	20 % sucrose
5 %	Triton X-100	5 ml	100 % Triton X-100
50 mM	Tris-Cl, pH 8	5 ml	1 M Tris-Cl, pH 8
50 mM	EDTA, pH 8	10 ml	0.5 M EDTA, pH 8
		100 ml	RO water

Mg-MES-AS buffer

1 ml	1 M $MgCl_2$
1 ml	1 M MES, pH 6
40 µl	0.5 M AS in DMSO

100 ml RO water.

Extraction buffer Exb7.5

50 mM	Tris-Cl, pH 7.5	0.5 ml	1 M Tris-Cl, pH 7.5
1 %	β-Mercapto ethanol	100 µl	β-ME (just before use)
	Protease inhibitors	1 tablet	Roche "Protease Mini EDTA free" protease inhibitor cocktail
		10 ml	MQ water

4xSB

100 mM	Tris-Cl, pH 6.9	2.5 mM	Tris-Cl, pH 6.8
20 %	Glycerol	5.8 ml	87 % Glycerol
4 %	SDS	1 g	SDS
10 %	β-Mercapto ethanol	2.5 ml	β-ME
0.2 mg/ml	Bromphenol blue	5 mg	Bromphenol blue
		25 ml	MQ water

Store aliquoted at -20 ºC.

10x Running buffer

30.3 g	Tris base

144.1 g Glycine
10 g SDS
add 1000 ml RO water
Dilute to 1x with RO water.

Transfer buffer

3.0 g Tris base
15.0 g Glycine
200 ml Methanol
add 1000 ml RO water

tTBS buffer

20 mM Tris-Cl, pH 7.5
150 mM NaCl
0.1 % Tween 20

20 ml 1 M Tris-Cl, pH 7.5
30 ml 5 M NaCl
1 ml Tween 20
1000 ml RO water
Pipette Tween 20 with a cut pipette tip.
Prepare 1 liter per gel.

Blocking buffer

5 % Nonfat dry milk powder

5 g Nonfat dry milk powder
100 ml tTBS
Mix with magnetic stirrer for 30 min.
Prepare 50 ml per gel.

Ponceau S stain

0.2 g Ponceau S
5 ml Glacial acetic acid
200 ml RO water
Keep at 4 ºC, the stain can be reused several times.

Coating buffer, pH 9.6

0.16 g Na_2CO_3

0.29 g $NaHCO_3$

Dissolve in 90 ml sterile MQ, adjust pH to 9.6 with HCl and fill up to 100 ml with sterile water.

10x PBS, pH 7.4

80 g NaCl

2 g KH_2PO_4

29 g $NaHPO_4$ $2H_2O$

2 g KCl

Dissolve in 900 ml sterile MQ, adjust to pH 7.4 and fill up to 1 liter.

Note: working solution is always 1x PBS.

PBST

Add 0.5 ml Tween 20 in 1 liter of 1x PBS.

Note: Tween 20 is very viscous and sticky, use a tip with a cut end and mix well.

PVP-PBST

Add 1 g of PVP to 50 ml PBST.

Mix well with stirring.

Antibody buffer

Add 250 mg dry skimmed milk powder to 10 ml PBST.

Mix well with stirring.