Characterization and Expression Analysis of Peroxidases and Glucan Synthase Like Genes in *Cucumis melo* L.

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ABSTRACT Powdery mildew (PM) is a severe fungal disease for melon cultivation worldwide. Stress resistance related genes could be important tools to address this problem. In this study, we retrieved defense related *peroxidase* and *glucan synthase* genes from Melon Genome Database 'Melonomics'. Thereafter, we analyzed the genes in silico. We conducted protein blast in the NCBI database and found a high degree of homology among them. Based on the highest protein homology we named two isoforms of *Cucumis melo* *peroxidase 2-like* genes (*CmPrx2-1* and *CmPrx2-2*) and one *glucan synthase1-like* gene (*CmGLS1*). In reverse transcription-polymerase chain reaction (PCR), all 3 genes showed organ specific expression in a *C. melo* line, SCNU1154. Real-time quantitative PCR expression of these 3 genes was conducted in the infected leaf samples by PM fungus *Podosphaera xanthii* and also treated leaf samples by exogenous phytohormones (salicylic acid and methyl jasmonate). The *CmPrx2-2* gene was up-regulated in response to all seven races of PM fungus whereas up-regulation or down-regulation of *CmPrx2-1* gene was race-specific. The *CmGLS1* gene was down-regulated in response to all races except one race. The *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes were up-regulated under both salicylic acid and methyl jasmonate treatments but their level of expression was higher in salicylic acid treated plants compared to methyl jasmonate. Therefore, we speculate that defense response of the three tested genes is largely mediated by the salicylic acid signaling pathway under PM infection. Taken together, the data presented herein may be useful resources in the development of PM stress resistant in *C. melo* L.

Keywords Melon, *Peroxidase*, *Glucan synthase*, Phytohormones, Powdery mildew

INTRODUCTION

Powdery mildew (PM) disease is severe threat for melon (*Cucumis melo* L.) cultivation around the world. PM fungus reduces the quantity as well as the quality of cucurbit crop yields (Jarvis *et al.* 2002). Although in Europe, two severe PM causal agents namely *Podosphaera xanthii* and *Golovinomyces cichoracearum* have been reported (Epinat *et al.* 1993; Křístková *et al.* 2004) but many research showed that *P. xanthii* alone is the major responsible causal agent for PM diseases for melon in many countries (Sowell 1982; Cohen *et al.* 1984; McCreight *et al.* 1987; Hosoya *et al.* 1999; 2000). Application of fungicide

is the common control strategy for PM disease in cucurbit crop production (Hewitt 1998). Due to frequent chemical use some PM fungi have gradually evolved their resistance to fungicides (Hollomon and Wheeler 2002). Therefore, development of PM-resistant cultivar is necessary to control this disease (Perchepped *et al.* 2005).

The biotrophic PM fungi are generally regulated by plant defense signaling pathway like salicylic acid (SA) (Glazebrook 2005; Pieterse *et al.* 2009) and jasmonate (Glazebrook 2005). This signaling molecules subsequently activate wide range of defense responses such as hypersensitive response (HR); phenylalanine ammonium lyase; cell wall reinforcing materials; and generation of

Received April 20, 2016; Revised May 23, 2016; Accepted May 24, 2016; Published May 31, 2016

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different antimicrobial products like pathogenesis-related (PR) proteins and phytoalexins resulting in incompatible plant-microbes interaction and hence plant resistance (Veronese *et al.* 2003; Glazebrook 2005; Pieterse *et al.* 2009). Furthermore, plant resistance responses to pathogens challenge results from a series of inter-related stages (Dangl and Jones 2001; Veronese *et al.* 2003). Recognition of pathogen by the host plant is the initial step that often consist of interaction between resistant (R) genes in the host plant and corresponding pathogen avirulence (Avr) genes that encode specific elicitors (Glazebrook 1999; Schulze-Lefert and Vogel 2000).

Peroxidase gene, linked to generate HR and cell wall reinforcement, was induced by phytohormones such as SA and methyl jasmonate (MeJA) (Durrant and Dong 2004; van Loon *et al.* 2006; Pieterse *et al.* 2009). Despite high sequence homology (from 28% to 100%) between two peroxidase proteins, there is significant difference observed in their functions such as lignification, suberisation, auxin catabolism, cross-linking of cell wall proteins, defense against pathogen attack, salt tolerance and oxidative stress (Penel *et al.* 1992; Hiraga *et al.* 2001). Callose, a (1,3)- β -glucan polymer, functions in many aspects of plant growth and development (Stone and Clarke 1992). Plant produces callose under a range of biotic and abiotic stresses including wounding, desiccation, metal toxicity, and microbial attack (Stone and Clarke 1992; Ellinger *et al.* 2013). However, previous reports showed that pathogen-induced callose could be involved negatively to regulate the SA signaling pathway of plants. In *Arabidopsis*, callose lacked *powdery mildew resistant 4* (*pmr4*) mutant enhanced the SA signaling and increased resistance to pathogen (Nishimura *et al.* 2003).

PM disease development in melon cultivars varied in response to different races of *P. xanthii* (Bardin *et al.* 1999). Moreover, a race shift was recorded during the prolonged production period (Sowell 1982; Thomas *et al.* 1984; Hosoya *et al.* 1999; 2000). Previous reports showed that *peroxidase* gene (*PR9*) in melon revealed defense response role on induced systemic resistance upon PM fungus inoculation (García-Gutiérrez, 2013). However, Glucan Synthase like-5 (*GSL5*)/*Powdery Mildew Resistant 4* (*PMR4*)/*Callose Synthase 12* (*CalS12*) generates callose

as a defense response upon pathogen challenge to plants. Conversely, due to loss of function mutant of *gsl5/pmr4/cals12* was unable to generate callose at papillae (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Dong *et al.* 2008). In addition, it is speculated that plant defense response is augmented through secondary defense signaling pathway (Vogel and Somerville 2000). However, there is no interaction information among different races of PM fungus and defense related *peroxidase* gene as well as *glucan synthase* genes in melon. Therefore, we planned this study to observe the expression pattern of defense related *peroxidase* and *glucan synthase* like genes against different races of PM fungus *P. xanthii*, and under exogenous applications of SA and MeJA in *C. melo* SCNU1154 line. Moreover, this study will be helpful for future functional characterization of these genes in the development of stress resistant *C. melo* L.

MATERIALS AND METHODS

In silico analysis of peroxidase and glucan synthase-like genes

We collected *peroxidase* gene (AY373372; García-Gutiérrez *et al.* 2013) and *Arabidopsis Powdery Mildew Resistant 4* (*PMR4*) gene encoding callose synthase (protein AEE82336.1; Huibers *et al.* 2013) from the literature. Then we blasted these two gene sequences in the Melon Genome Database ‘Melonomics’ (<https://melonomics.net/>) and retrieved two peroxidases (*Prx*) and one glucan synthase (*GLS*)-like genes. Protein homology analysis was done by the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/gene/>) (Table 1). Based on the highest protein homology, the retrieved two *peroxidase* and one *glucan synthase-like* genes were named as *C. melo* peroxidase 2-like isoforms 1 (*CmPrx2-1*), *C. melo* peroxidase 2-like isoforms 2 (*CmPrx2-2*), and *C. melo* glucan synthase 1-like (*CmGLS1*) (Table 1). The primary structures of the genes were studied using ProtParam software (<http://expasy.org/tools/protparam.html>). Web-based software ‘SMART’ from European Molecular

Table 1. Protein homology analysis of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes of *Cucumis melo* (analyzed using BLAST from NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>).

Gene name	Top matched clones	Name of protein	Identity (%)	E-value	Top homologous species	References
MELO3C014657 (<i>CmPrx2-1</i>)	NP_001284470.1	Peroxidase 2-like precursor	91	0.0	<i>C. melo</i>	Keren-Keiserman <i>et al.</i> 2004
	CAA76680.1	Peroxidase	74	3e-167	<i>Cucurbita pepo</i>	Carpin <i>et al.</i> 1999
	KG54102.1	Peroxidase	63	1e-129	<i>C. sativus</i>	Ren <i>et al.</i> 2009
	AAM66044.1	Peroxidase	56	2e-125	<i>Arabidopsis thaliana</i>	Haas <i>et al.</i> 2002; Alexandrov <i>et al.</i> 2006
MELO3C014656 (<i>CmPrx2-2</i>)	NP_001284470.1	Peroxidase 2-like precursor	99	0.0	<i>C. melo</i>	Keren-Keiserman <i>et al.</i> 2004
	CAA76680.1	Peroxidase	73	4e-164	<i>C. pepo</i>	Carpin <i>et al.</i> 1999
	KG54102.1	Peroxidase	62	4e-127	<i>C. sativus</i>	Ren <i>et al.</i> 2009
	AAM65211.1	Peroxidase	56	1e-121	<i>A. thaliana</i>	Haas <i>et al.</i> 2002; Alexandrov <i>et al.</i> 2006
MELO3C002603 (<i>CmGLS1</i>)	NP_567278.1 (AtGSL1)	Glucan Synthase-Like 1	70	0.0	<i>A. thaliana</i>	Mayer <i>et al.</i> 1999
	XP_007025627.1 (TcGSL1-1)	Glucan synthase-like 1 isoform 1	75	0.0	<i>Theobroma cacao</i>	Motamayor <i>et al.</i> 2013

Biology Laboratories (EMBL) (http://smart.emblheidelberg.de/smart/set_mode.cgi?Genomic=1) was used to identify the domains. Multiple alignments of three proteins were constructed with reference proteins using GeneDoc (www.psc.edu/biomed/genedoc) software (Nicholas and Nicholas 1997). Multiple genomic DNA sequence alignment was constructed among *CmPrx2-1* and *CmPrx2-2* with reference sequence using GeneDoc (www.psc.edu/biomed/genedoc) (Nicholas and Nicholas 1997). The number and arrangement of introns and exons were analyzed using Gene Structure Display Server (GSDS 2.0) by aligning the coding sequence (CDS) with the genomic sequences (Guo *et al.* 2007) (<http://gsds.cbi.pku.edu.cn/>). MEME software (Multiple Em for Motif Elicitation, V4.10.0) (<http://meme-suite.org/tools/meme>) was used to analyze the conserved motifs of the selected proteins along with the reference sequences (Bailey *et al.* 2006). The MEME search was set to identify maximum 10 motifs for each protein.

Phylogenetic analysis

We retrieved the *Arabidopsis*, cucumber and summer squash protein sequences homologous to three selected

defense-related proteins of *C. melo* through NCBI blast search (<http://www.ncbi.nlm.nih.gov/blast/>). ClustalW software was used to align all protein sequences of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* from four different crop species including the reference protein sequences (Thompson *et al.* 1997). A phylogenetic tree was generated with MEGA6.06 using the Neighbor-Joining algorithm (Saitou and Nei 1987; Tamura *et al.* 2007). Two distinct clades of peroxidase, and glucan synthase were identified which were named according to reference protein. Bootstrap analysis with 1,000 replicates was conducted to evaluate the significance of the nodes.

Plant materials

In this study, we used *C. melo* SCNU1154 line that is susceptible against all identified races of PM fungus. Seeds of SCNU1154 line were soaked overnight to enhance germination and were sown in plastic pots containing sterilized soil mixture. Then seedlings were grown in a growth chamber at 22°C, 16:8 hours light/dark with a photon flux density of 140 $\mu\text{molm}^{-2}\text{s}^{-1}$. The relative humidity was kept between 65% and 75%. The selected plant organs of fresh roots, stems, leaves at three leaf

stages, and flowers were harvested. The samples were snap frozen in liquid nitrogen and then kept at -80°C for organ-specific expression analysis.

Biotic stress treatments

We initially cultured the seven races of *P. xanthii* viz., *KPH19*, *KPH01*, *BN968*, *DH487*, *BN625*, *SN102*, and *BN103*, on detached leaves of SCNU1154 line in petri dishes containing fungal growth media (mixture of 5 g agar powder+20 g D-Mannitol+10 g saccharose in 1,000 ml distilled water) in a growth chamber at 22°C with 16:8 hours day: night photoperiodic cycle and a photon flux density of $140\ \mu\text{molm}^{-2}\text{s}^{-1}$. Thereafter, we performed biotic stress treatments using all seven races to the adaxial part of 5th leaf (from ground level) of the six-week-old seedlings using the sterilized pencil brushes. The 80% relative humidity was maintained during the stress period. To conserve moisture, the inoculated plants were wrapped with polythene leaving perforations to exchange air until the development of typical symptoms. Similarly, control plants were also brushed with a sterilized pencil brush without any fungal inoculum. Sampling from the 5th leaf of both infected and non-infected plants was done at 18 days post inoculation. The harvested samples were immediately shifted to liquid nitrogen and then stored at -80°C for RNA extraction.

Phytohormone stress treatments

We conducted two phytohormone stress treatments on seedlings with three true leaves. Exogenous application with 100 μM solutions of SA, and MeJA was performed at the adaxial position of the seedlings. Then, we wrapped the treated plants by polyvinyl bags up to 48 hours, leaving perforations to exchange air. A non-treated plant was sampled just before the commencement of treatments and considered as 'control (0 hour)'. Sterile water sprayed to the plants instead of phytohormones was regarded as 'mock' samples. The treated leaf samples (1st and 2nd leaves) at 0 hour, 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, and 48 hours were harvested after all treatment imposition including mock treatments. The harvested leaf samples were snap frozen and then stored at -80°C until RNA extraction.

RNA extraction

We isolated the total RNA from the different organs of melon plants, as well as from the control and all stress-treated samples using an RNeasy mini kit (Qiagen, Hilden, Germany). RNase-free DNase (Promega, Madison, WI, USA) treatment was performed to remove any contaminants of genomic DNA. The cDNA synthesis was subsequently done by using the Superscript[®] III First-Strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction expression analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using EmeraldAmp[®] GT PCR Master Mix (Takara, Shiga, Japan). Specific primers were designed for all genes for RT-PCR and *CmActin* primers from *C. melo* (Gene Bank Acc. AY859055; Cheng *et al.* 2012) were used as control (Supplementary Table 1). PCR was carried out using 50 ng of cDNA from the roots, leaves, stems or flowers of *C. melo* SCNU1154 line as template (1 μl), PCR master mix (8 μl), 20 pmol forward and reverse primers (2 μl), and double-distilled H_2O (9 μl) were mixed to make a total volume of 20 μl in 0.5 ml PCR tubes. The samples were then subjected to the following conditions: pre-denaturation for 94°C for 5 minutes, followed by 30 and 35 cycled 3-step amplification at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds, and a final extension for 5 minutes at 72°C in a PCR thermal cycler (Takara).

Quantitative PCR expression analysis

Real-time quantitative PCR was carried out using 1 μl of 50 ng cDNA with a 20- μl reaction volume using 2 $\times$ qPCRBIO SyGreen Mix Lo-Rox SYBR[®] Green Supermix with ROX (PCR Biosystems Ltd., London, UK). The primers used in RT-PCR were also used for real-time PCR (Supplementary Table 1). The conditions for real time PCR were as follows: pre-incubation for 5 minutes at 95°C , followed by 3-step amplifications at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 20 seconds for 40 cycles. The melting temperature was at 95°C for 10 seconds, 65°C

for 60 seconds, and 97°C for 1 second as a default setting. The fluorescence was recorded following the last step of each cycle, and three replications were counted for each sample. Amplification, detection, and data analysis were carried out using LightCycler96 (Roche, Mannheim, Germany). The relative gene expression was estimated on the basis of the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

RESULTS

Sequence analysis of peroxidase and glucan synthase-like genes

We retrieved two *peroxidase* and one *glucan synthase like* genes from the ‘Melonomics’ database (<https://melonomics.net/>). The deduced amino acid of the retrieved two peroxidase genes shared high similarity of 91 and 99%, respectively with the reference melon peroxidase 2-like protein while the retrieved glucan synthase gene accounted high similarity between 70% and 75% respectively with the reference *Arabidopsis* and *Theobroma* glucan synthase 1-like protein (Table 1). Therefore, we named these genes as *C. melo peroxidase 2-like isoforms 1*, *CmPrx2-1*, *C. melo peroxidase 2-like isoforms 2*, *CmPrx2-2*, and *C. melo glucan synthase 1-like*, *CmGLS1* (Table 1). In addition, *CmPrx2-1* and *CmPrx2-2* proteins also shared from 62% to 74% similarity with peroxidase protein of other plants (Table 1). The predicted sizes of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* proteins are 37.2, 37.1, and 204.8 kDa, respectively and predicted isoelectric points are 6.01, 5.69, and 9.08, respectively (Table 2).

Analysis of protein domain organization revealed that *CmPrx2-1* and *CmPrx2-2* proteins contained one short signal peptide in their N-terminal, and one large peroxidase domains (Fig. 1A). The peroxidase domain of *CmPrx2-1* and *CmPrx2-2* proteins is highly conserved with the reference peroxidase protein from *Arabidopsis*, melon and summer squash (Fig. 1A). Conversely, *CmGLS1* protein contained one FKS1_dom1 domain in their N-terminal, eight transmembrane domains and one glucan synthase domain (Fig. 1B). The glucan synthase domain of *CmGLS1* protein is also highly conserved with the reference glucan synthase protein from *Arabidopsis* and

Table 2. In silico analysis of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes collected from the Melonomics database²⁾.

Gene name	Locus ID	Domain	Genome position	Gene position		ORF (bp)	Length (aa)	Protein		No. of introns
				Start	End			MW (kDa)	pI	
<i>CmPrx2-1</i>	MELO3C014657	Peroxidase, signal peptide	CM3.5_scaffold00022	4820763	4822308	1038	345	37.1137	6.01	3
<i>CmPrx2-2</i>	MELO3C014656	Peroxidase, signal peptide	CM3.5_scaffold00022	4818139	4819883	1038	345	37.1876	5.69	3
<i>CmGLS1</i>	MELO3C002603	FKS1_dom1, transmembrane region, Glucan synthase	CM3.5_scaffold00001	4726334	4732316	5310	1769	204.8114	9.08	1

²⁾Melonomics database (<https://melonomics.net/>).

ORF: open reading frame, bp: base pair, aa: amino acid, MW: molecular weight, kDa: kilo Dalton, pI: iso-electric point.

Fig. 1. Sequence alignment of *Cucumis melo* peroxidase and glucan synthase proteins. (A) Peroxidase, CmPrx2-1, MELO3C014657; CmPrx2-2, MELO3C014656 and (B) glucan synthase, CmGLS1, MELO3C002603 with other plant peroxidase (*Arabidopsis*, *Arabidopsis thaliana*, AAM65211.1 and AAM66044.1; Melon, *C. melo*, NP_001284470.1; Summer squash, *Cucurbita pepo*, CAA76680.1; Cucumber, *C. sativus*, KGN54102.1) and glucan synthase (*Arabidopsis*, *A. thaliana*, NP_567278.1; *Theobroma*, *Theobroma cacao*, XP_007025627.1) proteins. The alignment was generated by CLUSTALW using default parameters. (A) Red colored box represents signal peptide; orange colored box designates peroxidase domain. (B) Orange colored box represents glucan synthase domain; red colored box indicates FKS1 dom1 domain and blue colored boxes indicates different transmembrane domain (TM).

Theobroma (Fig. 1B).

Furthermore, we performed multiple alignment among *CmPrx2-1*, *CmPrx2-2* and two reference peroxidase 2-like genes (*C. melo*, ASM31304v1; *C. sativus*, ASM407v2) (Supplementary Fig. 1) and found that genomic sequence of *CmPrx2-2* had a total of additional six adenine (A) both in intron and exon part (green colored box in Supplementary Fig. 1), while much nucleotide variation was observed in genomic sequence of *CmPrx2-1* compared to reference peroxidase 2-like genes (*C. melo*, ASM31304v1). Analysis of intron and exon distribution showed that *CmPrx2-1* and *CmPrx2-2* gene displayed similar splicing pattern where *CmGLS1* gene exhibited a quite different splicing pattern (Fig. 2A).

We observed that ten identical motifs were conserved in *CmPrx2-1* and *CmPrx2-2* proteins, ranging between 11 and 50 amino acids (Fig. 2B-I). Conserved motif 1, 2, 3, 4, 5, 6, 7, and 8 were present in all peroxidase proteins examined (Fig. 2B-I). Motif 9 and 10 were absent in two peroxidase proteins from *Arabidopsis* (AAM65211.1 and

AAM66044.1), and motif 10 was absent in cucumber (KGN54102.1) (Fig. 2B-I). However, there were 10 conserved motifs present in all 3 glucan synthase proteins examined with 50 amino acids (Fig. 2B-II). Phylogenetic analysis showed that *CmPrx2-1* and *CmPrx2-2* proteins are distributed in peroxidase clade and *CmGLS1* protein is in glucan synthase clade (Fig. 3).

Organ-specific expression analysis

The organ-specific RT-PCR expression pattern was much obvious at 35 cycles of amplification in contrast to 30 cycles (Fig. 4). The expression of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes was noticed in all organs tested in *C. melo* SCNU1154 line and varied widely in terms of the level of expression except *CmPrx2-2* gene which is not expressed in leaf tissue (Fig. 4).

qPCR expression analysis under biotic stress treatment

C. melo peroxidase 2-like isoform 1 (*CmPrx2-1*)

Fig. 2. (A) Schematic representation of intron-exon distribution of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes in *Cucumis melo*. Comparisons of motifs distribution of Fig. B-I. peroxidase and Fig. B-II. glucan synthase hormone related proteins in *C. melo* with other plant proteins, peroxidase (*Arabidopsis*, *Arabidopsis thaliana*, AAM65211.1 and AAM66044.1; Melon, *C. melo*, NP_001284470.1; Summer squash, *Cucurbita pepo*, CAA76680.1; Cucumber, *C. sativus*, KGN54102.1) and glucan synthase (*Arabidopsis*, *A. thaliana*, NP_567278.1; *Theobroma*, *Theobroma cacao*, XP_007025627.1).

Fig. 3. Phylogenetic relationships of the deduced amino acid sequences of CmPrx2-1, CmPrx2-2 and CmGLS1 proteins (peroxidase, CmPrx2-1, MELO3C014657; CmPrx2-2, MELO3C014656 and glucan synthase, CmGLS1, MELO3C002603) with other plant peroxidase (Arabidopsis, *Arabidopsis thaliana*, AAM65211.1 and AAM66044.1; Melon, *Cucumis melo*, NP_001284470.1; Summer squash, *Cucurbita pepo*, CAA76680.1; Cucumber, *C. sativus*, KGN54102.1) and glucan synthase proteins (Arabidopsis, *A. thaliana*, NP_567278.1; Theobroma, *Theobroma cacao*, XP_007025627.1). The scale represents the frequency of amino acid substitutions between sequences as determined by the Poisson evolutionary distance method.

Fig. 4. Expression of CmPrx2-1, CmPrx2-2, and CmGLS1 in different organs of *Cucumis melo*. Reverse transcription- polymerase chain reaction (PCR) analysis of 3 genes is shown for in roots, stems, leaves, and flowers of *C. melo*. PCR amplification was recorded at both 30 and 35 cycles.

displayed race-specific expression against seven races of PM fungus *P. xanthii* after infection (Fig. 5). CmPrx2-1 showed the highest level of expression against KPH19 followed by BN968, KPH01, BN103, DH487, and exhibited comparatively lower level of expression against BN625 and SN102 races compared to control (Fig. 5). The approximate relative expression of CmPrx2-1 gene varied between 1.2 and 2.2 fold against the up-regulated races (Fig. 5).

Conversely, *C. melo* peroxidase 2-like isoform 2 (CmPrx2-2) was up-regulated in response to all seven races

of *P. xanthii* infection compared to control (Fig. 5). The maximum up-regulation was recorded against KPH01 followed by BN968, KPH19, DH487, BN103, BN625, and SN102 races (Fig. 5). The approximate relative expression of CmPrx2-2 gene varied between 8.0 and 30.0 fold against all seven up-regulated races (Fig. 5).

However, CmGLS1 gene was down-regulated in response to six races of *P. xanthii* infection compared to control while this gene was almost inactive against BN625 race (Fig. 5). The maximum down-regulation was observed against BN103 followed by BN968, SN102, DH487, KPH19, and KPH01 races (Fig. 5).

qPCR expression analysis after exogenous phytohormone treatments

Furthermore, to explore the expression analysis of CmPrx2-1, CmPrx2-2, and CmGLS1 genes we exogenously sprayed the two defense signaling molecules viz. SA and MeJA in the current study. Under SA and MeJA treatment, two isoforms of peroxidase 2-like genes (CmPrx2-1 and CmPrx2-2) and one glucan synthase 1-like gene (CmGLS1) were up-regulated compared to control (Fig. 6). Peroxidase 2-like isoform 1 (CmPrx2-1) showed maximum up-regulation of 84 fold and 17 fold at 6 hours after exogenous application of SA and MeJA, respectively and thereafter

Fig. 5. Real-time quantitative polymerase chain reaction relative expression analysis of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes after infection with seven races of powdery mildew fungus *Podosphaera xanthii* in *Cucumis melo* SCNU1154 plants. The error bars represent the standard error of the means of three independent replicates.

Fig. 6. Real-time quantitative polymerase chain reaction relative expression analysis of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes after exogenous application of salicylic acid (SA) (A) and methyl jasmonate (MeJA) (B) in *Cucumis melo* SCNU1154 plants. The error bars represent the standard error of the means of three independent replicates.

declined upto the end of the treatment periods (Fig. 6). Similarly, peroxidase 2-like isoform 2 (*CmPrx2-2*) exhibited maximum up-regulations of 3.88 fold at 12 hours after SA treatment and 2.66 fold at 48 hours after MeJA treatment (Fig. 6). *CmGLS1* genes displayed maximum up-regulation of nearly 8 fold and 4.8 fold at 12 hours after exogenous application of SA and MeJA, respectively (Fig. 6).

DISCUSSION

To explore the expression profiling of defense-related

genes, two peroxidase 2-like genes viz. *CmPrx2-1*, *CmPrx2-2*, and one glucan synthase 1-like gene viz. *CmGLS1* genes were retrieved from Melon Genome Database 'Melonomics' (<https://melonomics.net/>). Protein domain organization revealed that *CmPrx2-1* and *CmPrx2-2* isoforms contain characteristic features of signal peptide domains as of class III peroxidases (E.C.1.11.1.7) in their N-termini (Fig. 1). Research data revealed that signal peptide targets the proteins into the secretory pathway via the endoplasmic reticulum (Penel 2000). The secreted plant peroxidases are very important to generate hydrogen peroxide (H_2O_2) that takes part in oxidation reactions

process needed for the cells (Penel 2000). Conversely, *glucan synthase-like* genes contain a typical glucan synthase domain that regulate callose synthases as plant defense mechanisms (Cui *et al.* 2001; Doblin *et al.* 2001; Hong *et al.* 2001; Østergaard *et al.* 2002). Apart from high homology of glucan synthase-like gene with yeast *FK506 hypersensitivity (FKS)* genes, this gene is also speculated to be linked to (1,3)- β -D-glucan biosynthesis (Douglas *et al.* 1994; Cabib *et al.* 2001; Dijkgraaf *et al.* 2002). Moreover, β -1,3-glucan synthase (UDP-glucose; 1,3-b-D-glucan 3-b-D-glucosyltransferase; EC 2.4.1.34) is involved to catalyze the formation of a β -1,3-glucan polymer, a main component of the fungal cell wall (Shematek *et al.* 1980). Furthermore, although there is high similarity exists (99%) between *CmPrx2-2* and reference peroxidase 2-like protein (*C. melo*, NP_001284470.1), we speculate that *CmPrx2-2* might be a distinct form of peroxidase 2-like gene in respect to reference one and might have a different function (Supplementary Fig. 1). This might be due to addition of adenine residues in *CmPrx2-2* genomic sequence (Supplementary Fig. 1).

The organ-specific expression analysis of defense related genes was done to select an appropriate organ for qPCR analysis (Fig. 3). Variable level of expression was observed in case of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes. This observation suggests that these three defense-related genes are differentially expressed in different organs and are involved in growth and developmental functions in melon.

CmPrx2-1 displayed race-specific expression against seven different races of PM fungus *P. xanthii* (Fig. 5). Previous results indicated that the higher the up-regulation of the peroxidase gene under PM fungus infection was associated with plant defense reaction against pathogen challenge (García-Gutiérrez *et al.* 2013). The race-specific up-regulation might be resulted in response to interaction between race-specific R genes in the host cell and a corresponding fungal Avr gene (Glazebrook 1999; Schulze-Lefert and Vogel 2000). In addition, the gene-for-gene interaction triggers defense reactions, such as the HR, to restrict pathogen growth and reproduction (Hammond-Kosack and Jones 1996). In plant-microbe interaction, PM fungus might generate microbial effectors that could interact only with host R genes for hyper-

sensitive reactions resulting PM resistance. However, the lack of resistant genes probably brings to a compatible disease reaction (Panstruga 2003). This indicates that microbial effectors protein generated by the different races of *P. xanthii* was variable and that different peroxidase isoforms and *glucan synthase like* genes were essential for their effective recognition.

Conversely, *CmPrx2-2* was up-regulated in response to all seven races of *P. xanthii* compared to control (Fig. 5). Although *CmPrx2-1* and *CmPrx2-2* genes contained the conserved signal peptide and peroxidase domain, their expression pattern and level varied noticeably against different races of PM fungus (Fig. 5). Research reports revealed that despite of high homology (from 28% to 100%) between two peroxidase proteins, the particular isoform function is quite different in defense responses against pathogen challenge (Penel *et al.* 1992; Hiraga *et al.* 2001). Furthermore, plant defensive *peroxidases*, induced by SA and JA signaling pathway, are involved to generate lignin and hydrogen peroxide as a HR upon pathogen challenge and thereby play roles in plant disease resistance mechanisms (Avdiushko *et al.* 1993; Niranjan Raj *et al.* 2012). Therefore, we speculate that highly induced *CmPrx2-1* and *CmPrx2-2* genes might be related to plant disease resistance against PM fungus *P. xanthii* in *C. melo* L.

CmGLS1 gene was down-regulated in response to six races of *P. xanthii* compared to controls while this gene was almost inactive against BN625 race (Fig. 5). Previous reports showed that *GSL5/Powdery Mildew Resistant 4 (PMR4)/CalS12* generates callose as a defense response in sporophytic tissue after pathogen challenge or wounding treatment in plants. Conversely, due to loss of function, mutant of *gsl5/pmr4/cals12* was unable to generate callose at papillae (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Dong *et al.* 2008). However, apart from callose synthesis, plant evolved multiple defense mechanisms against pathogen challenge (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Dong *et al.* 2008). Interestingly, callose generated upon pathogen challenge is involved negatively to controls the SA signaling pathway of plants (Nishimura *et al.* 2003). Conversely, the powdery mildew resistant 4 (*pmr4*) mutant in *Arabidopsis* reduce callose deposition after fungal penetration and could activate SA defense signaling and

thereby increased resistance to pathogen (Nishimura *et al.* 2003). Therefore, we speculate that our *CmGLS1* gene might be a mutant as the expression of this gene is down-regulated against almost all races of PM fungus after infection and might function in plant disease resistance response through this pathway.

Moreover, we also investigated the expression behavior of *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* genes under exogenous application of two defense signaling molecules viz. SA and MeJA. Importantly, we observed that all of three genes viz. *CmPrx2-1*, *CmPrx2-2*, and *CmGLS1* were highly induced by SA treatment over MeJA (Fig. 6) (Glazebrook 2005). Some notable reports revealed that plant defense responsive *peroxidase* genes are induced by both SA and jasmonate (JA) signaling pathways (Durrant and Dong 2004; van Loon *et al.* 2006; Pieterse *et al.* 2009). Likewise, plant defense genes against biotrophic pathogens were also highly induced by application of exogenous SA in *Arabidopsis* (Thomma *et al.* 2001). Signaling molecules MeJA plays a crucial role in plant defense, programmed cell death and leaf senescence (Reinbothe *et al.* 2009). Taken together, we propose that apart from growth and developmental roles, three defense responsive genes studied herein might function more effectively through SA-signal mediated pathway over MeJA against biotrophic PM fungus *P. xanthii*. However, this speculation needs further validation through simultaneous application of exogenous phytohormones and *P. xanthii* fungal inocula. The results of this study would be valuable resources for further functional characterization and development of PM stress resistant variety of *C. melo* L.

ACKNOWLEDGEMENTS

This research work was done by the funding of Export Promotion Technology Development Program (Grant No. 312065-05-4-HD030) and Golden Seed Project (Center for Horticultural Seed Development, No. 213003-04-4-SB110) of the Ministry of Agriculture, Food and Rural Affairs (MAFRA), Republic of Korea.

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