Identification and characterization of strawberry FvGELP1 (*Fragaria vesca* GDSL esterase/lipase)

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Abstract. *Witasari LD, Huang FC, Schwab W. 2022. Identification and characterization of strawberry FvGELP1 (*Fragaria vesca *GDSL esterase/lipase). Biodiversitas 23: 907-915.* Fruit softening is primarily the result of the hydrolytic enzymes activity. Esterases are reasonable candidates due to their putative role in cell wall components degradation. This study aimed to identify and characterize a new enzyme related to strawberry fruit ripening. Esterase activity was detected in the native PAGE of protein extract isolated from *Fragaria × ananassa* red fruit. Amino acid sequence analysis of the protein band revealed several esterases as possible candidates. GDSL esterase/lipase from *Fragaria vesca* (gene27964; *FvGELP1*) was chosen for cloning purposes and further analysis. Quantitative RT-PCR of *FvGELP1* in plant tissues of *F. vesca* indicated high expression levels in fruit, in particular in early developmental stages. The gene *FvGELP1* (1,161 bp) was amplified from *F. vesca* fruit cDNA and expressed as a 43 kDa HisTag fusion protein in *Saccharomyces cerevisiae*. FvGELP1 possesses four conserved residues Gly43-Asp44-Ser45-Asn46. FvGELP1 contains Ser45, Asp364, and His367 as the catalytic triad. Esterase assays of FvGELP1 resulted in high levels of the aromatic alcohol products by applying α-naphthyl acetate (αNA), p-nitrophenyl acetate (pNPA), phenyl acetate, and benzyl acetate as substrates. It could be suspected that FvGELP1 plays a role in strawberry fruit ripening and might be involved in the hemicellulose degradation, presumably by deacetylation of the polysaccharide.

Keywords: *Fragaria × ananassa*, *F. vesca*, fruit ripening, FvGELP1, GDSL esterase/lipase

Abbreviations: αNA: α-naphthyl acetate, αNB: α-naphthyl butyrate, pNPA: p-nitrophenyl acetate, pNPB: p-nitrophenyl butyrate, pNB: p-nitrobenzyl, GDSL esterase/lipase: a canonical Gly-Asp-Ser-Leu (GDSL) motif of esterase/lipase, FvGELP1: *Fragaria vesca* GDSL esterase/lipase, qRT PCR: Quantitative real-time PCR, Native PAGE: Native Polyacrylamide Gel Electrophoresis, SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, APS: Ammonium persulfate, TEMED: Tetramethylethylenediamine, FPLC: Fast Protein Liquid Chromatography, HPLC-UV: High Performance Liquid Chromatography-Ultraviolet

INTRODUCTION

Characteristics of the ripe fruit in non-climacteric fruits such as strawberry (*Fragaria* spp.) are the result of physiological and biochemical changes. Hydrolytic enzymes like hydrolases could modify the polysaccharide of the cell wall and trigger fruit softening. Esterases are known also involved in the cell wall component degradation, such as pectin esterase in strawberry fruits ripening (Castillejo et al. 2004; Xue et al. 2020). Acetylxylan esterase in combination with cellulases, mannanases, and xylanases may engage in the plant cell walls degradation. A carbohydrate esterase13 and a GDSL esterase/lipase protein (GELP) family member of plant deacetylases have been exhibited to cut acetyl groups from polysaccharides (Gou et al. 2012; Zhang et al. 2017). Recently, DARX1 (deacetylase on arabinosyl sidechain of xylan 1), rice (*Oryza sativa*) GDSL esterase regulates the conformation of arabinoxylan and the cross-linking mode with cell wall polymers of cellulose (Zhang et al. 2019).

GDSL esterases/lipases are hydrolytic enzymes with wide substrate specificity. Plant GDSL-lipases are known as acylhydrolases which might involve in plant development

and growth (Ma et al. 2018; An et al. 2019; Ding et al. 2019a; Watkins et al. 2019), and morphogenesis (Smyth 2017; Yadav et al. 2017; Zhang et al. 2017). EXL6 (extracellular lipase 6) expression in *Brassica rapa* explained that this gene plays a pivotal role in pollen development (Dong et al. 2016). ZmMs30, a GDSL lipase expressed in maize anthers required for pollen exine and anther cuticle development (An et al. 2019). Besides, the hydrolytic activity of GDSL esterases/lipases has been connected to pathogen defense (Lee et al. 2009; Kim et al. 2013, 2014; Rajarammohan et al. 2018; Ding et al. 2019b), seed germination (Clauss et al. 2008), secondary metabolism (Huang et al. 2015), and the stress tolerance (Naranjo et al. 2006). To facilitate extracellular exportation, GDSL-lipases are synthesized as preproteins restraining signal peptides (Kram et al. 2008). This extracellular exportation might facilitate the degradation of complex polysaccharides in cell walls.

Plants retain a large number of GDSL esterases/lipases family, such as 105 AtGELP in *Arabidopsis thaliana* (Lai et al. 2017), 114 OsGELP in *Oryza sativa* (Chepyshko et al. 2012), 121 BrGELPs in *Brassica rapa* (Dong et al. 2016), and 194 GmGELP in soybean (*Glycine max*) (Su et al.

2020). In addition, Volokita et al. (2011) investigated 57, 96, 126, and 130 GDSL-lipases family members in *Plutella xylostella*, *Vitis vinifera*, *Populus trichocarpa*, and *Sorghum bicolor*, respectively. The GDSL-lipase gene family is defined by the distinct GDSL amino acid motif and several highly conserved domains. Similar to SGNH hydrolases, GDSL esterases/lipases contained at least four conserved sequence blocks I, II, III, and V with respective catalytic residues of Ser, Gly, Asn, and His (Upton and Buckley 1995). GDSL-lipases have a flexible catalytic site. They could adjust their conformation in the existence of different substrates and thus, the enzymes have broadly diverse enzymatic activities, such as thioesterase, protease, arylesterase, and lysophospholipase activity (Akoh et al. 2004). In plants, GDSL esterases/lipases could show both transferase and hydrolase activities. A GDSL-lipase from *Tanacetum cinerariifolium* possesses transferase (in vivo) and esterase (in vitro) activities in pyrethrins biosynthesis (Kikuta et al. 2012). These activities are comparable to xyloglucan endotransglycosylases/hydrolases (XTHs) that exhibited transferase and hydrolase activities associated with strawberry fruit ripening (Witasari et al. 2019).

GDSL esterase/lipase in strawberry has not been reported. This study aimed to identify and characterize a new enzyme in strawberry related to fruit ripening. In this current study, we purified native proteins from *Fragaria × ananassa* fruit to investigate their hydrolytic activity and identified a candidate GDSL esterase/lipase from *F. vesca* namely *FvGELP1*, whose recombinant protein was produced in *S. cerevisiae* and biochemically characterized.

MATERIALS AND METHODS

Plant material and reagents

Diploid (*Fragaria vesca* cv. Hawaii 4) and octoploid (*Fragaria × ananassa* cv. Elsanta) strawberry were purchased from KRAEGE Beerenpflanzen, Telgte, Germany (https://kraege.de). These plants were cultivated in a glasshouse (16 h light/8 h dark) in Dünast, Freising, Germany. Chemicals were purchased from Carl Roth (https://www.carlroth.com), Sigma-Aldrich (https://www.sigmaaldrich.com), J.T. Baker (https://www.avantorsciences.com), and VWR International (https://www.vwr.com), unless otherwise indicated.

Protein extraction

Protein extraction was performed according to Niehaus and Gross (1997) method with modification. *Fragaria × ananassa* red fruits (50 g) were chilled in liquid nitrogen and then grounded in a rotor-stator homogenizer. The strawberry fruit powder was added PVP (10 g) in 100 mL of Tris HCl buffer (50 mM, pH 8.5) and then stirred. The mixture was filtered using muslin and subsequently centrifuged (20,000 g, 20 min). The filtrate was added protamine sulfate (2%) under stirring and then centrifuged (20,000 g, 20 min). The supernatant was collected and further fractionated using ammonium s sulfate (40-60%). The pellet contained protein was dissolved in 5 mL of Tris-HCl buffer (50 mM, pH 7.5),

clarified by centrifugation (20,000 g, 10 min), and then desalted through dialysis using the same buffer.

SDS PAGE

The Tris-glycine SDS-PAGE gel (12%) was set in a Bio-rad gel tank (Walker 2009). Ten microgram protein sample was added to 8 µL of 4x loading dye (Carl Roth). The protein samples and a PageRuler Plus Prestained Protein Ladder (Thermo Scientific) were loaded carefully in SDS PAGE gel. Electrophoresis was performed in 1x TAE running buffer at 160 V for 1.5 h. Commasie Blue solution was used to stain the proteins at room temperature (RT), 20 rpm overnight. Finally, the gel was de-stained using 50% methanol and 10% acetic acid solution at RT, 20 rpm for 3 h.

Western Blot analysis

SDS gel and filter paper was incubated in semidry blotting buffer (25 mM TRIS base, 200 mM Glycin, 20% (v/v) methanol) for 5-10 minutes. The PVDF membrane (Roth) was activated in methanol for 1 minute and then equilibrated in semidry blotting buffer for 5 minutes. From the bottom part (anode) of semidry blotter was placed in order: 4x filter papers, membrane, polyacrylamide gel, and then 4x filter papers. A current of 10 V (0.8 mA/cm2 gel) was applied for 60 min. The membrane was removed from the blotter and incubated in blocking buffer (5% BSA or milk powder in washing buffer) on a shaker for 60 min at room temperature. The membrane was washed three times for 5 min in washing buffer (20 mM TRIS base, 140 mM NaCl, 0.1% (v/v) Tween 20). The membrane was incubated in a 50 mL falcon tube with 3 ml blocking buffer and 1µl of Anti-His-AP (Abcam) monoclonal antibody with rotation overnight at 4°C. The membrane was washed three times 10 min in washing buffer and then two times 5 min in detection buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl2). The membrane was incubated in 20 mL of detection buffer containing 50 µL NBT substrate (5 mg NBT in 100 µL 70% DMF) and 25 µL BCIP (5 mg BCIP Na-salt in 100 µL H2O) for 15 min to 3 h in the dark with shaker until bands appear. Finally, the membrane was washed with water.

Native PAGE

Protein extract is dissolved in a non-denaturing sample buffer (Walker 2009). The 12% native PAGE separating gel (4 mL of acrylamide/bis-acrylamide (30%/0.8% w/v), 5.89 mL of Tris-HCl buffer (0.375 M, pH 8.8), 100µL of APS (10% w/v), and 10µL of TEMED) was set in a tris glycine buffer (25 mM Tris, 192 mM glycine). Ten microgram protein sample and 8 µL of 2x sample buffer (Tris-HCl (62.5 mM, pH 6.8), glycerol (25%), and bromophenol blue (1%) were loaded carefully into each well in a Bio-rad gel tank. Electrophoresis was carried out at 100 V for 3 h at 4°C. Right after electrophoresis, α-naphthyl acetate was loaded as a substrate for detection of esterase activity (Gabriel 1971). Afterward, a diazonium salt was added to visualize the product of α-naphthol.

Protein sequence analysis

The protein band in the native pages were partially sequenced using mass spectrometry as previously described (Franz-Oberdorf et al. 2017). The translated *F. vesca* genome database (Shulaev et al. 2011) was used to identify the detected amino acid sequences. Peptide quantification and identification was carried out as previously mentioned (Franz-Oberdorf et al. 2017).

Gene expression analysis and phylogenetic tree

Extraction of total RNA from *F. vesca* cv. Hawaii 4 fruits was performed based on the method described by Liao et al (2004). Afterward, cDNA and qRT-PCR were carried out according to Witasari et al. (2019). The expression levels of the interspacer 26S-18S RNA (IS) housekeeping gene were utilized to normalize the qRT-PCR data for *FvGELP1*. Interspacer (IS) primers and gene-specific primers were applied to amplify the IS gene and the target gene, respectively (Table 1). The dilutions of the cDNA of the IS gene and target gene were 8000× and 20×, respectively. Melting curve profiles were analyzed to confirm the absence of unspecific amplicon. The phylogenetic tree was created using Geneious software v. 2020 (https://www.geneious.com).

Cloning and expression of *FvGELP1*

The *FvGELP1* (1,161 bp) was amplified from *F. vesca* and *F. × ananassa* cDNA using specific primers (FP_GDSL 27964 and RP_GDSL 27964) (Table 1). First, initial denaturation was done at 95°C for 4 min. Denaturation was conducted at 95°C for 45 sec, followed by annealing at 55°C for 30 sec and elongation at 72°C for 1 min in 35 cycles. Finally, termination was performed at 72°C for 10 min. Cloning and transformation were performed using the PSK+ cloning vector for *Escherichia coli* NEβ 10 cells and the pYES2 expression vector for *S. cerevisiae* INVSc1 cells. FvGELP1 overexpression was induced by galactose. A HisTrap FF column, 5mL (Thermo Fisher Scientific) connected to a FPLC (ÄKTA system; GE Healthcare) was utilized to purify FvGELP1. His wash/bind buffer (sodium phosphate buffer (50 mm, pH 7.4), NaCl (0.3 m) and imidazole (30 mm)) at a flow rate of 0.5 mL min⁻¹ was used to equilibrate the column. The target protein was eluted using 1× His elution buffer (sodium phosphate buffer (50 mm, pH 7.4), NaCl (0.3 m), and imidazole (250 mm)) in a flow rate of 0.5 mL min⁻¹ for

20 min. Two milliliter fractions were collected for esterase assays.

Multiple sequence alignment

The full-length amino acid sequence of FvGELP1 was retrieved from the genome sequence of *F. vesca* ssp. *vesca* accession Hawaii 4 (Shulaev et al. 2011). The multiple sequence alignment of FvGELP1 and several GDSL esterase/lipase from different species was compiled using Clustal W software.

Esterase assay

Low molecular weight esters such as α-NA, pNPA, phenyl acetate and benzyl acetate were used as substrates based on Gilham and Lehner (2005) method with modification. The sample mixture was 10 μL of FvGELP1 crude extract and 5 μL of 20 mM substrate in 85 μL potassium phosphate buffer (0.1m, pH 6.0). The samples were incubated at 30°C for 24 h with constant shaking (300 rpm). The reaction was stopped using 50 μL methanol followed by centrifugation at 4°C (20,000 *g*, 10 min). The clear supernatant (50 μL) was used for HPLC-UV analysis.

HPLC-UV analysis

HPLC-UV was achieved using a reverse-phase column (Luna 3u C18(2) 100A, 150 × 2 mm; Phenomenex, https://www.phenomenex.com) in an Agilent 1100 HPLC-UV system (Agilent Technologies, https://www.agilent.com). Water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) was used as mobile phase. The injection volume was 5 μL. Flow rate was 0.2 mL min⁻¹. A gradient was applied from 0% B to 50% B in 30 min. Within the next 5 min, B was increased to 100% for 15 min. Afterward, B was decreased to 0% within 5 min. These initial conditions were kept for 10 min for system equilibration. UV signals were detected at 280 nm.

Data analysis

Data analysis for protein sequence was achieved using Scaffold 4, v.4.2.1 software (Searle 2010) provided by Chair of Proteomics and Bioanalytics, Technical University of Munich, Germany. qRT-PCR data analysis were performed using $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). HPLC-UV data was analyzed based on the method by Ring et al. (2013).

Table 1. Primers used for *FvGELP1* cloning

Primer name	Vector	Sequence (5'→ 3')
FP_GDSL 27964	-	ATGCTGGGAATGGAGATTTGGAAG
RP_GDSL 27964	-	GCTCCTATGACAAGCCTGAGTAATGG
FP_GDSL_PYES2	PYES2	CGGGGTACCAACACAATGTCTGGAATGGAG
RP_GDSL_PYES2_HIS	PYES2	TAAGCGGCCGCTTAATGATGATGATGATGATGGCTCCTATGAC
FP_GDSL_qPCR	-	ACCGGAGGATCAACAATTAGAAGGCCGA
RP_GDSL_qPCR	-	ATTTGGAAGTCTGCTTCTCTCATAGGGA
IS-RT_F	-	ACCGTTGATTCGCACAATTGGTCATCG
IS-RT_R	-	TACTGCGGGTCGGCAATCGGACG

RESULTS AND DISCUSSION

Hydrolase activity and protein sequence analysis

Native proteins were extracted from *Fragaria × ananassa* red fruit to search enzymes candidate associated with the fruit ripening. Target proteins from *F. × ananassa* red fruit were isolated using ammonium sulfate precipitation (40-60%). This protein extract was further separated through SDS PAGE (Figure 1a) and native PAGE (Figure 1b). An esterase assay in the native PAGE gel revealed a positive band indicating α-naphthol release from the respective ester and thus hydrolase activity. An insoluble yellow to red colored product was formed after the coupling of α-naphthol with a diazonium salt (Figure 1b). The protein band presenting esterase activity was cut from the native PAGE gel and then delivered to the Chair of Proteomics and Bioanalytics at Technical University of Munich, Germany for amino acid sequencing (Figure 1). The data interpretation revealed various esterases candidates for instance pectinesterase 1 (PE1), PE3, PE34, and GDSL esterase/lipase (Figure 1c). Castillejo et al. (2004) has characterized several pectinesterase genes from *F. × ananassa* identified as FaPE1, FaPE2, FaPE3, and FaPE4. Accordingly, GDSL esterase/lipase At5g14450, Precursor (putative), gene27964 (namely *FvGELP1)* was selected for cloning and biochemical analysis in this study.

Gene expression analysis and phylogenetic tree

Gene expression analysis of *FvGELP1* was performed in a transcriptome data set of *F. vesca* varieties including Hawaii 4, Yellow Wonder, and Reine de Vallées by Härtl et al. (2017). High expression levels of *FvGELP1* were found in green fruit of achenes and receptacles in all varieties (Figure 2). During strawberry fruit ripening, decreasing expression levels were detected. In addition, expression levels in receptacles were higher than in achenes. Thus, *FvGELP1* might involve in strawberry fruit ripening. Furthermore, *FvGELP1* showed the highest expression level among 25 candidate GDSL esterase/lipase genes in the receptacle of *F. vesca* Hawaii 4 fruits (Figure 3). Among these genes, *FvGELP1* showed high similarity with gene29811 (GDSL esterase/lipase At5g45920 (putative)) and gene28412 (GDSL esterase/lipase At5g14450, Precursor (similar to)) based on phylogenetic tree (Figure 4). The NGS data of *FvGELP1* in *F. vesca* var. Hawaii 4 fruit at different ripening stages was then verified by qRT-PCR.

Quantitative real-time PCR of *FvGELP1* in *F. vesca* Hawaii 4

The expression levels of *FvGELP1* in *F. vesca* fruit, leaf, and flower tissues, qRT-PCR analysis was presented in Figure 5. The qRT-PCR data of *FvGELP1* genes in different tissues and development stages could provide reference to molecular mechanisms of plant growth and development. The highest expression levels of the gene was detected in large green fruit. It elevated significantly from small green fruit to large green fruit but subsequently declined throughout fruit ripening. This suggested that *FvGELP1* may play pivotal roles in developmental stages. In other tissues, the expression of *FvGELP1* in flower was greater than in old and young leaves.

FvGELP1 expression in different tissues implied that *FvGELP1* might play diverse roles in various tissues and during development. Similarly, BrGELPs in *Brassica rapa* were expressed in various tissues, including floral organs (Dong et al. 2016). In soybean, *GmGELP22*, *GmGELP149*, and *GmGELP186* showed high transcription levels in most tissues throughout the growth period. Whereas *GmGELP10*, *79*, *115*, *124*, and *180* exhibited high expression in young leaves, flowers, pods, and pod shells (Su et al. 2020).

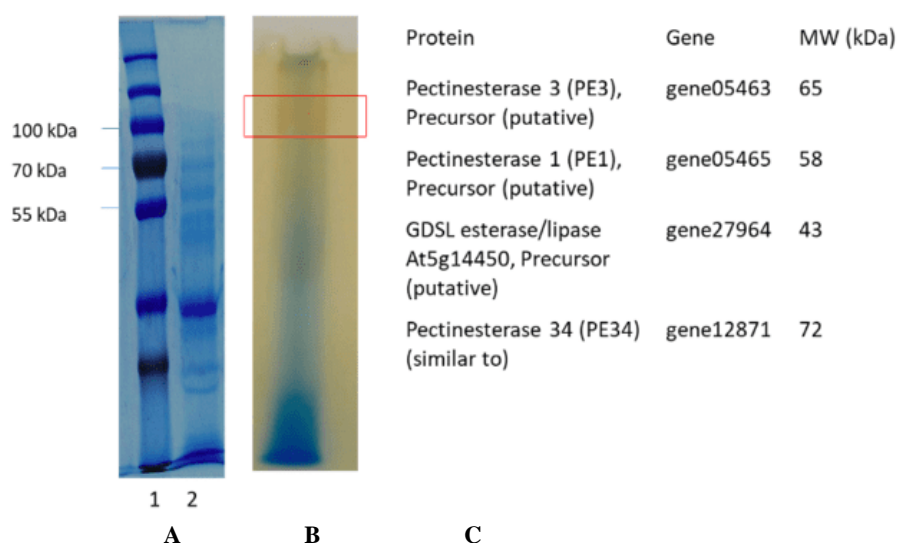| Protein	Gene	MW (kDa)
Pectinesterase 3 (PE3), Precursor (putative)	gene05463	65
Pectinesterase 1 (PE1), Precursor (putative)	gene05465	58
GDSL esterase/lipase At5g14450, Precursor (putative)	gene27964	43
Pectinesterase 34 (PE34) (similar to)	gene12871	72

Figure 1. Protein bands of an ammonium sulfate extract from *F. × ananassa* fruit: A. SDS PAGE, B. Native PAGE, C. Possible esterase candidates of targeted band. The yellow band (red box) indicated positive esterase activity
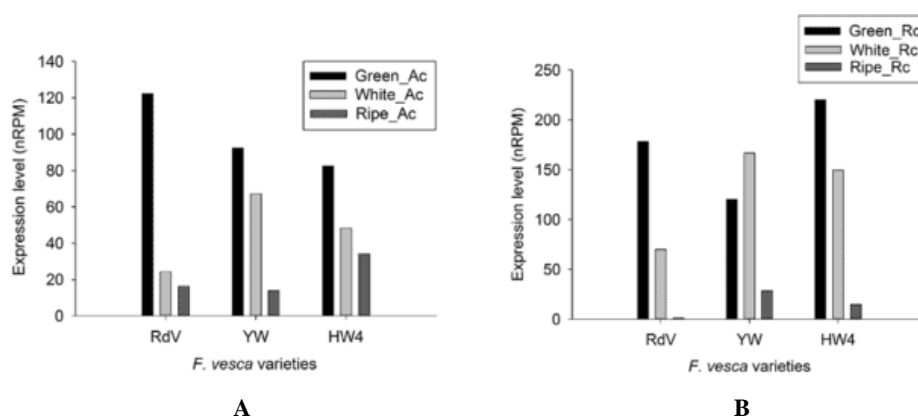
Figure 2. Gene expression levels of gene27964 in three *F. vesca* var. Hawaii 4 (HW4), Reine des Vallées (RdV), and Yellow Wonder (YW). A. Expression levels in achenes (Ac), B. receptacle (Rc) presented as nRPM (normalized read per million)
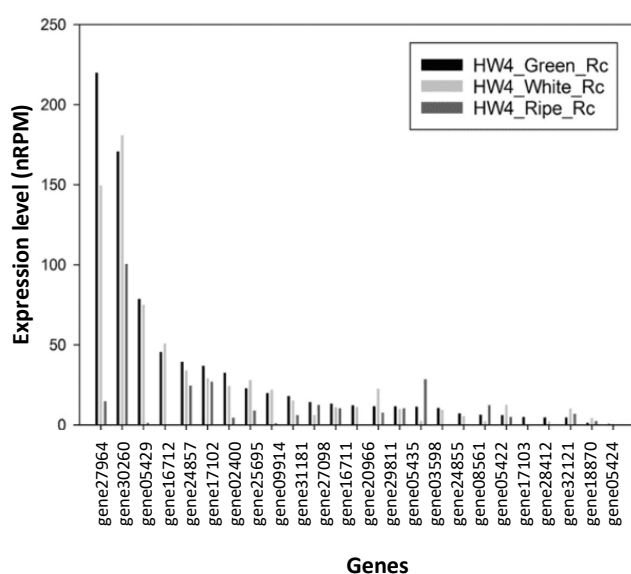

Figure 3. Gene expression level of candidate GDSL esterase/lipase in the receptacle of *F. vesca* var. Hawaii 4. Putative genes were arranged in decreasing order of their expression levels, presented as nRPM (normalized read per million)
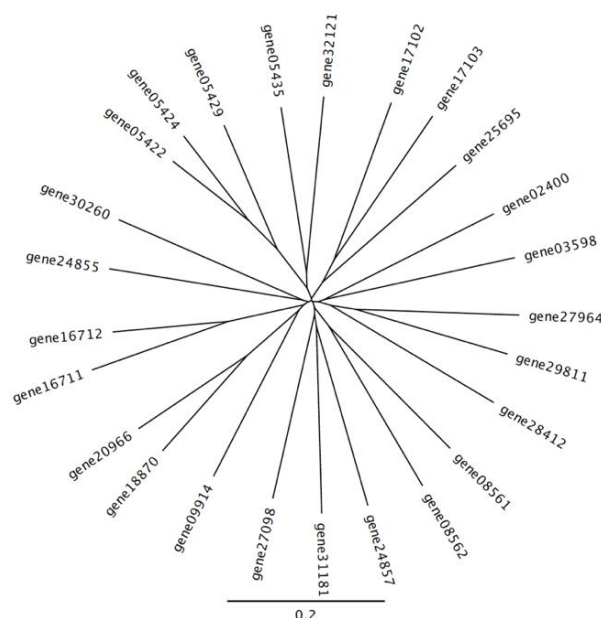

Figure 4. Phylogenetic tree of GDSL esterase/lipase candidate genes from *F. vesca*. Gene27964 (*FvGELP1*) is in black frame box
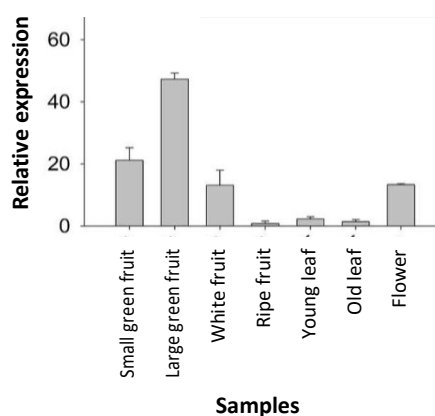

Figure 5. The expression levels of *FvGELP1* in *F. vesca* fruit, leaf, and flower tissues. Relative expression levels are normalized to the interspacer (IS) gene. qPCR data were obtained by analyzing three biological and three technical replicates of each sample

Multiple sequence alignment of FvGELP1

The primary amino acid sequence of FvGELP1 consists of specific conserved residues for the GDSL family. The catalytic triad and conserved motif of FvGELP1 were explained by multiple sequence alignment with others GDSL esterase/lipase enzyme (Figure 6). GDSL lipases established a lipolytic enzymes subclass indicated by a specific GDSL motif in the N-terminal of Gly-Asp-Ser-(Leu) (Akoh et al. 2004).

The respective residues in FvGELP1 are Gly^{43}-Asp^{44}-Ser^{45}-Asn^{46}. BnSCE3/ BnLIP2 (*Brassica napus*) and OsEst1 (*Oryza sativa*) have an Ile / Leu exchange (Clauss et al. 2008; Hamada et al. 2012). FvGELP1 contains Ser^{45}, Asp^{364}, and His^{367} as the catalytic triad similar to other GDSL-motif proteins (Upton and Buckley 1995). The seryl residue is located in block I, while aspartyl and histidyl residues are part of block V. In addition, the SGNH hydrolases are

designated as a subgroup of the GDSL family depending on the existence of Serine in block I, Glycine in block II, Asparagine in block III, and Histidine in block V residues (Mølgaard et al. 2000). FvGELP1 belongs to SGNH hydrolases within the GDSL subclass. It is characterized by the existence of the conserved residues Ser, Gly, Asn, and His in the conserved peptide block I, block II, block III and block V, respectively (Mølgaard et al. 2000; Ling et al. 2006). Each of the conserved residues shows an essential function in enzyme catalysis. The Ser residue acts as the catalytic nucleophile. In addition, the Ser together with the Gly and the Asn residues contribute as proton donors to the oxyanion hole. The His residue serves as a base which deprotonating the hydroxyl group, thus generating the active site Ser more nucleophilic (Akoh et al. 2004). Furthermore, GDSL esterase/lipases have a flexible active site that is able to change their conformation with the binding of different substrates.
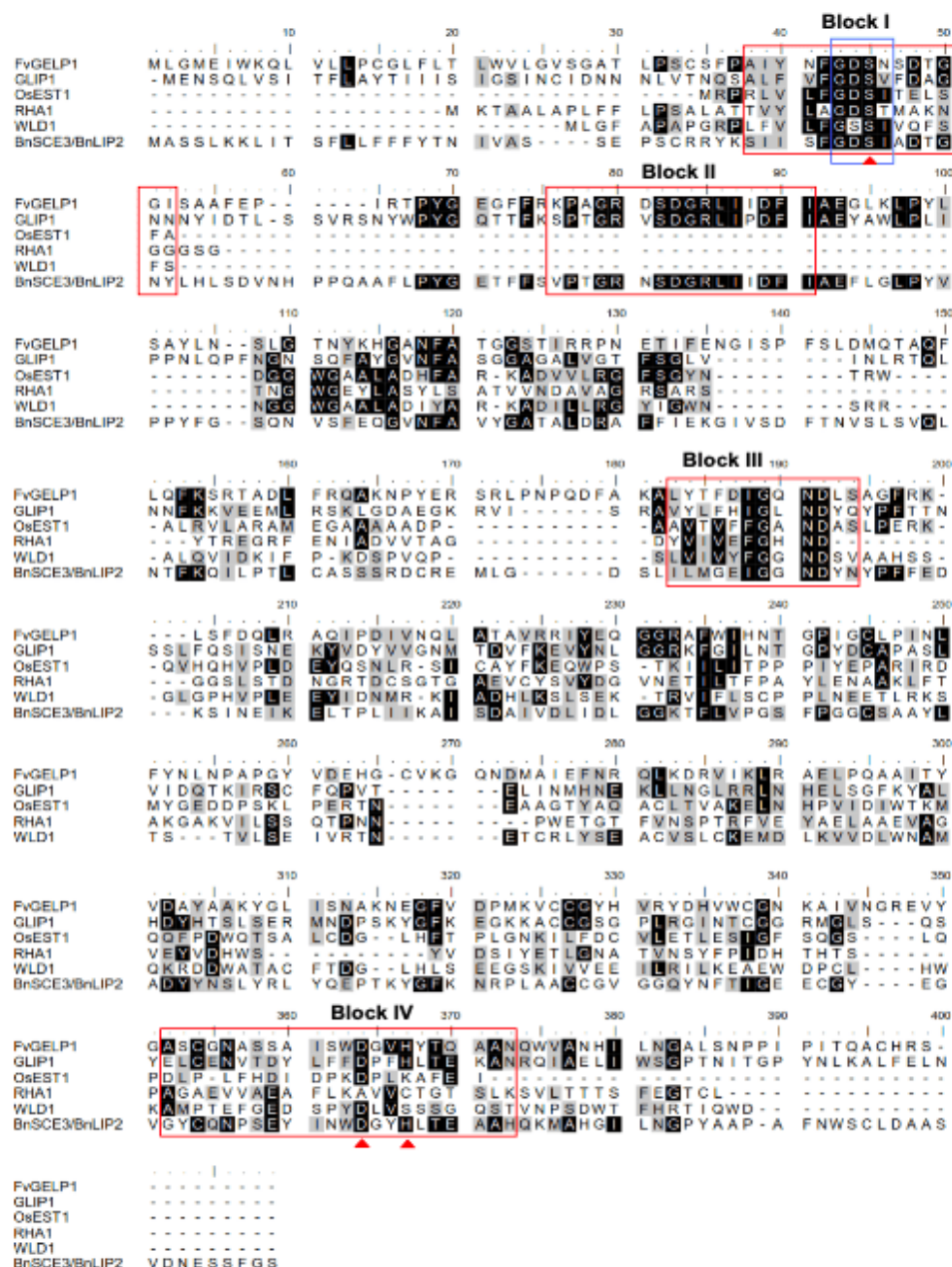

Figure 6. Multiple sequence alignment of FvGELP1. Sequences resemble the subsequent accession numbers and species: GLIP1 (NP_198915.1, *A. thaliana*), OsEST1 (AK061229.1, *Oryza sativa*), WLD1 (AK067429), RHA 1 (UniProtKB-Q00017, *Aspergillus aculeatus*), and BnSCE3 (AAX62802.1, *Brassica napus*). The blue lines box indicates a fully conserved residue. Four conserved peptide blocks (block I, block II, block III, and block V) in the SGNH-hydrolases family are in the red frame box (Ling et al. 2006). The catalytic triad of Ser, Asp, His residues in blocks I and V are marked by red triangles

Cloning and expression of *FvGELP1*

The correct size bands (1.2 kb) have been amplified from cDNA of *F. vesca* and *F. × ananassa* fruits (excluding from *F. × ananassa* small green fruit) (Figure 7). According to Shulaev et al. (2011), the gene27964 consists of 1,161 bp. These amplified fragments were cloned into PSK+ plasmid and transformed into *E. coli* NEβ10. The sequence alignment of the genes from *F. × ananassa* and *F. vesca* resulted in 51% and 100% identity with *FvGELP1*, respectively. In addition, translation analysis of the nucleotide sequence amplified from cDNA of *F. × ananassa* fruits revealed many stop codons. Therefore, *FvGELP1* amplified from *F. vesca* fruit cDNA was selected for further cloning and heterologous expression in *S. cerevisiae*. *FvGELP1* was expressed as a 43 kDa His-tagged fusion protein (Figure 8). GDSL esterases/lipases have a molecular weight of 22-60 kDa (Akoh et al. 2004).

Esterase assay of FvGELP1

Esterase assays were performed using small molecule esters such as α-NA, pNPA, phenyl acetate, and benzyl acetate as substrates to investigate the enzymatic activity of FvGELP1. GDSL esterases/lipases exhibit a broad range of substrate specificity. FvGELP1 esterase assays revealed positive results for phenyl acetate, benzyl acetate, pNPA, and αNA. It released high content of aromatic alcohol products (Figure 9). These suggest that FvGELP1 is a promiscuous hydrolase catalyzing a range of low molecular weight esters. The results also indicated that FvGELP1 shows the activity of carboxylesterases.

A p-nitrobenzyl (pNB) carboxy-esterase from *Bacillus subtilis* hydrolyzed simple organic esters for instance benzyl acetate, pNB-acetate and αNA (Chen et al. 1995). Cbes-AcXE2 (a *Caldicellulosiruptor bescii* acetyl xylan esterase) catalyzed phenyl acetate, p-nitrophenyl butyrate (pNPB), and pNPA with equivalent efficiency. Phenyl acetate as the most preferred substrate showed K_M and specific activity of 0.85 mM and 142 U/mg, respectively (Soni et al. 2017). OsEST1 from *Oryza sativa* strictly recognizes small molecule esters. It demonstrated high enzymatic activity for αNA with V_{max} of 63.7 μmol min^{-1} mg^{-1} and K_m of 172 μM. Its relative activity for α-naphthyl butyrate (αNB) was significantly lower than αNA. Furthermore, OsEST1 showed negligible activity using triglycerides as substrates such as tributyrin and olive oil. The latest implied that OsEST1 is not a lipase (Hamada et al. 2012). Enod8 from *Medicago sativa* exhibited esterase activity for acetyl and butyryl esters as substrates however not for longer chain aliphatic esters (Pringle and Dickstein 2004). GLIP2, GDSL lipase 2 protein from *Arabidopsis thaliana* showed lipase activity. It catalyzed pNP acetate, and pNP butyrate hydrolysis (Lee et al. 2009). Although the preliminary enzymatic activity of FvGELP1 has been determined, further research is needed to study the biological role of FvGELP1 throughout strawberry fruit ripening.
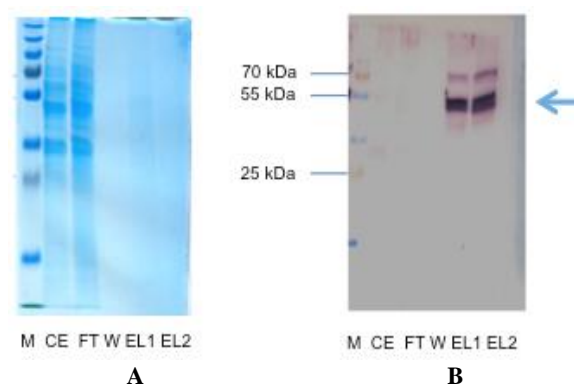

Figure 8. Recombinant FvGELP1 produced in *S. cerevisiae*: A. SDS PAGE, B. western blot analysis. M: protein marker, CE: crude extract, FT: flow through, W: wash, EL1 and EL2: elution
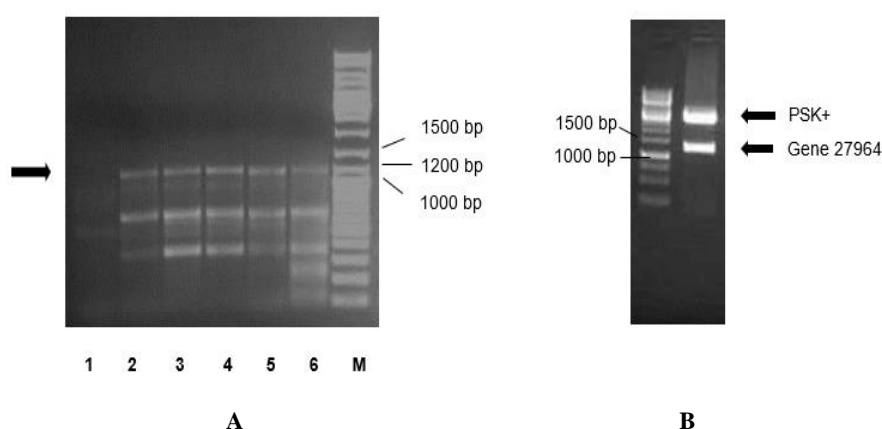

Figure 7. The amplified bands from cDNA of *F. vesca* and *F. × ananassa* fruits. A. Amplification of gene27964 from *F. × ananassa* (1-5) and *F. vesca* (6) fruit. 1: small green, 2: large green, 3: white, 4: tuning, 5: red fruit of *F. × ananassa*, 6: ripe fruit of *F. vesca*. M: DNA marker. Target gene27964 (1.161 bp) bands are shown by an arrow. B. Restriction enzyme analysis of recombinant *FvGELP1* using SmaI and XhoI. M: DNA marker, 1: gene27964 and PSK+ plasmid band
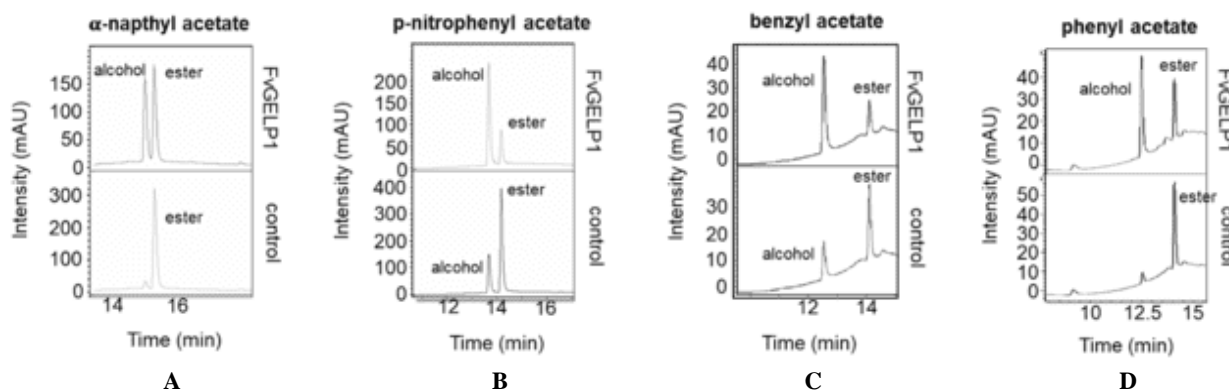
Figure 9. HPLC-UV esterase assay of FvGELP1. Esters including: A. αNA, B. pNPA, C. Benzyl acetate, D. Phenyl acetate were utilized as substrates. Negative control was a crude extract of *S. cerevisiae* transformed with empty pYES2 vector

In conclusion, this study is successfully identified and characterized a new enzyme in strawberry namely FvGELP1 (Fragaria vesca GDSL esterase/lipase). FvGELP1 showed hydrolase activity against small molecule esters such as α-NA, pNPA, benzyl acetate, and phenyl acetate. Therefore, FvGELP1 might involve in strawberry fruit ripening, possibly by deacetylation of the polysaccharide.

REFERENCES

Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. 2004. GDSL family of serine esterases/lipases. Prog Lipid Res 43 (6): 534-552. DOI: 10.1016/j.plipres.2004.09.002.

An X, Dong Z, Tian Y, Xie K, Wu S, Zhu T, Zhang D, Zhou Y, Niu C, Ma B, Hou Q, Bao J, Zhang S, Li Z, Wang Y, Yan T, Sun X, Zhang Y, Li J, Wan X. 2019. ZmMs30 encoding a novel GDSL lipase is essential for male fertility and valuable for hybrid breeding in maize. Mol Plant 12: 343-359. DOI: 10.1016/j.molp.2019.01.011.

Castillejo C, Fuente JI, Pietro Iannetta P, Botella MA, Valpuesta V. 2004. Pectin esterase gene family in strawberry fruit: Study of FaPE1, a ripening-specific isoform. J Exp Bot 55 (398): 909-918. DOI: 10.1093/jxb/erh102.

Chen Y, Usui S, Queener SW. 1995. Purification and properties of ap-nitrobenzyl esterase from *Bacillus subtilis*. J Ind Microbiol 15: 10-18. DOI: 10.1007/BF01570007.

Chepyshko H, Lai C-P, Huang L-M, Liu J-H, Shaw J-F. 2012. Multifunctionality and diversity of GDSL esterase/lipase gene family in rice (*Oryza sativa* L. *japonica*) genome: New insights from bioinformatics analysis. BMC Genomics 13: 309. DOI: 10.1186/1471-2164-13-309.

Clauss K, Baumert A, Nimtz M, Milkowski C, Strack D. 2008. Role of a GDSL lipase-like protein as sinapine esterase in Brassicaceae. Plant J 53: 802-813. DOI: 10.1111/j.1365-313X.2007.03374.x.

Ding LN, Guo XJ, Li M, Fu ZL, Yan SZ, Zhu KM, Wang Z, Tan XL. 2019a. Improving seed germination and oil contents by regulating the GDSL transcriptional level in *Brassica napus*. Plant Cell Rep 38: 243-253. DOI: 10.1007/s00299-018-2365-7.

Ding LN, Li M, Guo XJ, Tang MQ, Cao J, Wang Z, Liu R, Zhu KM, Guo L, Liu SY, Tan XL. 2019b. *Arabidopsis* GDSL1 overexpression enhances rapeseed *Sclerotinia sclerotiorum* resistance and the functional identification of its homolog in *Brassica napus*. Plant Biotechnol J 18: 1255-1270. DOI: 10.1111/pbi.13289.

Dong X, Yi H, Han CT, Nou IS, Hur Y. 2016. GDSL esterase/lipase genes in *Brassica rapa* L.: Genome-wide identification and expression analysis. Mol Genet Genomics 291: 531-542. DOI: 10.1007/s00438-015-1123-6.

Franz-Oberdorf K, Eberlein B, Edelmann K, Bleicher P, Kurze E, Helm D, Olbricht K, Darsow U, Ring J, Schwab W. 2017. White-fruited strawberry genotypes are not per se hypoallergenic. Intl Food Res J 100: 748-756. DOI: 10.1016/j.foodres.2017.07.076.

Gabriel O. 1971. Analytical disc gel electrophoresis. Methods Enzymol 22: 565-578. DOI: 10.1016/0076-6879(71)22041-3.

Gilham D, Lehner R. 2005. Techniques to measure lipase and esterase activity in vitro. Methods 36 (2): 139-47. DOI: 10.1016/j.ymeth.2004.11.003.

Gou JY, Miller LM, Hou G, Yu XH, Chen XY, Liu CJ. 2012. Acetylesterase-mediated deacetylation of pectin impairs cell elongation, pollen germination, and plant reproduction. Plant Cell 24 (1): 50-65. DOI: 10.1105/tpc.111.092411.

Hamada S, Hasegawa Y, Suzuki Y. 2012. Identification of a GDSL-motif carboxylester hydrolase from rice bran (*Oryza sativa* L.). J Cereal Sci 55: 100-105. DOI: 10.1016/j.jcs.2011.10.006.

Härtl K, Denton A, Franz-Oberdorf K, Hoffmann T, Spornraft M, Usadel B, Schwab W. 2017. Early metabolic and transcriptional variations in fruit of natural white-fruited *Fragaria vesca* genotypes. Sci Rep 7: 45113. DOI: 10.1038/srep45113.

Huang LM, Lai CP, Chen LO, Chan MT, Shaw JF. 2015. Arabidopsis SFAR4 is a novel GDSL-type esterase involved in fatty acid degradation and glucose tolerance. Bot Stud 56: 33. DOI: 10.1186/s40529-015-0114-6.

Kikuta Y, Ueda H, Takahashi M, Mitsumori T, Yamada G, Sakamori K, Takeda K, Furutani S, Nakayama K, Katsuda Y, Hatanaka A, Matsuda K. 2012. Identification and characterization of a GDSL lipase-like protein that catalyzes the ester-forming reaction for pyrethrin biosynthesis in *Tanacetum cinerariifolium*-A new target for plant protection. Plant J 71 (2): 183-193. DOI: 10.1111/j.1365-313X.2012.04980.x.

Kim HG, Kwon SJ, Jang YJ, Nam MH, Chung JH, Na YC, Guo H, Park OK. 2013. GDSL LIPASE1 modulates plant immunity through feedback regulation of ethylene signaling. Plant Physiol 163: 1776-1791. DOI: 10.1104/pp.113.225649.

Kim HG, Kwon SJ, Jang YJ, Chung JH, Nam MH, Park OK. 2014. GDSL lipase 1 regulates ethylene signaling and ethylene-associated systemic immunity in *Arabidopsis*. FEBS Lett 588: 1652-1658. DOI: 10.1016/j.febslet.2014.02.062.

Kram BW, Bainbridge EA, Perera MA, Carter C. 2008. Identification, cloning and characterization of a GDSL lipase secreted into the nectar

of *Jacaranda mimosifolia*. Plant Mol Biol 68: 173-183. DOI: 10.1007/s11103-008-9361-1.

Lai CP, Huang LM, Chen LO, Chan MT, Shaw JF. 2017. Genome-wide analysis of GDSL-type esterases/lipases in *Arabidopsis*. Plant Mol Biol 95: 181-197. DOI: 10.1007/s11103-017-0648-y.

Lee DS, Kim BK, Kwon SJ, Jin HC, Park OK. 2009. *Arabidopsis* GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. Biochem Biophys Res Commun 379: 1038-1042. DOI: 10.1016/j.bbrc.2009.01.006.

Liao Z, Chen M, Guo L, Gong Y, Tang F, Sun X, Tang K. 2004. Rapid isolation of high-quality total RNA from taxus and ginkgo. Prep Biochem Biotechnol 34 (3): 209-214. DOI: 10.1081/PB-200026790.

Ling H, Zhao J, Zuo K, Qiu C, Yao H, Qin J, Sun X, Tang K. 2006. Isolation and expression analysis of a GDSL-like lipase gene from *Brassica napus* L. J Biochem Mol Biol 39: 297-303. DOI: 10.5483/bmbrep.2006.39.3.297.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25:402-408. DOI: 10.1006/meth.2001.1262.

Ma R, Yuan H, An J, Hao X, Li H. 2018. A *Gossypium hirsutum* GDSL lipase/hydrolase gene (GhGLIP) appears to be involved in promoting seed growth in *Arabidopsis*. PLoS One 13: e0195556. DOI: 10.1371/journal.pone.0195556.

Mølgaard A, Kauppinen S, Larsen S. 2000. Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. Structure 8 (4): 373-383. DOI: 10.1016/s0969-2126(00)00118-0.

Naranjo MA, Forment J, Roldan M, Serrano R, Vicente O. 2006. Overexpression of *Arabidopsis thaliana* LTL1, a salt-induced gene encoding a GDSL-motif lipase, increases salt tolerance in yeast and transgenic plants. Plant Cell Environ 29: 1890-1900. DOI: 10.1111/j.1365-3040.2006.01565.x.

Niehaus JU, Gross GG. 1997. A gallotannin degrading esterase from leaves of pedunculate oak. Phytochemistry 45 (8): 1555-1560. DOI: 10.1016/S0031-9422(97)00261-6.

Pringle D, Dickstein R. 2004. Purification of ENOD8 proteins from *Medicago sativa* root nodules and their characterization as esterases, Plant Physiol Biochem 42: 73-79. DOI: 10.1016/j.plaphy.2003.10.004.

Rajarammohan S, Pradhan A.K, Pental D, Kaur J. 2018. Genome-wide association mapping in *Arabidopsis* identifies novel genes underlying quantitative disease resistance to *Alternaria brassicae*. Mol Plant Pathol 19: 1719-1732. DOI: 10.1111/mpp.12654.

Ring L, Yeh S, Hücherig S, Hoffmann T, Blanco-Portales R, Fouche M, Villatoro C, Denoyes B, Monfort A, Caballero JL, Muñoz-Blanco J, Gershenson J, Schwab W. 2013. Metabolic interaction between anthocyanin and lignin biosynthesis is associated with peroxidase FaPRX27 in strawberry fruit. Plant Physiol 163: 43-60. DOI: 10.1104/pp.113.222778.

Searle BC. 2010. Scaffold: A bioinformatic tool for validating MS/MS-based proteomic studies. Proteomics 10 (6): 1265-1269. DOI: 10.1002/pmic.200900437.

Shulaev V, Sargent DJ, Crowhurst RN, Mockler TC, Folkerts O, Delcher AL, Jaiswal P, Mockaitis K, Liston A, Mane SP, et al. 2011. The genome of woodland strawberry (*Fragaria vesca*). Nat Genet 43: 109-116. DOI: 10.1038/ng.740.

Smyth DR. 2017. Wrinkles on sepals: Cuticular ridges form when cuticle production outpaces epidermal cell expansion. Mol Plant 10: 540-541. DOI: 10.1016/j.molp.2017.02.008.

Soni S, Sathe SS, Odaneth AA, Lali AM. 2017. SGNH hydrolase-type esterase domain containing Cbes-AcXE2: A novel and thermostable acetyl xylan esterase from *Caldicellulosiruptor bescii*. Extremophiles 21 (4): 687-679. DOI: 10.1007/s00792-017-0934-2.

Su H-G, Zhang X-H, Wang T-T, Wei W-L, Wang Y-X Chen J, Zho Y-B, Chen M, Ma Y-Z, Xu Z-S, Min D-H. 2020. Genome-wide identification, evolution, and expression of GDSL-type esterase/lipase gene family in soybean. Front Plant Sci 11: 726. DOI: 10.3389/fpls.2020.00726.

Upton C, Buckley JT. 1995. A new family of lipolytic enzymes? Biochem Sci 20 (5): 178-179. DOI: 10.1016/S0968-0004(00)89002-7.

Volokita M, Rosilio-Brami T, Rivkin N, Zik M. 2011. Combining comparative sequence and genomic data to ascertain phylogenetic relationships and explore the evolution of the large GDSL-lipase family in land plants. Mol Biol Evol 28: 551-565. DOI: 10.1093/molbev/msq226.

Walker JM. 2009. The Protein Protocols Handbook. Humana Press, New Jersey.

Watkins JL, Li M, McQuinn RP, Chan KX, McFarlane HE, Ermakova M, Furbank RT, Mares D, Dong C, Chalmers KJ, Sharp P, Mather DE, Pogson BJ. 2019. A GDSL esterase/lipase catalyzes the esterification of lutein in bread wheat. Plant Cell 31: 3092-3112. DOI: 10.1105/tpc.19.00272.

Witasari LD, Huang F-C, Hoffmann T, Rozhon W, Fry SC, Schwab W. 2019. Higher expression of the strawberry xyloglucan endotransglucosylase/hydrolase genes FvXTH 9 and FvXTH 6 accelerates fruit ripening. Plant J 100: 1237-1253. DOI: 10.1111/tpj.14512.

Xue C, Guan SC, Chen JQ, Wen CJ, Cai JF, Chen X. 2020. Genome wide identification and functional characterization of strawberry pectin methylesterases related to fruit softening. BMC Plant Biol 20: 13. DOI: 10.1186/s12870-019-2225-9.

Yadav VK, Pant P, Singh, SP, Maurya R, Sable A Sawant SV. 2017. GhMYB1 regulates SCW stage-specific expression of the GhGDSL promoter in the fibres of *Gossypium hirsutum* L. Plant Biotechnol J 15: 1163-1174. DOI: 10.1111/pbi.12706.

Zhang B, Zhang L, Li F, Zhang D, Liu X, Wang H, Xu Z, Chu C, Zhou Y. 2017. Control of secondary cell wall patterning involves xylan deacetylation by a GDSL esterase. Nat Plants 3: 17017. DOI: 10.1038/nplants.2017.17.

Zhang L, Gao C, Mentink-Vigier F, Tang L, Zhang D, Wang S, Cao S, Xu Z, Liu X, Wang T,Zhou Y, Zhang B. 2019. Arabinosyl deacetylase modulates the arabinoxylan acetylation profile and secondary wall formation. Plant Cell 31: 1113-1126. DOI: 10.1105/tpc.18.00894.