Analysis of differentially expressed genes in Curcuma amada and Zingiber officinale upon infection with Ralstonia solanacearum by suppression subtractive hybridization

Article *in* Acta Physiologiae Plantarum · December 2013

DOI: 10.1007/s11738-013-1362-2

6 authors, including:

D. Prasath
Indian Institute of Spices Research
57 PUBLICATIONS **107** CITATIONS
SEE PROFILE

T. P. Prameela
Indian Institute of Spices Research
8 PUBLICATIONS **21** CITATIONS
SEE PROFILE

Manonmani Anandaraj
SAVEETHA ENGINEERING COLLEGE
70 PUBLICATIONS **288** CITATIONS
SEE PROFILE

Acta Physiol Plant (2013) 35:3293–3301
DOI 10.1007/s11738-013-1362-2

ORIGINAL PAPER

Analysis of differentially expressed genes in *Curcuma amada* and *Zingiber officinale* upon infection with *Ralstonia solanacearum* by suppression subtractive hybridization

D. Prasath · E. J. Suraby · R. Karthika · O. B. Rosana · T. P. Prameela · M. Anandaraj

Received: 9 June 2013 / Revised: 14 August 2013 / Accepted: 27 August 2013 / Published online: 11 September 2013

Abstract Bacterial wilt, one of the major diseases in ginger is caused by *Ralstonia solanacearum* and lack of resistant genotype adds constraints to the crop improvement programs and for the management of bacterial wilt. *Curcuma amada* is a potential donor for bacterial wilt resistance to *Zingiber officinale*, if the exact mechanism of resistance is understood. In this study, a PCR-based suppression subtractive hybridization (SSH) was used to identify *C. amada* genes that are differentially and early expressed in response to the *R. solanacearum* infection compared to *Z. officinale*. A forward subtracted library of differentially expressed genes was synthesized by cloning DNA fragments (300–1,200 bp) from subtracted sample and sequenced. Upon computational analysis of the 150 SSH clones sequenced, 119 produced suitable sequences of high homology with the genes of known functions. These were classified into three potential functional categories: proteins involved in biological processes (31 %), cellular component (43 %) and molecular function (26 %). Among these differentially expressed genes, sequences coding for putative proteins related to defense response (high homology between 85 and 100 % identity) were, cell wall-associated hydrolase, xyloglucan transglycosylase (XTG), cytochrome P 450, metallocarboxypeptidase inhibitor, peroxiredoxin, nitrilase-associated protein, leucine-rich protein (LRR) and glutathione-*S*-transferase (GST). The three transcripts were discriminative: expression of LRR, GST and XTG was much higher in resistant species (*C. amada*) than in susceptible species (*Z. officinale*) at every time point. The isolated ESTs reported here contribute to improve our understanding about this plant–pathogen interaction.

Keywords Ginger · *Curcuma amada* · Bacterial wilt · Suppression subtractive hybridization (SSH) · Differentially expressed genes

Introduction

Ginger (*Zingiber officinale* Roscoe) is an important spice crop belonging to the family *Zingiberaceae* which comprises 1,500 species all over the world. The dried underground stem of ginger is used as spice as well as condiment. Bacterial wilt caused by *Ralstonia solanacearum* and rhizome rot caused by several species of oomycetes pathogen, *Pythium,* incur serious economic loss in ginger producing countries. Apart from its natural incidence, the closely related Zingiberaceous members, *Curcuma amada*, *C. zedoria*, *C. longa*, *C. aromatica*, *Kaempferia galanga*, *Elettaria cardamomum* and *Zingiber zerumbet* were evaluated for resistance to *Ralstonia solanacearum* (Biovar3) and *Pythium* species (Kumar and Hayward 2005; Kumar et al. 2006). *C. amada*, the Indian mango ginger was found to be resistant to both pathogens and *Z. zerumbet*, a wild relative of cultivated ginger was found to be resistant to *P. aphanidermatum* (Kavitha and Thomas 2008). Recent studies revealed a strong expression of PR5 genes upon *R. solanacearum* inoculation in *C. amada* as compared to *Z. officinale* in which the expression is weak due to the presence of silencing element binding factor (Prasath et al. 2011). It is important to understand the network of genes that governs the resistance

Communicated by E. Kuzniak-Gebarowska.

D. Prasath (✉) · E. J. Suraby · R. Karthika · O. B. Rosana · T. P. Prameela · M. Anandaraj
Indian Institute of Spices Research, Indian Council of Agricultural Research, Kozhikode 673 012, Kerala, India
e-mail: prasath@spices.res.in

to *R. solanacearum* in *C. amada* as compared to *Z. officinale* to engineer ginger for bacterial wilt resistance.

Resistance responses to plant pathogens are the focus of intensive research, because current technologies offer the possibility of genetic engineering in the plants for broad-based effective resistance against pathogen (Veronese et al. 2003). Resistance responses can be divided into a series of interrelated stages (Veronese et al. 2003; Glazebrook 2001; Dangle and Jones 2001). The identification of genes expressed during compatible and incompatible plant–pathogen interactions has contributed to understanding molecular mechanisms involved in plant response, and defense process (Fekete et al. 2009; Nascimento et al. 2009; Mukherjee et al. 2009; Schlink 2010); furthermore, such genes have been employed successfully in the production of transgenic plants (Latha et al. 2005; Zuo et al. 2007).

Subtractive suppression hybridization (SSH) is a powerful tool for gene expression profiling that effectively identifies genes differentially expressed under different conditions or in different tissues (Diatchenko et al. 1996). This method is relatively simple and easy, it can be used with species for which there is little or no genomic information, and the cDNAs isolated are typically longer than 100 bp and can be effectively annotated using comparative genomics (e.g., BLAST analysis). This method is often used to isolate plant genes specifically expressed in response to pathogen infection and to identify differences in the transcript profiles of infected resistant and susceptible plants (Ros et al. 2004; Aslam et al. 2010; Fan et al. 2010; Gor et al. 2010; de Souza et al. 2011; Hirao et al. 2012). SSH selection reduces the cloning of abundantly expressed housekeeping genes or genes commonly expressed in both "tester" and "driver" samples, and, therefore, normalizes the expressed cDNA profiles during library construction. As a result, SSH selection significantly enhances the chances of cloning differentially expressed genes. A successfully combined SSH and cDNA microarray technology has been proved to be a powerful tool to develop a high throughput screening procedure in identifying differentially expressed genes and their expression profilings (Tian et al. 2003; Yang et al. 1999). We report here, by employing the SSH strategy, the genes associated with bacterial wilt resistance of *C. amada* at early stage and speculation of possible mechanisms implied by the transcription profiling of selected genes.

Materials and methods

Plant material and pathogen inoculation

Forty-five to 60-day-old *C. amada* and *Z. officinale* (IISR Rejatha) plants maintained in the nethouse of Indian Institute of Spices Research, Kozhikode were used for the experiment. The virulent colonies of *R. Solanacearum* (GRsMnt2), cultured on Casamino acid-Peptone-Glucose (CPG) medium (Kelman 1954), were multiplied in sucrose peptone broth (sucrose, 20 gl^{-1}; peptone, 10 gl^{-1}; K_2HPO_4, 0.5 gl^{-1}; $MgSO_4$, 0.25 gl^{-1}; pH 7.2) for 2 days. The bacteria was pelleted at 10,000 g for 20 min at 4 °C, re-suspended in water and poured around the base of the 45-day-old plants, as water suspension at a concentration of 10^9 cells ml^{-1} of water. The inoculated plants were monitored for occurrence of bacterial wilt. All the ginger plants wilted on seventh day after inoculation whereas no wilting symptoms on *C. amada* were found. (Fig. 1).

RNA isolation

Total RNA was isolated from *C. amada* and *Z. officinale* at 1, 4, 8, 16, 24, 48 and 126 h after inoculation (hai) with *R. solanacearum* and just before inoculation (0 hai) using TRI reagent (Sigma). Poly (A) RNA was separated from total RNA with Poly A purification Kit (MN, Germany) according to manufacturer's instructions.

SSH and construction of subtracted cDNA library

RNA samples derived from plants after 4 and 8 hai were pooled and used to understand the early genes involved in bacterial wilt resistance in *C. amada* by subtractive hybridization. Suppression subtractive hybridization and subtracted cDNA library construction were performed using

Fig. 1 The *C. amada* (**a**) and *Z. officinale* (**b**) plants non-inoculated and inoculated with *R. solanacearum* at 6 days after inoculation (dai) and 10 dai

the PCR-Select cDNA subtraction kit following the guidelines of the manufacturer (Clontech Laboratories, USA). Double stranded cDNA was produced from approximately 2 μg of poly (A+) RNA. The cDNA tester (*C. amada*) was synthesized from a sample created by combining equimolar poly (A) RNA from inoculated plants at 4 and 8 hai, while to synthesize the cDNA driver, we used a sample created by combining equimolar poly (A+) RNA from *Zingiber officinale* plants at 4 and 8 hai. After digestion with RsaI, the tester cDNA preparation was divided into two subpopulations, which were ligated to different adaptors and were then hybridized with an excess amount of driver cDNA, after which they were combined and hybridized again in the presence of driver cDNA, without denaturing the DNA before the second hybridization. The driver was not adaptor-ligated. Following the second hybridization, two rounds of PCR were performed that could amplify only those fragments which had two different adaptors to enrich and amplify the differentially expressed sequences.

A complete control subtraction was performed in parallel with the experimental subtraction using skeletal muscle cDNA provided as driver. The skeletal muscle tester cDNA was prepared by mixing control skeletal muscle cDNA with diluted *f*X 174/*Hae*III control DNA provided in the kit. After the preferential exponential amplification of differentially expressed sequences in the tester sample, the subtracted products were checked on a 1 % agarose/EtBr gel. The subtracted cDNAs were introduced into pGEM-T vector (Promega, USA) according to the manufacturer's instructions and inserted into chemocompetent *Escherichia coli* DH5α cells (Invitrogen, USA). Randomly selected recombinant white clones were checked for the presence of insert fragments by performing PCR amplifications using subtraction-specific adaptor primers (provided in the kit) and selected colonies are sequenced.

Sequencing and basic local alignment search tool (BLAST) analysis

Three hundred white positive clones from subtractive cDNA library were selected for sequencing. The vector and adaptor sequences were subsequently removed to obtain the expressed sequence tags (ESTs). ESTs were compared with database nucleotide collection (nr/nt) using blastx and blastn (http://www.ncbi.nih.gov).

Quantitative real-time PCR (qPCR) gene expression analysis

Three genes, LRR, GST and XTG with different transcript abundances and functions were selected for qRT-PCR, and their transcript levels were compared between resistant (*C. amada*) and susceptible (*Z. officinale*) upon inoculation with *R. solanacearum*. The housekeeping gene actin was used as an endogenous reference for relative quantification.

DNase-treated RNA (5 μg) was reverse transcribed in a total volume of 50 μl using SuperScript III Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). Real-time quantitative PCR was performed using 10 ng of total RNA in a 20 μl reaction volume with SYBR GREEN PCR MasterMix (Qiagen, Mississauga, ON, Canada) on a M×4000® multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA). M×4000® v 4.20 software (Stratagene, La Jolla, CA, USA) was used to design gene-specific primers (Table 1). For all the genes studied here, the optimal primer concentration was 200 nM. RT-PCR conditions were as follow: 95 °C (15 min), then 45 cycles of 95 °C (15 s) and 60 °C (30 s). The products were further analysed by a dissociation curve program at 95–60 °C (16 s). All RT-PCR experiments, for each gene, were run in triplicate with different cDNAs synthesized from three biological replicates. Each sample was run in three technical replicates on a 96-well plate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the β-actin as an internal standard. To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔCt was calculated for three genes, and β-actin genes, and was determined to be <0.1. To determine relative fold differences for each sample in each experiment, the Ct value for the four studied genes was normalized to the Ct value for β-actin and was calculated relative to a calibrator using the formula $2^{\Delta\Delta Ct}$.

Table 1 The real-time quantitative PCR primers used for expression analysis

Primer name	Sequence 5′–3′
Actin	
Forward (F)	TAGGTGCCCAGAGGTTCTATT
Reverse (R)	ACCGCTAAGCACCACATTAC
LRR	
Forward (F)	CAGCAGTTCTACCTCTGGTTC
Reverse (R)	CGTCCCGTCCACATAAATCA
GST	
Forward (F)	TGCCGTCTTTGGAGCATAAC
Reverse (R)	CGGGATCATCAGGCAGTAATTT
XTG	
Forward (F)	CAGCAGTTCTACCTCTGGTTC
Reverse (R)	CGTCCCGTCCACATAAATCA

The goal of this study was to identify differences in the transcript profiles of mango ginger and ginger-inoculated *R. solanacearum* to understand the difference in the defense responses of resistant and susceptible individuals. Three important experimental design elements enhanced the clarity and relevance of our findings: (1) We used the most resistant species of *Zingiberaceae* C. *amada*. (2) We sampled inoculated plants (both resistant and susceptible) (3) We used SSH, a powerful approach for identifying differentially expressed genes regardless of their abundance.

Results

Isolation of differentially expressed ESTs by SSH

A subtractive library constructed from pooled samples taken at two time points, 4 and 8 h hai, was used to identify genes that are differentially expressed in resistant and susceptible species as disease symptoms develop following *R. solanacearum* inoculation to understand the early genes involved in bacterial wilt resistance in *C. amada*. The subtracted products on gel analysis appeared as a smear ranging in size from 300 bp to 1.2 kb with 2–4 definite bands clearly distinguishing them from the un-subtracted sample control. The subtracted skeletal muscle sample showed DNA fragments corresponding to the *f*X174/*Hae*III digest. The control-subtracted sample provided in the kit gave an identical pattern, which confirms the efficiency of the control subtraction experiment. A forward SSH library containing transcripts differentially expressed in *C. amada* infected by *R. solanacearum* was constructed (350 colonies) using RNA from *C. amada* as a tester and RNA from *Z. officinale* as driver. Randomly selected subtracted clones were PCR-amplified using the adaptor primer pair of the subtraction. We sequenced nucleotides from the 5′-end of 150 cDNA inserts that were recovered in the library (Table 2). Insert length varied from 300 to 1,200 bp.

Functional classification of the expressed genes in SSH library and identification of differentially accumulated genes

To annotate the putative gene and functional Gene Ontology (GO) categories for the transcripts of the SSH library, we compared non-redundant ESTs of each SSH library with the GenBank non-redundant and EST databases using the search programs blastx and blastn using the Blast2GO program to understand comprehensive overview of *C. amada* subtracted transcriptome. The 137 SSH sequences showed high homology with the genes whose functions were known and they were classified into seven functional groups based on gene ontology terms (Fig. 2). The transcripts related to photosynthesis category, such as photosystem II cytochrome b559, photosystem II cp47 protein, photosystem II 32 kDa protein and mitochondrion, were present at a higher percentage (accounting for 28 %) in the subtracted library. This was followed by a "defense/stress" functions category (20 %). This group included genes showing a homology to leucine-rich repeats (LRR), glutathione-*S*-transferase (GST), cell wall-associated hydrolase (CWH), xyloglucan transglycosylase/hydrolase (XTG), cytochrome P 450, metallocarboxypeptidase inhibitor and peroxiredoxin (Prx). Other categories included functions involving cell wall-associated hydrolases (15 %), hypothetical proteins (13 %), senescence-associated protein (4 %), metabolism (3 %), and others (17 %) in the library. Putative functions of all ESTs were annotated based on the highest scoring matches by BLAST in NCBI.

Clones containing differentially expressed cDNAs of *C. amada* were sequenced using M13 forward and reverse primers. Of the 150 clones, sequenced, 137 produced suitable sequences and 13 had no identity with sequences in the databases. To evaluate the specificity and diversity of the transcripts that were specific in a time course, ESTs were selected from the SSH library. Among these differentially expressed genes, sequences coding for putative proteins related to defense response during *C. amada—R. solanacearum* interaction (high homology between 85 and 100 % identity) were, Leucine-Rich Repeats (LRR), glutathione-*S*-transferase (GST), cell wall-associated hydrolase, Xyloglucan transglycosylase (XTG), cytochrome P 450, metallocarboxypeptidase inhibitor and peroxiredoxin.

Quantitative real-time PCR (qPCR) analysis of genes potentially related to defense response processes

qPCR assays were performed to evaluate the expression pattern of three genes potentially related to defense response process in *C. amada—R. solanacearum* interaction. The expression of three ESTs recovered (length >400 bp) was assayed using qPCR; samples for the qPCR analysis were collected from bacterial wilt resistant *C. amada* and susceptible *Z. officinale* at 0, 8, 16, 24, 48 and 96 hai. The results of the qPCR analysis and SSH were consistent. It indicated that, three ESTs were differentially expressed (Fig. 3), expression levels of LRR gene showed peak expression at 8 hai and then down-regulated in *C. amada*, however, its response postponed at 16 hai in *Z. officinale*, and showed further down regulation (Fig. 3a). Expression of GST was up-regulated and reached a peak at 8 hai in *C. amada* and maintained higher expression up to 96 hai, however, in contrast, GST activity decreased dramatically in *Z. officinale,* except 48 hai (Fig. 3b). Levels of XTG gene expression showed significant changes in *C. amada* after inoculation with *R. solanacearum* at 8 hai

Table 2 Differentially expressed cDNAs in *C. amada* infected by *R. solanacearum*

Accession number	GenBank hit	Best match in database	*E*-value	Sequence identity (%)
Pathogenesis related proteins/plant defense				
A13	ABH09321.1	Leucine-rich protein [*Arachis hypogaea*]	8E−12	65
A98	ACQ85266.1	Xyloglucan transglucosylase/hydrolase [*Musa accuminate* AAA Group]	3E−29	78
A39	ACA58349.1	Putative nitrilase-associated protein [*Sandersonia aurantiaca*]	4E−11	100
A102	XP_003616487.1	Metallocarboxypeptidase inhibitor [*Medicago truncatula]*	2e−30	94
A30	XP_003616487.1	Metallocarboxypeptidase inhibitor [*Medicago truncatula]*	6e−31	92
A16	XP_003616487.1	Metallocarboxypeptidase inhibitor [*Medicago truncatula]*	5E−50	77
SSHCa35	XP_003616487.1	Metallocarboxypeptidase inhibitor [*Medicago truncatula]*	4E−52	73
A132	ACF06618.1	Meloidogyne-induced giant cell protein-like protein [*Elaeis guineensis*]	2E−67	72
A21	XP_002326305.1	Peroxiredoxin [*Populus trichocarpa*]	1E−30	80
A1	XP_003614400.1	Cytochrome P450 likeTBP [*Medicago truncatula*]	6.00E−20	80
SSHCa26	XP_003614380.1	Cytochrome P450 likeTBP [*Medicago truncatula*]	1.00E−23	81
A4	AAA34096.1	Plasma membrane H+ ATPase, partial [*Nicotiana plumbaginifolia*]	5.00E−84	97
Stress related proteins				
SSHCa1	ACF06541.1	Glutathione -s-transferase [*Elaeis guineensis*]	3E−56	76
Senescense-related proteins				
A14	BAB33421.1	Putative senescence-associated protein [*Pisum sativum*]	8E−39	77
A18	XP_003616487.1	Putative senescence-associated protein [*Cupressus sempervirens*]	6E−53	92
SSHCa4	ACA30301.1	Senescense associated protein [*Cupressus sempervirens*]	2E−55	92
Metabolism				
A3	XP_002278829.1	Triose phosphate/phosphate translocator TPT, chloroplastic [*Vitis vinifera*]	4.00E−79	95
SSHCa7	XP_003612185.1	Ferredoxin-NADP reductase [*Medicago truncatula*]	4.00E−36	86
SSHCa50	ABS82569.1	ATPase subunit III [*Olea europaea*]	4.00E−11	100
SSHCa20	BAD94694.1	Fructose bisphosphate aldolase like protein [*Arabidopsis thaliana*]	0.031	80
Cell wall-associated hydrolase				
A164	XP_003637074.1	Cell wall-associated hydrolase, partial [*Medicago truncatula*]	2.00E−33	96
A152	XP_003637074.1	Cell wall-associated hydrolase, partial [*Medicago truncatula*]	2.00E−61	90
A9	XP_003637074.1	Cell wall-associated hydrolase, partial [*Medicago truncatula*]	9.00E−44	97
A2	XP_003637074.1	Cell wall-associated hydrolase, partial [*Medicago truncatula*]	2.00E−91	96
A8	XP_003614400.1	Cell wall-associated hydrolase, partial [*Medicago truncatula*]	3E−22	96

and then down-regulated. In contrast, the activity of the XTG in *Z. officinale* peaked at 8 hai and then down-regulated (Fig. 3c).

Discussion

The present study constitutes the first genome wide effort to understand the molecular basis of a host—*R. solanacearum* interaction in *C. amada* compared to *Z. officinale*. Suppression subtractive cDNA libraries from two species (resistant and susceptible) from leaf tissues at the early infection stage were constructed to identify spatial and temporal transcriptional changes resulting from *R. solanacearum* infection. Because a whole ginger genome sequence has not yet been completed, ESTs could serve as an efficient alternative approach to the discovery of novel genomic information. SSH library synthesized in this work generated 137 SSH sequences and in silico analysis revealed that some of the SSH genes isolated show high identity with proteins playing known functional roles in plant defense response against pathogen attacks.

Understanding how plant morphological and developmental remodeling and pathogen cell wall targeted virulence influence infections provides new perspectives about plant–pathogen interactions (Cantu et al. 2008). *R. solanacearum* recognition by *C. amada* is expected to be the first event after pathogen contact and can involve the participation of receptor molecules, such as the putative leucine-rich repeat protein isolated here. Most plant disease resistance (R) proteins contain a series of leucine-rich repeats (LRRs), a nucleotide-binding site (NBS), and a putative

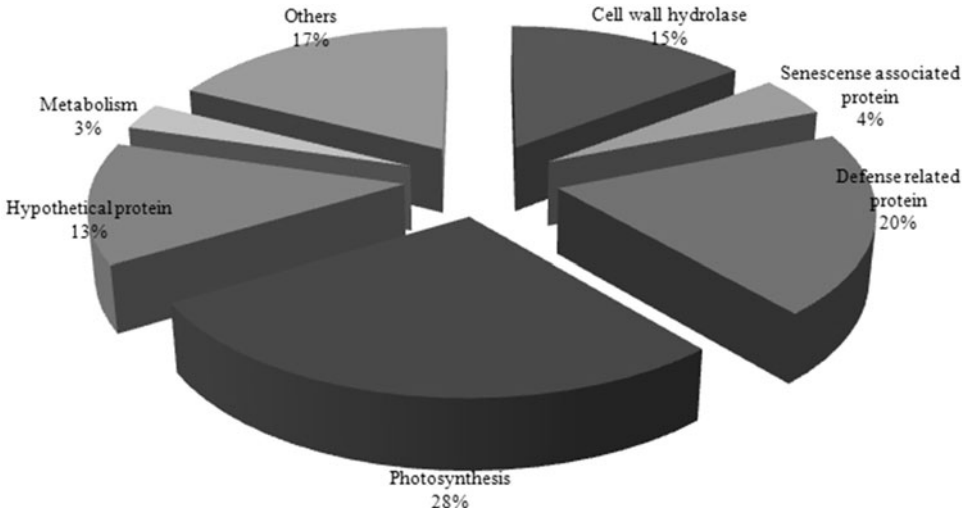

Fig. 2 *Pie charts* showing functional categories of 137 ESTs from SSH library in *C. amada* in response to *R. solanacearum*

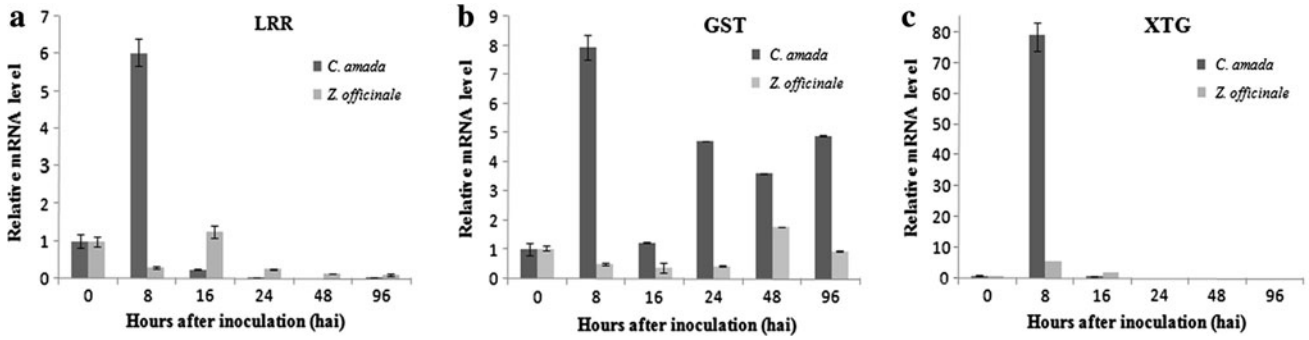

Fig. 3 Quantitative real-time PCR of transcripts differentially expressed in resistant and susceptible species following *R. solanacearum* inoculation. The putative functional genes from **a** to **c** were clearly discernible ESTs in resistant SSH libraries

amino-terminal signaling domain. Genetically, the LRRs of plant R proteins are determinants of response specificity, and their action can lead to plant cell death in the form of the familiar hypersensitive response (Belkhadir et al. 2004). Therefore, based on our results, it is possible that LRR sequences participate in recognition and signal transduction processes, leading to the activation of defense mechanisms. Resistance and susceptibility do not depend not only upon qualitative differences in the activated defense genes, but also on differences in the timing and magnitude of their expression (Thompson and Goggin 2006).

Plant cell walls are made of cellulose and other polysaccharide, which are long molecules of sugars. Some bacteria and plants, such as rice, cabbage and mustard, produce glycoside hydrolases and polysaccharide lyases. When produced by bacteria, these enzymes degrade the cellulose molecule in the cell wall, causing plant diseases. However, plant-produced glycoside hydrolases are essential in cell wall polysaccharide metabolism. Among 137 sequences, we found at least twenty cell wall-associated hydrolases (CWHs) sequences potentially involved in cell wall polysaccharide metabolism. CWHs are less prevalent among plant glycoside hydrolases that cleave structural polysaccharides; however, several CBM49 and CBM22 modules are present in plant cellulases and xylanases, respectively. The CBM49 modules are located in a subfamily of GH9 plant endoglucanases, and one of these modules was shown to bind tightly to crystalline cellulose (Urbanowicz et al. 2007). It is possible that the GH9 CBM49-containing endoglucanases play a role in modulating the structure of crystalline cellulose. Before encountering the intracellular defense, a pathogen has to face the cell wall and upon attack, various defense-related polymers are synthesized to reinforce the cell wall at the sites, of pathogen entry. One of the *C. amada* transcripts isolated in this study has shown similarity to xyloglucan transglycosylase (XTG) which is involved in the rearrangement of the cellulose/xyloglucan architecture in the cell wall (Nishitani 1998; Sharmin et al. 2012). In conclusion, it is possible that both the putative CWHs and XTG enzymes can play important roles in avoiding *C. amada* cellular disintegration during infection by *R. solanacearum*.

Invading pathogenic bacteria may produce stress-inducing chemicals, such as phytotoxins, resulting in

significant stress and damage to the host cells. Glutathione-*S*-transferase (GST) participates not only in detoxifying oxidative-stress metabolites, but also it is known to be induced by infection or by treatments that invoke plant defense reactions or by osmotic stress. The expression of glutathione-*S*-transferase has been detected in incompatible interactions (Hammond-Kosack and Jones 1996). Dean et al. (2005) showed induction of two glutathione-*S*-transferase genes in tobacco upon infection by *Colletotrichum destructivum* and *Colletotrichum orbiculare* and found statistically significant increase in susceptibility of tobacco to *C. orbiculare* infection when one of the GST genes was silenced by virus-induced silencing. Seeming induction of a glutathione-*S*-transferase homolog in *C. amada* SSH library is reasonable because previous studies associated induction of this gene with plant disease resistance. The gene that is represented by the clone SSHCa1 may have been induced due to the resistance response in the tester plants, which were resistant.

One of the transcripts (A39) has 100 % similarity to putative nitrilase-like protein which is reported to interact with GCC Box DNA-binding proteins involved in ethylene and defense responses to regulate PR gene expression (Xu et al. 1998). The detoxification of ROS occurs in various cellular compartments, such as chloroplasts, mitochondria, peroxisomes and cytosol and the enzymes like ascorbate peroxidase, catalase, glutathione peroxidase and superoxide dismutase are prominent antioxidant enzymes (Noctor and Foyer 1998). The peroxiredoxins (Prx) are reported as new components in the antioxidant defense network of barley (Stacy et al. 1996). The subtracted transcriptome of *C. amada* also shows similarity to peroxiredoxins. We also isolated genes related to defense response, including putative cytochrome P 450 which has been clearly related to plant defense response against pathogens in compatible and incompatible interactions (Luo et al. 1990; Oh et al. 1999; Kim et al. 2006; Ghose et al. 2008).

H+-AtPases can be functional in either disease or resistance. Since pH of compartments is critical in signaling events, H+ transporting ATPases may be regulated to adjust compartment pH to direct signaling events. The induced transcripts in this case may belong to genes that function in regulating pH of cytoplasm or other compartments to direct necessary defense signaling pathways. Fusicoccin, the major phytotoxin from peach and almond pathogen *Fusicoccum amygdale*, also stimulates H+-ATPase (Yang et al. 1997). One of the transcripts of *C. amada* subtracted transcriptome library, A4 has 95 % identity to auto inhibited H+-ATP ases of Populus × canadensis.

Our qPCR results showing the up-regulation of putative LRR, GST and XTG genes at 8 hai indicate that the *C. amada* defense response was activated during infection by *R. solanacearum*. Thus, the very early and robust accumulation of *C. amada* transcripts further indicates a crucial role for these genes in the *R. solanacearum* resistance machinery in *C. amada*.

Since many studies have reported the expression of genes involved in plant defense in the early stages of infection as a crucial step for an effective mechanism of resistance to diseases, the identification of such genes induced at a late stage of infection can help to explain the success of *R. solanacearum* in causing the bacterial wilt in ginger and its resistance mechanism in *C. amada*.

Using the SSH approach, we have also come across several unknown/hypothetical genes. Either no homology was found for these 18 genes using the NCBI database or, despite homology to other genes, their function is still unclear.

Conclusion

In this study, the first large-scale screening was performed for genes preferentially expressed in *C. amada* challenge inoculated by *R. solanacearum*. Some of those genes were found up-regulated in *C. amada* and may play important role in tolerating to *R. solanacearum*. This study will undoubtedly shed light on defense signaling pathways in *C. amada* and identifying genes in disease resistance pathway. This is the first report of analysis of differentially expressed transcripts in ginger by SSH technique. The ESTs identified in this study should provide a useful genomic resource for biologists and plant breeders in developing new strategies for improving *R. solanacearum*-resistant ginger cultivar.

Author contribution Conceived and designed the experiments: DP, EJS, MA. Performed the experiments: DP, EJS, RK, TPP. Analyzed the data: EJS, OBR. Wrote the paper: DP, EJS.

Acknowledgments Funding from Indian Council of Agricultural Research (ICAR) as ORP (PhytoFuRa) is gratefully acknowledged. Authors are grateful to Director, IISR; Head, Crop Improvement and Biotechnology, IISR; DISC, IISR; Head, Crop Protection, SBI, Coimbatore for technical help and support.

References

Aslam M, Sinha VB, Singh RK, Anandhan S, Pande V, Ahmed Z (2010) Isolation of cold stress-responsive genes from *Lepidium latifolium* by suppressive subtraction hybridization. Acta Physiol Plant 32:205–210

Belkhadir Y, Subramaniam R, Dangl JL (2004) Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr Opin Plant Biol 7(4):391–399

Cantu D, Vicente AR, Labavitch JM, Bennett AB, Powell ALT (2008) Strangers in the matrix: plant cell walls and pathogen susceptibility. Tren Plant Sci 13:610–617

Dangle JL, Jones JDG (2001) Plant pathogens and integrated defence response to infection. Nature 411:826–833

de Souza CRB, Brı′gida ABS, Santos RCD, de Nazare′ Monteiro Costa C, Darnet SH, Harada ML (2011) Identification of sequences expressed during compatible black pepper—*Fusarium solani* f. sp. *piperis* interaction. Acta Physiol Plant 33:2553–2560

Dean JD, Goodwin PH, Hsiang T (2005) Induction of glutathione-*S*-transferase genes of *Nicotiana benthemiana* following infection by *Colletotrichum destructivum* and *C. orbiculare* and involvement of one in resistance. J Exp Bot 56:1525–1533

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025–6030

Fan F, Li X-W, Wu Y-M, Xia Z-S, Li J–J, Zhu W, Liu J-X (2010) Differential expression of expressed sequence tags in alfalfa roots under aluminum stress. Acta Physiol Plant 33(2):539–546

Fekete C, Fung RWM, Szabo′d Z, Qiu W, Chang L, Schachtman DP, Kova′cs LG (2009) Up-regulated transcripts in a compatible powdery mildew–grapevine interaction. Plant Physiol Biochem 47:732–738

Ghose K, Dey S, Barton H, Loake GJ, Basu D (2008) Differential profiling of selected defence-related genes induced on challenge with *Alternaria brassiciola* in resistant white mustard and their comparative expression pattern in susceptible India mustard. Mol Plant Pathol 9:763–775

Glazebrook J (2001) Genes controlling expression of defense responses in *Arabidopsis*-2001 status. Curr Opin Plant Biol 4:301–308

Gor MC, Ismail I, Mustapha WAW, Zainal Z, Noor NM, Othman R, Hussein ZAM (2010) Identification of cDNAs for jasmonic acid responsive genes in polygonum minus roots by suppression subtractive hybridization. Acta Physiol Plant 33:283–294

Hammond-Kosack KE, Jones JDG (1996) Resistance gene dependent plant defense responses. Plant Cell 8:1773–1791

Hirao T, Fukatsu E, Watanabe A (2012) Characterization of resistance to pine wood nematode infection in *Pinus thunbergii* using suppression subtractive hybridization. BMC Plant Biol 12:13. doi:10.1186/1471-2229-12-13

Kavitha PG, Thomas G (2008) Population genetic structure of the clonal plant *Zingiber zerumbet* (L.) Smith (Zingiberaceae), a wild relative of cultivated ginger, and its response to *Pythium aphanidermatum*. Euphytica 160:89–100

Kelman A (1954) The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathol 44:693–695

Kim YC, Kim SY, Paek KH, Choi D, Park JM (2006) Suppression of CaCYP1, a novel cytochrome P450 gene, compromises the basal pathogen defense response of pepper plants. Biochem Biophys Res Commun 345:638–645

Kumar A, Hayward AC (2005) Bacterial diseases of ginger and their control. In: Ravindran PN, Babu KN (eds) Monograph on Ginger. CRC Press, USA, pp 341–366

Kumar A, Bhai RS, Sasikumar B, Anandaraj M, Parthasarathy VA (2006) Curcuma amada Roxb. A bacterial wilt evading species in Zingiberaceae—A potential source of valuable genes for bacterial wilt resistance. In: The 4th International Bacterial wilt symposium, 17–20th July 2006. The Lakeside conference centre, Central Science Laboratory, York, UK

Latha AM, Rao KV, Reddy VD (2005) Production of transgenic plants resistant to leaf blast disease in finger millet (*Eleusine coracana* (L.) Gaertn.). Plant Sci 169:657–667

Luo M, Kong X, Huo N, Zhou R, Jia J (1990) Gene expression profiling related to powdery mildew resistance in wheat with the method of suppression subtractive hybridization. Chin Sci Bull 47:1990–1994

Mukherjee AK, Lev S, Gepstein S, Horwitz BA (2009) A compatible interaction of *Alternaria brassicicola* with *Arabidopsis thaliana* ecotype DiG: evidence for a specific transcriptional signature. BMC Plant Biol 9:31

Nascimento SB, Cascardo JCM, de Menezes IC, Duarte MLR, Darnet SH, Harada ML, de Souza CRB (2009) Identifying sequences potentially related to resistance response of *Piper tuberculatum* to *Fusarium solani* f. sp. *piperis* by suppression subtractive hybridization. Prot Pept Lett 12:1429–1434

Nishitani K (1998) Construction and restructuring of the cellulose-xyloglucan framework in the apoplast as mediated by the xyloglucan related protein family: a hypothetical scheme. J Plant Res 111:159–166

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Ann Rev Plant Physiol Plant Mol Biol 49:249–279

Oh BJ, Ko MK, Kim YS, Kim KS, Kostenyuk I, Kee HK (1999) A cytochrome P450 gene is differentially expressed in compatible and incompatible interactions between pepper (*Capsicum annuum*) and the anthracnose fungus, *Colletotrichum gloeosporioides*. Mol Plant Microbe Interact 12:1044–1052

Prasath D, El-Sharkawy I, Tiwary KS, Jayasankar S, Sherif S (2011) Cloning and characterization of PR5 gene from *Curcuma amada* and *Zingiber officinale* in response to *Ralstonia solanacearum* infection. Plant Cell Rep 30(10):1799–1809

Ros B, Thummler F, Wenzel G (2004) Analysis of differentially expressed genes in a susceptible and moderately resistant potato cultivar upon *Phytophthora infestans* infection. Mol Plant Pathol 5:191–201

Schlink K (2010) Down-regulation of defense genes and resource allocation into infected roots as factors for compatibility between *Fagus sylvatica* and *Phytophthora citricola*. Func Integ Genom 10:253–264

Sharmin S, Azam MS, Islam MS, Sajib AA, Mahmood N, Hasan AM, Ahmed R, Sultana K, Khan H (2012) Xyloglucan endotransglycosylase/hydrolase genes from a susceptible and resistant jute species show opposite expression pattern following *Macrophomina phaseolina* infection. Comm Integ Biol 5(6):1–9

Stacy RAP, Munthe E, Steinum T, Sharma B, Aalen RB (1996) A peroxiredoxin antioxidant is encoded by a dormancy-related gene, Per1, expressed during late development in the aleurone and embryo of barley grains. Plant Mol Biol 31:1205–1216

Thompson GA, Goggin FL (2006) Transcriptomics and functional genomics of plant defense induction by phloem-feeding insects. J Exp Bot 57:755–766

Tian ZD, Liu J, Xie CH (2003) Isolation of resistance related-genes to Phytophthora infestans with suppression subtractive hybridization in the R-gene free potato. Acta Genet Sin 30:597–605

Urbanowicz BR, Catala C, Irwin D, Wilson DB, Ripoll DR, Rose JK (2007) A tomato endo-beta-1,4-glucanase, SlCel9C1, represents a distinct subclass with a new family of carbohydrate binding modules (CBM49). J Biol Chem 282:12066–12074

Veronese P, Ruiz MT, Coca MA, Hernandez-Lopez A, Lee H, Ibeas JI (2003) In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. Plant Physiol 131:1580–1590

Xu P, Narasimhan ML, Samson T, Coca MA, Huh G, Zhou J, Martin GB, Hasegawa PM, Bressan RA (1998) A nitrilase-like protein interacts with GCC box DNA-binding proteins involved in ethylene and defense responses. Plant Physiol 118(3):867–874

Yang T, Shah J, Klessig DF (1997) Signal perception and transduction in plant defense responses. Gen Dev 11:1621–1639

Yang GP, Ross DT, Kang WW, Brown PO, Weigel RJ (1999) Combining SSH and cDNA microarray for rapid identification of differentially expressed genes. Nucl Acids Res 27:1517–1523

Zuo KJ, Qin J, Zhao JY, Ling H, Zhang LD, Cao YF, Tang KX (2007) Over-expression of GbERF2 transcription factors in tobacco enhanced brown spots disease resistance by activating expression of downstream genes. Gene 391:80–90