Differential Expression of β -1,3-Glucanase Transcripts Induced by Pathogens in the Leaves of *Vitis flexuosa*

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ABSTRACT The differential expression of β -1,3-glucanase genes in the leaves of *Vitis flexuosa* induced by fungal and bacterial pathogen infections was investigated. The nucleotide and deduced amino acid sequences of β -1,3-glucanase genes from the transcripts of *V. flexuosa* were compared. The percentage similarity of deduced amino acid ranged from 22.0% between VfGlu34359 and VfGlu48103 to 96.9% in VvGlu2735 and VvGlu48103. To demonstrate the differential expression pattern of β -1,3-glucanase genes, primers specific to unique regions in each gene were obtained by alignment of the sequences. Accumulation patterns of β -1,3-glucanase mRNAs in the leaves of *V. flexuosa* were induced differentially and were dependent on the pathogens used including *Botrytis cinerea*, *Colletotrichum acutatum*, *Elsinoe ampelina*, and *Rhizobium vitis*. This study provides useful information that will improve our understanding of grapevine defense responses to various pathogen attacks.

Keywords Defense response, Fungal pathogen, Gene expression, PR protein, *Rhizobium vitis*

INTRODUCTION

In host-pathogen interactions, plants regulate defenses through physical and chemical responses that may occur either before or after pathogen infection. Induced defense mechanisms are involved in the transduction of various signals involved in the production of reactive oxygen species, phytoalexin biosynthesis, reinforcement of plant cell walls, and the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial properties (Kombrink and Somssich 1995; Fritig *et al.* 1998; Rivera *et al.* 2002; Gill and Tuteja 2010; Ibraheem *et al.* 2010; Huffaker *et al.* 2011). PR proteins that are induced by pathogen infection have been reported in wheat (Li *et al.* 2001), grapevine (Hatmi *et al.* 2013; Renault *et al.* 1996), mango (Ebrahim *et al.* 2011), banana (Harish *et al.* 2009), European plum (El-kereamy *et al.* 2011), and apple (Li *et al.* 2014).

β -1,3-glucanases are PR-2 proteins that catalyze endotype hydrolytic cleavage of 1,3- β -D glycosidic linkages in β -1,3-glucans present in the cell walls of many pathogenic

fungi (Simmons 1994). Induction of β -1,3-glucanase has been reported in various plants infected with fungal pathogens including tomato (Joosten and De Wit 1989), wheat (Kemp *et al.* 1999), peach (Zemanek *et al.* 2002), rubber tree (Thanseem *et al.* 2005), apple (Venisse *et al.* 2002), rucola (Gupta *et al.* 2013), *V. flexuosa* (Ahn *et al.* 2012), and strawberry (Fang *et al.* 2012). It has been suggested that these compounds play multiple roles in plant defense response. They can degrade the cell wall of the pathogen directly or exert indirect defenses such as induction of the release of cell wall-derived components that act as elicitors of active defense reactions (Mauch *et al.* 1988; Yoshikawa *et al.* 1993).

Among *Vitis* species native to Korea, *V. flexuosa* was reported to show resistant responses such as expression of β -1,3-glucanase and glutathione-S-transferase genes (Ahn *et al.* 2012). In this study, β -1,3-glucanase transcripts expressed in different levels in the transcriptome from *V. flexuosa* inoculated with *E. ampelina* were obtained. The objectives of this study were to compare nucleotide and deduced amino acid sequences by alignment of β -1,3-

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glucanase genes, and to analyze the differential expression against different pathogens such as *Botrytis cinerea*, *C. acutatum*, *Elsinoe ampelina* (culture filtrates and spore), and *Rhizobium vitis* in *Vitis flexuosa*.

MATERIALS AND METHODS

Plant materials, pathogen preparation, and inoculation

A Korean wild grape, *Vitis flexuosa* VSKO001, was obtained from the grapevine germplasm collection field of Yeungnam University, Gyeongsan. Cuttings of *V. flexuosa* VSKO001 were cultivated in an experimental glasshouse at 25°C under a 16 h photoperiod. The upper fourth or fifth leaves from the shoot apex of the plant were individually used for the pathogen inoculation, which was accomplished after lightly scratching the epidermis with a pencil tip.

B. cinerea was grown in petri-dishes at 25°C on potato dextrose agar (PDA, 0.4% potato starch, 2% dextrose, and 1.5% agar) under 12/12 h light/dark conditions. Spores of *B. cinerea* were collected from the plates, suspended in 0.24% potato dextrose broth at a concentration of 10^6 spores/ml and then centrifuged at 3,000 g for 5 min to remove mycelia debris. Leaves were inoculated by dropping 30 μ l of spore suspension on the wounded areas.

Virulent strain of *C. acutatum* Co601 isolated from infected leaves at the National Academy of Agricultural Science, RDA, Suwon, Korea was used for plant inoculation. *C. acutatum* were grown on PDA medium at 25°C under 12/12 h light/dark conditions, after which 30 μ l of spore suspensions (10^5 spores/ml) were dropped onto the abaxial portion of the leaf.

Several colonies of *E. ampelina* were incubated in a shaking incubator (140 rpm) at 28°C for ten days. The cultures were then harvested by centrifugation, ground in a homogenizer in sterile distilled water, and poured onto V-8 juice agar medium (20% V-8 juice, 2% agar) and incubated at 28°C under a near ultraviolet lamp for two days to produce spores of the pathogen (Yun *et al.* 2003). Spores of *E. ampelina* were harvested by scraping-off of plates with sterile distilled water. The concentration was then adjusted to 10^5 spores/ml and sprayed onto leaves. Next, the *E.*

ampelina was incubated in PDB medium (potato dextrose broth) at 28°C for 21 days, after which cell-free culture filtrates (CFCF) were collected from the supernatant by centrifugation and sterilized by ultra-filtration (0.2 μ m pore diameter). Finally, 30 μ l of *E. ampelina* culture filtrate was dropped onto the wounded portion of leaves.

A single colony of bacteria was placed in YEP medium (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.05% MgSO_4 , pH7.2) and cultivated at 28°C in a shaking incubator, after which it was spun down by centrifugation and resuspended with sterile water. *R. vitis* cell suspension (30 μ l) was dropped onto the wounded portion of the leaves.

Leaves inoculated with the pathogens were placed on two layers of moist paper towel, then incubated in a closed box in a dark, moist chamber (100% RH) at 25°C or 28°C for two days. Five leaves were used for each treatment, and the experiment was repeated twice. Controls were harvested from water-treated leaves under the same conditions. Leaves were harvested at the indicated time points (0, 1, 6, 24, and 48 h) after inoculation, immediately frozen in liquid nitrogen, and then stored at -80°C for future use.

RNA isolation and cDNA synthesis

Frozen grapevine leaves (1 g) were ground in liquid nitrogen using a mortar and pestle. RNA was then isolated using a modified version of the method described by Chang *et al.* (1993). The quality of total RNA was measured based on the absorbance at 230, 260, and 280 nm using a Nano Drop spectrophotometer (ND-1000, Technologies Inc., Wilmington, DE, USA). First-strand cDNA was then synthesized from the total RNA (500 ng) using a GoScriptTM Reverse Transcription System (GoScriptTM Reverse Transcription System, Promega, Madison, WI, USA) and subsequently used as a template for PCR.

Cloning and sequence analysis

Nucleotide sequences were translated using the Translate program hosted by the ExPASy Proteomics Server (<http://au.expasy.org>). Pairwise comparisons and multiple alignments of nucleotide and deduced amino acid sequences were performed using ClustalX (Thompson *et al.* 1997). A

phylogenetic tree was constructed by the neighbor-joining method using Mega 4.0 (Fig. 2).

Real-time PCR analysis

Real-time PCR was performed on a C1000™ Thermal Cycler (CFX96™ Real-Time System, BioRad, Hercules, CA, USA) using SYBR Premix Ex (SYBR Premix Ex Taq, TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye. Amplification was conducted by subjecting the samples to one cycle at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Transcript levels were calculated using the standard-curve method and normalized against the grapevine actin gene (AB372563) as an internal control and non-treated leaves (at time zero) as the reference sample, after which melting curves of the amplified products

were recorded. For each gene, the reference sample was defined as the 1x expression level, and results were expressed as the fold increase in mRNA over the reference sample. All reactions were performed in triplicate.

Among 28 DEGs identified upon transcriptome analysis of *V. flexuosa* inoculated with *E. ampelina* (Ahn *et al.* 2014), six loci showing variation with more than 100 transcripts were selected for evaluation of their differential expression against pathogen attacks. Four of these β -1,3-glucanase genes showed differential expression in response to *E. ampelina* inoculation of grapevines, therefore, specific primers for real-time PCR were designed by alignment of the nucleotide sequences obtained from the *V. flexuosa* transcriptome data. Nucleotide sequence data of the *V. flexuosa* transcript loci 2735, 5391, 34359, and 48103 have

Table 1. Sequences of primers used for real-time PCR analysis in this study.

Name	Accession no. or NABIC ²⁾ ID	Primer sequences
β -1,3-glucanase 1	DQ267748.1	5'-GGGTTTTTATTGGCAACCCTTC-3' 5'-GCTACCACTTGTGATGCTGGTG-3'
β -1,3-glucanase 2 (Locus 2735- VfGlu2735)	NS-0399-000001	5'-CGTTCAGAGTGCAATAACCTCCT-3' 5'-CGAGGACGGCTGAATATGTTG-3'
β -1,3-glucanase 1 (Locus 5391- VfGlu5391)	NS-0400-000001	5'-GCTTGACCTCAGGGTAGTGGAA-3' 5'-TCAAGGTGGGCAGGCTTAGTAG-3'
β -1,3-glucanase 1 (Locus 34359- VfGlu34359)	NS-0402-000001	5'-GAATCTCGATCAGGCCTTGAGA-3' 5'-GAACTGCAGTGGAAGCTGGT-3'
β -1,3-glucanase 1 (Locus 48103- VfGlu48103)	NS-0401-000001	5'-GCGATGTAGCGGAACCTTCACTT-3' 5'-AGCTACCTGGGTGCAAAACAAC-3'
VACT	AB372563	5'-ACGAGAAATCGTGAGGGATG-3' 5'-ATTCTGCCTTTGCAATCCAC-3'

²⁾NABIC - National Agricultural Biotechnology Information Center, RDA, Korea.

Table 2. Identity of deduced amino acid from alignment of β -1,3-glucanase (Glu) genes of *V. flexuosa* transcripts. Glu proteins and accession numbers: VvGlu (DQ267748.1), VfGlu2735 (Locus 2735, NS-0399-000001), VfGlu5391 (Locus 5391, NS-0400-000001), VfGlu34359 (Locus 34359, NS-0402-000001), and VfGlu48103 (Locus 48103, NS-0401-000001).

	VvGlu	VfGlu2735	VfGlu5391	VfGlu34359	VfGlu48103
VvGlu		59.8	58.4	49.0	55.3
VfGlu2735	59.8		59.3	50.4	96.9
VfGlu5391	58.4	59.3		48.5	46.0
VfGlu34359	49.0	50.4	48.5		22.0
VfGlu48103	55.3	96.9	46.0	22.0	

been deposited in the NABIC (National Agricultural Biotechnology Information Center, RDA, Korea) database with the respective accession numbers NS-0399-000001, NS-0400-000001, NS-0402-000001, and NS-0401-000001. The β -1,3-glucanase primer sequences are listed in Table 1. The deduced amino acid sequences of β -1,3-glucanase genes between *V. flexuosa* transcripts were compared using the CLC Main Workbench (Ver. 4.0, CLC Bio, Aarhus, Denmark).

RESULTS

The deduced amino acid sequences of β -1,3-glucanase genes in *V. flexuosa* transcripts are shown in Fig. 1. Using the deduced amino acid sequences of β -1,3-glucanase genes, the percent similarity ranged from 22.0% for VfGlu34359 and VfGlu48103 to 96.9% for VvGlu2735 and VfGlu48103.

The similarity value of the other transcript pairs were 59.8% between VfGlu and VfGlu2735, 50.4% between VvGlu2735 and VfGlu34359, and 46.0% in VfGlu5391 and VfGlu48103 (Table 2).

Based on pairwise comparisons and multiple alignments of nucleotide and deduced amino acid sequences, a phylogenetic tree was constructed by the neighbor-joining method using Mega 4.0 (Fig. 2). The results revealed a close relationship between VvGlu, VfGlu5391, VfGlu2735, and VfGlu48103.

Specific primers for β -1,3-glucanase genes designed based on alignment of nucleotide sequences from *V. flexuosa* transcripts were used to detect differential expression in grapevine leaves inoculated with pathogens by real-time PCR. The accumulation patterns of β -1,3-glucanase mRNA were induced differentially and were dependent on several pathogens tested in this study (Fig. 3). In grapevine leaves inoculated with *B. cinerea*, the expression of VvGlu and VfGlu2735 was rapidly up-regulated, peaking at 24 h. The

VvGlu	1	MA-----AMVLLLGFLLATLQITGVKSVGVCYGMGLNNLPPASQVVALYKSRNIDRMRIYDF	57
VfGlu2735	1	MALT---RNKP-L-VVVLGGEVIMSTLTIGAQSIGVCYGTNGDNLPSASQVINLYKSNIGISMRIYDF	64
VfGlu5391	1	MTVNSYVKSSPRMTTVLLLLGLL-MTSLHLTETQIGVCYGTLDNLFPPEEVVELYTRNNFQRMRLYDF	68
VfGlu34359	1	-----	0
VfGlu48103	1	MALT---RNKP-L-VVVLGGEVIMSTLTIGAQSIGVCYGTNGDNLPSASQVINLYKSNIGISMRIYDF	64
VvGlu	58	NQAALQALRGSNIQMLGVFNSDLQGLATNPSSQAQSWVQRNVRNYWPGVSFRYIAVGNEVSPVNGGTSR	126
VfGlu2735	65	NSDTLQALKGSGIELILDVPNTSLQSLASDASDASTWVQNNVNYASDVKFRYIAAGNEVLFT-GSNAQ	132
VfGlu5391	69	NLAALRALGGSNIELLLSVFNNDLQDIAASQGIANLWVQAYVTNYT-KVKFRYIAVGNIIVSPS-D---S	132
VfGlu34359	1	-----	0
VfGlu48103	65	NSDTLQALKGSGIELILDVPNTDLQSLASDASAAATWVQNNVNYASEVKFRYIAVGNEVLFT-GSNAQ	132
VvGlu	127	FAQFVLFPAMRNIRAAASAGLQDRVKVSTAIIDLTLGNISYPPSQGAFRGDVRGYLDPIIRFLVDNKSPL	195
VfGlu2735	133	YAQYVLPAMQNVQSAITSAGLQGGQIKVSTATYSSVLGTSYPPSAGSFSSDVSSFINPIISFLAENGSPF	201
VfGlu5391	133	FAQELLFAMQNNINNAISAGLHNQIKVSTAINTVGLTGYPPSQGSFQTQARSFLDPIISLLVENRAPL	201
VfGlu34359	1	-----MQNLDQALREAQLPYSIPVSTSFSTAVLGTSYPPSQGEFSMDIDFIMRSITEFLAANGSPF	61
VfGlu48103	133	YAQYVLPAMQNVQSAIT-----	149
VvGlu	196	LANIYPYFGYSGNPKDISLPYALFTANSVVVWDGQRGYKNLFDAMLDALYSALGRAGGASLEVVSSESG	264
VfGlu2735	202	LANIYPYFSYTGTNTQSIQLDYALFTSPEVVVKDGSYQYQNLFDALLDALYALGKAGGSNLKIVVSESG	270
VfGlu5391	202	LVNLYPYLSYSANTQDVSLDYALFTANEVTVQDGGQIGYRNLFDMSVDAFYSALEKAGGSLSLEIVSESG	270
VfGlu34359	62	LVNVYPYFSYINDEPVNVPLDYALFNSSRVVVRDGELEYKNLFDADATYTALEKAGGASVKVVTESG	130
VfGlu48103	150	-----	149
VvGlu	265	WPS-AGGFGTTVDNARTYNSNLIRHVKG--GTPKRPGRAIETYLFAFMFDENKKEP-QLEKHFGLEFFPNK	329
VfGlu2735	271	WPS-EGGTAATVDNARTYYKNLINHVKG--GTPKRSG-AIETYLFAFMFDENQKTGLETEKHFGLEFTPSQ	335
VfGlu5391	271	WPS-AGGTGATLDNARIYNTNLIQHVKG--GTPKRPGKAIETVVFAMFDENRKTPELERHWGLFLEPNK	335
VfGlu34359	131	WPSNENGQIATIENTARMYNNNLVAHLSGAKGTPKKPGESIEAYVFAIFNEDLKPR-GTEQNFGLYYPNM	198
VfGlu48103	150	-----	149
VvGlu	330	QPKYSINFSGEKPWDVSTENDTNEESLKSIDI	360
VfGlu2735	336	ESKYQISF-----S	344
VfGlu5391	336	QSKYPLLCR-----IPSATQIVYSIV	356
VfGlu34359	199	TEVIHVE-----FIN	208
VfGlu48103	150	-----S	150

Fig. 1. Multiple amino acid sequence alignment of *V. flexuosa* β -1,3-glucanase (Glu) transcripts using the CLC Main Workbench (Ver. 4.0, CLC Bio, Aarhus, Denmark). Vv: *Vitis vinifera*, Vf: *Vitis flexuosa*. Glu proteins and accession numbers: VvGlu (DQ267748.1), VfGlu2735 (Locus 2735, NS-0399-000001), VfGlu5391 (Locus 5391, NS-0400-000001), VfGlu34359 (Locus 34359, NS-0402-000001), and VfGlu48103 (Locus 48103, NS-0401-000001).

expression of VfGlu5391, VfGlu48103, and VfGlu34359 was also up-regulated, peaking at 6 h after pathogen inoculation.

The mRNA levels of VvGlu, VfGlu5391 and VfGlu34359 were upregulated, reaching the peak values at 1 h and 24 h after inoculation with *C. acutatum*. In both leaves inoculated with spore suspensions of *E. ampelina* and then treated with their culture filtrates, the expression of VfGlu34359 was down-regulated while VvGlu and VfGlu2735 was

up-regulated, peaking at 48 h and 6 h, respectively. In the case of leaves inoculated with *R. vitis*, the mRNA level of VfGlu34359 was down-regulated, which is similar to the results observed with *E. ampelina* inoculation treatment. The VvGlu showed the maximum level of transcripts at 24 h after *R. vitis* inoculation, while VfGlu2735 and VfGlu48103 showed two peaks at 1 h and 24 h after pathogen inoculation.

Fig. 2. Phylogenetic tree of VvGlu (DQ267748.1) and other β -1,3-glucanase proteins from *Vitis flexuosa* transcripts VfGlu2735 (Locus 2735, NS-0399-000001), VfGlu5391 (Locus 5391, NS-0400-000001), VfGlu34359 (Locus 34359, NS-0402-000001), and VfGlu48103 (Locus 48103, NS-0401-000001).

Fig. 3. Expression of β -1,3-glucanase (Glu) genes by quantitative real-time PCR analysis of *V. flexuosa* leaves inoculated with pathogens. Transcript levels were calculated using the standard curve method from triplicate data with the grapevine actin gene as an internal control and non-treated leaves (at time zero) as a reference sample. The results represent the mean fold increase in mRNA level over non-treated leaves relative to the 1x expression level. Results are the means of triplicate data from three experiments. Bars indicate the standard deviation. VvGlu (DQ267748.1), VfGlu2735 (Locus 2735, NS-0399-000001), VfGlu5391 (Locus 5391, NS-0400-000001), VfGlu34359 (Locus 34359, NS-0402-000001), and VfGlu48103 (Locus 48103, NS-0401-000001).

DISCUSSION

Accumulation of PR proteins, such as β -1,3-glucanase, is one of the most common markers of actively induced plant defense response. The fact that β -1,3-glucanase is induced by a broad range of biotic and abiotic factors suggests that it plays a general role in plant defense (Zemanek *et al.* 2002). However, there is a limited number of studies focused on expression of various β -1,3-glucanase transcripts with differences in nucleotide sequences within a single plant species responding to different pathogens to date. In this study, five β -1,3-glucanase genes were used to demonstrate their expression in response to five different pathogens which are causal agents of the major grapevine diseases in Korea.

To investigate the differential expression patterns of β -1,3-glucanase genes, primers specific to unique regions in each gene were obtained by alignment of the sequences. Real-time PCR analysis showed that pathogen inoculation resulted in upregulated mRNA levels of β -1,3-glucanase genes in *V. flexuosa* leaves. Induction of β -1,3-glucanase in response to various pathogens such as *Plasmopara viticola* (Kortekamp 2006), *B. cinerea* (Renault *et al.* 1996), *Sphaerotheca fusca* (Rivera *et al.* 2002) and *Erysiphe necator* (Giannakis *et al.* 1998) has been thoroughly investigated in several plants, and the timing and level of transcripts has been reported to vary between pathogens and genotypes. In this study, even though there was little variation in the expression level and onset of response, β -1,3-glucanase mRNA levels were shown to increase in response to inoculation of five pathogens.

Culture filtrates (CF) have been used in screening for resistance (Jayasankar *et al.* 2000; Yun *et al.* 2006). Zemanek *et al.* (2002) reported that mRNA levels increased following treatment of CFs with bacterial and fungal pathogens. The CF of *E. ampelina* used in the present study has been found to induce β -1,3-glucanase mRNA transcripts of *V. flexuosa*. Different or multiple production pathways of β -1,3-glucanases may be involved in different responses to the diseases caused by several fungal pathogens (Cheong *et al.* 2000; Krishnaveni *et al.* 1999; Roulin and Buchala 1995). Zemanek *et al.* reported (2002) that β -1,3-glucanase can act as a preformed protein or be induced as a specific or

nonspecific defense protein against different pathogens. In the present study, we elucidated nucleotide and deduced amino acid sequences specific for β -1,3-glucanase genes differentially expressed in *V. flexuosa* in response to several pathogens.

β -1,3-glucanases genes comprise a large gene family that has been subdivided into three major classes based on their primary structures (Neale *et al.* 1990; Payne *et al.* 1990; Ward *et al.* 1991). It has been reported that plants express β -1,3-glucanase genes (I, II, and III) in response to fungal and viral infections (Dobnik *et al.* 2013; Linthorst *et al.* 1990; Sela-Buurlage *et al.* 1993; Ward *et al.* 1991; Zavaliev *et al.* 2011). In *V. vinifera*, 16 β -1,3-glucanases are present, but only five are basic proteins, and one of them contains both the N- and C-terminal signal peptide sequences (Jaillon *et al.* 2007). Accordingly, β -1,3-glucanase genes can be classified into classes based on previous reports and it will be possible to compare their differential expression patterns in grapevines in response to pathogens in the near future.

In this study, five β -1,3-glucanase genes with differences in nucleotide sequences were found to exhibit differential expression in response to five different pathogens in *V. flexuosa*. These results provide useful information regarding grapevine defense responses at the molecular level and analysis of differential expression patterns of pathogenesis-related genes under various pathogen infections. The data presented here will facilitate future investigations of modes of action and interaction between grapevines and pathogens that are also useful for breeding programs of grapevines resistant to diseases.

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