

Identification and Expression Profiles of Six Transcripts Encoding Carboxylesterase Protein in *Vitis flexuosa* Infected with Pathogens

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Plants protect themselves from pathogen attacks via several mechanisms, including hypersensitive cell death. Recognition of pathogen attack by the plant resistance gene triggers expression of carboxylesterase genes associated with hypersensitive response. We identified six transcripts of carboxylesterase genes, *Vitis flexuosa* carboxylesterase 5585 (*VfCXE5585*), *VfCXE12827*, *VfCXE13132*, *VfCXE17159*, *VfCXE18231*, and *VfCXE47674*, which showed different expression patterns upon transcriptome analysis of *V. flexuosa* inoculated with *Elsinoe ampelina*. The lengths of genes ranged from 1,098 to 1,629 bp, and their encoded proteins consisted of 309 to 335 amino acids. The predicted amino acid sequences showed hydrolase like domains in all six transcripts and contained two conserved motifs, GXSXG of serine hydrolase characteristics and HGGGF related to the carboxylesterase family. The deduced amino acid sequence also contained a potential catalytic triad consisted of serine, aspartic acid and histidine. Of the six transcripts, *VfCXE12827* showed upregulated expression against *E. ampelina* at all time points. Three genes (*VfCXE5585*, *VfCXE12827*, and *VfCXE13132*) showed upregulation, while others (*VfCXE17159*, *VfCXE18231*, and *VfCXE47674*) were down regulated in grapevines infected with *Botrytis cinerea*. All transcripts showed upregulated expression against *Rhizobium vitis* at early and later time points except *VfCXE12827*, and were downregulated for up to 48 hours post inocula-

tion (hpi) after upregulation at 1 hpi in response to *R. vitis* infection. All tested genes showed high and differential expression in response to pathogens, indicating that they all may play a role in defense pathways during pathogen infection in grapevines.

Keywords : *Botrytis cinerea*, disease resistance, *Elsinoe ampelina*, hypersensitive response, *Rhizobium vitis*

Plants have evolved several mechanisms to protect themselves against pathogen attacks. Hypersensitive response (HR) is one of the most common and efficient plant reactions to pathogens, although its precise role is not yet clear (Macro et al., 1990). The characteristics of hypersensitivity include rapid local cell death at the site of infection to limit further spread of invading micro organisms (Hammond-Kosack and Jones, 1997). Hypersensitive cell death occurs via the recognition of pathogen avirulence (avr) genes by plant resistance (R) genes (Liu et al., 2007). Although hypersensitive cell death is triggered by direct R genes, several hypersensitive related genes have been identified and characterized (Baudouin et al., 1997; Bézier et al., 2002; Macro et al., 1990; Pontier et al., 1998b; Tronchet et al., 2001).

Hypersensitivity-related genes, including *hsr203J* (tobacco), *Lehr203* (tomato), *BIG8.1* (*Botrytis*-induced grapevine), *PepEST* (pepper), and *SOBER1* (*Arabidopsis*), were characterized as carboxylesterase members of the serine hydrolase family (Baudouin et al., 1997; Bézier et al., 2002; Ko et al., 2005; Tronchet et al., 2001). Serine hydrolases comprise a large number of enzymes, including esterases, lipases, proteases and transferases (Kaschani et al., 2009). The motif 'GXSXG' is characteristic of many members of the serine hydrolase family, including lipases, esterases and proteases (Baudouin et al., 1997). Pentapeptide 'HGGGF' is observed in many lipase sequences (Baudouin et al., 1997). Carboxylesterases (es-

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terases and lipases) catalyze the hydrolysis of compounds containing an ester bond, while esterases hydrolyze water-soluble compounds such as short acyl chain esters and are inactive against water-insoluble long chain triacylglycerols, which, in turn, are specifically hydrolyzed by lipases (Chahinian and Sarda, 2009). Several esterase and lipase genes have been characterized from various plants such as tobacco, tomato, grapevine, *Arabidopsis*, and pepper.

Cultivated grapevines (*Vitis* sp.) are exposed to many pathogenic fungi, such as *Elsinoe ampelina* and *Botrytis cinerea*, which cause anthracnose and gray mold, respectively, as well as bacteria such as *Rhizobium vitis*, which is responsible for crown gall and viruses (Wang et al., 2011). The development of new varieties resistant to diseases can result in the cost saving and convenient management of various diseases in grape production. Several carboxylesterase genes from various plants including *hsr203J*, *Lehsr203*, *EDS1*, *PAD4*, *DAD1*, *PRLIP*, *SABP2*, and *PepEST* have been shown to function in different developmental mechanisms and resistant pathways against pathogens. In this study, we isolated six carboxylesterase genes in *Vitis flexuosa*, analyzed their structural features and homology, evaluated their phylogenetic relationship with other plants, and investigated expression patterns against infection of *E. ampelina*, *B. cinerea*, and *R. vitis*.

Materials and Methods

Plant materials and pathogens. Plants of *V. flexuosa* VSKO001 were cultured in a grapevine germplasm collection field of Yeungnam University, Gyeongsan, Korea for leaf production. Leaves were used for gene expression analysis following pathogen inoculation. The pathogens used in this study were virulent strains of *E. ampelina* (EA-1) and *B. cinerea* (B1035) isolated from infected grapes by Dr. W.K. Kim, National Academy of Agricultural Science, Rural Development Administration, Korea, as well as *R. vitis* (strain Cheonan 493) kindly provided by Prof. J.S. Cha, Chungbuk National University, Korea.

Inoculation of pathogens. Spores of *E. ampelina* (10^6 spores/ml) were sprayed onto leaves after scraping off of plates with sterile distilled water according to the method described by Yun et al. (2003). Spore suspensions (10^6 spores/ml) of *B. cinerea* in making by 0.24% potato dextrose broth solution were sprayed onto the leaves for inoculation. Additionally, to induce defense responses against bacteria in the leaves, 20 μ l cell suspensions ($OD_{600} = 1$) of *R. vitis* grown in YEP medium (yeast extract 10 g, bacto-peptone 5 g, NaCl 5 g/l, and pH 7.0) at 28°C in a shaking incubator for 16 to 18 hours were dropped onto the wounded portion of leaves that had been injured slightly with a pencil tip (Choi et

al., 2010). Leaves inoculated with spore suspensions were then incubated in a moist box at 22–28°C for 48 hours to induce early hypersensitive responses by pathogen infections. Leaves were subsequently harvested at the indicated time points (0, 1, 6, 12, 24, and 48 hours post inoculation [hpi]), immediately frozen in liquid nitrogen and then stored at –80°C for future use.

RNA isolation and real-time PCR analysis. For RNA isolation, desired leaf samples were ground in liquid nitrogen using a mortar and pestle and total RNA was extracted by the modified pine tree method (Chang et al., 1993). The RNA quality was determined based on the absorbance at 230, 260, and 280 nm, which was measured using a Nano Drop spectrophotometer (ACTGene ASP-3700; ACTGene Inc., Piscataway, NJ, USA). The GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) was used to synthesize first-strand cDNA from the total RNA (500 ng), which was subsequently used as a template for PCR. Real-time PCR was performed on a C1000™ Thermal Cycler (CFX96™ Real-Time System; Bio-Rad, Hercules, CA, USA) using SYBR Premix Ex Taq (TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye. Amplification was conducted by subjecting the samples to 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The standard-curve method was employed to determine the transcript levels. Transcripts were normalized against the grapevine actin gene (AB372563) as an internal control and non-treated leaves (at time zero) as a reference. Melting curves of the amplified products were also recorded. For each gene, the reference sample was defined as the 1 \times expression level and the results were expressed as the fold increase in mRNA level over the reference sample. To minimize error, all reactions were replicated three times. The specific primers used in real-time PCR are listed in Table 1.

Sequence analysis of genes. To verify the presence of the hydrolase like domain, the VfCXE protein sequences were analyzed using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1). Nucleotide sequences of genes were transmuted to amino acid sequences using translation software (<http://web.expasy.org/translate/>). The primary structure analysis of genes was performed using protParam (<http://web.expasy.org/protparam/>), while the secondary structure was analyzed by the Self-Optimized Prediction Method with Alignment (SOPMA; https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The tertiary structure of the protein was predicted by SWISS-MODEL (<http://>

swissmodel.expasy.org/interactive). Multiple alignment of protein sequences was performed using ClustalW (<http://www.genome.jp/tools/clustalw/>). Homologue protein sequences of the genes were identified by a BLAST (Basic Local Alignment Search Tool) search of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by the BLASTp tool using the “nr” database. The serine hydrolase genes were blasted (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against each other to check for gene duplication events. Phylogenetic neighbor-joining analyses of gene sequences were performed using Molecular Evolutionary Genetics Analysis (MEGA) ver. 6.0. The tree branches were evaluated using the bootstrap method.

Results and Discussion

Identification and sequence analysis of six carboxylesterase genes in *V. flexuosa*. Six full length transcripts of the carboxylesterase gene were selected from transcriptome analysis (NABIC, NN-0197-000001) by next generation sequencing (NGS) of *V. flexuosa* VSKO001 inocu-

lated with *E. ampelina* (Ahn et al., 2014), and verified as serine hydrolase-like genes by detection of the hydrolase like domain using SMART. In this study, these genes were characterized and referred to as *V. flexuosa* carboxylesterase 5585 (*VfCXE5585*), *VfCXE12827*, *VfCXE13132*, *VfCXE17159*, *VfCXE18231*, and *VfCXE47674* and deposited in the National Agricultural Biotechnology Information Center (NABIC), Rural Development Administration, Korea under accession numbers NABIC NS-0001-1 to NS-0006-1, respectively. The VfCXE genes were then compared by BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of each other to investigate gene duplication events. The maximum similarity of the predicted amino acid sequences (77%) was observed between VfCXE5585 and VfCXE12827 (Table 2), while the similarity between two amino acid sequences among other genes ranged from 28% to 58%. All six VfCXE genes tested in this study were confirmed to be independent upon gene duplication analysis based on the index proposed by Kong et al. (2013). The primary structure and the characteristics of the six VfCXE genes were analyzed using the bioinformatic tool protParam (<http://web.expasy.org/protparam/>) (Table 3). The size of the six VfCXE genes extended from 1,098 to 1,629 bp, while the open reading frame varied from 930 to 1,008, encoding 309 to 335 amino acids (34.2 to 37.7 kDa) with predicted isoelectric points ranging

Table 1. Specific primers based on alignment of six VfCXE genes used for real-time PCR

Gene	Primers	Sequences
<i>VfCXE5585</i>	FP	CCTCAGATTCAGGATGGAGCAT
	RP	GCTGGTGGAATCTGAAGGAGAA
<i>VfCXE12827</i>	FP	ATTGCTATCAGGGCCATTCAAA
	RP	CTTGGCTGATTCACTTGCCTTT
<i>VfCXE13132</i>	FP	GCTGTGGTACCAGTGGCATT
	RP	CGACCTCATGAATAACTTTTGGC
<i>VfCXE17159</i>	FP	ACTACGACCGGAGGTTCAATCAG
	RP	ATACAATGGTCCCCCTTCCACT
<i>VfCXE18231</i>	FP	GCCTTGTGCTGACTATGCCTTT
	RP	TTTGCCCCATATAGGTGTTTCGT
<i>VfCXE47674</i>	FP	GCCGCTTCCTTGTAAGATTTTC
	RP	CTTAAAGCGATAAATGCGCTTC

FP, forward primer; RP, reverse primer.

Table 2. Percent identities of six predicted proteins from the VfCXE gene

	1	2	3	4	5	6
1	-	77	35	36	39	35
2	77	-	37	37	39	38
3	35	37	-	33	53	54
4	36	37	33	-	28	30
5	39	39	53	28	-	58
6	35	38	54	30	58	-

1, VfCXE5585; 2, VfCXE12827; 3, VfCXE13132; 4, VfCXE17159; 5, VfCXE18231; 6, VfCXE47674.

Table 3. Predicted primary structure of six CXE genes from *Vitis flexuosa*

Gene	Nucleotide length (bp)	ORF (bp)	Protein length (aa)	MW (kDa)	pI	Instability index
<i>VfCXE5585</i>	1,098	936	311	34.66	5.16	45.59
<i>VfCXE12827</i>	1,299	930	309	34.28	5.41	43.60
<i>VfCXE13132</i>	1,286	960	319	35.03	5.63	29.84
<i>VfCXE17159</i>	1,629	1008	335	37.75	8.16	53.96
<i>VfCXE18231</i>	1,334	972	323	35.46	5.15	33.53
<i>VfCXE47674</i>	1,468	972	323	35.69	5.18	40.65

ORF, open reading frame; MW, molecular weight; pI, isoelectric point.

Table 4. Predicted secondary structures of six proteins from the VfcXE gene

Protein	Random coil	Alpha helix	Beta turn	Extended strand
VfcXE5585	35.69	30.87	11.58	21.86
VfcXE12827	37.86	31.39	8.74	22.01
VfcXE13132	33.54	31.66	10.66	24.14
VfcXE17159	41.79	27.16	8.06	22.99
VfcXE18231	41.18	30.65	9.91	18.27
VfcXE47674	36.84	26.01	11.46	25.70

from 5.15 to 8.16. The predicted isoelectric points of these genes showed basic characters except *VfcXE17159*. The VfcXE13132 and VfcXE18231 proteins predicted from nucleotide sequences of the VfcXE transcripts showed an instability index of less than 40 (Table 3), which was considered stable based on the instability index (Guruprasad et al., 1990). The secondary structures of the proteins predicted from the six loci of the VfcXE gene were analyzed by SOPMA (Table 4). The results revealed variations in the proportion of random coils (33.54–41.79%), alpha helices (26.01–31.66%), beta turns (8.06–11.58%), and extended strands (18.27–25.70%) in amino acids from

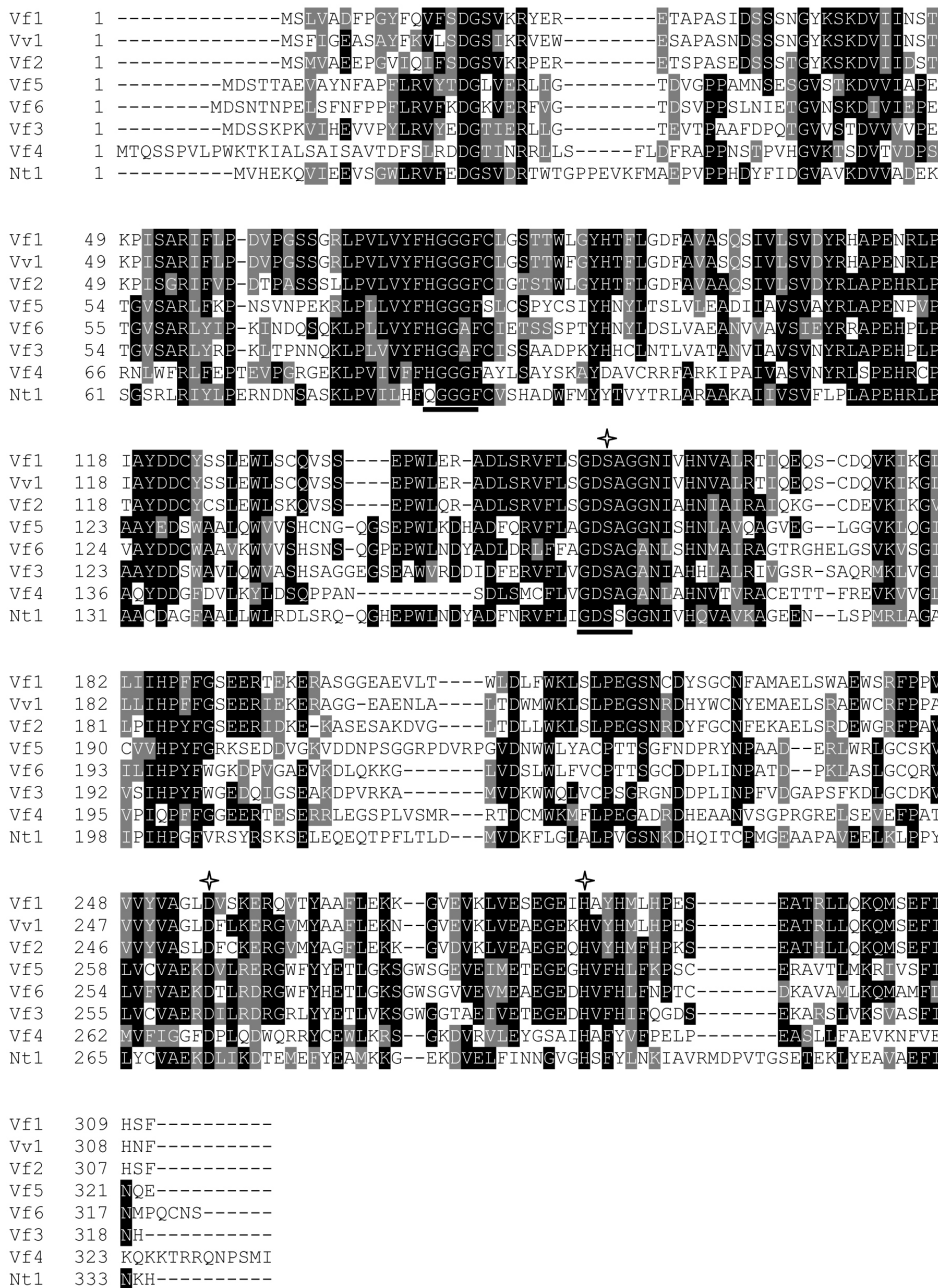


Fig. 1. Multiple alignment of six predicted proteins from the VfcXE gene and two other CXE proteins. The two motifs characteristic of carboxylesterase are shown by bars under the motifs and asterisks indicate the positions of the catalytic triad inferred from HSR203J. Data from the article of Baudouin et al. (1997) (*Eur. J. Biochem.* 248:700–706). Vf1, VfcXE5585; Vf2, VfcXE12827; Vf3, VfcXE13132; Vf4, VfcXE17159; Vf5, VfcXE18231; Vf6, VfcXE47674; Vv1, BIG8.1; Nt1, HSR203J. The position of the residue of S among VfcXE ranged from 154–167th, D varied from 253–269th, and H ranged from 283–299th amino acids (asterisks).

each locus. These results suggest that all six loci of VfCXE genes formed abundant random coils in their amino acid sequences, which are important elements influencing protein flexibility and stabilization of protein folding (Sharmin et al., 2011).

Multiple sequence alignment of the predicted proteins from the six loci of VfCXE genes including *Vitis* and tobacco serine hydrolase proteins was conducted using ClustalW to analyze the sequence characteristics. Many lipases and esterases contained the consensus motif ‘HGGGF’ in their upstream regions (Baudouin et al., 1997; Bézier et al., 2002) and the motif (GXSXG) is a highly conserved sequence located around the middle of amino acid sequences that contain an active side residue of serine hydrolase family genes, including lipases, esterases and proteases (Baudouin et al., 1997; Bézier et al., 2002; Lee et al., 2010; Reina et al., 2007). The alignment showed that all proteins predicted from VfCXE genes contained two consensus sequence motifs, ‘HGGGF’ related to the lipase/esterase group and a highly conserved serine hydrolase motif (GXSXG) in the middle of the amino acid sequences, which contain an active side residue of serine (Fig. 1). In addition, the catalytic triad, serine (S), situated in the motif ‘GXSXG’, aspartic acid (D), and histidine (H) were also strictly conserved at the C-terminal of all proteins predicted from the six VfCXE genes (Fig. 1). The position of the residue of S among VfCXE ranged from 154–167th, D varied from 253–269th, and H ranged from 283–299th amino acids. The residues of the catalytic triad were dispersed in primary amino acid sequences, but came together in the tertiary structure (Fig. 1, 2), which is essential for activity of hormone sensitive lipase in humans (Osterlund et al., 1996). The integrated results of sequence

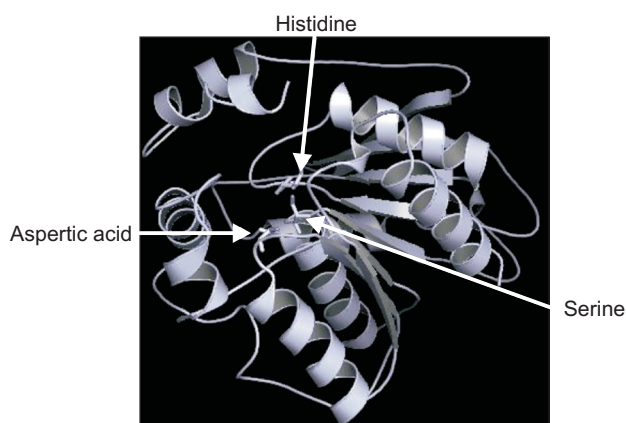


Fig. 2. Representative tertiary structure of VfCXE protein using VfCXE5585 amino acid sequence created by PyMOL (Schrodinger, New York, NY, USA). Arrows indicate conserved catalytic triad.

analyses revealed that the proteins predicted from the CXE genes of *V. flexuosa* belong to the carboxylesterase family in the serine hydrolase superfamily.

Amino acid sequence comparison. To investigate the genetic relationships of VfCXEs with other *Vitis* species proteins, homologous protein sequences of VfCXE genes were collected by BLAST searches of the NCBI database. A phylogenetic tree was then constructed from the CXE amino acid sequences of *Vitis* by the neighbor joining method using MEGA ver. 6.0 (Fig. 3). The CXE proteins predicted from the VfCXE transcripts tested in this study were divided into two groups (group I and II) with strong bootstrap support for the monophyly of each clade. VfCXE18231, VfCXE47674, and VfCXE13132 were clustered into group I, whereas VfCXE17159, VfCXE12827, and VfCXE5585 were clustered into group II. A BLAST search of the NCBI database also indicated that the deduced protein sequences of the six loci of the VfCXE gene showed a high degree of similarity to CXE protein sequences from other plants. All VfCXE proteins were highly homologous to protein sequences that originated from *Vitis vinifera*, with greater than 92% query coverage values and *E* values of 0.0, indicating their relatively conserved evolutionary relationship at the protein level (Table 5).

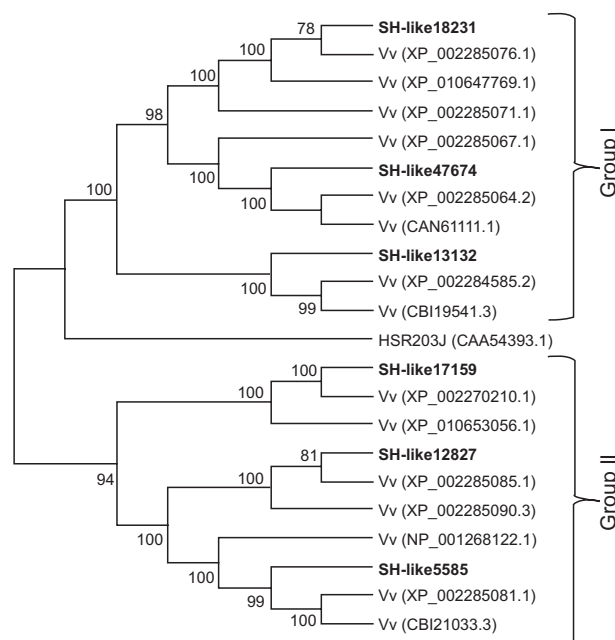
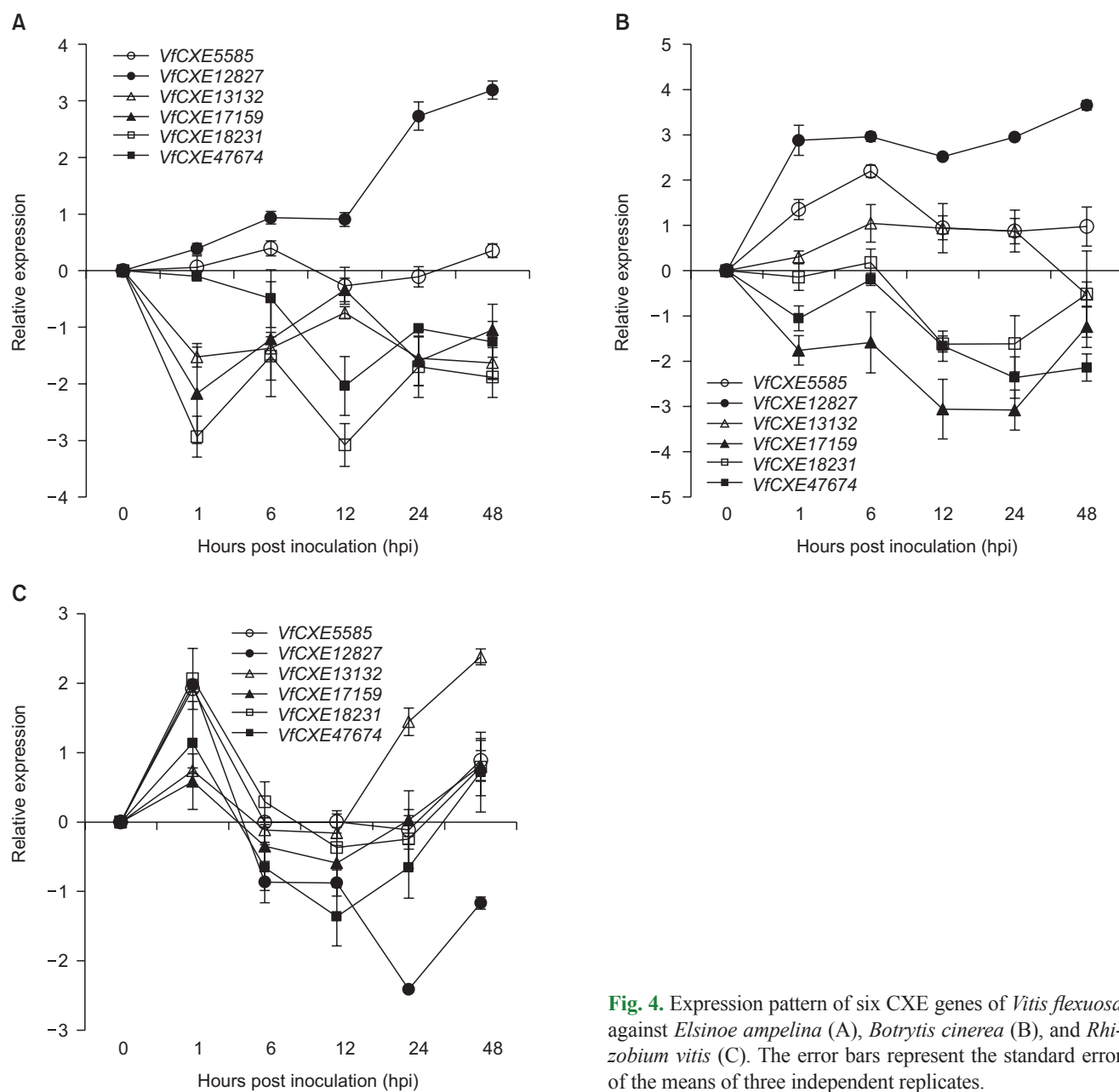


Fig. 3. Phylogenetic tree of predicted proteins from the VfCXE genes with other CXE proteins. The unrooted tree was generated using the MEGA ver. 6.0 software and the neighbor-joining method. Bootstrap values (above 70%) from 1,000 replicates are indicated at each node.

Table 5. Homology analysis of six predicted proteins from the VfCXE gene of *Vitis flexuosa*

Protein	Top matched clones	Top homologous species	Identity (%)	Query cover (%)	E value
VfCXE5585	XP_002285081.1	<i>Vitisvinifera</i>	92	100	0.00
VfCXE12827	XP_002285085.1	<i>Vitisvinifera</i>	98	100	0.00
VfCXE13132	XP_002284585.2	<i>Vitisvinifera</i>	97	100	0.00
VfCXE17159	XP_002270210.1	<i>Vitisvinifera</i>	99	100	0.00
VfCXE18231	XP_002285076.1	<i>Vitisvinifera</i>	98	100	0.00
VfCXE47674	XP_002285064.2	<i>Vitisvinifera</i>	99	100	0.00

**Fig. 4.** Expression pattern of six CXE genes of *Vitis flexuosa* against *Elsinoe ampelina* (A), *Botrytis cinerea* (B), and *Rhizobium vitis* (C). The error bars represent the standard error of the means of three independent replicates.

Expression analysis of VfCXE genes. To investigate the expression patterns of VfCXE genes against several pathogen infections, expression analysis was performed by quantitative real-time PCR using gene specific primers based on nucleotide sequence alignment. The expression levels of tested genes in grapevine leaves infected with pathogens were examined as expression relative to uninoculated control samples. The expression levels of six VfCXE genes against *E. ampelina* pathogens are shown in Fig. 4A. *VfCXE12827* was upregulated at all time points, whereas the remaining genes (except for *VfCXE5585*) were downregulated at all time points during infection of *E. ampelina*. The highest expression was observed in *VfCXE12827* at 48 hpi, which was upregulated 3-fold relative to the control. The responses of VfCXE genes to *B. cinerea* infection are presented in Fig. 4B. All genes showed active response against *B. cinerea*, with three genes (*VfCXE5585*, *VfCXE12827*, and *VfCXE13132*) being upregulated and the remaining three (*VfCXE17159*, *VfCXE18231*, and *VfCXE47674*) being downregulated. *VfCXE12827* showed the highest expression, which was 3.5 fold greater than the control at 48 hpi with *B. cinerea*. All transcripts showed active responses against *R. vitis* inoculation (Fig. 4C), and all genes showed upregulated expression at 1 hpi and abruptly decreased expression at 6 hpi. At 48 hpi with *R. vitis*, all genes were again upregulated, except for *VfCXE12827*, which was downregulated. The highest expression was observed at 24 hpi in *VfCXE12827* by *R. vitis* inoculation, which was downregulated by 2-fold relative to the control.

Generally, hypersensitive cell death through the recognition of pathogen avr genes by plant R genes occurs at early stage of resistant response against pathogen infections (Liu et al., 2007). Bézier et al. (2002) reported that *hsr203J* (tobacco) showed highest response at 48 hpi. It also reported that *Ralstonia solanacearum* induced the development of HR response on tobacco leaves within 18–24 hpi (Pontier et al., 1998a). Therefore, we were to analyze expression pattern of HR related genes up to 48 hpi of pathogens in the grapevine leaves.

Carboxylesterases belong to the serine hydrolase superfamily, which catalyzes the hydrolysis of compounds containing an ester linkage (Baudouin et al., 1997). Carboxylesterase proteins share a conserved motif of pentapeptide (GX SXG). The main feature of carboxylesterases is the conserved catalytic triad composed of an active site serine surrounded by the conserved consensus sequence, GX SXG, an aspartic acid or a glutamic acid and a histidine (Marshall et al., 2003). In the present study, the molecular structure was analyzed to detect common characteristic features in six VfCXE transcripts based on their predicted amino acid sequences in *V. flexuosa*. Hydrolase like do-

mains in all six VfCXE proteins were detected by the SMART program in this study. All VfCXE proteins tested in this study contained two conserved motifs (GX SXG and HGGGF), as well as a catalytic triad of serine, aspartic acid and histidine (Fig. 1, 2), which are characteristic features of carboxylesterase in the serine hydrolase superfamily.

Resistance to plant diseases is characterized by either partial or complete suppression of pathogen growth at the site of infection. To protect themselves, plants employ several defense mechanisms against invading pathogens. One typical feature of disease resistance is death of plant cells at the site of infection to confine the growth of pathogens, known as the HR (Greenberg, 1996). Generally, HR occurs in response to interaction between plant R genes and corresponding pathogen avr genes (Ade et al., 2007; Hammond-Kosack and Jones, 1996). In addition to R genes, some genes belonging to the carboxylesterase family are reportedly expressed during HR (Baudouin et al., 1997; Bézier et al., 2002; Marshall et al., 2003; Tronchet et al., 2001). Carboxylesterase genes such as *EDS1*, *PAD4*, *HSR203J*, *PRLIP1*, and *PepEST* have been isolated from plant-microbe interactions (Ko et al., 2005). *HSR203J* is reportedly a tobacco carboxylesterase gene that is rapidly activated in a highly localized and specific manner to enable incompatible interactions between tobacco and the bacterial pathogens *R. solanacearum*, *Pseudomonas syringae* pv. *lisi*, and *Erwinia amylovora* (Pontier et al., 1998a). Pontier et al. (1998a) also reported that *Lehsr203*, a tomato carboxylesterase gene, was rapidly and transiently induced in leaves of the tomato containing *Cf-9* disease resistance gene following Avr9 product infiltration, but not in the absence of *Cf-9*. A pepper carboxylesterase gene *PepEST* showed induced expression in fruit following infection with *Colletotrichum gloeosporioides* fungus (Ko et al., 2005).

HSR203J was considered to be a HR-specific marker because its expression was strongly correlated with programmed cell death in response to different HR inducing pathogens and elicitors, as well as in response to various cell death triggering extra cellular agents such as heavy metals, but not following exposure to virulent pathogens and elicitors that did not induce cell death (Pontier et al., 1994, 1998b). In *Arabidopsis*, *EDS1* is a signaling component of disease resistance pathways activated by the TIR-NBS-LRR class of R genes (Aarts et al., 1998; Parker et al., 1996). The *PAD4* gene was found to be required for expression of multiple defense genes against pathogens (Jirage et al., 1999).

It was reported that HR activation in plants is mediated by different rapid changes, such as the production of reactive oxygen species (ROS) such as H₂O₂ (Levine et al.,

1994), ionic fluxes (Mittler et al., 1995; Nürnberger et al., 1994), and protein phosphorylation (Dunigan and Madlener, 1995). In contrast, Zurbriggen et al. (2010) reported that it was not clear if ROS participate in triggering localized cell death, in the induction of pathogenesis-related genes, or in both pathways and that ROS-independent processes also contributed to the HR. Tada et al. (2004) reported that ROS are not essential mediators for the initiation of hypersensitive cell death. Pontier et al. (1998b) suggested that a pathway not requiring H₂O₂ (ROS) is involved in the *hsr203J* gene activation or that ROS is insufficient as a cell death activator under these conditions. Like *hsr203J* gene, hypersensitivity related genes studied in this experiment, may have no strong relations with ROS. Although HR responses including ROS accumulation in plants were not analyzed resistant response in plants, expression genes related with HR in initial stages of resistant responses suggested that HR was induced by pathogen infections in grapevines.

Thomma et al. (1998) reported that over expression of *PepEST* in transgenic *Arabidopsis* plants showed restriction of *Alternaria brassicicola* colonization by inhibiting spore production. Ko et al. (2005) reported that *PepEST* accumulation was localized in epidermal and cortical cell layers in infected ripe fruit during immunochemical examination. Lee et al. (2010) also reported that some *Brassica oleracea* chlorophyllase isozymes which are serine hydrolase family enzymes degraded chlorophyll. Specifically, it has been reported that a detoxifying esterase from the bacterium *Pantoea dispersa* abolished the function of albicidin phytotoxin, which is a potent pathogenicity factor produced during the infection of sugarcane by *Xanthomonas albilineans* that causes a devastating disease known as leaf scald (Zhang and Birch, 1997).

It has been reported that a grape carboxylesterase gene, *BIG8.1*, was induced in grape leaves during the infection of *B. cinerea* (Bézier et al., 2002). Additionally, it was reported that a conserved carboxylesterase inhibit resistant phenotypes triggered by AvrBsT, a type III effector from *Xanthomonas campestris* pv. *vesicatoria* that is translocated into plant cells during infection in *Arabidopsis* (Cunnac et al., 2007). Tronchet et al. (2001) reported that antisense suppression of a tobacco gene *HSR203J* showed accelerated HR against *R. solanacearum* and increased resistance against *P. syringae* pv. *Pisi* and *Phytophthora parasitica*, suggesting that *HSR203J* may be involved in the suppression of HR cell death. It suggests that carboxylesterases are involved in HR in plants infected with pathogens, and have roles in synthesis or degradation of a molecule in signal transduction pathways for disease resistance responses in plants. The exact functional mechanisms of CXEs in plants is not well understood, even though sev-

eral studies have characterized carboxylesterase activity (Gershtater and Edwards, 2007).

We attempted to investigate the response of six VfCXE genes against infection with fungi and bacteria in grapevines. In the expression study, all VfCXE genes showed changes in their expression in response to infection by all pathogens. *VfCXE5585* and *VfCXE12827* commonly showed upregulation and *VfCXE17159*, *VfCXE18231*, and *VfCXE47674* were commonly downregulated, whereas *VfCXE13132* showed reverse expression by fungal pathogens tested in this study (Fig. 4A, B). In the case of *R. vitis* infection, all transcripts showed upregulation at early and later time points, except *VfCXE12827*, which decreased rapidly after showing upregulation at 1 hpi and downregulation for up to 48 hpi (Fig. 4C). Among all genes, *VfCXE12827* exhibited strong and interesting expression against fungal and bacterial pathogens as indicated by upregulation in response to *E. ampelina* and *B. cinerea* at all time points and downregulation after 1 hpi against *R. vitis*. Although it is well known that pathogen responsive carboxylesterase genes are upregulated against pathogen attacks, we observed both up and down regulated response of VfCXE genes in response to plant pathogens. It has also been reported that *V. vinifera* *HSR1* transcripts were undetectable at 18 to 48 hpi and were upregulated at later time points after inoculation of *Plasmopara viticola* (Chong et al., 2008), which is similar to the expression of VfCXE transcripts against *R. vitis*. Islam et al. (2015a, 2015b) also reported that several receptor-like protein kinases and CC-NBS-LRR genes, which are considered potential plant resistance genes, showed up- and down-regulated expression against plant pathogens. Therefore, the change in expression level of all tested VfCXE genes after pathogen infection indicated that VfCXE might play a role in signal transduction pathways for disease resistance or a correlated response, similar to other characterized carboxylesterase genes.

In conclusion, six transcripts of the VfCXE gene were identified and characterized in this study that showed various expression levels in the transcriptome of grapevines inoculated with *E. ampelina*. The molecular structure was analyzed for characteristic features in the six VfCXE genes based on their predicted amino acid sequences in *V. flexuosa*, and the expression pattern of VfCXE transcripts was investigated by real-time PCR using gene specific primers in response to pathogen infection in grapevines. Six non-duplicated VfCXE genes were identified from transcriptome analysis by NGS of *V. flexuosa* inoculated with *E. ampelina*. Structural analysis and a comparison study confirmed that the tested genes are members of the carboxylesterase gene of the serine hydrolase superfamily. All of these genes also showed a high degree of

homology with other carboxylesterase genes. Expression analysis revealed that all tested genes showed responses to differential expression against all tested pathogens, indicating that these genes may be related to plant resistance responses against different pathogens in grapevines. Among tested genes, *VfCXE12827* showed upregulated expression at 1 hpi against all tested pathogens and showed upregulation against *E. ampelina* and *B. cinerea* at all time points. *VfCXE5585* and *VfCXE13132* showed upregulation in grapevines infected with *B. cinerea*. All transcripts showed upregulated expression at early time points in response to *R. vitis* infection. Therefore, it suggests that *VfCXE12827* was commonly upregulated gene against fungal and bacterial pathogens, *VfCXE5585* and *VfCXE13132* were upregulated specifically in response to *B. cinerea*, and all tested VfCXE genes may involve in signaling role at early stage in resistant responses against *R. vitis* infection. Taken together, these results provide valuable information for elucidating the complex molecular mechanisms of responses resistant to diseases in grapevines.

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