Targeting the Peroxisomes for Synthesizing Polyketides by Type-III PKSs Enzymes in Tobacco and Petunia

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Tiivistelmä — Referat — Abstract

Secondary metabolites are bioactive compounds that help the plant to adapt in different adverse environmental conditions but are not an essential part of plant developmental processes and also secondary metabolites have pharmaceutical value because of their antioxidant, anticancer, antibacterial and antifungal properties. Type-III polyketide synthases (PKSs) are a group of polyketide synthases that produce secondary metabolites with diverse biological activities in plants. The main objective was to localize the PKSs G2PS1, G2PS2, FvCHS2-1, HlVPS into the peroxisomes for synthesizing secondary metabolites in plants. The experiment was performed by amplifying the genes with specific Px-targeting signal. Then the genes cloned into destination vector pEAQ-HT-DEST1. The plasmid constructs were transformed to *Agrobacterium tumefaciens* and agro-infiltrated to *Nicotiana benthamiana* and *Petunia hybrida* leaf tissue. Western blotting results revealed that all proteins were expressed in infiltrated leaves of both tobacco and petunia but HPLC chromatograms showed that only the protein FvCHS2-1 produced novel peaks for metabolites in tobacco.

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Secondary metabolites, Type-III PKSs, Peroxisomes, Tobacco, Petunia

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Abbreviations

ACP	Acyl carrier protein
AT	Acyltransferase
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	Coenzyme A
F3H	Flavanone 3-hydroxylase
FNS	Flavone synthase
FvCHS2-1	*Flagaria vesca* chalcone synthase 2-1
G2PS1	Gerbera 2-pyrone Synthase 1
G2PS2	Gerbera 2-pyrone Synthase 2
HlVPS	*Humulus luplus* valerophenone synthase
HMC	4-Hydroxy-5-methylcoumarin
HPLC	High performance liquid chromatography
kD	Kilodaltons
KS	Ketosynthase
LB	Lysogeny broth
NADPH	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
PKSs	Polyketide synthases
PTS	Peroxisomal targeting signal
Px	Peroxisomes
Rubisco LS	Ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit
SDS	Sodium dodecyl sulfate
STS	Stilbene synthase
TAL	Triacetic acid lactone
TE	Tris-EDTA

1. Introduction

Plant secondary metabolites are bioactive compounds that play vital roles in the agriculture, food, pharmaceutical, cosmetic and other sectors because of their health promoting properties and also they have some function for the prevention of plant diseases (Aguirre-Becerra *et al.* 2021). Secondary metabolites are different types of molecules that help in adaptation of plants to their specific environment but they are not directly related to plant growth and development (Verpoorte *et al.* 2002; Makkar *et al.* 2007). Plant secondary metabolites can be categorized into three groups: terpenoids, alkaloids, and phenylpropanoids based on their biosynthetic origin (Kabera *et al.* 2014) and many polyketides belong to the subgroup of phenylpropanoids (Croteau *et al.* 2000; Yu & Jez 2008). Polyketides are diverse natural products that involved in biological and physiological functions in plants, bacteria and also protect the structural components of pollen walls in plants (Funabashi *et al.* 2008; Grienenberger *et al.* 2010; Dao *et al.* 2011; Zeng *et al.* 2012). At the same time, they have pharmaceutical value because of their antimicrobial, antifungal, antiparasitic, antitumor, and agrochemical properties (Staunton & Weissman 2001; Chooi & Tang 2012).

Polyketides are derived from the sequential condensation reaction of two-carbon acetate units from malonyl-CoA on the acyl group of a starter substrate (Ames *et al.* 2008; Hertweck 2009). This condensation reaction is performed by polyketide synthases (PKSs). Based on their domain architecture and subunit organization PKSs are classified into three categories: Type-I, type-II, and type-III (Shimizu *et al.* 2017). A type-I PKS is complex in structure. The protein complex has large subunit that is characterized by a series of multiple domains including ketosynthase (KS), acyl carrier protein (ACP) and acyltransferase (AT). Type-II PKS is composed of a heterodimeric KS and an ACP but the type-III PKSs are simple structures of a homodimer subunit with single KS domain (Hertweck 2009; Weng & Noel 2012). Type-III PKSs have the ability to perform reiterative malonyl-CoA condensation reactions on different types of starter molecules to make a diverse array of polyketides (Liu *et al.* 2007). Plant polyketides such as chalcones, phloroglucinols, acyl-phloroglucinols, stilbenes, chromones, coumarins and curcuminoids are natural secondary metabolites produced by plant type-III polyketide synthases (Weng *et al.* 2012; Kontturi 2017; Pandith *et al.* 2020).

In this study we are working with four PKSs genes (*G2PS1*, *G2PS2*, *FvCHS2-1*, *HlVPS*). These PKSs enzymes use acetyl-CoA, isovaleryl-CoA, isobutyryl-CoA as the starter substrate and malonyl-CoA or methylmalonyl-CoA as a extender substrate for producing

secondary metabolites in plants (Chan *et al.* 2009; Yu *et al.* 2012; Abe 2020; Pandith *et al.* 2020). In the peroxisomes, beta oxidation of fatty acid takes place so they are rich in at least acetyl-CoA and also other starter and extender substrate (Keegstra & Cline 1999; Incarbone *et al.* 2018). We are expecting that in peroxisomes PKS enzymes are able to produce secondary metabolites with the help of this substrate and extender substrate.

2. Literature Review

2.1 Secondary Metabolites

Secondary metabolites are naturally synthesized compounds that help the plant to adapt in different adverse environmental conditions but are not an essential part of plant developmental processes (Aguirre-Becerra *et al.* 2021). Plants synthesize different types of secondary metabolites such as alkaloids, flavonoids, phenolics, steroids, anthocyanins etc., which have great impact in agrochemical and pharmaceuticals industry (Kabera *et al.* 2014). Some secondary metabolites also protect the plants from insects, pests, herbivores and phytopathogens' attack (Taghizadeh *et al.* 2019). In recent years plant biologists are more focusing their research interest on secondary metabolites because of various positive effects including antioxidant, anticancer, antibacterial, antifungal effects.

2.1.1 Groups of secondary metabolites

There are three main groups of secondary metabolites: phenolics, terpenoids and alkaloids (Verpoorte 1998).

Phenolic: In phenolic compounds a hydroxyl group (-OH) is directly connected with an aromatic hydrocarbon group. Based on the number of phenol units phenolic compounds are grouped into two categories such as simple phenols and polyphenols. According to Staniek *et al.* (2013), almost 8000 phenolic compound are available in plant species. Phenolic compounds have also great diversity. According to Vogt (2019), coumarins, flavonoids, hydroxycinnamic acids, suberins, monolignols, lignanas, phenylpropenes, stilbenes, lignins, vanillin ect. are considered as a phenolic compounds. Among these compounds, vanillin and curcumin are mostly used in food and pharmaceutical industry, respectively.

Terpenoids: Terpenoids are a diverse group of naturally synthesized organic aromatic compounds formally derived from five carbon isoprene units. Terpenoids play a vital role in pharmaceutical industries as well as in plant biotechnology (Ashour *et al.* 2010). According to Kandi *et al.* (2015), terpenoids are responsible for scent of eucalyptus, flavours of cloves, cinnamon and ginger and are important for the colour of tomato and sunflowers.

Alkaloids: Alkaloids are bioactive compounds that present at least one nitrogen atom. They may also contain some neutral and acidic properties (Manske & Shin 1965). In addition to nitrogen atom, oxygen, sulphur, chlorine, bromine and phosphorous can also be present in alkaloids. The large group of organism like bacteria, fungi, plants and animals are responsible for the production of alkaloids (Wink & Roberts 1998). Alkaloids have various uses in

pharmacological industries including anticancer, antimalarial, antibacterial activities (Cushnie *et al.* 2014; Kittakoop *et al.* 2014).

2.1.2 Biosynthesis of secondary metabolites

Biosynthesis pathways are responsible for the production of both primary and secondary metabolites in plants (Michal & Schomburg 2013). Biosynthesis is energy consuming and this energy is produced in plants directly from photosynthesis or by the breakdown of glucose from carbohydrates. In this step ATP is produced by the oxidation reaction of glucose, fatty acids and amino acids. Catabolism is responsible for oxidation reactions of molecules and anabolism is accountable for the reduction reactions. NADPH is essential for this reduction reaction and its considered as a coenzyme (Kabera *et al.* 2014). The most usual pathway occupied for biosynthesis are implemented through the pentose for glycosides, polysaccharides; acetate-malonate for phenols and alkaloids and mevalonic acid for terpenes, steroids and alkaloids (Dewick 2002).

2.2 Polyketide synthases

Polyketide synthases (PKSs) are a group of an enzyme complex that plays a crucial role in the production of large class of secondary metabolites in plants, fungi and bacteria (Khosla *et al.* 1992; Austin & Noel 2003). According to chemical structure, PKSs are divided into three categories: Type-I, type-II and type-III. Type-I and type-II are complex in structure. On the other hand, type-III PKSs are comparatively simple structures of small homodimeric proteins.

2.3 Type-III Polyketide synthases

The type-III polyketide synthases (PKSs) are homodimeric enzymes that contribute to the production of diverse secondary metabolites like chalcones, phloroglucinols, acyl-phloroglucinols, stilbenes and coumarins in plants (Kontturi 2017). These compounds are involved in the protection of plants against different types of abiotic and biotic stress as well as UV irradiation (Abe 2020). Type-III PKSs perform a condensation reaction with starter molecules of a variety of CoA thioesters and acetate croups derived from malonyl-CoA. The type-III PKSs have 30-95% similarity in their amino acid sequence to each other. On the other hand, only 21-31% to the type-I and types-II PKSs (Abe & Morita 2010). Almost 20 diverse groups of type-III PKSs have been characterised. Chalcone synthase (CHS) is considered as an archetypical type-III plant specific PKS superfamily enzyme but later CHS-related enzymes were revealed from fungi and bacteria (Funa *et al.* 1999; Moore & Hopke 2001). The biosynthesic reactions catalyzed by PKSs enzymes are closely related to fatty acid biosynthesis

(Austin & Noel 2003). Structural analysis and description of different types of type-III PKSs are given below.

2.3.1 Chalcone synthase (CHS)

The first characterised type-III PKS was CHS in the flavonoid biosynthesis pathway in plants that produces, the naringenin chalcone scaffold by catalyzing the condensation reaction of three acetate units from malonyl-CoA to the *p*-coumaroyl-CoA starter molecule that originates from phenylalanine via the general phenylpropanoid pathway (Ferrer *et al.* 1999; Austin & Noel 2003). The chalcone generates a heterocyclic ring of the flavanone naringenin through the isomerisation with the help of chalcone isomerase (CHI) and the flavanone naringenin can be used for the flavone synthesis by flavone synthase (FNS). This flavanone helps to produce dihydroflavonols by flavanone 3-hydroxylase (F3H) that is a precursor molecule for flavonols catalyzed by flavonol synthase (FLS). This flavonoid compound protects plants from the harmful UV-A radiation. Meanwhile, plants use these compounds to protect from the pest attack and also its attract the beneficial insects for pollination (Holton & Cornish 1995; Harborne & Williams 2000). Dihydroflavonols are also precursors for anthocyanins that give color to many flowers and fruits.

FvCHS2-1 used in this study is a bifunctional CHS with isovaleryl-CoA as a preferred substrate and producing acylphloroglucinols in strawberry plants (Song *et al.* 2015). Similarly, HlVPS is a CHS-related enzyme from hops that uses isobutyryl-CoA and isovaleryl-CoA as starters (Xu *et al.* 2013).

2.3.2 Stilbene synthase (STS)

Stilbenes are considered as a group of phenylpropanoids that are produced in a number of distinct plant species. Stilbene performs defense mechanisms in plants as well as it contains pharmacological properties such as resveratrol (Hain *et al.* 1993; Jang *et al.* 1997; Parage *et al.* 2012). Stilbene synthase (STS) produces the stilbene backbone by catalyzing the condensation reaction with linear tetraketide intermediate from p-coumaroyl CoA and three molecules of malonyl-CoA. In this reaction, the STS catalyzes the carbon C2 and C7 in an aldol condensation of the same tetraketide intermediate as CHS. The first crystal structure of an STS was exposed from *Pinus sylvestris* at 2.1 Å resolution (Austin *et al.* 2004). The STS enzyme active site embraces a complementary hydrogen bonding network for producing the thioesterase catalytic activity and it helps to cleave the thioester linkage between C1 of the tetraketide intermediate (Austin & Noel 2003; Parage *et al.* 2012).

2.3.3 2-pyrone synthase (2PS)

2-Pyrone synthases are a specific group of PKSs derived from *Gerbera hybrida* that produce a precursor of phytoalexin called methylpyrone by using condensation reaction with acetyl-CoA and also malonyl-CoA. This compound protects the plant from insect and pathogen attack (Helariutta *et al.* 1995). The chemical structure of 2-PS and CHS quite similar but a major constriction is that in 2-PS the variation in the three residues tightly lining the active-site cavity. CHS considered as a model of Claisen type of condensation reaction, STS considered as an aldol type reaction whereas 2-PS reflected as a typical example of lactonization reaction. This 2-PS enzyme also accepts other small groups of aliphatic starter substrate including propionyl-CoA, buturyl-CoA and hexanoyl-CoA instead of acetyl-CoA and malonyl-CoA (Elomaa *et al.* 1996; Thaisrivongs *et al.* 1996).

2.4 Protein import into the Peroxisomes

Peroxisomes are eukaryotic organelles bounded by a single lipid membrane that play vital roles for metabolic activities (Kaur *et al.* 2009). Peroxisomes are considered as a house of enzymes that help to perform an oxidation reaction, including a fatty acid oxidation as well as enzymes that protect cells from oxidative damage, such as catalase (Gould *et al.* 1988a). Peroxisomes are able to import both their matrix proteins and membrane proteins from the cytosol. Peroxisomal matrix proteins are nuclear encoded and contain either carboxy-terminal (PTS1) or amino-terminal (PTS2) peroxisomal targeting signal. The peroxisomal targeting signal (PTS) are recognized by specific receptors that deliver them to the membrane translocation site (Gould *et al.* 1988a; Gould *et al.* 1990).

Most of the targeting of peroxisomal protein is accomplished by a conserved C-terminal tripeptide of the sequence serine-lysine-leucine or a derivative consensus sequence. The con C-terminal tripeptide SKL was found and that is vital and sufficient to target a protein to peroxisome (Hettema *et al.* 1999; Incarbone *et al.* 2018). However, there are different types of C-terminal sequences with a two-out-of- three fit with the consensus sequence found in peroxisomal matrix protein. In the consensus tripeptide sequence the C-terminal amino acids highly bind with the receptor and it does not depend on the interaction with PTS1 receptor. In addition, the non-consensus tripeptide depends on the PTS1 receptor for their interaction. Therefore its concluded that the peroxisomal protein are imported in a definite way by specific receptor to recognize the C-terminal tripeptide and its accessory sequences (Purdue & Lazarow 1994).

3. Objectives

In this study, the main objective was to localize four PKSs into the peroxisomes for synthesizing secondary metabolites because beta oxidation of fatty acid in the peroxisomes would produce more substrates for the PKS enzymes to produce metabolites. So to test this hypothesis, the specific aims of this study were to:

i. Clone the Px-targeting PKSs genes in the vector pEAQ-HT-DEST1 for localizing into the peroxisomes in tobacco and petunia
ii. Analyse the expression of protein in leaves tissue in tobacco and petunia
iii. Investigate the secondary metabolites from agrobacterium infiltrated tobacco and tetunia leaves by HPLC

4. Materials and Methods

4.1 PCR of non-targeted and Px-Targeted PKS genes

PCR reaction was done by two steps with PKS genes *G2PS1, G2PS2, FvCHS2-1* and *HlVPS* for generating the complete Gateway attB site. In the first step, we used gene specific primers and second step we used the following adapter primers that generate attB sites. The initiation and stop codons are marked by underlining and the Px-targeting signal SKL coding nucleotides by purple colour.

GER29F: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and
GER30R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'

List of gene specific primers:

G2PS1 (F): 5'-AAAAAGCAGGCTCCATGGCGTCCTCCGTTGA-3'
G2PS1 (R): 5'-AGAAAGCTGGGTTCACAGTTTAGAGTTTCCATTGGCAACCG-3'
G2PS2 (F): 5'- AAAAAGCAGGCTCCATGGGATCATCTTACCC-3'
G2PS2 (R): 5'-AGAAAGCTGGGTTCACAGTTTAGAAATTCCGTTGGCCACCA-3'
FvCHS2-1 (F): 5'-AAAAAGCAGGCTCCATGGTGACCGTTGAGGA-3'
FvCHS2-1 (R): 5'-AGAAAGCTGGGTTCACAGTTTAGAAGCAGATACACTGTGAA-3'
HlVPS (F): 5'-AAAAAGCAGGCTCCATGGCGTCCGTAACTGT-3'
HlVPS (R): 5'-AGAAAGCTGGGTTCACAGTTTAGAGACGTTTGTGGGCACGC-3'

All PCR reactions were executed by using Phusion High-Fidelity PCR Kit (Thermo Scientific) and by following the manufacturer's instructions. The PCR product was purified by Roche PCR purification kit.

4.2 Gateway reactions and transformation of *E. coli* DH5α

The purified second PCR products were cloned to the entry vector pDONRzeo (Invitrogen by Thermo Fisher Scientific) by BP reaction using the following protocol: Purified PCR fragment (150 ng), pDONRzeo (150 ng) in 8 µl 10 mM Tris-Cl, 1 mM EDTA, pH 8 (TE) buffer. 2 µl BP clonase II enzyme mix (Invitrogen by Thermo Fisher Scientific) was added and incubated overnight at 24˚C. 1 µl Proteinase K (Invitrogen by Thermo Fisher Scientific) was added and the mixture was incubated at 37˚C for 10 minutes. 10 µl reaction mixture was added to *E. coli* DH5α competent cells and kept on ice for 30 minutes and then heat-shock was done at 42˚C for 30 seconds. The samples were kept on ice for 5 minutes, then 1 ml SOC medium was added to the mixture and incubated at 37˚C for one hour for phenotypic expression of the selection

marker. The mixtures were centrifuged at 2600rpm for 2 minutes and most of the supernatant was removed, leaving ca. 200 µl. The cells were resuspended with the remaining supernatant and spread on a low-salt LB agar plate with 50 µg/ml zeocin and incubated at 37˚C for overnight. After pure culture of the bacterial colonies, plasmid isolation was done by GenElute Plasmid Miniprep Kit (Thermo Fisher Scientific). The plasmids DNA were analysed by digestion with *Pvu*II and *Hin*dIII restriction enzymes. The plasmids were also sequenced for final confirmation.

In LR reaction the entry clones from the BP reaction were cloned to the destination vector pEAQ-HT-DEST1 with kanamycin resistance. The cloning was done using the same protocol that was followed in BP reaction but with LR clonase II enzyme mix (Invitrogen by Thermo Fisher Scientific). Then the reaction mixtures were transformed to *E. coli* DH5α. After that, plasmid isolation was done by GenElute Plasmid Miniprep Kit (Thermo Fisher Scientific) and checked by restriction digestion with *Eco*RI and *Pvu*II enzymes.

4.3 *Agrobacterium* transformation and expression in tobacco and petunia

The confirmed plasmids from the LR reaction were transformed to *A. tumefaciens* strain C58C1(pGV2260) with rifampicin, carbenicillin, and kanamycin resistance and incubated at 28°C for 3 days. After transformation, pure culture of Agrobacterium was done and inoculated in 5 ml LB medium and kept at 28°C overnight with shaking. The OD600 value was adjusted to 0.5 in 10 mM $MgCl_2$, 10 mM MES-KOH pH 6.0, 200 µM acetosyringone buffer. The mixtures were injected to six-week old *Nicotiana benthamiana* and ca. two-month old *Petunia hybrida* leaves using the agro-infiltration method (Li & Qu 2011).

4.4 Protein extraction and western blotting

About 10 mg leaf tissue from the three days old infiltrated leaves was collected in 1.5 ml Eppendorf tubes. The tubes were kept on ice and 75 µl of extraction buffer (50 mM Tris-Cl pH 7.5, 1 tablet/10 ml Roche Protease Mini EDTA free inhibitor, 0.2% β-mercapto ethanol) was added in each tube. The tissues were grinded by a small E-tube fitting pestle. The cell debris was centrifuged at maximum speed for 10 minutes at 4°C. 50 µl supernatant was transferred to new E-tube and total protein concentration was measured at 595 nm optical density in enzyme-linked immunosorbent assay (ELISA) plate according to the method described by Bradford (1976). Before running the gel 17 µl of 4xSB (100 mM Tris-Cl pH 6.9, 20% glycerol, 4% SDS, 10% β-mercapto ethanol, 0.2 mg/ml Bromphenol blue) was added in each tube and heated them at 98°C for 5 minutes. The cell debris was centrifuged for 5 minutes at maximum speed. Around 5 µg of soluble protein was loaded in Bio-Rad ready-made gel with 10x Running buffer

(30.3 g Tris base, 1441.1 g Glycine, 10 g SDS for 1000 ml RO water) and run at 200 V for 30 minutes.

For transferring the protein to the membrane, at first 4 pieces of filter papers were wetted with transfer buffer (3 g Tris base, 15 g Glycine, 200 ml methanol for 1000 ml RO water) and placed on the lower plate of the Bio-Rad Trans-Blot tray. The wetted Amersham Protran Premium membrane also placed on the filter paper stack and gel was placed on the membrane. Again, 4 pieces of wetted filter papers were placed on the membrane and then locked the lid of the tray in place and the tray was putted in the Trans-Blot Turbo machine for 30 minutes. After transferring the protein to the membrane Poinceau S staining was done to visualize the transferred protein bands.

For blocking the membrane, the membrane was washed with tTBS (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 5 minutes ar room temperature and pour off the tTBS. 25 ml blocking buffer (5% NONFAT dry milk powder) was added in the membrane and kept on shaker for one hour at room temperature. The membrane was washed with 100 ml tTBS for 10 minutes and placed in a 50 ml falcon tube by rolling. The primary antibody (anti-2-PS) with 10 ml blocking buffer was added in the tube and closed the cap properly. The tube was placed in the roller at 4°C for overnight. The membrane was washed 3 times with 100 ml tTBS. The membrane was incubated with HRP-conjugated secondary antibody (Anti-rabbit, HRP linked antibody) with 10 ml blocking buffer and kept on shaker for one hour at room temperature. The membrane was washed with 4 times with 100 ml tTBS. The membrane was taken from the tTBS and dried by two paper towels and placed in on the glass plate. The Amersham ECL Western Blotting Detection Reagent was added on the membrane and spread it properly by roller pin. The membrane was placed between th plastic sheets in a film cassette and the cassette closed. The Amersham Hyperfilm ECL film was used for detecting bands of PKS proteins from the membrane.

4.5 Metabolites analysis with HPLC

100-200 mg leaf tissue from one week old infiltrated leaves were collected in 15 ml falcon tubes and crushed by glass rod with liquid nitrogen. Methanol was added into the tube (0.5 ml per 100 mg) and vortexed properly. The mixtures were sonicated for 15 minutes in a dish sonicator. The mixtures were spined at 4000 rpm for 10 minutes and transferred the supernatant to the E-tube. The supernatant was centrifuged again at 13K for 10 minutes in a microcentrifuge and the supernatants except cell debris was transferred into HPLC ampules for each sample and analysed by HPLC with standard compounds.

5. Results

5.1 Cloning and *Agrobacterium* transformation of Px-targeting PKS genes

In the first step of cloning, PCR was done by two steps reaction with PKS genes for making the complete attB site for gateway cloning. PCR reaction was performed by amplifying the gene with specific Px-targeting signal. The primers were designed based on this SKL px-targeting targeting signal. Non-targeted (cytoplasmic) versions of the genes had been done earlier in the laboratory. The gene specific primers were used in first PCR and second step we used the adapter primers that generate attB sites. In gateway cloning, entry clones were constructed by BP reaction and all clones were checked by restriction digestion with *Hin*dIII and *Pvu*II restriction enzymes (Figure 1 and Table 1). The entry clones were also checked by sequencing. After the BP reaction, the expression clones were constructed by LR reaction. The expression plasmids were confirmed by the restriction digestion with *Eco*RI and *Pvu*II restriction enzymes (Figure 2 and Table 2) and transferred to C58C1(pGV2260) *Agrobacterium* strain, finally checked by restriction digestion with *Eco*RI and *Pvu*II enzymes (Figure 3 and Table 3).

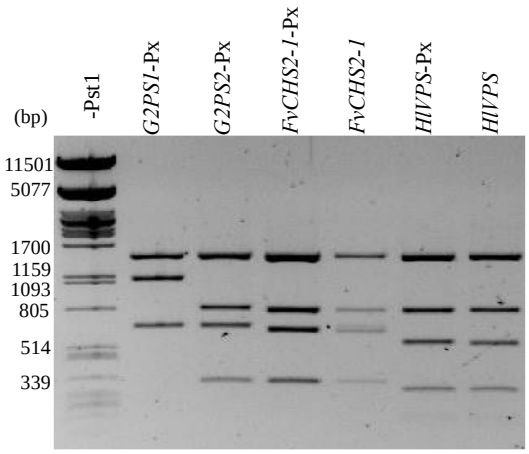

Figure 1. Restriction digestion with *Hin*dIII and *Pvu*II of the constructs isolated from *E. coli* after BP reaction. Ladder Lambda Pst1 (λ-Pst1).

Table 1. Constructs isolated from *E. coli* and their restriction fragments after digestion with *Hin*dIII and *Pvu*II enzymes (The bold numbers represent identical bands).

Name of constructs	Size of band (bp)
pMAM 1 (*G2PS1*-Px)	1462, **1148**, 674
pMAM 2 (*G2PS2*-Px)	1462, **826**, **674**, 325
pMIN 11 (*FvCHS2-1*-Px)	1462, 808, **650**, 325
pMIN 5 (*FvCHS2-1*)	1462, 808, **641**, 325
pMIN 12 (*HlVPS*-Px)	1462, 814, **569**, 289, 126
pMIN 6 10 (*HlVPS*)	1462, 814, **560**, 289, 126

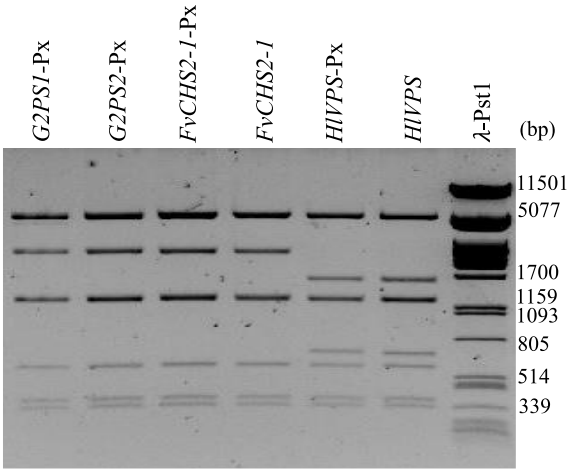

Figure 2. Restriction digestion with *Eco*RI and *Pvu*II of the constructs isolated from *E. coli* after LR reaction. Ladder Lambda Pst1 (λ-Pst1).

Table 2. Constructs isolated from *E. coli* and their restriction fragments after digestion with *Eco*RI and *Pvu*II enzymes (The bold numbers represent identical bands).

Name of constructs	Size of band (bp)
pMAM 3 (*G2PS1*-Px)	4916, **2333**, 1277, 1265, 599, 382, 344, 104
pMAM 4 (*G2PS2*-Px)	4916, **2336**, 1277, 1265, 599, 382, 344, 104
pMIN 7 (*FvCHS2-1*-Px)	4916, **2294**, 1277, 1265, 599, 382, 344, 104
pMIN 9 (*FvCHS2-1*)	4916, **2285**, 1277, 1265, 599, 382, 344, 104
pMIN 8 (*HlVPS*-Px)	4916, **1604**, 1277, 1265, **705**, 599, 382, 344, 104
pMIN 10 (*HlVPS*)	4916, **1604**, 1277, 1265, **696**, 599, 382, 344, 104

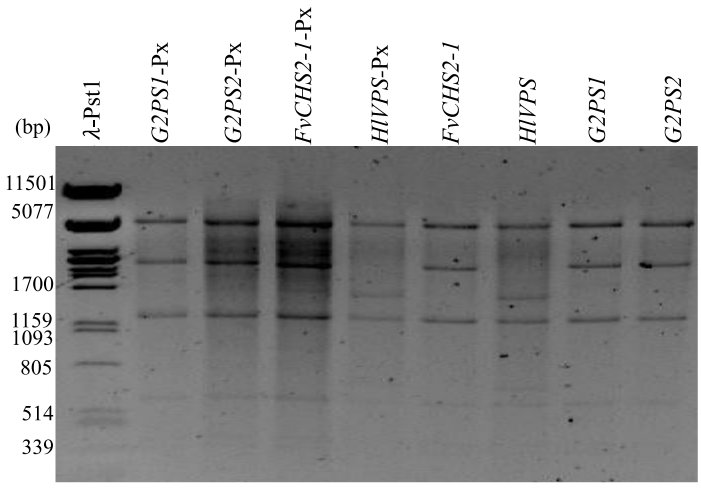

Figure 3. Restriction digestion with *Eco*RI and *Pvu*II of the constructs isolated from *Agrobacterium*. Ladder Lambda Pst1 (λ-Pst1).

Table 3. Constructs isolated from *Agrobacterium* and their restriction fragments after digestion with *Eco*RI and *Pvu*II enzymes. (The bold numbers represent identical bands).

Name of constructs	Size of band (bp)
AGMA 3 (*G2PS1*-Px)	4916, **2333**, 1277, 1265, 599, 382, 344, 104
AGMA 4 (*G2PS2*-Px)	4916, **2336**, 1277, 1265, 599, 382, 344, 104
AGMI 7 (*FvCHS2-1*-Px)	4916, **2294**, 1277, 1265, 599, 382, 344, 104
AGMI 8 (*HlVPS*-Px)	4916, **1604**, 1277, 1265, **705**, 599, 382, 344, 104
AGMI 9 (*FvCHS2-1*)	4916, **2285**, 1277, 1265, 599, 382, 344, 104
AGMI 10 (*HlVPS*)	4916, **1604**, 1277, 1265, **696**, 599, 382, 344, 104
TATU 1 (*G2PS1*)	4916, **2324**, 1277, 1265, 599, 382, 344, 104
TATU 2 (*G2PS2*)	4916, **2327**, 1277, 1265, 599, 382, 344, 104

5.2 Measurement of protein yields

Leaf tissues at the infiltrated spots were used as material for protein extraction. At the same time total protein from leaves with uninfiltrated and infiltrated with *Agrobacterium* strain as negative control were also extracted to explore if the infiltration treatment itself caused any changes in their protein content, mainly in PKSs. The total amount of crude protein and concentration was measured by comparing the absorbance at 595 nm with Bovine Serum Albumin (BSA) standard (Table 4).

Table 4. Concentration of crude protein extracted from inoculated Tobacco and Petunia leaves.

Constructs	Tobacco		Petunia	
	Absorbance (A_{595})	Concentration (µg/µl)	Absorbance (A_{595})	Concentration (µg/µl)
TATU 1 (G2PS1)	0.270	1.08	0.326	1.30
TATU 2 (G2PS2)	0.400	1.60	0.288	1.15
AGMA 3 (G2PS1-Px)	0.338	1.35	0.425	1.70
AGMA 4 (G2PS2-Px)	0.320	1.28	0.459	1.84
AGMI 7 (FvCHS2-1-Px)	0.277	1.11	0.460	1.84
AGMI 8 (HlVPS-Px)	0.386	1.54	0.540	2.16
AGMI 9 (FvCHS2-1)	0.245	0.98	0.475	1.90
AGMI 10 (HlVPS)	0.316	1.26	0.534	2.14
GV 2260	0.211	0.84	0.650	2.60
Uninfiltrated leaves	0.243	0.97	0.556	2.22

The concentration of protein from uninfiltrated leaves in tobacco and petunia were 0.97 and 2.2 µg/µl, respectively whereas infiltrated leaves with GV2260 *Agrobacterium* strain were 0.84 and 2.60 µg/µl, respectively. In tobacco, the concentration of protein from infiltrated leaves with *Agrobacterium* carrying the Px-targeting G2PS1, G2PS2, FvCHS2-1 and HlVPS were 1.35, 1.28, 1.11 and 1.54 µg/µl, respectively. whereas in petunia, the concentration of protein

from the infiltrated leaves with *Agrobacterium* carrying the Px-targeting G2PS1, G2PS2, FvCHS2-1 and HlVPS were 1.70, 1.84, 1.84 and 2.16 µg/µl, respectively.

5.3 Protein Expression by Western Blotting

In order to study the function of Px-targeting G2PS1, G2PS2, FvCHS2-1 and HlVPS, these genes were transiently expressed in *N. benthamiana* and *P. hybrida*. Both the uninfiltrated leaves of tobacco and petunia and leaves infiltrated with *Agrobacterium* strain were used as a control. After extraction, proteins from infiltrated tobacco and petunia leaves were separated in SDS-PAGE and analyzed by western blotting (Figure 4) to confirm the expression of the PKS proteins in cells. The size of the proteins G2PS1, G2PS2, FvCHS2-1 and HlVPS were 43, 44, 42.5 and 43 kD, respectively. So, the expression of these proteins should be at that size. In tobacco, the expression of Px-targeting G2PS1, G2PS2 and HlVPS were higher compare to Px-targeting FvCHS2-1 (Figure 4a). However, this seems to be due to lower loading of thegel (Figure 5). Signal in the lane with empty agrobacterium (GV2260) could be an induced CHS in tobacco, or simply a spill-over in loading.

In petunia, the Px-targeting G2PS1, G2PS2 and HlVPS were also highly expressed than Px-targeting FvCHS2-1 (Figure 4b). However, here lower loading is not an explanation (Figure 5). Low signal for G2PS1 is also not due to low loading of the gel. The expression of all proteins for both tobacco and petunia was at between 41 to 45 kD size. In uninfiltrated leaves of both tobacco and petunia, there was no expression of protein.

In Ponceau S staining the strong band of Rubisco LS protein was observed at 55 kD size for both tobacco and petunia but the intensity of band was higher in petunia compare to tobacco (Figure 5).

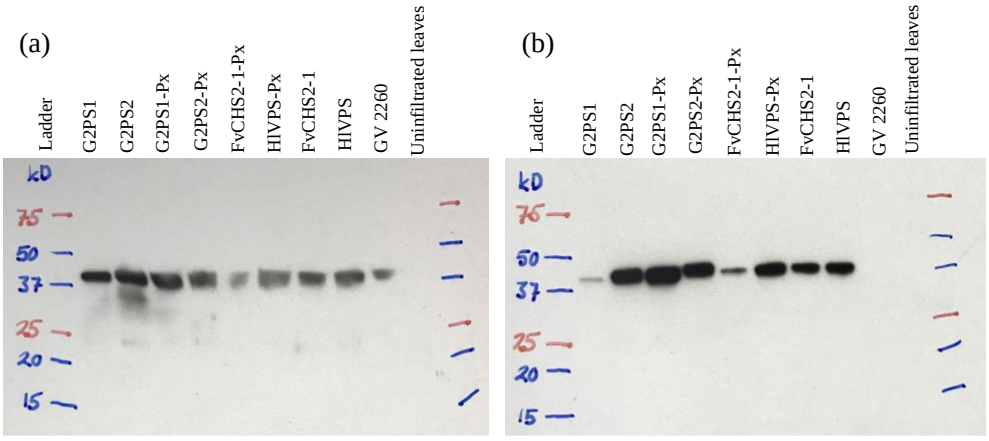

Figure 4. Expression of protein extracted from tobacco (a) and petunia (b) leaves infiltrated with *Agrobacterium* carrying with and without Px-targeting G2PS1, G2PS2, FvCHS2-1 and HlVPS.

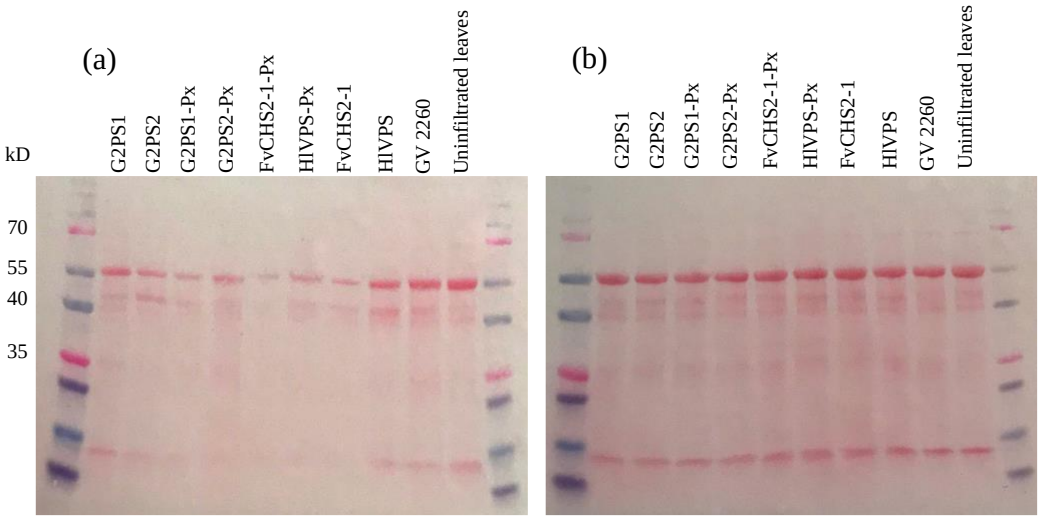

Figure 5. Ponceau S staining of the of Nitrocelulose membrane (NC) after protein transfer of tobacco (a) and petunia (b) leaves. The Rubisco LS of size 55 kD serves as a loading control.

5.4 Analysis of secondary metabolites by HPLC

The secondary metabolites profile analysis of tobacco and petunia leaves expressing with and without Px-targeting G2PS1, G2PS2, FvCHS2-1 and HlVPS were performed from the methanol extracts of infiltrated leaves by HPLC (Figure 6-9). Two standard compounds 4-hydroxy-5-methylcoumarin (HMC) and triacetic acid lactone (TAL) was also used to compare the peaks because the G2PS1 and G2PS2 enzymes are responsible for the biosynthesis of these metabolite compounds (Pietiäinen *et al.* 2016). The Px-targeting protein FvCHS2-1 expressed in tobacco produced two peaks in HPLC with the retention time of 7.2 and 8.1 at 280 nm wavelength (Figure 6c) that were not produced in control infiltration with *Agrobacterium* strain (Figure 6f). Even the protein FvCHS2-1 without Px- targeting expressed in tobacco produced similar peaks in HPLC with the same retention time of 7.2 and 8.1 (Figure 7c) but we cannot conclude with the data we have which compounds they are. Moreover, the others Px-targeting and without Px-targeting proteins G2PS1, G2PS2 and HlVPS did not produce novel peaks at 280 nm wavelength in tobacco as compared to control with *Agrobacterium* strain.

In petunia, these proteins with and without Px-targeting did not produce novel peaks for metabolite compounds at 280 nm wavelength as compared to control *Agrobacterium* strain (Figure 8-9). The uninfiltrated leaves of both tobacco and petunia were also analyzed to show whether *Agrobacterium* alone causes any changes in metabolites production. As compared to uninfiltrated leaves of petunia, the empty *Agrobacterium* strain GV2260 produced a peak in HPLC with the retention time of 7.08 at 280 nm wavelength (Figure 10d).

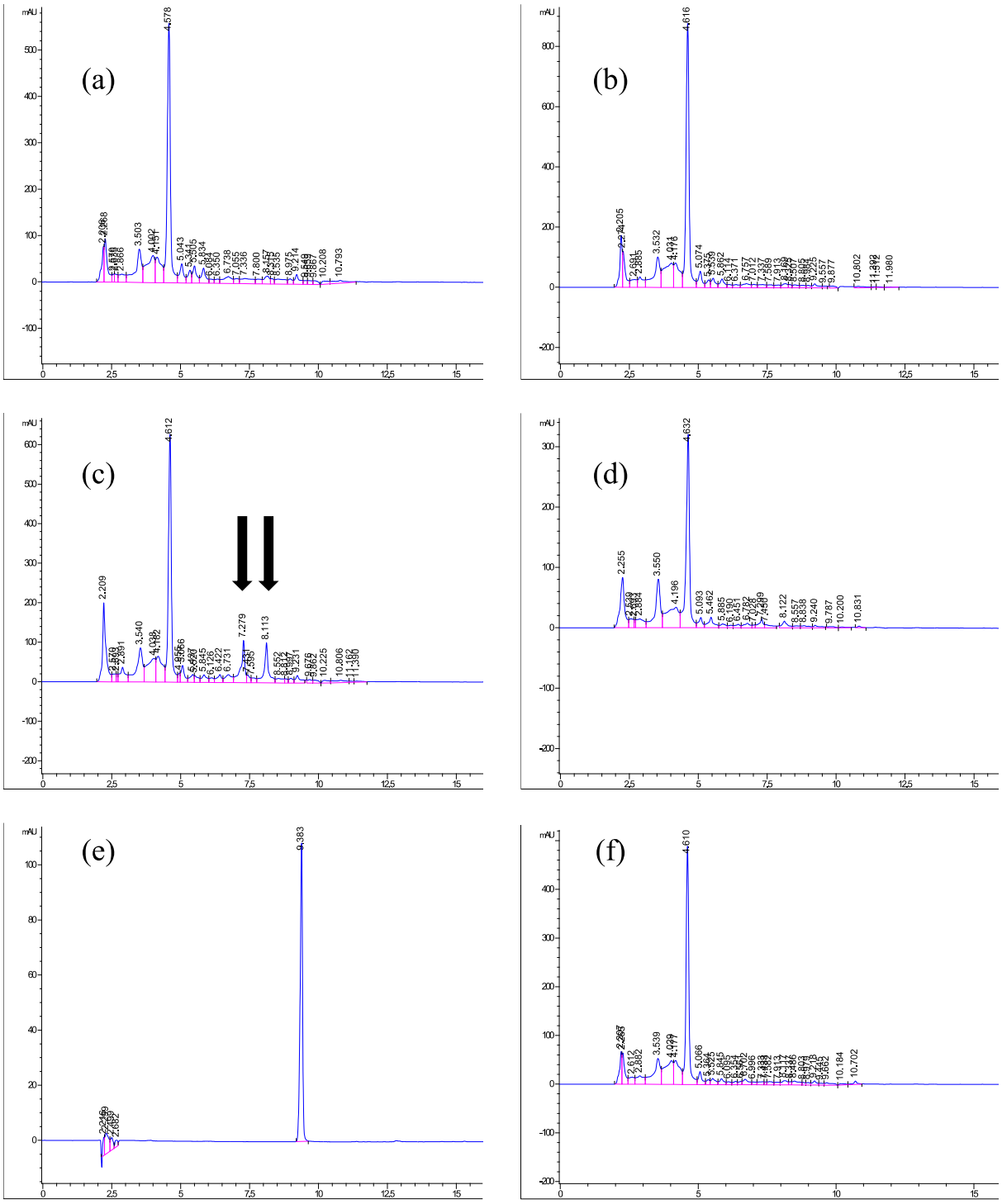

Figure 6. HPLC chromatograms analysis of Px-targeting G2PS1, G2PS2, FvCHS2-1, HlVPS (a-d), respectively at 280 nm wavelength in tobacco; (e): standard compound 4-hydroxy-5-methylcoumarin-HMC and (f): GV2660 *Agrobacterium* strain as a control.

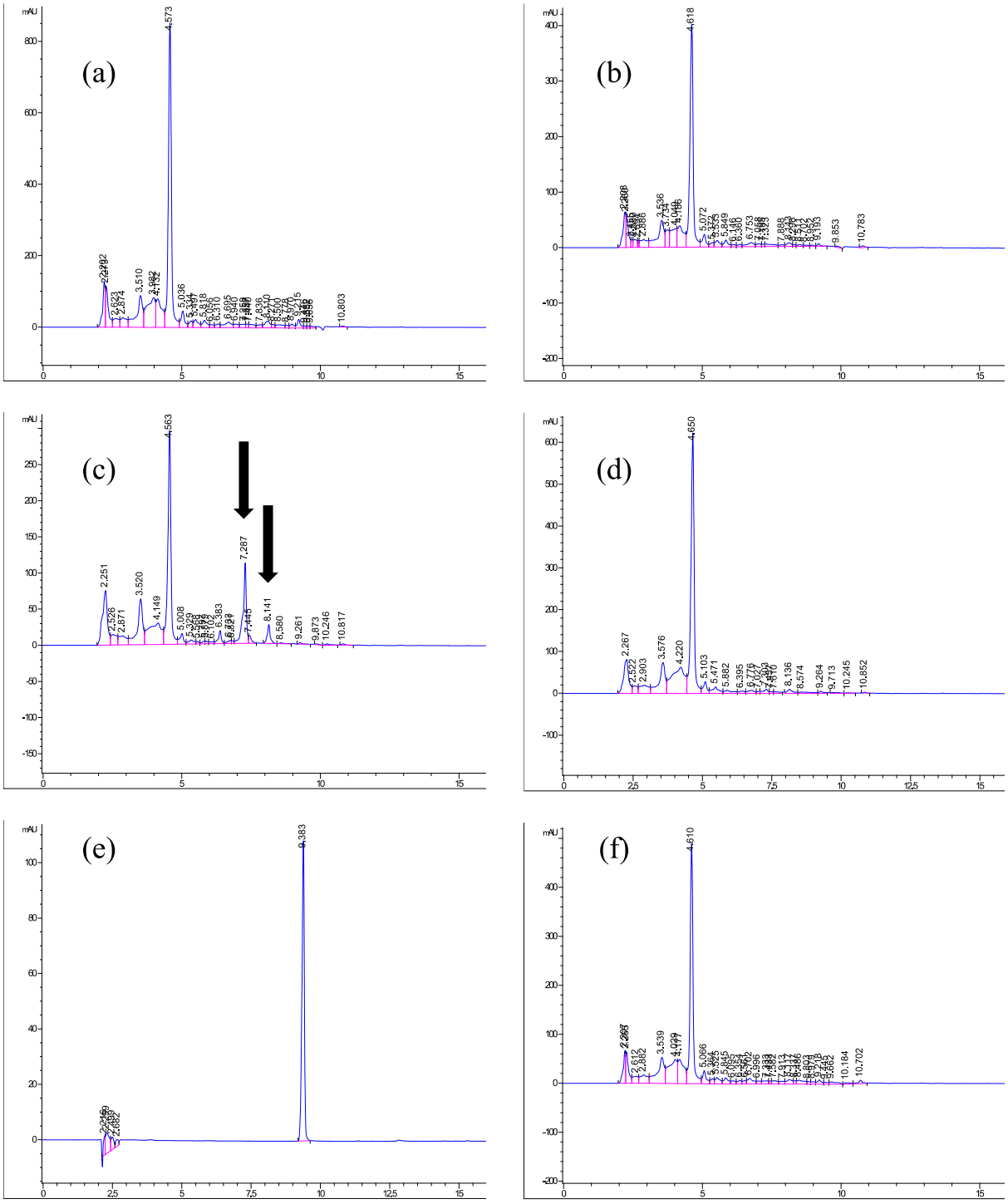

Figure 7. HPLC chromatograms analysis of without Px-targeting G2PS1, G2PS2, FvCHS2-1, HlVPS (a-d), respectively at 280 nm wavelength in tobacco; (e): standard compound 4-hydroxy-5-methylcoumarin-HMC and (f): GV2660 *Agrobacterium* strain as a control.

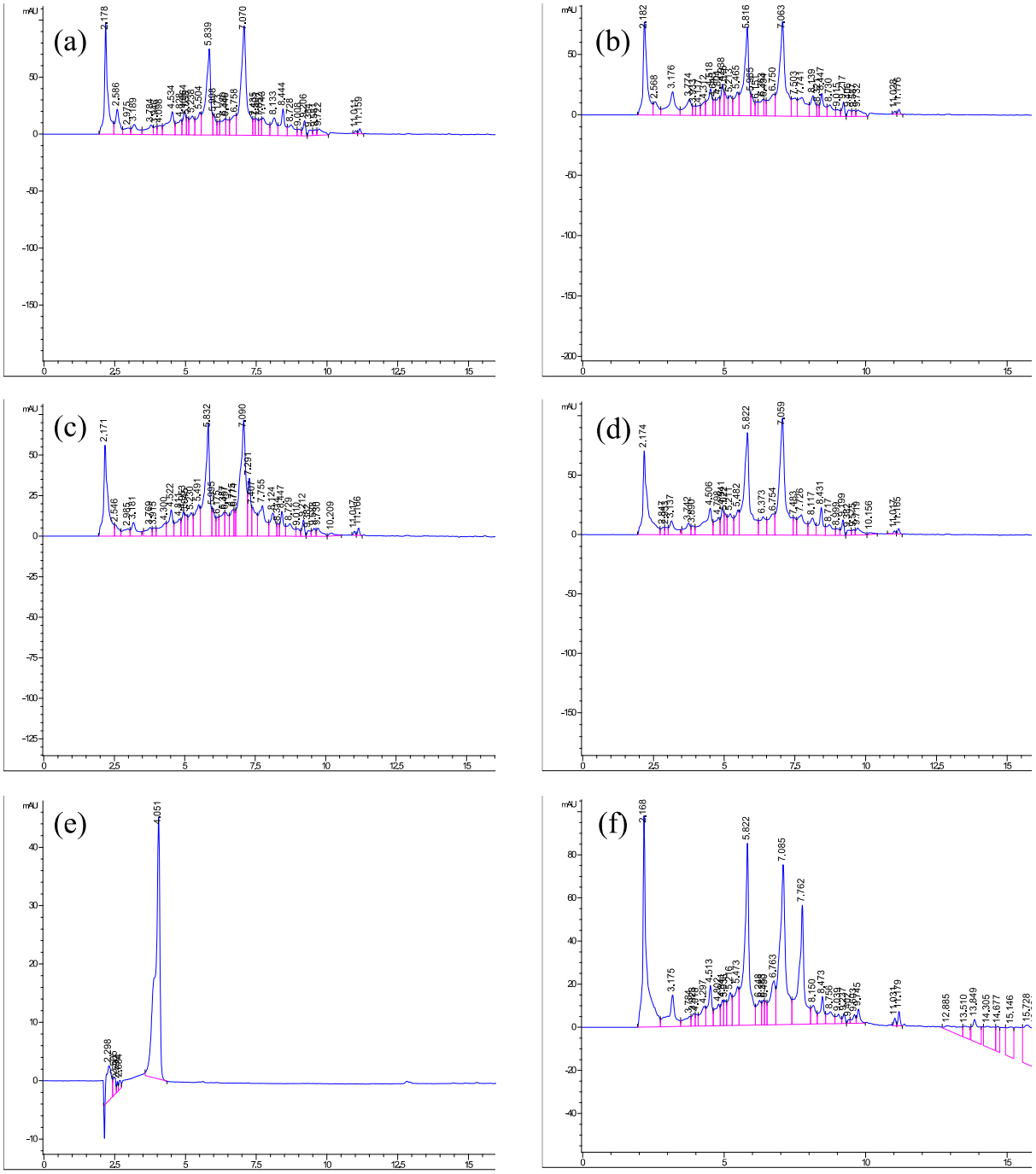

Figure 8. HPLC chromatograms analysis of Px-targeting G2PS1, G2PS2, FvCHS2-1, HlVPS (a-d), respectively at 280 nm wavelength in petunia; (e): Standard compound Triacetic acid lactone-TAL and (f): GV2660 *Agrobacterium* strain as a control.

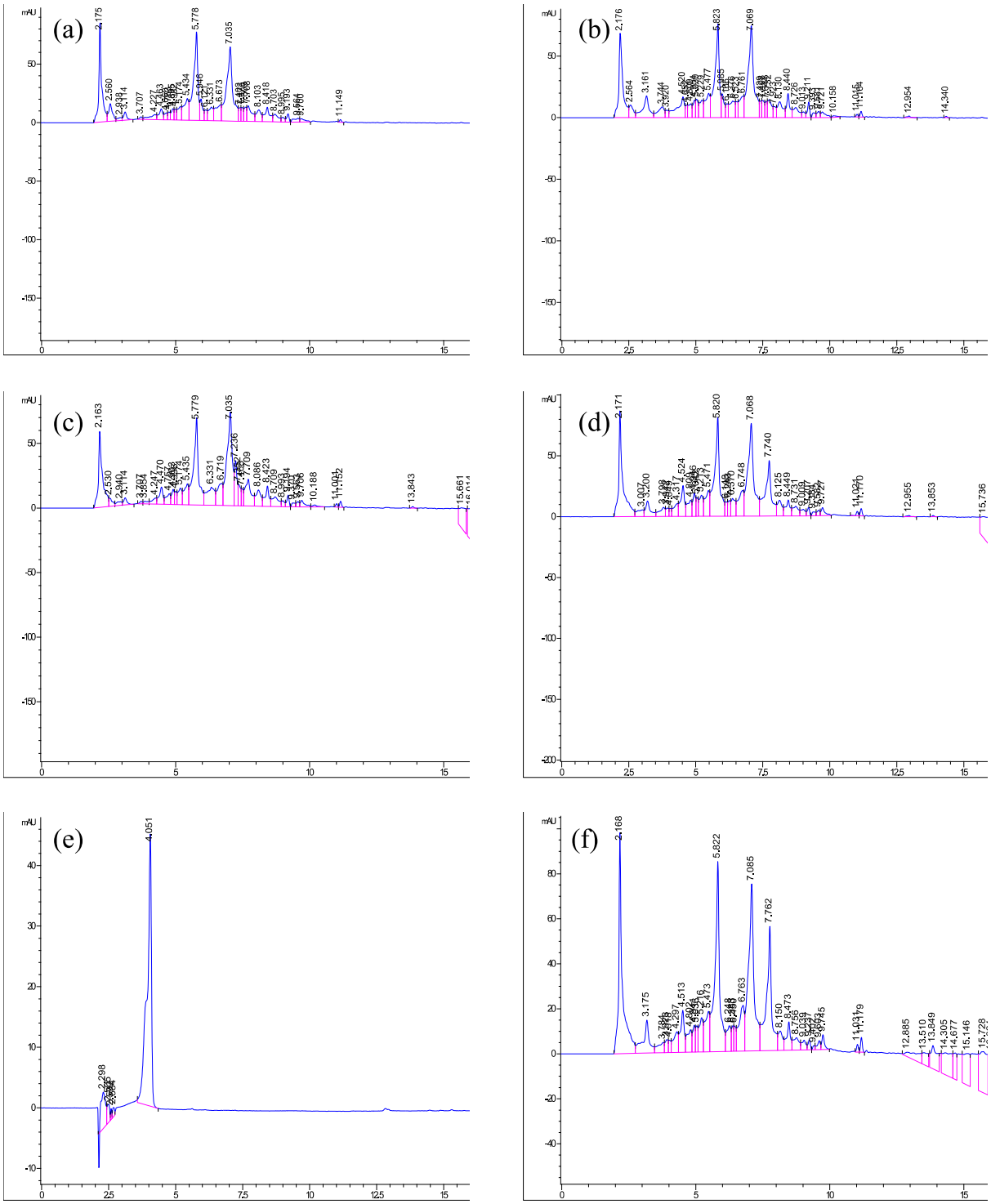

Figure 9. HPLC chromatograms analysis of without Px-targeting G2PS1, G2PS2, FvCHS2-1, HlVPS (a-d), respectively at 280 nm wavelength in petunia; (e): Standard compound Triacetic acid lactone-TAL and (f): GV2660 *Agrobacterium* strain as a control.

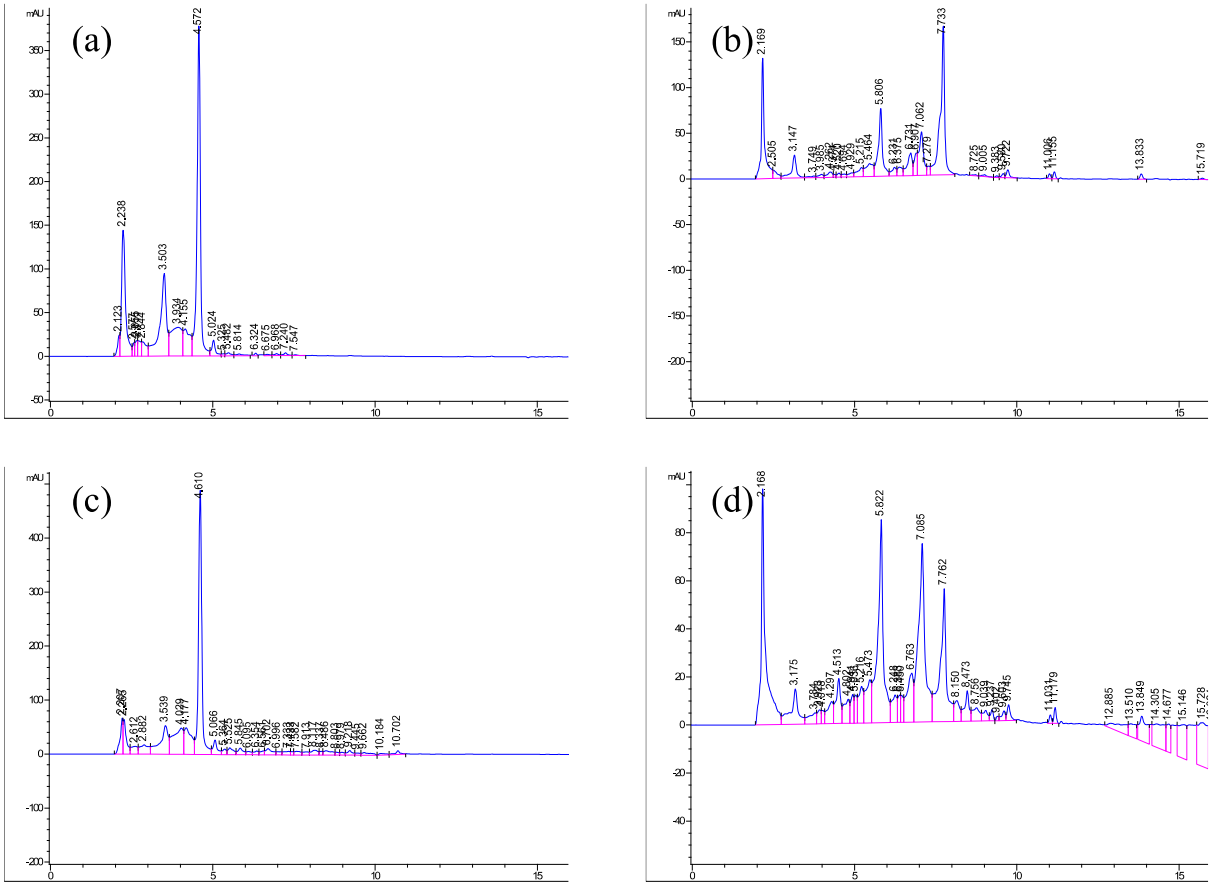

Figure 10. HPLC chromatograms analysis of uninfiltrated tobacco and petunia leaves (a-b); GV2260 *Agrobacterium* strain in tobacco and petunia, respectively (c-d).

6. Discussion

Peroxisomes are eukaryotic organelles that participate in different types of metabolic activities including beta-oxidation of fatty acid in plants. In this study, we localized PKSs genes into the peroxisomes by agroinfiltration methods. At first amplification of PKSs genes with SKL peroxisomes targeting signal was done by PCR reactions. A previous study by Gould *et al.* (1988b) reported that the C-terminal tripeptide SKL was found to target a protein to peroxisomes. Gateway cloning was done for producing the expression clone of Px-targeting genes. Hartley *et al.* (2000) reported that Gateway cloning is a more useful and precise ways to clone the DNA sequence into multiple vectors for analyzing the protein expression and functional analysis. After the Gateway reaction, agroinfiltration was done to study the function of protein expression and metabolites analysis in tobacco and petunia.

Western blotting was done to confirm the expression of the PKSs proteins in cells of infiltrated tobacco and petunia. Western blotting results showed that all the proteins are expressed in both infiltrated tobacco and petunia leaves but the expression of Px-targeting G2PS1, G2PS2 and HlVPS were higher compared to Px-targeting FvCHS2-1. In petunia, the expression of G2PS1 and px-targeting FvCHS2-1 was lower but it's totally unexpected because the primary antibody that we used in the membrane work well with these enzymes. So we are thinking that it might be missed sampling from the infiltration area or infiltration did not work well. In Poinceau S staining, the strong band of nonspecific protein (Rubisco LS) was found in the membrane at around 55 kD size.

The secondary metabolites profile analysis from both infiltrated tobacco and petunia leaves was done by HPLC. HPLC results revealed that the FvCHS2-1 produced two peaks at 7.2 and 8.1 retention time but we don't know which compounds they are. It should be investigated in detail. However the other proteins G2PS1, G2PS2, HlVPS did not produce novel peaks in HPLC for secondary metabolites in tobacco. According to Pietiäinen *et al.* (2016), G2PS1 and G2PS2 are responsible for the synthesis of triacetolactone (TAL) and 4-hydroxy-5-methylcoumarin (HMC) in gerbera. Even in petunia, these enzymes did not produce novel peaks for the metabolite compounds. We are thinking that in peroxisomes the substrates are not sufficient for the production of metabolites with these enzymes and maybe a place where there is more substrates is a better localization of these PKS genes. In case of FvCHS2-1 and HlVPS, these enzymes considered as chalcone synthase but they have shown to make acyl phloroglucinols at least in vitro and maybe also in the plant but as leaves do not usually

have active phenylpropanoid pathway, we cannot expect any chalcone product from these enzymes.

Meanwhile, the uninfiltrated leaves of both tobacco and petunia were also analyzed to show whether *Agrobacterium* alone makes any changes in metabolites production and we found that in petunia the *Agrobacterium* strain produced a peak in HPLC. Based on this result we can say that in leaves tissue some metabolites compounds always produced in different stress conditions.

7. Conclusion

Secondary metabolites are naturally synthesized bioactive compounds that play pivotal roles in agriculture, food and pharmaceutical industries because of their agrochemical, antifungal, anticancer, anti-bacterial properties. Type-III polyketide synthases (PKSs) produce secondary metabolites with diverse biological activities in plants as well as in bacteria. In this study, we cloned the PKS genes with specific Px-targeting signals for transforming into the peroxisomes to study the functions of these enzymes for metabolites production. At first, we found that all proteins were expressed in western blotting but after HPLC chromatograms we revealed that only the protein FvCHS2-1 was producing novel peaks for metabolites compounds in tobacco. Their identity should be investigated in detail in a future study. Also, other PKS proteins were expressed well in western blotting but they were producing no new metabolite compounds. Further research is still required to solve these questions.

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