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Transcriptome-wide identification and characterization of resistant gene analogs (RGAs) of ginger (*Zingiber officinale Rosc.*) and mango ginger (*Curcuma amada Roxb.*) under stress induced by pathogen

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ABSTRACT

Resistance genes (*R*-genes) are responsible for activation of plant defense signaling involving the recognition of pathogen effectors by R-proteins resulting in the onset of the hypersensitive response (HR). *R*-gene analogs (RGAs) have been characterized in numerous plants and efficiently used for plant breeding purposes. Ginger (*Zingiber officinale* Rosc.) is an asexual spice crop which is affected by many diseases of which bacterial wilt caused by *Ralstonia solanacearum* is one of the most important production constraints. Out of the Zingiberaceous species screened for bacterial wilt resistance, mango ginger (*Curcuma amada* Roxb.) was found to be resistant. With an aim to study the disease resistance mechanism in mango ginger, RGAs have been identified from the ginger transcriptome database (gTDB) and characterized. Screening for the RGAs using bioinformatics-driven methods resulted in the identification of 160 clusters in mango ginger and 212 clusters in ginger with similarity to known *R*-genes that were classified based on the presence and organization of conserved domains. A large number of the clusters with gene ontology related to disease resistance were identified to be of NBS-LRR type. The gene expression studies of selected NBS-LRR transcripts identified that they got highly expressed in mango ginger in the initial hours post inoculation indicating their possible role in disease resistance. The study paves a way to understand the existing RGAs in ginger and mango ginger. Unfolding the mechanism that confers the high level of resistance in mango ginger may provide new insights for developing bacterial wilt resistance in ginger.

1. Introduction

Ginger (*Zingiber officinale* Rosc.) is an economically important spice crop widely used in herbal medicines and as a flavoring agent in foods and beverages. It is cultivated in many tropical and sub-tropical regions of the world, the most important ginger producing nations being India, China, Nepal, Indonesia and Nigeria (FAOSTAT, 2017). Diseases are one of the major constraints in the production of ginger of which bacterial wilt is one of the most serious, that inflicts serious economic losses on small and marginal farmers who depend on this crop for their livelihood (Kumar and Hayward, 2005). Pesticides provide an effective means of protection but their environmental effects are adverse, and they can lead to the emergence of resistant pathogen strains (McDowell and Woffenden, 2003). Incorporating *R*-genes from disease resistant wild relatives is an environment-friendly approach to develop disease resistance in asexually reproducing spice crops. This can be achieved through conventional breeding techniques or through molecular biology techniques such as genetic transformation. As ginger is an obligatory asexual plant, there is lack of genetic variability among the accessions for disease resistance (Ravindran et al., 2005). Genetic transformation with specific disease resistance traits from the wild relatives is the only opportunity for plant breeders to overcome the constraints imposed by the sterility of these rhizomatous plants. Despite an extensive search no resistance sources to Ralstonia solanacearum induced bacterial wilt could be located in ginger (Kumar et al., 2006; Prasath et al., 2011). The search for resistance has been extended to other closely related genera in the family Zingiberaceae for their response to *R. solanacearum*. The Indian mango ginger (*Curcuma amada* Roxb.) was the only plant found to be resistant to bacterial wilt (Kumar et al., 2006; Prasath et al., 2011). To understand the molecular basis of disease resistance in mango ginger, the transcriptome sequences of ginger and mango ginger deposited in the ginger transcriptome database (Prasath et al., 2014) were used to identify and classify members of major class of disease-resistance genes.

Plant cells autonomously maintain constant vigilance against pathogens by expressing large arrays of *R*-genes (Dangl and Jones, 2001).

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R-genes recognize specific pathogen effectors, "avirulent (avr) proteins" leading to the activation of plant defense signaling pathways resulting in the onset of hypersensitive response (Belkhadir et al., 2004). *R*-genes encode proteins of several classes which can be broadly classified into five major categories based on their conserved protein signatures (Song et al., 1997; Ellis and Jones, 1998). They incorporate certain motifs in common *viz*., serine/threonine protein kinases (PKs), leucine-rich repeats (LRRs), nucleotide binding sites (NBSs), and transmembrane domains (TMs) that are combined in different orders of arrangements with the following signatures, PK, LRR-TM-PK, NBS-LRR and LRR-TM (Ellis and Jones, 1998). NBS-LRR proteins are categorized into two types *viz.*, LRR proteins with a nucleotide binding site (NBS) and a coiled-coil (CC) or leucine-zipper (LZ) motif and NBS-LRR proteins with N-terminal region showing similarity to the Toll and interleukin-1 receptor (TIR) proteins (Pan et al., 2000).

In our earlier studies, the transcriptomes of ginger and mango ginger challenge inoculated with *R. solanacearum,* were sequenced and compared (Prasath et al., 2014). The study resulted in identification of several genes associated with abiotic and biotic stress resistance. A large set of candidate genes assumed to confer resistance to bacterial wilt pathogen in mango ginger were identified and validated *in vitro*. The transcriptome data has been deposited in ginger transcriptome database (gTDB) (http://220.227.138.213/GTDB/). The web resource provides public access to the data. The present work we aimed *in silico* analysis to screen the gTDB for identification of RGAs with the hypothesis that they might play a role in resistance to bacterial wilt.

2. Materials and methods

2.1. Identification of RGAs from the transcriptome

The transcriptome of ginger and mango ginger were sampled over 72 h period after pathogen inoculation and sequenced using Illumina genome analyzer technology (Illumina, San Diego, CA, USA) (Prasath et al., 2014). The raw reads thus generated were subjected to *de novo* assembly using the CLC Genomics Workbench ver. 6.0 (CLC Bio, Swansea, UK). A total of 26,387,032 and 22,268,804 paired-end reads obtained after quality filtering of ginger and mango ginger were assembled into 36,359 and 32,312 transcript sequences respectively. The transcriptome data thus obtained has been deposited in gTDB that provides an overview of the transcriptome sequencing, research group, and other related information of the ginger transcriptome data.

The amino acid (aa) sequences of previously reported *R*-genes (Table S1) were used to mine out similar sequences using tblastn (Altschul et al., 1990) with an E-value cut-off of $1e^{-10}$. The extracted sequences were *in silico* translated to protein sequences and potential coding areas within transcript sequences were identified using TransDecoder using default parameters (Haas et al., 2013).The transcriptome sequences were clustered using hierarchical clustering using CD-HIT (Li et al., 2001) with sequence identity cut-off of 40%. The ginger and the mango ginger unigenes showing matches to known *R*-genes were subjected to Blast2GO analysis (Conesa et al., 2005). The functional analysis and prediction of the domains of the extracted RGAs were carried out using InterProScan (Quevillon et al., 2005) that combined data from PROSITE, PRINTS, Pfam, ProDom, SMART, TIGRFAMs, PIRSF, SUPERFAMILY, GENE 3D, COILS, TMHMM, and PANTHER. Each of the clusters was classified into different categories based on the presence and organization of conserved domains, predicted by NCBI conserved domain search service (CD-search) (Marchler-Bauer and Bryant, 2004) and the InterProScan motif signatures. The redundancies were checked manually.

2.2. Gene expression studies of selected RGAs

Bacterial inoculum was prepared by transferring virulent colonies of *R. solanacearum* identified on Casamino acid-Peptone-Glucose (CPG) medium (Kelman, 1954) and incubated at 28 °C for 16 h with constant shaking. The bacterial cells were pelleted by centrifugation. The pellet was suspended in sterile water and adjusted to a concentration of 10^9 cells ml^{-1}. The bacterial suspension was poured onto the base of ginger and mango ginger plants. Total RNA was isolated using Tri reagent (Catalog No. T9424) (Sigma-Aldrich, USA) as per the manufacturer's instructions using leaf tissues of mango ginger and ginger at 0, 1, 4, 8, 24, 48, 72 and 96 h post inoculation (hpi) with *R. solanacearum*. The total RNA was quantified and 1 µg of RNA samples were treated with TURBO DNA-free kit (Invitrogen, USA) for 30 min at 37 °C to remove the contaminating genomic DNA. After DNase treatment, first strand cDNA synthesis was carried out utilizing 1 µL of RevertAid Reverse Transcriptase (200U/µL) in 4 µL of 5X reaction buffer (Thermo Scientific), 1 µL of $Oligo(dT)_{18}$ primer (Thermo Scientific), 0.5 µL of RiboLockRNase Inhibitor (40U/µL) (Thermo Scientific) and 2 µL of dNTP mix (Thermo Scientific), incubated for 60 min at 42 °C and 70 °C for 5 min. The expression of three NBS-LRR type RGAs with lower E-values in mango ginger (Contig-17109, Contig-35377 and Contig-17103) and ginger (Contig-4736, Contig-18964 and Contig 89073) was validated using the primers designed from PrimerQuest tool, Integrated DNA Technologies (Table 1). Quantitative real-time PCR (qPCR) reactions were carried out in Rotor-Gene Q (Qiagen) with a 20 µL reaction mixture containing 10 µL of QuantiFast SYBR Green PCR Mastermix (Qiagen), 1 µL of each primer (10 mM), 3 µL of the template (3 ng) and 5 µL of nuclease free- distilled water (Invitrogen, USA). The thermal conditions were as follows: initial holding stage at 95 °C for 5 min and followed by 40 cycles at 95 °C for 15 s and a final step at 60 °C for 45 s. All the reactions were performed in triplicate in 72-well reaction plates with cDNAs synthesized from three biological replicates. Fold change of the transcripts was calculated relative to the control (0 hpi) using $2^{-\Delta\Delta Ct}$ method.*β*-actin was used as an internal control.

3. Results

3.1. Data mining and identification of RGAs from transcriptomic sequences

The tblastn search against the ginger transcriptome database resulted in extraction of 365 similar transcripts in mango ginger and 549 transcripts in ginger showing similarity to known *R*-genes. The E-value was lowered to a scale of e^{-10} to prevent the exclusion of important candidate genes. ORF sizes ranged from 100 to 1424 amino acids for mango ginger and 102 to 959 amino acids for ginger respectively. The clustering using CD-HIT resulted in 160 clusters in mango ginger and 212 clusters in ginger. Functional characterization of extracted sequences using Blast2GO and InterProScan resulted in identification of putative functions of the extracted sequences combined with the identification of motif signatures required to classify the RGAs. The search for conserved domains using Conserved Domain Database (CDD)

Table 1
Primers used to amplify NBS-LRR type RGAs for qRT-PCR experiments.

Primer sets		Sequence (5' - 3')
CaNBS-17103	Forward (F)	GGTGGACCAGATTGAAGAAG
	Reverse (R)	GAGGGTACAAAGAGCAATAGAG
CaNBS-35377	Forward (F)	GCAAAGACGGTAGGAGGATTAT
	Reverse (R)	GGCAGAACGTCACTCTCATT
CaNBS-17109	Forward (F)	GTGCATGGATCTCTGTGTCTAA
	Reverse (R)	GAGTTATCTGTTCCTTCCTCTCAC
ZoNBS-89073	Forward (F)	TTGGATTCCGGAGGACAAAC
	Reverse (R)	CATCCTAAGAGTGGCCAAGAAG
ZoNBS-18964	Forward (F)	ACGTGTGGAGCGATGATAAG
	Reverse (R)	ATGTCCCGAGTTGTAACAATGA
ZoNBS-4736	Forward (F)	GGAAGATGGGCTGGCTTAAT
	Reverse (R)	GCTGGCTTCTCTTTCCTCTAAT
Actin	Forward (F)	TAGGTGCCCAGAGGTTCTATT
	Reverse (R)	ACCGCTAAGCACCACATTAC

Table 2
Representation of the transcripts of ginger (Zo) and mango ginger (Ca) similar to kinase class, with GO terms related to defense response.

Class	Contigs	(aa)	E-value	GO names list
Ca-Kinase	Comp_81719_c0_seq1	373	0.00E + 00	protein kinase activity; defense response; response to bacterium; phosphorylation; single-organism cellular process; regulation of cellular process cellular response to stimulus
Ca-Kinase	Comp_5850_c0_seq10	384	0.00E + 00	systemic acquired resistance; salicylic acid mediated signaling pathway; protein serine/threonine kinase activity;
Ca-Kinase	Comp_73744_c0_seq4	350	0.00E + 00	systemic acquired resistance; protein serine/threonine kinase activity; regulation of defense response
Ca-Kinase	Comp_8280_c0_seq13	314	2.80E-154	protein serine/threonine Kinase activity; defense response
Zo-Kinase	Comp_21703_c0_seq1	464	0.00E + 00	ATP binding; defense response to bacterium, incompatible interaction; protein serine/threonine kinase activity
Zo-Kinase	Comp_17617_c0_seq3	423	0.00E + 00	protein serine/threonine kinase activity; defense response
Zo-Kinase	Comp_81719_c0_seq1	373	0.00E + 00	systemic acquired resistance; salicylic acid mediated signaling pathway; salicylic acid biosynthetic process; protein serine/threonine kinase activity
Zo-Kinase	Comp_31115_c0_seq1	644	0.00E + 00	protein serine/threonine kinase activity; defense response
Zo-Kinase	Comp_28187_c0_seq1	490	0.00E + 00	protein serine/threonine kinase activity to defense response, response to hydrogen peroxide

predicted 154 clusters of mango ginger and 198 clusters of ginger bearing the conserved domains characteristic of each *R*-gene class (Fig.S1).

The tblastn alignment against the ginger transcriptome database extracted 34 clusters of mango ginger and 36 clusters of ginger bearing a kinase domain. Post-translational phosphorylation of proteins by protein kinases is known to play a prominent role in many signal transduction pathways especially in defense responses. A total of 29 clusters of mango ginger and 39 clusters of ginger included a transmembrane domain along with a kinase domain. The genes associated with the GO term 'defense response' were also identified (Table 2). These classes of R-proteins resemble the cytoplasmic protein kinase domain of LRR-kinase. The kinase domain is shared by members of kinase and LRR-kinase classes.

The R proteins belonging to the NBS-LRR class are the most functionally defined and characterized forms, whose main role is in disease resistance (Ellis and Jones, 1998). Twenty nine clusters of mango ginger and 31 clusters of ginger possessed an NB-ARC domain. They were categorized into NBS, CC-NBS, CC-NBS-LRR and NBS-LRR depending on their variability. The predicted RGAs with NBS-domain were aligned with related crops like *Kaempferia galanga*, *Curcuma longa, C. angustifolia, C. aromatica, C. zedoaria and Z. zerumbet* using ClustalW (Thompson et al., 1994) with default parameters. The resulting alignment was manually curated using Jalview (Waterhouse et al., 2009) to remove the regions of poor alignment. Multiple sequence alignment of deduced amino acid sequences revealed the presence of kinase-1a (P-loop), kinase-2, kinase-3a (RNBS-B), and the GLPL motif in the NBS-spanning region (Fig. 1). Genes that contained no or partial NB-ARC domain excluded from alignment. The presence of the TIR domain was not detected in any of the clusters. In plants, the TIR motif is possibly absent in monocotyledons and found only associated with NBS regions of dicotyledons (Meyers et al., 1999). The blast search revealed that all the NBS-LRR RGAs identified in mango ginger (Table 3) and ginger (Table 4) were aligned to disease resistance genes from other plant species. The Blast2GO annotation revealed that they were involved in nucleotide binding, signal transduction, protein binding, and defense response.

The rice disease resistance gene *Xa21* encodes a protein which carries an LRR domain along with a serine/threonine kinase-like domain in which the LRR domain is predicted to be extracellular (Song et al., 1995). About 25 clusters of mango ginger and 22 clusters of ginger possessed both LRR and kinase domains. The GO's pertaining to disease resistance has been identified in four transcripts in mango ginger and twelve transcripts in ginger (Table 5). *Arabidopsis* also carries homologs of other *R*-gene classes, many of which are likely to play a role in development rather than defense (Jones, 2001). The clusters that showed similarity to both kinase and LRR-kinase class were categorized into MIX I (LRR, PK, LRR-PK), MIX II (PK, LRR-PK), MIX III (LRR, LRR-PK) and MIX IV (PK, LRR) based on the domains identified in each cluster.

The *Cf-9* class of plant proteins contains only the extra-cytoplasmic leucine rich repeats (eLRRs). Thirty seven clusters of mango ginger and 70 clusters of ginger contained LRR domain only. The LRRs that clustered separately from NBS-LRR and LRR-kinases were included as a separate group. This domain is present in all the classes of *R*-genes except kinases. Out of these LRR clusters, seven clusters of mango

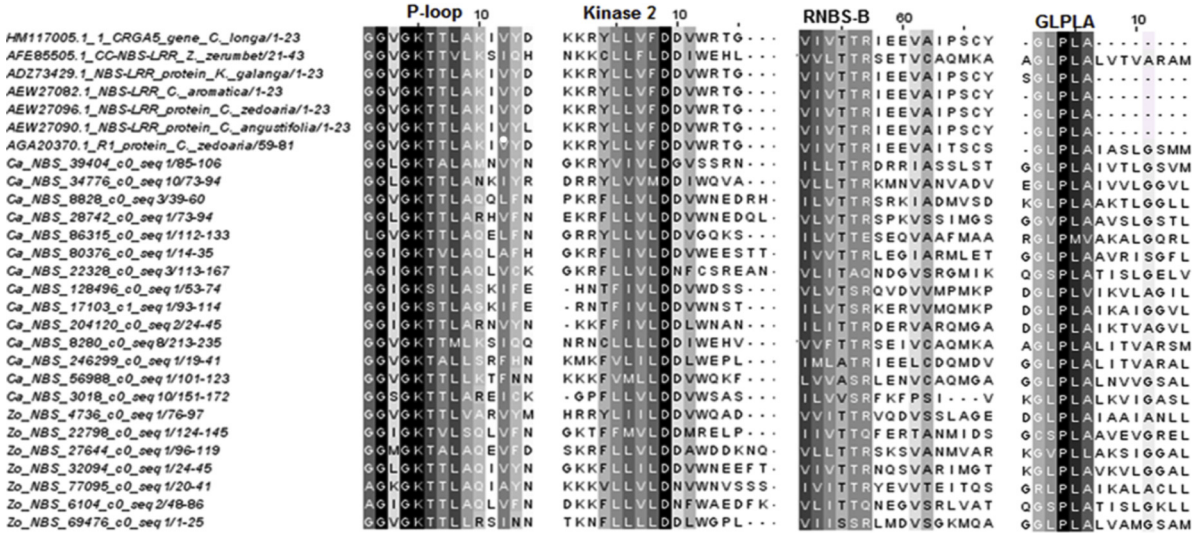

Fig. 1. Multiple sequence alignment of deduced amino acid sequences of RGAs in mango ginger (Ca) and ginger (Zo) with closely related Zingiberaceous members. Only specific motifs of P-Loop, Kinase-2, RNBS-B and GLPL hydrophobic domain within the NBS-domain are highlighted.

Table 3
The NBS-LRR sequences in mango ginger identified using the templates of well-known *R*-genes are indicated with contig IDs, annotations, the length (aa) and E-value.

CA Contig ID	Annotation	(aa)	E-value
Comp_10433_c1_seq1	disease resistance RPM1-like	240	1.60E-63
Comp_108916_c0_seq1	disease resistance RGA3	583	2.80E-130
Comp_128496_c0_seq1	disease resistance RGA3	337	4.80E-100
Comp_136872_c0_seq1	disease resistance RPP13	303	1.50E-98
Comp_137771_c0_seq1	TMV resistance N-like isoform X1	354	9.20E-50
Comp_142683_c0_seq1	disease resistance RGA3	143	2.70E-55
Comp_14397_c0_seq62	disease resistance RPP13	110	2.80E-29
Comp_17103_c1_seq1	disease resistance RGA3	728	0.00E + 00
Comp_184990_c0_seq1	disease resistance RGA1	260	1.30E-79
Comp_195923_c0_seq1	disease resistance RPP13	311	2.50E-68
Comp_204120_c0_seq2	disease resistance RGA3	228	1.80E-77
Comp_22328_c0_seq3	disease resistance RGA3	962	0.00E + 00
Comp_235242_c0_seq1	disease resistance RPM1-like	208	1.80E-58
Comp_245410_c0_seq1	Os05g0492600	240	6.80E-71
Comp_246299_c0_seq1	disease resistance RPS2-like	340	3.50E-171
Comp_27474_c0_seq1	disease resistance RGA3	114	2.90E-34
Comp_280573_c0_seq1	disease resistance RPM1-like	249	1.40E-103
Comp_28742_c0_seq1	disease resistance RGA3	911	0.00E + 00
Comp_3018_c0_seq10	disease resistance At5g47280	924	0.00E + 00
Comp_33519_c0_seq13	NBS-LRR, partial	134	2.20E-43
Comp_34502_c0_seq1	disease resistance RGA3	142	1.60E-41
Comp_34776_c0_seq10	disease resistance RPP13	788	0.00E + 00
Comp_352483_c0_seq1	disease resistance At3g14460	105	3.60E-26
Comp_39404_c0_seq1	disease resistance RPM1-like	918	0.00E + 00
Comp_412050_c0_seq1	resistance, partial	113	1.20E-35
Comp_43443_c0_seq10	disease resistance RGA1	116	3.90E-43
Comp_4606_c0_seq12	disease resistance RPP13	316	9.60E-67
Comp_56988_c0_seq1	Disease resistance RPS2	934	0.00E + 00
Comp_62740_c0_seq1	disease resistance At1g50180	637	2.90E-127
Comp_6982_c1_seq1	disease resistance RGA3	223	6.10E-67
Comp_79043_c0_seq2	disease resistance RGA1	238	4.50E-81
Comp_80376_c0_seq1	disease resistance RGA1	542	3.10E-141
Comp_82086_c0_seq1	NBS-LRR, partial	257	3.40E-57
Comp_8280_c0_seq8	probable disease resistance	799	0.00E + 00
Comp_86315_c0_seq1	disease resistance RGA3	423	3.30E-97
Comp_8828_c0_seq3	disease resistance RGA1	697	0.00E + 00
Comp_162565_c0_seq1	disease resistance RGA3	294	1.70E-97
Comp_179158_c0_seq1	disease resistance RGA1	547	5.40E-131
Comp_319566_c0_seq1	disease resistance RGA3	130	8.20E-37
Comp_35377_c0_seq1	disease resistance RGA1	786	1.10E-147
Comp_162565_c0_seq1	disease resistance RGA3	294	1.70E-97
Comp_179158_c0_seq1	disease resistance RGA1	547	5.40E-131
Comp_319566_c0_seq1	disease resistance RGA3	130	8.20E-37
Comp_35377_c0_seq1	disease resistance RGA1	786	1.10E-147
Comp_80298_c0_seq1	disease resistance RGA3	370	3.70E-117
Comp_53249_c0_seq1	disease resistance RPP13	708	9.50E-91
Comp_34776_c0_seq3	disease resistance At1g50180	906	0.00E + 00

ginger and 13 clusters of ginger had a transmembrane domain. These sequences are imperfect and difficult to recognize with available *in silico* tools (Barbosa-da-Silva et al., 2005). Upon blast search, the sequence description pertained to LRR receptor-like serine/threonine kinase, leucine-rich repeat receptor-like kinase groups. Some of the LRRs were annotated to be LEPR3 like receptor like kinases involved in blackleg resistance, *Verticillium* wilt resistance proteins, and *Cf-2.1* class (Table 6). The details of the RGAs and the domains identified and the predicted number of clusters showing similarity to the known *R*genes has been included in Table 7.

InterProScan analysis of the protein clusters predicted specific motif signatures for each class of RGAs *viz*., PR00364 (Disease resistance protein signature), IPR002182 (NB-ARC), IPR027417 (P-loop containing nucleoside triphosphate hydrolase), PR00019 (Leucine-rich repeat signature), IPR032675 (Leucine-rich repeat domain, L domain-like), IPR011009 (Protein Kinase-like domain), IPR013320 (Concanavalin A-like lectin/glucanase domain), IPR001245 (serine-threonine/tyrosine-protein kinase catalytic domain), IPR002290 (serine/threonine-protein kinase domain).

Table 4
The NBS-LRR sequences of ginger extracted with templates of well-known *R* genes are indicated with contig IDs, annotations, the length (aa) and E-value.

ZO Contig ID	Annotation	(aa)	E-value
Comp_18964_c0_seq1	disease resistance RGA1	608	7.6E-97
Comp_231926_c0_seq1	resistance, partial	141	3.2E-31
Comp_11610_c0_seq6	disease resistance RGA1	171	2.3E-44
Comp_11612_c0_seq1	disease resistance RGA4	649	1.2E-64
Comp_120653_c0_seq2	disease resistance RPP13 1	345	7.5E-78
Comp_120740_c0_seq1	disease resistance RPP13 1	108	3.7E-22
Comp_146739_c0_seq1	disease resistance RPP13 1	113	2.1E-25
Comp_148925_c0_seq1	disease resistance RGA1	177	1.3E-44
Comp_15598_c1_seq1	disease resistance At1g50180	761	0.00E + 00
Comp_166758_c0_seq1	disease resistance RPP13 1	102	2.1E-20
Comp_185826_c0_seq1	disease resistance RGA1	324	5.6E-110
Comp_18964_c0_seq64	disease resistance RGA1	250	1.2E-76
Comp_210198_c0_seq1	disease resistance RGA3	194	5.8E-85
Comp_219909_c0_seq1	disease resistance RGA1	126	2.2E-29
Comp_22798_c0_seq1	disease resistance RPP13 1	680	5.3E-107
Comp_235427_c0_seq1	disease resistance RGA3	156	1.9E-53
Comp_26006_c1_seq6	disease resistance RGA1	331	4.2E-74
Comp_263518_c0_seq1	disease resistance protein RGA2-like	119	1.1E-18
Comp_27644_c0_seq1	disease resistance RGA1	564	9.3E-89
Comp_31624_c0_seq1	disease resistance RPP13 1	746	4.3E-101
Comp_32094_c0_seq1	disease resistance RPP13 1	886	0.00E + 00
Comp_402805_c0_seq1	disease resistance RGA3, partial	100	4.3E-34
Comp_42019_c0_seq1	disease resistance RPP13 1	721	0.00E + 00
Comp_42060_c0_seq1	TMV resistance N-like isoform	189	3.4E-29
Comp_45287_c0_seq1	disease resistance RPP13 1	317	3.3E-93
Comp_46834_c0_seq2	Disease resistance family, isoform 1	697	0.00E + 00
Comp_4736_c0_seq1	disease resistance RPM1-like	919	0.00E + 00
Comp_48166_c0_seq3	disease resistance RGA3	252	5E-82
Comp_55445_c0_seq2	disease resistance RGA3	108	1.6E-16
Comp_55782_c0_seq1	disease resistance RPM1-like	472	1.4E-77
Comp_6104_c0_seq2	disease resistance RGA3	930	0.00E + 00
Comp_69476_c0_seq1	disease resistance RPS2-like	717	0.00E + 00
Comp_72647_c0_seq1	disease resistance RPS2-like	663	7.1E-102
Comp_77095_c0_seq1	disease resistance RGA3	781	3E-172
Comp_79460_c0_seq1	disease resistance RGA1	99	1.8E-24

In addition to these well-studied five *R*-gene classes, *R*-gene clusters with *RPW8* (resistance to powdery mildew) domain was identified in contig-3018 (895 aa) at the N-terminal of the NBS-LRR protein in mango ginger and contig-3496 (906 aa) in ginger. The *RPW8* domain (IPR008808) is found in several broad-spectrum mildew resistance proteins from *A. thaliana* and other dicots (Xiao et al., 2001). The sequences with this domain contain a predicted N-terminal transmembrane region or possibly a signal peptide, and a coiled-coil (CC) motif. The mango ginger contig-157,074 (E-value 3.97e-54) and contig-153545 of ginger (E-value 3.70e-89) showed similarity to *Verticillium* wilt resistance protein *Ve2* of *Solanum lycopersicum* that is known to impart resistance to *Verticillium dahlia*. The *Ve* proteins (*Ve1* and *Ve2*) encode cell surface glycoproteins with signals for receptor-mediated endocytosis and leucine-zipper or Proline-Glutamine-Serine-threonine (PEST) sequences that belong to the extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins (Kawchuk et al., 2001; Wang et al., 2008).

3.2. Quantitative real-time PCR (qPCR) analysis of selected RGAs

The qPCR assays were performed using cDNA of mango ginger and ginger prepared from RNA isolated at 0, 1, 4, 8, 24, 48, 72 and 96 hpi and relative fold changes with respect to control (0 hpi) plants were calculated. The expression of the contig-17103, contig-17109 and contig-35377 of mango ginger peaked up at 8 hpi (3.51), 96 hpi (1.91) and 48 hpi (2.67) respectively (Fig. 2-a). The contig-17103 showed a comparatively higher fold change in the initial hours of pathogen encounters. In both mango ginger and ginger, the expression pattern varied with time-course with the expression being up- and down-regulated at various time points. In ginger contig-89703 and contig-

Table 5
Blast2GO results of the contigs of ginger (Zo) and mango ginger (Ca) similar to LRR-kinase class, with GO terms related to defense response.

Class	Contigs	aa	E-value	GO names list
Ca-LRR- Kinase	Comp_11169_c0_seq1	408	8.10E-78	Protein kinase activity; defense response to fungus; protein phosphorylation
Ca-LRR-Kinase	Comp _16283_c0_seq1	977	0.00E + 00	Protein serine/threonine kinase activity; defense response to bacterium defense response to fungus
Ca-LRR-Kinase	Comp_141070_c0_seq1	248	0.00E + 00	Response to other organism; regulation of programmed cell death; protein kinase activity; protein phosphorylation; response to stress; nucleotide binding; regulation of defense response
Ca-LRR-Kinase	Comp_183922_c0_seq1	398	0.00E + 00	Protein kinase activity; immune system process protein phosphorylation; signal transduction response to endogenous stimulus; regulation of defense response; regulation of programmed cell death single-organism metabolic process; negative regulation of biological process; detection of stimulus; cellular response to acid chemical; cellular response to organic substance; defense response to other organism; cellular response to oxygen-containing compound
Zo-LRR-Kinase	Comp_272629_c0_seq1	109	1.90E-59	Protein serine/threonine kinase activity ATP binding defense response to bacterium defense response to fungus
Zo-LRR-Kinase	Comp_288330_c0_seq1	110	1.60E-40	Protein serine/threonine kinase activity non-membrane spanning protein tyrosine kinase activity defense response to bacterium, incompatible interaction
Zo-LRR-Kinase	Comp_53474_c0_seq4	385	4.20E-171	Protein serine/threonine kinase activity; defense response
Zo-LRR-Kinase	Comp_17617_c0_seq3	423	0.00E + 00	Transmembrane receptor protein; serine/threonine kinase activity; defense response to bacterium
Zo-LRR-Kinase	Comp_8231_c0_seq1	736	0.00E + 00	Transmembrane receptor protein kinase activity; defense response to bacterium; defense response to fungus
Zo-LRR-Kinase	Comp_31115_c0_seq1	644	0.00E + 00	Protein kinase activity; defense response; response to bacterium; single-organism cellular process; cellular response to stimulus
Zo-LRR-Kinase	Comp_5282_c0_seq1	249	1.60E-138	Protein serine/threonine kinase activity; respiratory burst involved in defense response protein targeting to membrane; detection of external stimulus; detection of biotic stimulus; systemic acquired resistance, salicylic acid mediated signaling pathway jasmonic acid mediated signaling pathway; regulation of hydrogen peroxide metabolic process; regulation of plant-type hypersensitive response; negative regulation of defense response; defense response to bacterium; negative regulation of programmed cell death; defense response to fungus; defense response by callose deposition
Zo-LRR-Kinase	Comp_136090_c0_seq1	461	0.00E + 00	Transmembrane receptor protein kinase activity; regulation of anthocyanin biosynthetic process; defense response to bacterium; defense response to fungus; protein kinase activity; response to stress; regulation of defense response; regulation of programmed cell death; single-organism cellular process
Zo-LRR-Kinase	Comp_138281_c0_seq1	125	5.70E-58	Protein kinase activity; protein phosphorylation; response to stress; regulation of defense response; regulation of programmed cell death; single-organism cellular process; response to other organism
Zo-LRR-Kinase	Comp_53474_c0_seq1	597	0.00E + 00	Serine/threonine kinase activity; signal transduction response to wounding; regulation of defense response innate immune response
Zo-LRR-Kinase	Comp_136090_c0_seq1	461	0.00E + 00	Response to other organism; regulation of programmed cell death; response to stress; regulation of defense response
Zo-LRR-Kinase	Comp_53474_c0_seq1	597	0.00E + 00	Response to bacterium; protein kinase activity; defense response

Table 6
Contigs with LRR domains having possible role in disease resistance in mango ginger (Ca) and ginger (Zo).

Class	Contigs	Annotation	(aa)	E-value
Ca-LRR	Comp_164248_c0_seq2	putative disease resistance protein LEPR3	184	4.9E-34
Ca-LRR	Comp_157074_c0_seq2	*Verticillium* wilt disease resistance protein Ve2	216	3.4E-68
Zo-LRR	Comp_15371_c0_seq1	disease resistance protein Cf-2.1-like	462	0.00E + 00

Table 7
Representation of the number of clusters of RGAs identified with their predicted protein domains namely NBS, LRR, CC, PK, TM.

R gene class	Predicted protein domains	Mango ginger	Ginger
NBS-encoding genes	NBS	6	13
	CC-NBS	2	3
	CC-NBS-LRR	9	5
	NBS-LRR	11	9
LRR	LRR	30	57
	LRR-TM	7	13
Kinases	PK	34	36
	PK-TM	29	39
LRR-kinases	LRR-PK	6	14
MIX I	LRR, PK, LRR-PK	6	3
MIX II	PK, LRR-PK	5	3
MIX III	LRR, LRR-PK	4	1
MIX IV	PK, LRR	4	1

4736 were largely downregulated. The contig-18964 showed higher expression at 72 hpi (1.09) (Fig. 2-b). The specificity of the qPCR amplified products was confirmed by using melt curve analysis in mango ginger and ginger. Using RNA-Seq mapping tools of CLC Genomic Workbench version 6.0, the raw reads were mapped back to the *de novo* transcriptome assemblies of ginger and mango ginger (Prasath et al., 2014). The change in gene expression was calculated based on the number of reads mapped and RPKM (Reads per kilo base million). Based on the transcriptome data, the expression of contig-35377, contig-17103 and contig-17109 were found to be 8.58, 3.35 and 1.45-folds higher than ginger.

4. Discussion

In the past 2 decades, numerous *R*-genes have been identified and cloned from a large number of plant species, with the majority encoding for NBS-LRR proteins (Richter and Ronald, 2000; Holt et al., 2003; Ameline-Torregrosa et al., 2008; Li et al., 2010; Guo et al., 2011; Kang et al., 2012; Frazier et al., 2016). The strikingly similar structure among different *R*-genes conferring resistance to diverse pathogens indicates the highly conserved nature of the plant disease resistance mechanism (Dangl et al., 1996; Maekawa et al., 2012; Sharma et al., 2014). The RGAs have been utilized to develop molecular markers for tagging and mapping disease resistance traits in many plant species (Gururani et al., 2012). Utilizing plant *R*-genes for development of disease resistant varieties is an eco-friendly alternative to the employment of chemical control measures for disease control (Gururani et al., 2012).

RGAs hold a great promise for the improvement of genetically less amenable asexual crops like ginger. In ginger, RGAs have been isolated using a PCR based approach employing the primers specific to the four

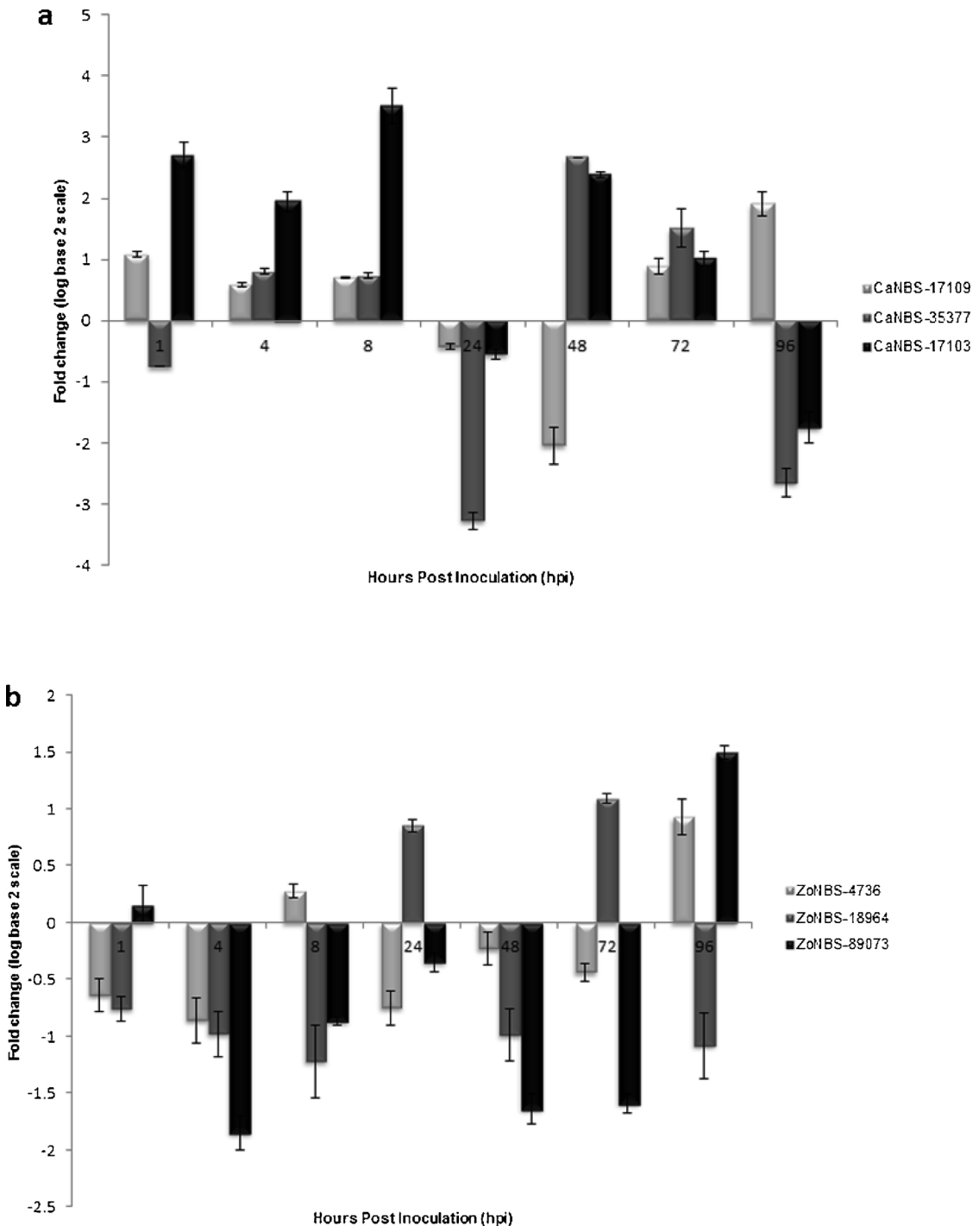

Fig. 2. Normalized relative gene expression levels of the selected three sets of NBS-LRRgenes in (a) mango ginger (b) ginger.

classes of RGAs namely NBS-LRR, LRR-TM-kinase, kinases and LRR-TM (Nair and Thomas, 2007a). NBS-LRR sequences are abundant in plant genomes and our results reveal that a large number of NBS-LRR-type sequences are also present in mango ginger and ginger which got clustered into 29 clusters in mango ginger and 31 clusters in ginger. The presence of TIR type NBS-LRR genes were not detected in any of the transcripts in ginger and mango ginger. The TIR-type NBS-LRR class *R*-genes were reported to be present in early land plants but their numbers diminished significantly in monocots and magnolids during evolution (Tarr and Alexander, 2009). In *Arabidopsis*, the genome was found to encode 159 NBS-LRR genes, and 113 of these genes occurred in 38 clusters (Guo et al., 2011). The NBS domain consists of several conserved and strictly ordered motifs including the phosphate binding loop (P-loop), the kinase-2 domains, which are ATP and GTP binding sites, the kinase-3a domain and the hydrophobic membrane-spanning GLPL domain (Meyers et al., 1999; Baldi et al., 2004). These motifs have been reported to play a significant role in signal transduction and activation of the plant defense responses (Hammond-Kosack and Jones, 1997). The alignment of deduced amino acid sequences of mango ginger and ginger NBS-LRR type RGAs exhibited the typical motifs of the NBS domain (P loop, Kinase 2, RNBS-B and GLPL domains), which indicates their possible function in disease resistance (McHale et al., 2006). The NBS-LRR type RGAs have been isolated, characterized and expression studies have been carried out in ginger and its wild relatives

from *Zingiber*spp (Nair and Thomas, 2007b). Further *ZzR1*, a resistance gene belonging to CC-NBS-LRR has been isolated from *Z. zerumbet* which has the potential for imparting resistance to soft rot caused by *Pythium aphanidermatum* (Nair and Thomas, 2013). The NBS-LRR type *R*-genes are constitutively expressed at low levels in healthy uninoculated plants and gets rapidly upregulated only after infection by pathogens (McHale et al., 2006). Induction of *R*-genes in plants following a pathogen attack has been observed for many plant species including rice (Wang et al., 1999), sunflower (Radwan et al., 2004), cucumber (Wan et al., 2010), *Arabidopsis* (Xiao et al., 2001) and ginger (Nair and Thomas, 2013). In mango ginger, there was significant upregulation of RGAs in the earlier hours compared to ginger. Further detailed studies with these RGAs such as sequencing and characterization of full length of the selected contigs and further genetic transformation studies in ginger could possibly infer the possible involvement of the upregulated RGAs in imparting disease resistance.

The serine/threonine kinase class of *R*-genes are known to play a central role in defense signaling during pathogen recognition and subsequent activation of the *PR* gene expression, an increased level of SA, and occurrence of minute microscopic lesions similar to the hypersensitive response (Xiao et al., 2001; Tang et al., 1999). Annotation using Blast2GO revealed that they were probable receptor kinases, probable serine-threonine kinases, receptor-like serine-threonine kinases, probable leucine-rich repeat receptor kinases, wall-associated receptor kinases, PTI1-like tyrosine kinases, pto-interacting 1-like kinases, cysteine-rich receptor kinase. The *Pto* genes with GO terms related to 'defense response to bacterium', and 'systemic acquired resistance' have been identified in both ginger and mango ginger. These contigs could be further explored for their role in imparting resistance against bacterial wilt.

The *Xa21* gene from rice confers broad spectrum resistance to the bacterial blight caused by *X. oryzae* pv. *oryzae* races, which is the most destructive disease of rice(Song et al., 1995; Wang et al., 1995). The LRR-kinases identified in the study possessed the GO terms related to 'negative regulation of programmed cell death', 'negative regulation of defense responses', 'systemic acquired resistance', 'salicylic acid mediated signaling pathway', 'jasmonic acid mediated signaling pathway' and 'defense response by callose deposition'. It was reported that *Xa21* could mediate negative regulation of cellular signaling which may play a role in the *Xa21*-mediated defense response (Gan et al., 2011).

The R-proteins belonging to *Cf9* gene family encodes an extracellular LRR with a transmembrane domain (Jones et al., 1994). They do not possess significant intracellular region that could act as a defense signaling component (Ellis and Jones, 1998). The blast search with these LRRs showed hits to LRR-receptor like kinases. However, as the LRRs clustered separately from NBS-LRR and LRR-kinases they were included as a separate group. InterProScan motif signatures predicted that they possessed motif signatures of LRR domain only.

The results of the present study provide important information on different classes of resistance genes in mango ginger and ginger. The data mining information generated expands our understanding of the possible defense response triggered in mango ginger and ginger in response to pathogens. The highly promising resistance genes from mango ginger need to be identified and transgenic technology could be used in introducing several resistance alleles into susceptible plants which could prove effective against a wide range of pathogen species affecting ginger (Gururani et al., 2012).

5. Conclusion

Using bioinformatics tools it was possible to mine out the RGAs from the transcriptome of mango ginger and ginger post challenge inoculation with *R. solanacearum*. The present work brought some light to existing *R*-gene group in these bacterial wilt resistant and susceptible crop species. Further *in silico, in vitro* and *in vivo* evaluations of the genome may bring promising results.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.01.003.

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