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Transcriptional profiling of watermelon during its incompatible interaction with *Fusarium oxysporum* f. sp. *niveum*

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Abstract Transcriptome profiling of watermelon during its incompatible interactions with *Fusarium oxysporum* f.sp. *niveum* (FON) was performed using an Agilent custom microarray, which contains 15,000 probes representing approximately 8,200 watermelon genes. A total of 24, 275, 596, 598, and 592 genes showed significant differential expression in FON-infected plant roots, as compared with mock-inoculated roots, at 0.5, 1, 3, 5 and 8 days post inoculation (dpi), respectively. Bioinformatics analysis of these differentially expressed genes revealed that during the incompatible interaction between watermelon and FON, the expression of a number of pathogenesis-related (PR) genes, transcription factors, signalling/regulatory genes, and cell wall modification genes, was significantly induced. A number of genes for transporter proteins such as aquaporins were down-regulated, indicating that transporter proteins might contribute to the development of wilt symptoms after FON infection. In the incompatible interaction, most genes involved in biosynthesis of jasmonic acid (JA) were expressed stronger and more sustained than those in a compatible interaction in FON-infected tissues. Similarly, genes associated with shikimate-phenylpropanoid-lignin biosynthesis were also induced during the incompatible interaction, but expression of these genes were not changed or repressed in the compatible interaction. Those results demonstrate that JA biosynthesis and shikimate-phenylpropanoid-lignin pathways might play important roles in watermelon against FON infection and thus provides new insights in understanding the molecular basis and signalling network in watermelon plants in response to FON infection. We also performed confocal imaging of watermelon roots infected with the green fluorescent protein (GFP)-tagged FON1 to revealed histological characteristics of the infection.

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Introduction

Watermelon (*Citrulls lanatus*) is one of the most important vegetable crops in the world and accounts for ~6% of the world's total area devoted to vegetable production (http://faostat.fao.org/). Fusarium wilt caused by the soil-borne vascular fungal pathogen *Fusarium oxysporum* f. sp. *niveum* (FON), is the most destructive disease in watermelon worldwide, causing approximately 30–50% yield losses (Martyn and Netzer 1991). Symptoms include wilting due to the disruption of water transport within the vascular system, vascular browning, and eventual death of the infected plant. Annual losses caused by FON account for 10–15% of the total watermelon yield, which sometimes can escalate to total loss in some regions under specific conditions (Martyn and Netzer 1991; Zhang et al. 2005).In recent years; progress has been made in understanding the responses of watermelon to FON at the physiological and biochemical level. Most resistant varieties have strengthened cell structures with thickened xylem compared to susceptible plants (Chang et al. 2008). During the early response against pathogens, parenchyma cells of vascular tissues in resistant varieties are also reinforced by the polysaccharides, callose and cellulose. Typically, reinforcement of parenchyma cells in roots of resistant varieties can be observed within 1 to 4 days post inoculation (dpi), while they can be seen later in roots of susceptible lines (Wang et al. 2002) . Biochemical and histochemical studies have revealed that phytoalexins can accumulate and be maintained at relatively high levels for several days in vascular tissues of resistant plants after FON infection and contribute to inhibition of hyphae expanding (Wu et al. 2010). Several metabolic enzymes such as chitinase, β-1, 3-glucanase, phenylalanine ammonia-lyase (PAL) and protective enzyme systems may also be involved in watermelon resistance against FON (Chang et al. 2008; Wang et al. 2002; Xu et al. 2000). In addition, increased concentrations of lignin and hydroxyproline-rich glycoprotein (HRGP) substances have been detected in roots of resistant lines (Xu et al. 2000; Chang et al. 2008). However, little is known about the molecular changes in watermelon plants after FON infection.

Genome-wide expression profiling of plants infected with *Fusarium oxysporum* has been reported in several crop plant species, including cotton (Dowd et al. 2004), chickpea (Gupta et al. 2010) and tomato (Amaral et al. 2008). However, no similar study has been reported in watermelon. The objective of this study was to elucidate the molecular basis of the incompatible interaction between watermelon and FON through a gene expression profiling analysis and to identify novel genes that are involved in watermelon-FON incompatible interaction process. Results from these studies may provide insights into the genetic basis of watermelon defence against FON. To this end, we employed the Agilent custom gene expression microarrays, which contain probes derived from more than 8,000 watermelon expressed sequence tag (EST) unigenes, and investigated gene expression changes in watermelon at different stages of a resistant line after FON infection. Expression patterns of genes in jasmonic acid (JA) and shikimate-phenylpropanoid-lignin biosynthesis pathways in incompatible and compatible interactions between watermelon and FON was further investigated using quantitative real-time PCRs (qRT-PCRs). We also performed confocal imaging of watermelon roots infected with the green fluorescent protein (GFP)-tagged FON1 to reveal histological characteristics of the infection. The FON-regulated genes identified in this study provide new insights into the understanding of the molecular mechanism in response of watermelon plants to FON infection and will be helpful in molecular breeding of FON resistant varieties and the development of rational strategies for control of Fusarium wilt disease in watermelon and other crops.

Materials and methods

Agrobacterium tumefaciens-mediated transformation of FON 1 and microscopy

The *F. oxysporum* f. sp. *niveum* isolate FON1, a race 1 isolate recovered from a symptomatic watermelon plant in a field in Tongxian, Beijing, China, was used in this study (Xu et al. 2000). Conidia of FON1 were prepared and transformed with the pCH-sGFP transformation vector using *Agrobacterium tumefaciens* AGL1 (provided by Dr. Bingyan Xie at CAAS),

which harbours the sGFP gene under the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene from *Cochliobolus heterostrophus*. The bacterial hygromycin B phosphotransferase gene (*HPH*), under the *Aspergillus nidulans* trpC promoter, was used as the selectable marker. *A. tumefaciens* strain AGL-1, containing the pCH-sGFP transformation vector was grown at 28°C for 2 days in the minimal medium (MM) supplemented with kanamycin (50 μg/ml) and rifampicin (50 μg/ml). *A. tumefaciens* cells were diluted to an OD600 nm of 0.15 with the induction medium (IM) containing 200 μM acetosyringone (AS) and grown for an additional 6 h before mixing them with an equal volume of a conidial suspension from strain BEIJING1 (1×10^6 conidia per ml). This mix (200 μl per plate) was plated on 90 mm diameter filter paper and placed on the co-cultivation medium which was same as IM except that it contained 5 mM glucose instead of 10 mM glucose. Following incubation at 26°C for 2 days, the filter paper was transferred to potato dextrose agar (PDA) containing hygromycin B (150 μg/ml) as the selection agent for transformants and cefotaxime (200 μM) to kill the *A. tumefaciens* cells. Individual transformants were transferred into PDA plate amended with hygromycin B (150 μg/ml) and incubated until conidiogenesis. To create monoconidial cultures, one germinating conidium from each transformant was picked and transferred to PDA containing hygromycin B (150 μg/ml). These monoconidial cultures were stored at −80°C till use.

Transformants were examined for morphology and GFP expression under a Nikon compound microscope (450 to 490 nm excitation, 590 nm long pass emission), and their pathogenicity in watermelon was determined and compared to that of the wild-type isolate. The FON1-GFP transformant was inoculated into the watermelon PI296341-FR cultivar. At 0.5, 1, 3, 5, and 8 days post inoculation (dpi), the plants were carefully taken out of the pots and their roots were gently swirled a few times in tap water to wash away soil particles. Roots were then placed directly on glass slides in drops of water, covered with a cover slide, and examined for the process of colonization and infection by the GFP-tagged FON1 isolate under a Leica TCS SP confocal laser scanning microscope (Leica Microsystems Inc., Bannockburn, IL). For each time point, roots from at least 10–20 different plants were analyzed.

Plant material and pathogen inoculation

FON1 hyphae from the potato-dextrose agar (PDA) plate were inoculated on the potato lactose medium and incubated on a shaker (140 rpm) at 28°C for 5–8 days. Subsequently, the culture was filtered through two layers of sterile gauze and the filtrate containing the conidia were centrifuged at $6{,}000\times g$ for 15 min. The conidial suspension was adjusted to 5.0×10^6 spore/ml with sterile distilled water before inoculation. Seeds of two FON1 susceptible watermelon cultivars, Black Diamond and Sugar Baby, one FON1 moderately susceptible cultivar, crimson sweet, two FON1 resistant cultivars, Calhoun Gray and PI296341-FR, and one FON1 moderately resistant cultivar, Charleston Gray, were first sterilized with 1.5% sodium hypochlorite, soaked in distilled water for 4 h , and then put at 30°C for germination. The germinated seedlings were grown in sterilized vermiculite and cultured under a cycle of 14 h-artificial light at 26°C and 10 h-dark at 22°C with 70% relative humidity (RH). Seedlings with two cotyledons were infected with FON1 by dipping their roots into a suspension of fungal conidia for 15 min. Seedlings inoculated with water were used as the mock controls. All the inoculated seedlings were replanted in 10×10 cm pots with sterilized soil (peat soil: garden soil=1:1) in a controlled environment of 26°C, 70% RH and a 12-h diurnal light cycle, and watered with Hoagland culture liquid every 3 days for 25 days. Disease response of plants was rated on a scale from 0 to 4 and recorded every day after the inoculation for 25 days. Disease index of each inoculated plant was calculated according to the formula as shown in (Swiader et al. 2002):

$$\text{Disease index} = \sum(\text{nv})100/\text{NV},$$

Where: n = degree of infection rate according to the 5-grade scale, v = number of plants in a category, V = total number of plants screened, N = highest degree of infection rate.

Roots of FON1 inoculated Black Diamond and PI296341-FR, as well as their corresponding mock controls, were harvested at 4 h, 8 h, and 12 h post inoculation (hpi), and 1, 2, 3, 5, and 8 dpi, respectively, quickly frozen in liquid nitrogen, and stored at 70°C for RNA extraction.

RNA isolation

Total RNA was isolated from roots using the Trizol Kit (Invitrogen, CA) and then purified using the RNeasy Kit (Qiagen, CA) according to the manufacturers' instructions. RNA quality and quantity were determined using a spectrophotometer and agarose gel electrophoresis.

Microarray design and fabrication

A total of 8,242 watermelon EST-derived unigenes were collected from public domains. Among these unigenes, 1,756 were from a normalized and subtracted cDNA library representing nine different time points of an incompatible interaction between watermelon and FON1 (Xu et al. 2008), 4,719 from the Cucurbit Genomics Database (http://www.icugi.org), and 1,767 from the NCBI dbEST database. Oligonucleotide probes with length of 60 bp were designed for each of the 8,242 watermelon unigenes using the eArray system (Agilent, Santa Clara, CA). These probes were used to design and manufacture high-density custom watermelon microarrays. A single watermelon microarray chip contained eight independently-hybridizable and identical mini-arrays, and each mini-array contained ~15,000 probes. Each of the 8,242 unigenes was represented by one or two 60-mer probes on the array. The probes were synthesized in situ by an ink-jet oligonucleotide synthesizer on glass slides using a computer-generated randomized pattern on the array.

Microarray hybridizations and data analyses

Root RNAs of watermelon cultivar PI296341-FR inoculated by FON1 and the corresponding mock controls,were used for the microarray experiment. Double-stranded cDNA was synthesized from RNAs using the T7 Promoter primer and M-MLV reverse transcriptase. The synthesized products were used as templates to produce cRNA using the T7 RNA Polymerase and then labelled with Cy3 (Cy3 NHS ester, GE healthcare). Labeled cRNAs were then purified using the RNeasy® Mini Kit (QIAGEN). Aliquots of Cy3-labelled cRNAs (1 μg each) of the inoculated and control samples were hybridized to the custom-made Agilent watermelon oligo microarrays following manufacturer's instructions (Agilent, Santa Clara, CA). Microarray hybridizations were performed for the following five time points: 0.5, 1, 3, 5, and 8 dpi, and three independent biological replicates were used for each time point. Raw data from the arrays was normalized using the quantile normalization algorithm (Bolstad et al. 2003). Significance of differential gene expression between FON1 inoculated plants and mock controls at same time points was determined using the LIMMA test (Smyth 2004) and raw p values of multiple tests were corrected using False Discovery Rate (FDR). Genes with FDR$<$0.05 and fold change no less than two were identified as differentially expressed genes.

Unigene sequences of probes on the watermelon array were compared against Swiss-Prot/TrEMBL protein databases using the BLAST program and Pfam domain database using the HMMER program, with an E-value cutoff of 1e-5. Gene Ontology (GO) terms were assigned to watermelon array probes using the Gene Ontology Annotation Database based on their top Swiss-Prot/TrEMBL hits and using the Pfam2GO mappings based on Pfam domains they contain. GO terms assigned to each gene were further mapped to a set of plant specific GO slims. Differentially expressed genes were classified into different functional categories using the plant-specific GO slims.

The microarray data described in the present study was deposited into the NCBI GEO database under accession number GSE29804.

qRT-PCR

First-strand cDNA was synthesized by reverse transcribing 2 μg of total RNA using M-MLV Reverse Transcriptase (Promega) and the Oligo$(dT)_{18}$ primer. qRT-PCR reactions were run on a Roche LightCycler® 480 Real-Time PCR system (Roche, Germany). Each 20 μl reaction consisted of 8 ng of cDNA, 1 μl of primer mix (5 μM of each forward and reverse primer), and 10 μl of LightCycler® 480 SYBR Green I Master Mix (Roche, Germany). Individual reactions were run with each primer pair with annealing temperatures ranging from 55°C to 60°C. Cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, gradient from 55°C to 60°C for 20 s, and 72°C for 30 s. Primer pairs of selected genes for qRT-PCR analysis were listed in Supporting Information Table S1. Melting

curves were performed at the end of each reaction run to detect primer-dimers and secondary products. Conditions were 95°C for 5 s, followed by 65°C for 1 min and then kept at 97°C. Quantification was achieved by normalizing the number of target gene copies to an endogenous reference gene using the comparative Ct method. The ΔCt was calculated by subtracting the average Ct value of each tissue type from the average Ct values of 18S rRNA. The ΔΔCt was calculated by subtracting the ΔCt of each of the FON1 inoculated plants from the ΔCt of the corresponding mock controls. The formula $2^{-(\Delta\Delta Ct)}$ was used to calculate a relative fold change between FON1 inoculated and mock plants. This relative fold change was determined by assuming a near perfect amplification resulting in a doubling of amplification product per cycle. Three biological replicates for each sample were used for real-time PCR analysis and real-time PCRs were performed with same RNA samples used for microarray analysis. Statistical analysis was performed using the Student's t test.

Results

Establishment of watermelon-FON interaction system for transcriptome profiling analysis

Three races (0, 1, and 2) have been previously identified in FON based on their abilities to cause disease on different watermelon genotypes (Martyn and Netzer 1991). Race 1 is the most widespread FON throughout the world (Martyn and Netzer 1991; Xu et al. 2000; Zhang et al. 2005; Chang et al. 2008), while the recently identified race 3, represented by the Maryland of USA isolates is the most virulent FON on watermelon (Zhou and Everts 2010). In this study, we selected isolates belonging to race 1 of FON (FON1) to establish a watermelon-FON interaction system for transcriptome profiling analysis. The race belonging to the selected isolates was confirmed by their ability to cause disease on watermelon cultivars Black Diamond, Sugar Baby, Crimson Sweet, Charleston Gray, Calhoun Gray, and PI296341-FR using a root-dip inoculation method (Xu et al. 2000). As shown in Table 1, susceptible cultivars Black Diamond and Sugar Baby showed approximately 90–100% wilt at 16 days post inoculation (dpi), while highly resistant cultivars Calhoun Gray and PI296341-FR showed no obvious wilt with disease indexes less than 5.0. Thus, the interaction between PI296341-FR and race 1 isolates represents an incompatible interaction. Based on these results, the incompatible interaction between PI296431-FR and FON1 isolates was used for transcriptome profiling analysis toward understanding of the molecular basis of watermelon-FON interactions.

We further revealed the colonization and infection behavior of watermelon root by a GFP-tagged FON1 isolate on roots of PI296341-FR. At 12 h post inoculation (hpi), the pathogens attached to and grew on the root surface, with <10% of the spores germinated (Fig. 1a). At 1 dpi, more than 50% of the spores on the root surfaces germinated (Fig. 1b) and hyphae from germinated spores continued to grow parallel to the longitudinal axis of the root. At 3 dpi, the hyphae grew parallel along the longitudinal groove of the root, expanded to larger areas, penetrated into epidermal cells at some sites, and appressoria were formed at the penetration sites (Fig. 1c). At 5 dpi, most of the root surface was covered with densely developed mycelium, especially at the cell borders, forming a fluorescent green pattern around the cells (Fig. 1d). At 7–8 dpi, the hyphae embraced

Table 1 Disease index of different watermelon cultivars inoculated with FON1

Cultivar	Days after inoculation					Disease response
	3 day	5 day	8 day	11 day	16 day	
Black Diamond	0	6.17	85.00	92.92	96.67	S
Sugar Baby	0	1.26	73.86	79.29	88.57	S
Crimson Sweet	0	0	13.33	34.51	63.86	MS
Charleston Gray	0	0	9.52	18.86	34.76	MR
Calhoun Gray	0	0	0	2.82	3.55	R
PI296341-FR	0	0	0	3.39	4.34	R

S Susceptible; *R* Resistant; *MS* Moderate Susceptible; *MR* Moderate Resistant

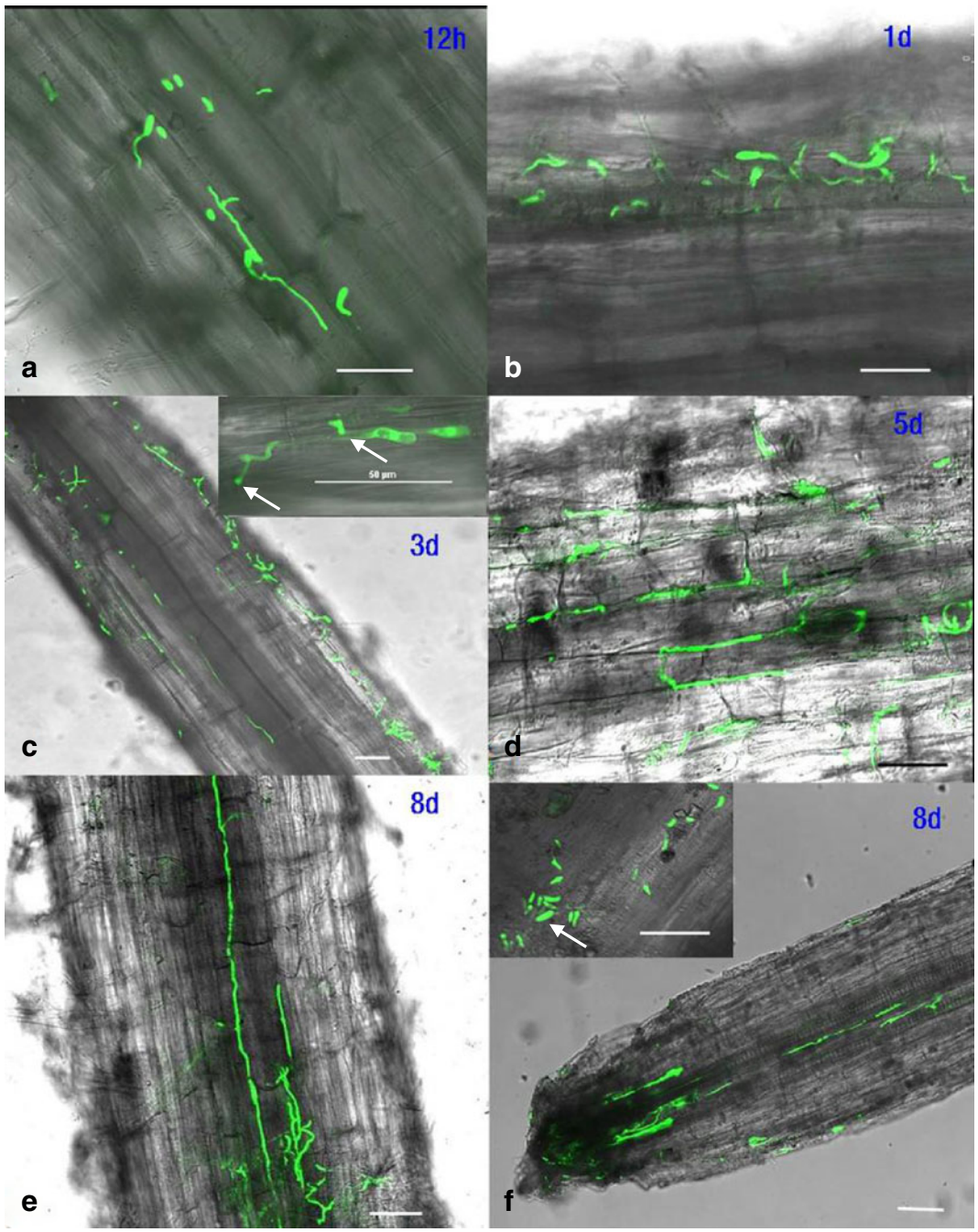

Fig. 1 Colonization and infection of watermelon (cv.PI296341-FR) root by GFP-tagged isolate of FON1 at 12 hpi (**a**), 1 dpi (**b**), 3 dpi (**c**), 5 dpi (**d**), and 8 dpi (**e, f**). Arrows in (**c**) point to appressoria and arrow in (**f**) points to conidia. Scale bar= 50 μm; *hpi* hours post inoculation; *dpi* days post inoculation

extensively most of the root surface and expanded to the cortical cells and tips (Fig. 1e and f). At this stage, the fungus sporulated on the roots (Fig. 1f). By contrast, in a compatible interaction between watermelon (cultivar Black Diamond) and FON1, hyphae colonized in the central cylinder of the roots, grew in the xylem vessels, fully colonized and destroyed the roots at 7 dpi (data not shown).

Transcriptome profiling analysis of watermelon-FON1 interaction

To monitor changes of gene expression and biological processes in watermelon after FON1 infection, we investigated global transcriptome profiles in an incompatible interaction between PI296341-FR, a resistance watermelon cultivar, and FON1, at different stages of infection. An Agilent custom watermelon microarray, which contains ~15,000 probes representing 8,242 watermelon genes, was designed and employed for gene expression profiling. In our microarray experiments, correlations between different biological replicates were very high (r=0.96–0.99), indicating that data obtained from microarray analysis were robust. From the microarray analysis, we identified a total of 24, 275, 596, 598, and 592 genes that were differentially expressed (fold change >= 2 and FDR<0.05) in FON1-inoculated plants as compared with those in mock-treated controls at 0.5, 1, 3, 5 and 8 dpi, respectively.

To validate the data from the microarray analysis, qRT-PCR assays were performed on 10 randomly selected genes. The results showed that although exact fold changes of selected genes at several data points varied between microarray and qRT-PCT analyses, trends of gene expression changes detected by the two different approaches were largely consistent and showed a high correlation with r=0.87 (Table 2). This further confirmed the robustness of our microarray data.

Biological processes and stage-specific expression of genes involved in watermelon-FON1 incompatible interaction

A total of 1,494 genes that were differentially expressed in FON1-infected plants, as compared with those in mock-inoculated plants, were identified in at least one of the five stages mentioned above and these differentially expressed genes were designated as FON1-responsive genes. The FON1-

Table 2 Verification of microarray results by qRT-PCR

TargetID	Gene annotation	Method	Fold change				
			12 h	1 day	3 day	5 day	8 day
Cluster502contig1	Expansin	Microarray	1.30	1.99	**3.87**	**167**	**34.74**
		qRT-PCR	1.98	2.15	**10.64**	**95.5**	**43.13**
08052303_T_15_66_B09	β-1,3-glucanse	Microarray	**2.40**	−1.67	−1.52	1.97	**19.35**
		qRT-PCR	**2.34**	−1.69	−1.52	1.62	**22.30**
Cluster302contig1	Chitinase class V	Microarray	−1.49	−1.59	1.08	**9.13**	**23.41**
		qRT-PCR	−1.01	1.07	1.47	**10.70**	**71.05**
WMU3685	Sodium transporter hkt1-like protein	Microarray	1.08	**−6.62**	**2.82**	**−5.28**	**−7.81**
		qRT-PCR	1.21	**−3.78**	1.75	**−4.47**	**−6.81**
08081903T1O_95_G12	MYB transcription factor	Microarray	**2.25**	−1.09	**−4.60**	1.04	1.76
		qRT-PCR	**3.27**	1.13	**−3.32**	1.57	**2.57**
08081903T1N_04_D01	Lipoxygenase	Microarray	1.15	1.17	**−3.57**	**−3.44**	−1.88
		qRT-PCR	1.71	−1.05	**−8.22**	**−5.98**	−1.57
Cluster71contig1	Silverleaf whitefly induced protein	Microarray	1.66	**−3.03**	**−2.79**	−1.89	**−2.21**
		qRT-PCR	1.91	**−3.45**	**−3.01**	−1.28	**−2.78**
08081903T1R_53_E07	Allene oxide cyclase	Microarray	1.73	1.01	**−4.35**	**−2.75**	**−3.54**
		qRT-PCR	1.45	−1.04	**−4.81**	**−3.25**	**−3.91**
Cluster35contig1	Shikimate kinase	Microarray	1.38	−1.22	**−3.15**	−1.47	**−2.46**
		qRT-PCR	1.42	−1.20	**−2.75**	−1.47	−1.71
Cluster362contig1	Early nodulin 93	Microarray	−1.22	1.76	**3.79**	1.04	**14.3**
		qRT-PCR	1.36	2.28	**4.57**	1.68	**15.81**

Significant difference (p<0.05 and fold change >= 2) in relative level is shown in bold

responsive genes were classified into different functional categories using a set of plant-specific GO slims (http://www.geneontology.org/GO.slims.shtml), which provide a broad overview of the ontology content. Figure 2 shows functional classifications of the FON1-responsive genes into plant specific GO slims within the biological process category, among which cellular process, response to stress and metabolic process were the most highly represented groups. This indicates that the watermelon plants undergo rapid and comprehensive cellular and metabolic changes in response to FON1 infection. Notably, GO annotations identified 135 and 130 FON1-responsive genes that are involved in biosynthetic process and transport process, respectively. Furthermore, a number of FON1-responsive genes involved in other important biological processes such as response to different stimuli, signal transduction and transcription were also identified through GO annotations.

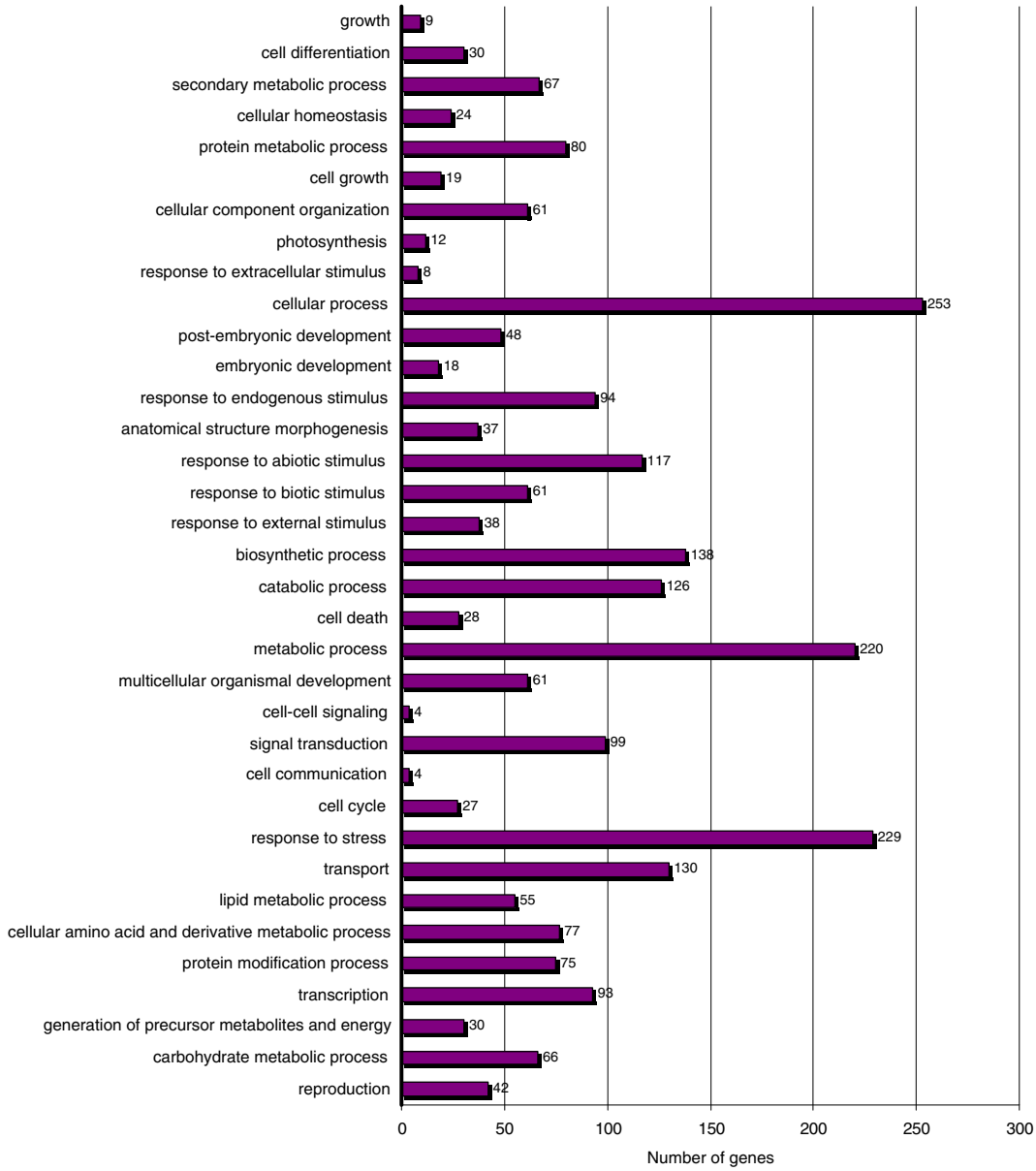

Fig. 2 Functional classification of differentially expressed genes

Collectively, a large number of the FON1-responsive genes encoded transcription factors, signalling/regulatory proteins, and proteins related to cell wall modification, host defence, transport, growth and phenylpropanoid biosynthesis (Table 3), some of which are known pathogen-responsive genes or postulated to be involved in defence response. Interestingly, the expression of the FON1-responsive genes showed dynamic patterns at different stages after FON1 infection.

At 12 h after FON1 infection of cultivar PI296341-FR, the fungus attached and started to germinate on the root surface (Fig. 1a). At this early stage, very few FON1-responsive genes were identified. These early FON1-responsive genes included genes encoding signalling/regulatory components such as calmodulin and genes in ethylene and jasmonic acid (JA) biosynthesis/signalling pathways.

At 1 dpi, expression of genes encoding for transport-related proteins such as aquaporins, sulphate transporter and sodium transporter was down-regulated by FON1 infection. Down-regulation of the aquaporins and drought-responsive genes may contribute to the development of wilt symptoms (Dowd et al. 2004; Amaral et al. 2008; Sade et al. 2009; Zhou and Wu 2009). At this stage, several transcription factors were significantly induced, e.g., ERF-like proteins, Myb family transcription factors, ethylene-responsive transcriptional coactivator, and RING-H_2 finger protein precursor.

At 3 dpi, expression of genes in the phenylpropanoid biosynthesis pathway was significantly induced by FON1 infection. The phenylpropanoid biosynthesis pathway is known to synthesize many secondary metabolites, some of which act as antimicrobial compounds or are important intermediates for lignin biosynthesis during cell wall thickening. In addition, several genes that are involved in cell wall modification were also up-regulated and these genes included expansins and xyloglucan endotransglucosylase, which act cooperatively to disassemble the polymer networks and thereby contribute to pathogen infection (Cosgrove 2000; Ding et al. 2008). At this stage, expression of most growth-related genes was significantly down-regulated; suggesting that growth of watermelon root was inhibited.

At 5 dpi, a large number of signalling and regulatory genes such as calmodulins and genes involved in ABA signalling, oxidative burst, and ethylene biosynthesis/signalling, and some pathogenesis-related genes were up-regulated by FON1 infection. However, expression of genes involved in cell wall modifications such as alpha galactosidases, pectin acetylesterase and methylesterase was significantly down-regulated, while expression of expansin genes was up-regulated. Differential expression of these genes indicates that at this stage of infection, the watermelon plants initiated defence signalling and started to synthesize defence-related proteins to protect from FON1 infection.

At 8 dpi, a number of genes encoding proteins involved in generation or homeostasis of reactive oxygen species (ROS), including peroxidase, oxidase, cytochrome P450, glutathione S-transferase and ferredoxin, were induced by FON1. Meanwhile, expression of genes for chitinases and β-1, 3-glucanases, which are known to catalyze the hydrolysis of chitin and β-1, 3-glucan, respectively, was also highly induced by approximately 20-fold. It is well known that β-1, 3-glucanases and chitinases act either directly by degrading the fungal cell wall or indirectly by releasing oligosaccharide elicitors of defense reactions, and thus play important roles in defence response against fungal infection. Differential expression of chitinases and β-1, 3-glucanases has been observed during compatible plant-fungal interactions in other plant-fungus interaction systems (Cheong et al. 2000; Bieri et al. 2003; Amaral et al. 2008). Therefore, it is likely that at this stage of infection, up-regulation of pathogenesis-related proteins genes especially chitinases and β-1, 3-glucanases indicated that a comprehensive network of defence response was activated in watermelon plants upon FON1 infection.

Importance of jasmonic acid biosynthesis and shikimate-phenylpropanoid-lignin pathways in watermelon-FON1 incompatible interactions

Data from transcriptome profiling analysis revealed that expression of a number of genes in the jasmonic acid (JA) and shikimate-phenylpropanoid-lignin biosynthesis pathways was significantly up-regulated or down-regulated in the incompatible interaction between PI296341-FR and FON1 (Table 3), indicating that JA and the shikimate-phenylpropanoid-lignin biosynthesis pathways may play important roles in watermelon defence response against FON1 infection. To test this hypothesis, we further investigated and compared expression patterns of these pathway genes

Table 3 Differentially expressed genes in interesting functional categories

Unigene	Description GenBank hits	Fold change				
		12 h	1 day	3 day	5 day	8 day
Transcription factors						
DV737703	AP2/ERF domain-containing transcription factor [Populus trichocarpa]	−1.47	−1.43	−1.33	**2.76**	1.85
AA660044	ERF-like protein [Cucumis melo]	−1.18	**2.51**	−1.79	−1.54	−1.09
_D_69_E09	Ethylene-responsive transcriptional coactivator, [Arabidopsis thaliana]	1.69	**3.02**	1.22	**2.56**	**2.43**
WMU2748	Myb-like transcription factor [Nicotiana tabacum]	1.39	**3.07**	−1.47	1.19	−1.92
Cluster133contig1	MYB24 [Malus x domestica]	1.34	1.68	−1.49	−1.56	**−2.65**
WMU4220	RING-H2 finger protein ATL5M precursor, putative [Ricinus communis]	−1.82	**2.01**	**2.01**	1.40	**4.06**
08042807T_5_33_A05	WRKY transcription factor, putative [Ricinus communis]	1.01	1.10	1.95	**2.47**	**2.17**
Signaling and regulation genes						
07122002T_A_68_D09	1-aminocyclopropane-1-carboxylate oxidase, [Ricinus communis]	1.14	1.68	1.19	1.80	**3.97**
AI563272	1-aminocyclopropane-1-carboxylate synthase [Citrullus colocynthis x Citrullus lanatus var. lanatus]	**2.03**	**2.29**	−1.30	**4.59**	**6.66**
DV737827	RecName: Full=Ethylene receptor 1	−1.12	−1.05	**2.41**	−1.21	1.01
DV737018	Calcium ion binding protein, putative [Ricinus communis]	−1.08	1.72	−1.01	−1.05	**2.11**
WMU183	Calcium lipid binding protein, putative [Ricinus communis]	1.05	−1.05	**2.18**	**2.57**	1.02
Cluster280contig1	Calcium sensor calcineurin B-like protein [Solanum lycopersicum]	1.16	1.36	**2.10**	1.45	1.44
AI563090	Calmodulin binding protein, putative [Ricinus communis]	1.07	−1.37	1.27	−1.39	**2.15**
WMU2242	Calmodulin, putative [Ricinus communis]	−1.54	1.40	1.87	**2.84**	**5.72**
WMU4160	CBL-interacting protein kinase 19 [Vitis vinifera]	1.01	1.22	−1.30	**2.96**	−1.05
Cluster249contig1	13S-lipoxygenase [Cucumis melo var. inodorus]	1.22	1.12	**−3.58**	**−2.74**	−1.85
08081903T1R_53_E07	Allene oxide cyclase 4, [Ricinus communis]	1.73	1.01	**−4.35**	**−2.75**	**−3.54**
07122002T_A_58_B08	Allene oxide synthase [Cucumis sativus]	**2.04**	1.16	0.67	−1.25	−1.30
Cluster515contig1	Jasmonate induced protein [Hordeum vulgare subsp. vulgare]	1.04	−1.64	1.07	1.11	**2.34**
Cluster228contig1	RecName: Full=23 kDa jasmonate-induced protein	1.12	−1.11	1.03	**4.67**	**3.18**
AI563098	Leucine-rich repeat transmembrane protein kinase, [Ricinus communis]	−1.14	1.32	1.29	1.91	**2.54**
DV737866	Nodulation receptor kinase precursor, putative [Ricinus communis]	−1.64	−1.33	1.49	−1.72	**2.10**
WMU761	lrr receptor-linked protein kinase, putative [Ricinus communis]	−1.16	1.06	−1.06	**2.31**	1.40
cluster301Contig1	Serine/threonine-protein kinase SAPK3, putative [Ricinus communis]	1.04	1.18	−1.05	1.87	**2.21**
Cell wall modification						
WMU221	Xyloglucan endotransglucosylase/hydrolase protein 2 precursor [Ricinus communis]	−1.61	−1.82	**2.45**	**2.01**	−1.19
WMU838	Beta-galactosidase, putative [Ricinus communis]	−1.08	−1.22	1.52	**3.11**	1.53
WMU1966	Acid alpha galactosidase 2 [Cucumis sativus]	−1.27	−1.28	1.49	**−4.08**	**−3.41**
WMU263	Glycosyl hydrolase family 38 protein [Arabidopsis thaliana]	1.04	−1.28	1.00	**2.19**	1.32
WMU3465	Beta-glucosidase, putative [Ricinus communis]	−1.27	1.33	2.21	**5.25**	**26.49**
Cluster70contig1	Glucanase [Rosa roxburghii]	2.07	−1.75	−1.52	1.82	**9.30**
Cluster502contig1	Beta-expansin 1a precursor, putative [Ricinus communis]	1.30	1.99	**3.87**	**167.1**	**34.74**
07122002T_B_49_A07	Expansin-like protein precursor [Solanum lycopersicum]	1.21	**2.69**	**5.51**	**10.02**	**12.92**
cluster415Contig1	Beta-expansin 1a precursor, putative [Ricinus communis]	−1.12	**2.34**	**2.39**	**9.87**	**64.27**
WMU681	Fiber protein Fb19 [Gossypium barbadense]	1.04	**2.07**	1.09	1.25	−1.04
WMU382	Cellulose synthase-like protein CslE [Nicotiana tabacum]	−1.11	1.20	**2.97**	**3.46**	1.56
WMU3337	PAE(pectin acetylesterase) [Litchi chinensis]	−1.21	−1.61	**−2.39**	−1.23	−1.22
WMU4106	Pectate lyase [Prunus persica]	−1.30	1.10	**−2.11**	−1.67	−1.82
WMU453	Pectin acetylesterase, putative [Ricinus communis]	−1.21	−1.11	−1.52	**−2.20**	1.21

Table 3 (continued)

Unigene	Description GenBank hits	Fold change				
		12 h	1 day	3 day	5 day	8 day
WMU751	Pectin methylesterase [Nicotiana tabacum]	−1.09	−1.18	−1.21	**−2.05**	−1.30
Pathogenesis-related genes						
Cluster362contig1	Early nodulin, putative [Ricinus communis]	−1.22	1.76	**3.79**	1.04	**14.96**
07122002T_A_50_B07	Heat shock factor protein HSF30, putative [Ricinus communis]	1.71	**3.36**	1.67	1.48	1.18
08081903T1G_27_C04	Heat shock protein 70 [Gossypium hirsutum]	1.55	**3.73**	**4.16**	**2.68**	1.38
08052303_T_15_66_B09	Beta-1,3-glucanase [Citrus sinensis]	2.40	−1.67	−1.52	1.97	**19.35**
cluster302Contig1	Class V chitinase [Momordica charantia]	−1.49	−1.59	1.08	**9.13**	**23.41**
AI563545	Pathogen induced 4 protein [Cucumis sativus]	−1.28	−1.01	**2.26**	**3.30**	**3.55**
08052303_T_16_59_C08	RecName: Full=Pathogenesis-related protein P2; Flags: Precursor	1.37	1.17	1.18	1.84	**4.56**
DV737471	Pathogen-related protein 1 [Cucumis melo var. inodorus]	−1.10	1.04	1.02	1.11	**6.04**
WMU372	Protein disulfide isomerase (PDI)-like protein 3 [Cucumis melo]	−1.43	−1.70	**2.10**	**3.83**	**7.08**
Cluster69contig1	Putative alcohol dehydrogenases [Cucumis melo]	1.06	1.59	**2.29**	**−2.62**	**3.58**
WMU3841	Heavy-metal-associated domain-containing protein [Arabidopsis thaliana]	1.32	1.64	1.05	**2.36**	**2.31**
Cluster615contig1	Silverleaf whitefly-induced protein 3 [Cucurbita pepo]	−1.89	−1.43	−1.33	**3.93**	**5.38**
Oxidative burst						
08031801D_01_22_F03	Cytochrome oxydase subunit III [Homo sapiens]	1.67	**3.00**	−2.17	1.14	−1.25
WMU502	1-Cys peroxiredoxin [Fagopyrum esculentum]	1.10	**2.05**	1.02	1.22	−1.35
AA660098	Polyphenol oxidase [Populus trichocarpa]	1.01	**2.15**	1.06	1.17	1.36
08080507T1A_48_H06	Glutathione S-transferase [Cucurbita maxima]	1.56	**2.63**	**2.40**	**2.23**	1.31
WMU2700	Cytochrome P450 [Populus trichocarpa]	−2.86	−1.47	**4.47**	**3.43**	**3.81**
cluster477Contig1	Netting associated peroxidase [Cucumis melo]	1.29	1.02	1.62	**2.07**	**2.82**
Cluster37contig1	Peroxidase [Cucumis sativus]	1.21	−1.25	1.32	1.54	**2.27**
WMU101	Amine oxidase [copper-containing] precursor [Ricinus communis]	−1.28	−1.41	1.64	1.57	**2.06**
WMU3570	RecName: Full=Catalase isozyme 3	−1.67	−1.30	1.99	1.34	**2.11**
WMU2372	Protoporphyrinogen IX oxidase [Glycine max]	1.23	1.62	1.00	1.42	**2.55**
08080507T1A_58_B08	Cucumber peroxidase.; peroxidase; putative [Cucumis sativus]	1.21	−1.30	1.31	1.48	**2.47**
WMU2681	Ferredoxin-1, putative [Ricinus communis]	−1.39	−1.47	1.74	−1.43	**2.02**
Phenylpropanoid						
Cluster528contig1	3-deoxy-d-arabino-heptulosonate 7-phosphate synthetase [Populus trichocarpa]	−1.15	−1.28	−1.32	−1.49	**−3.11**
Cluster35contig1	Putative shikimate kinase [Fagus sylvatica]	1.38	−1.22	**3.15**	−1.64	**2.46**
AI563248	Phenylalanine ammonia-lyase, putative [Ricinus communis]	1.33	−1.41	**2.09**	−1.96	**2.35**
Cluster563contig1	4-coumarate:CoA ligase [Eucalyptus camaldulensis]	1.45	−1.28	**2.41**	−1.52	−1.15
Cluster325contig1	Cinnamate 4-hydroxylase CYP73 [Citrus sinensis]	1.25	−1.32	**2.03**	**2.68**	−1.45
08052303_T_16_64_H08	Cinnamoyl CoA reductase [Populus trichocarpa]	1.23	−1.19	**2.10**	−1.14	−1.64
WMU150	CCoAMT; caffeoyl-CoA O-methyltransferase [Arabidopsis thaliana]	1.11	1.22	1.25	1.87	**2.24**
Growth						
08081903T1N_78_F10	Phloem protein 2 [Malus × domestica]	1.22	1.59	**−2.06**	**−2.02**	**−3.27**
WMU16	Phloem filament protein [Cucurbita maxima]	−1.33	−1.21	−1.21	**−2.10**	−1.33
DV737894	Epicotyl-specific tissue protein [Striga asiatica]	−1.70	**−2.36**	−1.70	−1.43	−1.59
AA660064	Chlorophyll A/B binding protein, putative [Ricinus communis]	1.52	−1.37	**−3.27**	**−4.18**	1.01
08042807T_1_69_E09	Chloroplast NADP-malic enzyme precursor [Nicotiana tabacum]	1.73	1.65	**−3.23**	**−2.78**	−1.61
AI563073	Chloroplast photosystem II 10 kDa polypeptide [Jatropha curcas]	1.17	−1.21	**−2.20**	−1.06	1.30

Table 3 (continued)

Unigene	Description GenBank hits	Fold change				
		12 h	1 day	3 day	5 day	8 day
AA660032	Photosystem I reaction center subunit III [Vigna radiata]	1.11	1.33	**−2.06**	−1.11	−1.18
cluster356Contig1	Auxin-induced protein 5NG4, putative [Ricinus communis]	1.65	1.75	**−5.88**	**−2.13**	−1.05
Transportation						
WMU161	Aquaporin, MIP family, TIP subfamily [Populus trichocarpa]	1.26	**−2.04**	1.08	**−2.37**	−1.79
WMU3685	Sodium transporter hkt1-like protein [Populus trichocarpa]	1.08	**−6.62**	**2.82**	**−5.28**	**−7.81**
WMU2003	Oligopeptide transporter, putative [Ricinus communis]	−1.64	**−2.19**	1.21	**−3.27**	**−3.40**
WMU1324	Sulfate transporter, putative [Ricinus communis]	−1.45	**−2.26**	**−2.33**	**−2.74**	**−2.12**
08042807T_3_46_F06	ATP-binding cassette transporter, putative [Ricinus communis]	1.14	−1.64	**−2.14**	**−2.07**	**−2.65**
07122002T_A_95_G12	Early-responsive to dehydration protein-related [Arabidopsis thaliana]	1.01	1.16	**−2.40**	**−2.07**	−1.70
WMU2474	Metal transport protein [Medicago truncatula]	−1.21	−1.64	1.46	−1.49	**−4.06**

Significant difference (FDR<0.05 and fold change >= 2) in relative level is shown in bold

in incompatible and compatible inactions between watermelon and FON1 using qRT-PCR. 13S-lipoxygenase (LOX), allene oxide cyclase (AOC), and allene oxide synthase (AOS) and phospholipase (PL) are involved in different steps of the JA biosynthesis pathway (Fig. 3). Results from qRT-PCRs showed that expression of LOX, AOC, AOS and PL was significantly induced at 8 and 12 hpi after FON1 infection in incompatible interaction (Fig. 3). Although expression of these genes was also induced at 4 hpi in the compatible interaction, their expressions were not significantly affected in later stages (Fig. 3).

Expression of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), shikimate kinase (SK), 4-hydroxycinnamoyl-CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), caffeovl-CoA O-methyltransferase (CCOMT), cinnamoyl-CoA reductase(CCR), pheammonialyase(PAL) and peroxidase (POD), which act at different steps of the shikimate-phenylpropanoid-lignin biosynthesis pathway, was up-regulated at 3, 5 and 8 dpi in incompatible interactions, while their expression was repressed or not significantly affected in the compatible interaction at these stages (Fig. 4). Differential expression patterns of these genes between incompatible and compatible interactions suggested that the shikimate-phenylpropanoid-lignin biosynthesis pathway, probably through its metabolic products and cell wall modification, may play an important role in the incompatible interaction between watermelon and FON1.

Discussion

In the present study, we performed transcriptome profiling analysis of watermelon during its incompatible interactions with FON1 and our data generated from this analysis indicated that a complex molecular network, which involves functions of a large set of transcriptional factors, signalling genes, cell wall modification genes, and JA and shikimate-phenylpropanoid-lignin biosynthesis genes, is involved in watermelon responses to FON1 infection. Our study also identified a number of genes that are FON1 responsive and further functional analysis of these FON1-responsive genes will provide novel insights into the molecular mechanism of defence responses in watermelon against FON1 infection.

Transcriptional activation of gene expression, which is achieved through functions of different type of transcription factors, is one of the most important molecular events in plants in response to pathogen attack (Baldo et al. 2010). In our transcriptome profiling analysis, we identified a large number of genes encoding transcription factors belonging to AP2/ERF, WRKY and MYB families and found that their expression was significantly changed upon FON1 infection (Table 3). The ERF transcription factors have been demonstrated to play crucial roles in regulating plant responses to environmental stresses (Fujimoto et al. 2000) and overexpression of individual ERF genes in transgenic plants can confer enhanced disease resistance against different types of

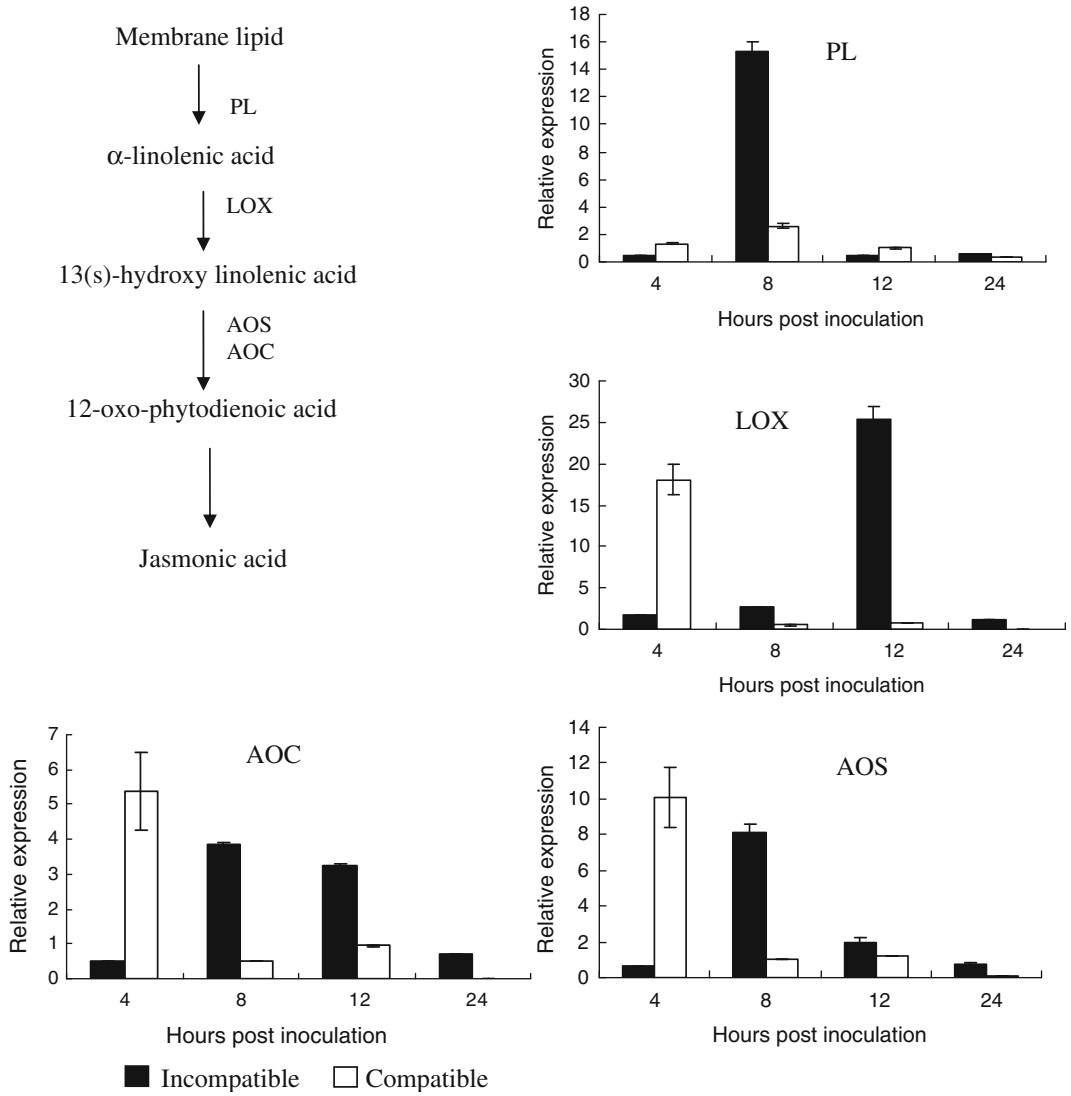

Fig. 3 Expression of JA biosynthesis genes in incompatible and compatible interactions between watermelon and FON1 by qRT-PCR. The JA biosynthetic pathway was adopted from Creelman and Mullet (1995) *PL* Phospholipase; *LOX* Lipoxygenase; *AOS* Allene oxide synthase; *AOC* Allene oxide cycle

pathogens (Berrocal-Lobo et al. 2002; Huang et al. 2004). The ERF transcription factors are known to be involved in ethylene signalling (Huang et al. 2004). In agreement with the differential expression of some of ERF genes, we also found in our study that FON1 infection also up-regulated a number of genes that are involved in ethylene biosynthesis such as 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) and 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Table 3). Gene expression analysis in cotton also implicated that ethylene was involved in the process of *Fusarium* wilt (Dowd et al. 2004). In this study, we observed that different MYB genes show diverse expression patterns in watermelon plants after FON1 infection. One MYB gene, WMU2748, is similar to the MYB gene from tobacco (*Nicotiana tabacum*) that is also induced by wounding and elicitors and known to positively regulate pathogenesis-related gene expression (Sugimoto et al. 2000). However, the other two homologues of MYB transcription factors were suppressed. These results suggested that different MYB transcription factors might play diverse functions upon FON1 infection. WRKY proteins were originally defined as

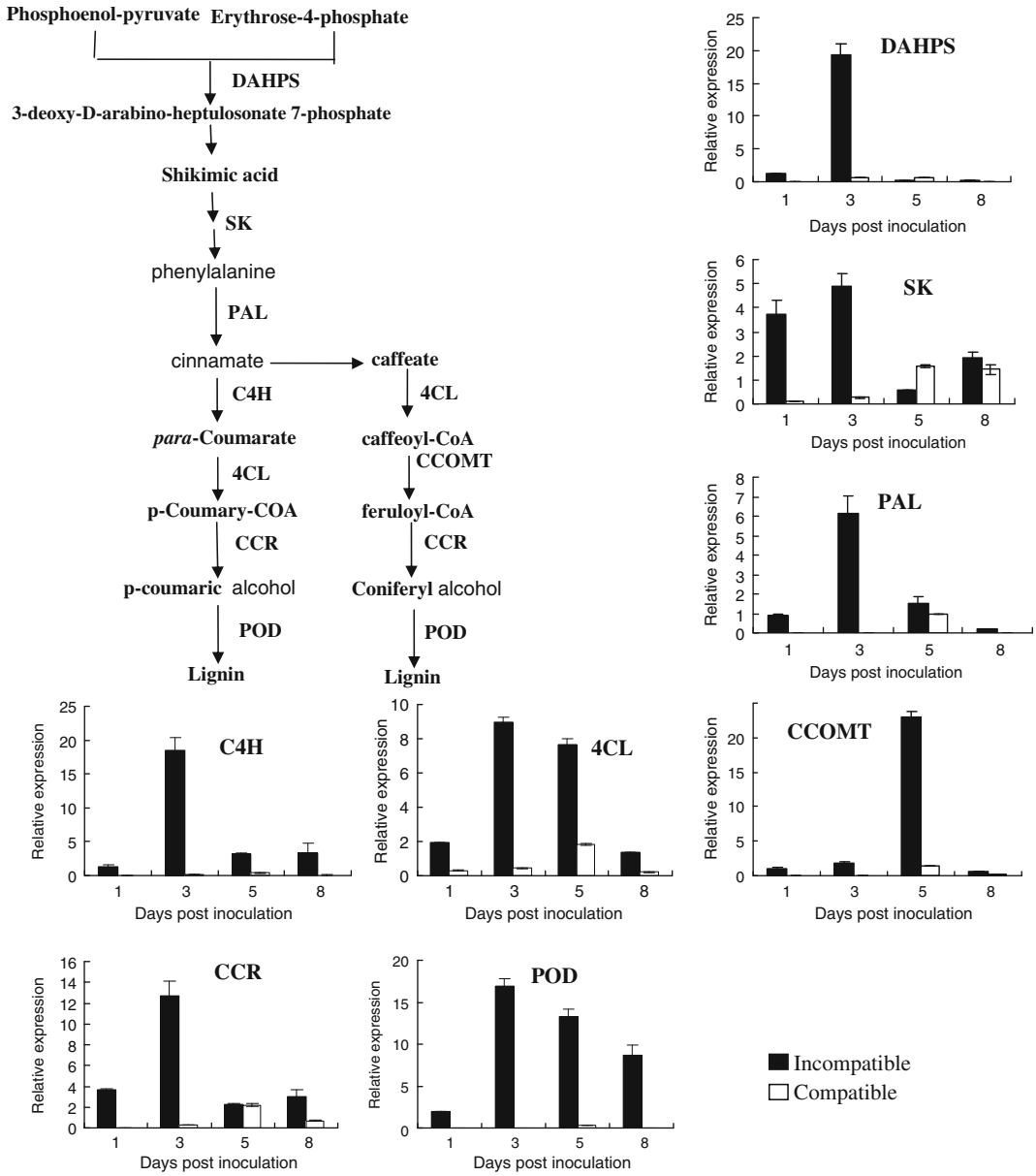

Fig. 4 Expression of shikimate-phenylpropanoid-lignin biosynthesis genes in incompatible and compatible interactions between watermelon and FON1 by qRT-PCR. The shikimate-phenylpropanoid-lignin biosynthetic pathway was adopted from Whetten and Sederoff (1995) *DAHPS* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; *SK* Shikimate kinase; *4CL* 4-hydroxycinnamoyl-CoA ligase; *C4H* Cinnamate 4-hydroxylase; *CCOMT* Caffeovl-CoA O-methyltransferase; *CCR* Cinnamoyl-CoA reductase; *PAL* Pheammonialyase; *POD* Peroxidase

transcription factors that target genes regulated by pathogen attack, fungal elicitors, and SA (Pandey and Somssich 2009). In our study, FON1 induced the expression of a WRKY transcription factor (08042807T_5_33_A05), further supporting that WRKY genes appear to be general regulators of plant defence in response to pathogens.

The cell wall is not only a physical barrier but also a defence barrier against pathogen penetration. In many cases of plant-pathogen interactions, transcriptional profiling studies have revealed the activation of genes that are involved in the biosynthesis and modification of cell wall components (Rinaldi et al. 2007; Stephens et al. 2008). Genes involved in cell

wall biosynthesis and modifications were also shown to be differentially expressed by FON1 (Table 3). These genes encode enzymes involved in biosynthesis of cell wall components, such as cellulose, pectin and hemicellulose. Plant hemicelluloses include xyloglucans, xylans, glucuronoarabinoxylan, glucomannans. Cellulose microfibrils are interconnected by hemicelluloses to form the network that is actively remodelled by plant enzymes (e.g. xyloglucan endotransglycosylase) during cell expansion (Vissenberg et al. 2000). In this study, the homologues of cellulose and hemicellulose related-genes were induced by FON1 infection. These polysaccharides are thought to act as a physical reinforcement at sites of infection. On the other hand, expression of genes in the shikimate-phenylpropanoid-lignin biosynthesis pathway was induced in the later stages of incompatible infection (3 dpi), but did not change or was repressed in the compatible interaction (Fig. 4). This is consistent with the previous report that lignin content of watermelon roots was apparently higher in incompatible than in compatible interactions between watermelon and FON (Xu et al. 2000). In the wheat–*Pst* pathosystem, the enzymes that participate in lignin biosynthesis induced at 24 hpi, and peaked at 48 hpi in the incompatible interaction (Wang et al. 2010). During *Medicago truncatula–Phymatotrichopsis omnivore*, a set of genes including *PAL, 4CL, CHS, CHR* was induced during both early (3 dpi) and later (5 dpi) stages of infection (Uppalapati et al. 2009). The biosynthesis of lignans was identified as potential new defence responses in cotton infected with *Fusarium oxysporum* f. sp. *vasinfectum* (Dowd et al. 2004). These results indicated that lignification appears to be also an active resistance mechanism in later (2–8 dpi) stages of host-pathogen interactions.

It is well known that ROS as a signal or oxidant is directly or indirectly involved in a wide range of biotic and abiotic stresses. A number of genes encoding enzymes involved in generation or homeostasis of ROS were activated in watermelon in response to FON1 infection, indicating a role for oxidative burst in the defence response of watermelon to FON. This is in agreement with previous observations that a number of oxidative stress-related genes were found to be differentially expressed in cotton and cucumber after the infection of wilt fungal pathogen (Dowd et al. 2004; Zhou and Wu 2009). ROS acts not only as a local signal for hypersensitive cell death but also as a second messenger for the induction of defence genes. Wang et al. (2010) reported that expression of two genes involved in ROS generation was down-regulated in the compatible interaction between wheat and stripe rust fungus. Differential expression of genes related to ROS generation in FON1-infected watermelon roots indicated that ROS may also be involved in the incompatible interaction between watermelon and FON1. However, this hypothesis needs to be further examined through measurement of ROS changes in watermelon roots after infection of FON.

Biosynthesis and signalling of JA play important roles in plant response to pathogen attack and herbivore feeding (Wasternack 2007). In our study, most of the JA biosynthetic genes were induced during the early stage of FON1 infection. This is consistent with previous reports that biotic and abiotic stresses, such as pathogen infection, wounding and insect feeding, can trigger the JA biosynthesis through direct activation of genes encoding the relevant biosynthetic enzymes (Creelman and Mullet 1995). The JA biosynthetic genes might be induced in both susceptible and resistant interactions, but the timing and levels of their induced expression differ between incompatible and compatible interactions. In the resistant watermelon lines, most of the JA biosynthetic genes were induced strongly and the expression levels were maintained longer as compared with those in susceptible plants (Fig. 3). In *Arabidopsis*, it has been reported that JA- and ethylene-mediated defence responses are implicated in defence against necrotrophic fungal pathogens, including *F. oxysporum* (Glazebrook 2001). During *Medicago truncatula–Phymatotrichopsis omnivore*, a set of genes including *AOC2, OPR3, OPR5,OPR12* were induced in response to initial entry of the fungal hyphae as early as 3 dpi (Uppalapati et al. 2009). Our results show the timing of induced expression was the early (4–12 hpi) stages of infection. These results suggest a role for JA in defence response of host-pathogen interaction.

Because most of the molecular mechanisms involved in the incompatible interactions between watermelon and FON1 are yet to be determined, the large number of genes identified in this study will serve as candidates for further studies to determine their functions and dissect the molecular networks involved in the plant-pathogen interactions.

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