Genome-wide identification, characterization and expression analysis of non-RD receptor like kinase gene family under *Colletotrichum truncatum* stress conditions in hot pepper

R. Srideepthi\(^1\) · M. S. R. Krishna\(^1\) · P. Suneetha\(^2\) · R. Sai Krishna\(^3\) · S. Karthikeyan\(^1\)

Received: 9 October 2019 / Accepted: 8 September 2020 / Published online: 12 September 2020
© Springer Nature Switzerland AG 2020

**Abstract**

Receptor like kinases (RLKs) are preserved upstream signaling molecules which regulate several biological processes from plant development to various stress adaptation programs. Non arginine aspartate (non-RD) a prominent class of RLKs plays a significant role in disease resistance and apoptosis in plants. In present investigation, a comprehensive in silico analysis for non-RD Kinase gene family as well as identification of gene structures, sequence similarity, chromosomal localization, gene duplication analysis, promoter analysis, transcript expression profiles and phylogenic studies were done. In this study, twenty-six genes were observed on nine out of twelve chromosomes. All these genes were clustered into five subfamilies under large monophyletic group termed as Interleukin-1 Receptor-Associated Kinase (IRAK) family. Some of the important physiochemical properties of twenty-six proteins are determined and ranged in the following order: (a) Amino acids size ranged from (620 to 1781) (b) Molecular weight ranged as of (70.11 to 197.11 KDa) and (c) Theoretical PI ranged from (5.69 to 8.63) respectively. Structural diversity in genomic structure among non-RD kinase gene family was identified and presence of pathogen induced *cis* regulatory elements including STRE, MYC, MYB, and W box were found. Expression profiles revealed the potential ability of three genes CaRLK1 from LRRXII and CaRLK15,16 from stress antifung subfamily were pointedly upregulated beyond the severe stress time period (9 DAI) in anthracnose resistant genotype PBC-80 in response to *Colletotrichum truncatum* infection. Subsequently, in silico studies from the available genome sequencing data helped us to identify candidate genes tangled in inducing disease resistance.

**Keywords** Pattern recognition receptors (PRRs) · Auto phosphorylation · Downstream signaling · *Colletotrichum truncatum* · Defense responses

**Introduction**

One of the major challenges in the twenty-first century, to worldwide food security and agricultural sustainability, is to develop economically important high yielding varieties that are stable with broad-spectrum of resistance. Hot pepper is a commercially important vegetable crop grown worldwide for its indispensable nutritional and therapeutic values, yet the annual production is low owing to numerous biotic and abiotic stresses (Ridzuan et al. 2018). Among various biotic stresses, anthracnose is one the utmost devastating fungal diseases hot pepper. This disease is characterized by sunken necrotic lesions all over the pod which are observed both at pre and post harvesting stages. This disease is instigated by *Colletotrichum* species and results in huge yield loss of about 20 to 70% every year (Roy et al. 2019). This fungal disease spreads very rapidly during moist-humid conditions and the protection measures are very meager. Existence of morphological and genetic, variability within the *Colletotrichum truncatum* species and its erratic pathogenic ability to infect different hosts had made it a herculean task to sort solution to these problems. Moreover, variations in climatic conditions and usage of commercial pesticides remained as...
an unsatisfactory measure for its effective control (Saxena et al. 2016). In this scenario, development of anthracnose resistant hot pepper remained as one of the major challenges for agriculture researchers worldwide. However, knowledge in understanding the defensive signaling mechanisms in resistant plants to encounter the pathogens by advanced molecular and computational techniques paved a ray of hope to address these challenges (Roy et al. 2019).

RLKs are surface localized receptor like protein kinases employed by Pattern Recognition Receptor (PRR) proteins of plants innate immune system as a primary defensive response (Saijo et al. 2018). These are involved in signal perception from pathogen by ectodomain, then transduction of signal by transmembrane region and then activation/deactivation of signaling cascade by kinase domain. Classification of extracellular domain was done based on type of ligand binding specificities (Haffani et al. 2004) and intracellular domain based on presence/absence of conserved arginine residues present preceding to aspartate in catalytic domain VI of kinase domain (Dardick and Ronald 2006). Among various types of RLKs, predominantly non-RD RLKs were allied with native immune receptors that identify conserved microbial signatures and activate pattern triggered immunity (PTI) tangled in disease resistance (Dardick et al. 2012). In silico studies of these conserved signaling molecules holds dynamic importance for identification of resistant genes in various plants. So far, 35 genes in arabidopsis and 328 genes in rice were identified to possess non-RD class of kinase receptor proteins. Functionally characterized non-RD kinases like, XA21, BSR1 and XA26 from Oryza sativa and FLS2, EFR from Arabidopsis thaliana were known to be effective against bacteria (Zipfel et al. 2006), whereas, Pi-D2 gene of lectin (non-RD) kinase was found to express broad spectrum resistance against fungus (Chen et al. 2006). LecRK-VI.2 gene from Arabidopsis thaliana (Singh et al. 2012) and WRKY protein from Lycopersicum esculentum serve as a potential link in providing resistance in contradiction of bacterial and fungal pathogens (Bai et al. 2018). However, due to inadequate literature on non-RD kinases in hot pepper, efforts were made through this study to identify candidate non-RD genes that are associated with disease resistance by comparative genomics analysis. Further characterization and validation of these genes would be a pioneering step for future crop improvement programs.

Materials and methods

Identification of non-RD RLK genes in hot pepper

The annotated non-RD RLKs from sequenced plant genomes like Arabidopsis and tomato (Sakamoto et al. 2012) were collected as query sequences from the NCBI and protein database, and were fed to a BLAST search against the hot pepper CM-334 variety genome datasets within version 1.55 of Capsicum annuum (https://www.solgenomics.net). The hits share showing 50% similarity to the selected Arabidopsis and tomato sequences were prioritized and further screened for the presence of typical Receptor like kinase domains (Delteil et al. 2016) like signal peptide through signal P 4.0 server (https://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011). Ligand-binding ectodomain, transmembrane, and intracellular kinase domain were further searched in the selected sequences with the SMART (https://smart.embl-heidelberg.de/) (Letunic et al. 2011) and Pfam libraries through HMMER (Bateman et al. 2002). For sequences encoding the kinase domain, evolutionarily conserved non-RD kinase motifs were subsequently localized with the multiple sequence comparison through log- expectation (MUSCLE) alignment using MEME (https://meme-suite.org/index.html) (Bailey et al. 2009). Naming to each gene was specified with the first two letters indicating Capsicum annuum (Ca) and the third letter representing the non-RD class of transmembrane receptor-like kinase (RLK) family followed by Arabic numerals in serial (Qin et al. 2018).

Phylogenetic analysis of non-RD RLK genes

To establish an evolutionary relationship among the annotated/predicted CaRLK proteins, the IQTree server (https://iqt.tree.cibiv.univie.ac.at/) was deployed using the gamma-model of the free-rate heterogeneity (Trifinopoulos et al 2016). Deploying the ultrafast bootstrap methodology for 10,000 iterative cycles over 10,000 bootstrap alignments and considering a minimum correlation coefficient of 0.9 as the convergence criterion along with the other default parameters. Phylogenetic tree was constructed for the same. The iTol server (https://itol.embl.de/) was further used to visualize and represent the consensus evolutionary tree (Letunic et al. 2011).

Physiochemical properties of non-RD RLK genes

Physiochemical parameters of putative CaRLK gene including molecular weight, isoelectric point, number of amino acids, aliphatic index, and grand average of hydropathicity (GRAVY) score was determined using online ExPASy programs (https://www.expasy.org/). Subcellular locations of CaRLK proteins were predicted using the online web server tool Plant-PLoc (https://www.csbio.sjtu.edu.cn/bioinf/plant-multi/).
Chromosomal locations and duplications of non-RD RLK genes

Information regarding the position and location of chromosomes on which CaRLK gene members were present was derived from the hot Pepper Genome Platform (PGP) (https://peppergenome.snu.ac.kr/). Genes were mapped onto chromosomes at their genomic position and were drawn manually. Duplicated genes were identified by Blast P search against each other when both identity and query coverage was > 80% of their partner sequence. Tandem duplication in genes was identified by occurrence of homologous genes located in single region (< 100 kb) within a chromosome, while segmental duplication occurred among homologous or non-homologous genes with > 1 kb in length and more than 90% sequence similarity dispersed but present on the same or different chromosomes from the same clade as described by Feng et al. (2017). Subsequently, non-synonymous (Ka) and synonymous substitution (Ks) rate ratio calculation in hot pepper among duplicated CaRLK gene pairs using online web server PAL2NAL (https://www.bork.embl.de/pal2nal/). The divergence time of the duplicated gene pairs were calculated as described by Koch et al. (2000).

Gene structure and Cis-regulatory element analysis

Gene structure was elucidated based on the relationship of the coding sequence and its corresponding genomic DNA sequence by GSDS 2.0 (https://gsds.cbi.pku.edu.cn/). Cis-acting regulatory elements of genomic DNA sequences of 3000 bp 5′ upstream region was mined from the Sol Genomics Network database. Promoter sequences obtained were submitted in (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).online server of Plant Care Database Following Diao et al. (2018) Conserved biotic-stress responsive elements in hot pepper were predicted.

Primer design

Genomic and its Coding sequences (CDS) of deduced RLK hot pepper proteins were retrieved from Sol Genomics Network (https://solgenomics.net/). Primer sets for RT-qPCR were designed in 3 and 5 untranslated regions of individual genes to avoid non-specific amplification using Prime Quest Tool as described by Barzana et al. (2014) (https://eu.idtdna.com/PrimerQuest/Home/Index). For all primers the Ubiquitin 3 was used as a reference gene. Genes with accession numbers and code assigned in this study were given in Supplementary Table S) with full details.

Plant material, fungal strains and stress treatments

Seeds of two hot pepper genotypes PBC-80 (anthracnose resistant) from National Bureau of Plant and Genetic Resources (NBPG), Hyderabad and Pusa Jwala (anthracnose susceptible) from Horticulture Research Station, Lam Farm, Guntur, Andhra Pradesh were used in the study. Seeds were scattered in black trays containing autoclaved blend of peat and vermiculite (2:1 v/v) along with micronutrients. Seedlings were raised and watered regularly in greenhouse under controlled conditions i.e., 16 h light/8 h dark photo-period at 27 °C throughout the day and 21 °C during night. Fungi were isolated from fruit rot infected hot pepper sample by single spore isolation technique. Isolate Colletotrichum truncatum was cultured on Oat meal agar medium with pH 7.0 at 25 ± 2 °C. Spore suspension was prepared from seven days old culture and sprayed on seedlings by artificial inoculation method as described by Mishra et al (2017) with 5 × 10^5 spores/ml concentration. Three-week-old hot pepper seedlings were taken for experimental studies. Genotypes PBC-80 and Pusa Jwala sprayed with conidial suspension were considered as test samples and those trays sprayed with autoclaved distilled water were treated as control. Each treatment was maintained with three replications.

RNA extraction

Leaf tissue was used as a source for extraction of RNA from stressed and control genotypes as per acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 2006). Hundred milligrams of leaf tissue were made into fine powder in mortar and pestle using liquid nitrogen. Fine homogenous powder was transferred carefully into 2 ml Eppendorf tubes with extraction buffer and centrifuged at 12,000 rpm. Supernatant was collected and equal aliquots of chloroform was added to it and centrifuged for 10 min at 12,000 rpm. To upper aqueous layer ice cold propanol and 1.5 M NaCl was supplemented and incubated at 4 °C for 10 min. Supernatant was collected and equal aliquots of chloroform was added to it and centrifuged for 10 min at 12,000 rpm. To upper aqueous layer ice cold propanol and 1.5 M NaCl was supplemented and incubated at 4 °C for 5 min. Then tubes with solution were allowed to centrifuge for 10 min at 12,000 rpm and supernatant was discarded. Resultant pellet was subjected to ethanol wash and allowed for air drying. Pellet was dissolved in DEPC water. Samples of RNA were rinsed with RNase-free water called DNase-I (Takara- cat # 2270B) to remove any residual genomic DNA. Purity of RNA was determined by calculating A260/A230 and A260/280 ratio of absorbance. RNA was visualized in 1.5% agarose gel after electrophoresis.

Quantitative real time PCR

Complementary DNA (cDNA) was produced from prime script 1st strand cDNA synthesis kit (Takara-cat # 6110A),
in triplicates from three µg of RNA extracted previously. Expression of genes was scrutinized in reverse transcription quantitative polymerase chain reaction (RT-qPCR) manufactured by QuantStudio3-Applied Bio systems. Twelve micro liter reaction mixture constituted with Syber Green (6 µl), cDNA (2 µl), Forward Primer (1 µl), Reverse Primer (1 µl) and DEPC water (2 µl) was loaded in 96-well reaction plate enclosed by MicroAmp Optical Adhesive Film supplied by Applied Bio systems. Essential cycling parameters and baseline thresholds were set manually while UBI-3 was used as a house keeping gene. CT values were determined by using intended software. Ability of RT-qPCR reaction for every single CaRLK gene was deliberated by standard curve attained by serial dilutions of collective cDNA. Calculation for relative expressions of individual sample was done using 2-DDCT method (Livak and Schmittgen 2001). Estimated level of gene expression relay purely on 100% PCR efficiency with respect to reference and target genes. Particularly 2% agarose gels were used to visualize RT-qPCR products. A separate band with predicted amplicon size was considered to be specific amplification (Hachez et al. 2006).

Statistical analysis

Relative gene expression studies with respect to four samples (PBC-80 control, PBC-80 stressed, Pusa Jwala control and Pusa Jwala stressed) were carried out with three biological replicates, each with two technical replicates retained at each point of time intervals. Data was represented as mean ± standard deviation. Significance among the resistant and susceptible genotypes were analyzed using analysis of variance (ANOVA) at 5% probability. Furthermore, post hoc test to compare mean separation of relative expression values, Fishers Least significance difference (LSD) was performed. Moreover, relative gene expression was envisaged using heat mapper (https://heatmapper.ca/) software (Babicki et al. 2016).

Results

Identification and annotation of non-RD RLK genes in hot pepper

Among 35 and 72 identified non-RD sequences from Arabidopsis (Dardick and Ronald 2006) and Tomato (Sakamoto et al 2012) only 24 and 31 genes possessed typical RLK domain organization from IRAK family were selected for the study. These known 55 sequences were used as query and subjected to Blast P homology searches with entire CM334 cultivar of pepper genome database. Homology searches revealed 2,705 hits, among them 1,251 hot pepper sequences were found to share more than 50 percent homology (He 2015). Non redundant sequences within 500–2000 amino acids sequence length were considered for further study while remaining redundant sequences were removed manually. A total of 138 putative sequences deduced from the pepper genome database were then subjected to multiple sequence alignment and checked manually for the presence of typical non-RD RLK conserved residues.

Identification of conserved residues and motifs in non-RD RLK genes

Multiple sequence alignment revealed only twenty-six among 138 protein sequences to possess conserved residues in specific kinase domains. Alignment of all these 26 protein sequences was given in Supplementary Fig. S1. Substitution of arginine with any one of these Leucine, cysteine, phenylalanine, Glycine, or Serine residues adjacent to aspartic acid (D) in kinase subdomain VI along with the presence of conserved lysine (K) residue and aspartic acid (D) in subdomain II and VII (Dardick and Ronald 2006). Presence of conserved residues was tabulated in Supplementary Table S2. Graphical representation of highly conserved amino acids at successive positions was depicted by sequence logos in Fig. 1. Possible number of amino acids at each position was represented in stack of letters. Size of the letter indicates the frequency of amino acid occurring at each position. Height of the letter indicates the mode of conservation present at that position. Information about total stack heights shows conservation and was measured in bit score where maximum score indicates completely conserved position and minimum to be completely random position.

Phylogenetic analysis of non-RD RLK genes in hot pepper

The selected dataset of 81 query and non-RD sequences were constructed for the three plant species (Arabidopsis, Tomato and Hot pepper) and was used to construct a phylogenetic tree. The deployed methodology yielded an evolutionary solution with the log-likelihood score and the total tree length was found to be −117,315.807 and 48.303 respectively. Three plant species Arabidopsis, Tomato and hot pepper were represented as AT, Solyc and CaRLK structure. The sequences were found to be clustered into five groups with eight subfamilies marked in specific colors. Among them major part of the tree was erected with LRR family members and clustered into group 1. At this juncture genes from diverse subfamily LRRXII, LRRXI, LRRII and LRRIII were clustered under same group from all three species appearing to be paraphyletic with a common ancestor.
Predominantly subfamily LRR-XII was comprised of 36 sequences (AT-7, Solyc-15 and CaRLK-14) represented in pink color. While a mixture of 6 sequences from subfamily LRR-XI with 2 sequences (AT-2) marked in Grey, LRR-III with three sequences (AT-2, Solyc-1) in Green and LRR-II with 1 sequence (AT-1) in Orange color as shown in Fig. 2 respectively. Consequentially 3 protein sequences (CaRLK-2, Solyc-1) from WAK_Like subfamily in violet color in group II and 2 sequences from stress-antifung subfamily particularly in hot pepper were bunched into group III and are represented in yellow color. Moreover 14 sequences (AT-7, Solyc-4, and CaRLK-3) from LRK10L2 subfamily and 20 sequences (AT-5, Solyc-10, and CaRLK-5) from SD2b subfamily were bunched into group IV and V visualized in red and blue color respectively.

**Physiochemical properties of non-RD RLK genes in hot pepper**

Non-RD receptor like protein kinases vary significantly with respect to their structural and physical properties. Genes CaRLK6 with highest number (1781) of amino acids and CaRLK25 with lowest number of amino acids (620) was observed while CaRLK 6 with high molecular weight (197.11 kDa) and CaRLK 23 with low molecular weight (70.11 kDa) respectively. Consequently, a wide variation was observed in their isoelectric point (PI) ranging from 5.69 (CaRLK14) to 8.63 (CaRLK10). Instability index ranged from (28.84 to 46.15) where 19 genes were considered to be stable as they exhibit instability index value less than 40, whereas the rest 7 among 26 non-RD RLKs were considered as unstable. Grand average hydrophaticity (Gravy) values ranged from −0.345 (CaRLK1)
to + 0.12 (CaRLK10) inferring the presence of both hydrophilic and hydrophobic amino acids. All 26 proteins exhibited aliphatic index more than 40 indicating that all are thermally stable with more number of hydrophobic amino acids in their structure. Among them CaRLK19 with lowest of 79.07 and CaRLK7 with highest of 110.0 index value was found. Most of them were found to be localized in extracellular spaces of cell membrane followed by chloroplast, nucleus and mitochondria. Characteristics of each non-RD proteins were given at Supplementary Table S3.

Chromosomal localization of non-RD RLK genes in hot pepper

The inherent ability of a host to defend against pathogens mostly depends on the occurrence of resistance genes and activation of their signaling mechanism present on chromosomes. A total of twenty-six putative genes belongs to the non-RD class were aligned in the 9 out of 12 chromosomes (Fig. 3). Chromosomes 3, 9 and 10 were devoid of non-RD genes, while in contrast a maximum number of seven genes...
were observed on chromosome 2. Thirteen genes—CaRLK 9, 11, 12, 13 on second, CaRLK 6, 7 on fourth, CaRLK 2, 8, 10 on sixth while CaRLK 3, 4, 5 on fifth and CaRLK 1 on the eleventh chromosome belongs to LRRXII subfamily. Five non-RD genes CaRLK 17, 18, 20, and 19 from the same subfamily SD2b were aligned on diverse chromosomes 7, 8, and 1. While in contrast, two genes CaRLK 25, 26 from WAK family on eleventh chromosome and two genes CaRLK 15, 16 from CRK family on second chromosome were dispersed.

**Duplication analysis of CaRLK genes in hot pepper**

Evolution of gene duplication reveals family expansion and occurrence of novel genes in a genome. Two pairs of genes showed tandem duplication viz., CaRLK 25/26 and CaRLK 9/12. While, only one paralog pair CaRLK 15/16 from CRLK was identified to exhibit segmental duplication. Details of duplication pairs were given in Supplementary Table S4. Genes with Ka/Ks ratios < 1.0 may experience purifying selection pressure and genes with Ka/Ks ratios > 1.0 exhibits positive mode of selection. Divergence time of non-RD
genes exposes the duplication events started from 5.40 Mya and continued up to 5.86 Mya in evolution.

**Gene structure analysis of non-RD RLK Genes in hot pepper**

Gene length varied from 2,224 bp (CaRLK 24) to 21,556 bp (CaRLK 6). Moreover, genes with either a positive or negative sense strand as a template to the coding regions are depicted in Supplementary Table S3. Various exon–intron positions were compared to gain insight into possible mechanisms of structural diversity existing among non-RD kinases in *Capsicum annuum* (Fig. 4). In this study, introns varied from 0 to 12 in number. CaRLK 6 gene from the LRRXII family showed a maximum of 12 introns. While in contrast a total of four genes (CaRLK 17, 18, 20 and 21) from G-type lectin and single gene CaRLK 13 from LRR type family were found to occur without introns in their structure. Genes from the same CRK subfamily members showed similar intron organization. While contrarily group I followers from LRRXII subfamily exposed varied introns organization like ten genes (CaRLK 1, 2, 3, 4, 5, 8, 9, 14, 19 and 23) with single intron. Five genes (CaRLK 10, 11, 12, 22 and 24) with two introns and three genes (CaRLK 7, 25, 26) with three introns were observed in their structural organization.

**Cis-regulatory element analysis of non-RD RLK genes in hot pepper**

*Cis*-regulatory element analysis promotes a good insight to understand the expression patterns of a gene under various stress conditions, whose validation needs to be warranted. Major pathogen-induced *cis*-regulatory elements identified in hot pepper (Fig. 5). Among twenty-six non-RD genes, the highest number of *cis*-regulatory elements (TGACG, STRE) known to be involved in defense and stress responses were found in CaRLK 3, 11 from LRR and CaRLK 16 from CRK subfamily. Fungal elicitor and oxidative responsive *cis* regulatory elements viz., W-box, F-box, As1 and box4 were observed majorly in CaRLK 1, 7, 8 from LRR-XII subfamily and CaRLK 22 from LRRK10L2 and CaRLK 26 from WAKLRK10L1 subfamily respectively. Correspondingly, elicitor responsive element G-box and Abscisic acid signifying region ABRE were observed in abundance among promoter regions of CaRLK3, 15, 21 and 24 genes. Whereas, Myb and Myc binding sites responsible for triggering stress-responsive metabolic pathways were found to occur predominately in CaRLK19 and CaRLK 1. Moreover, only 32 percent of genes showed the presence of TC-rich repeat and TCA elements linked with salicylic acid and methyl jasmonic acid pathway. When compared to all genes CaRLK1 from LRR family and CaRLK16 from CRK family were identified to hold more number of *cis*-regulatory elements and may associate intensely with defense responsive mechanism.

**Heat map analysis**

Graphical representation of non-RD RLK genes relative expression data congregated from RT-qPCR from hot pepper was used to generate heat map by complete linkage hierarchical clustering method as shown in Fig. 6.

**Expression profiles of CaRLK genes at various stress treatments**

In this investigation, we had analyzed differential expression patterns of particular gene during various temporal stages of disease progression. During various infection stages of *Colletotrichum truncatum* like spore adhesion, germination...
and penetration by appresorium (24hai), expression levels of CaRLK genes was increased in both the resistant and susceptible genotype with respect to the control. Later during subcuticular colonization of hyphae (48hai), among 26 genes only eighteen genes in PBC-80 showed upregulation as follows CaRLK1,18,21,23 genes with fourfold increase, CaRLK 3,11,15,22 genes with threefold change, CaRLK 2,4,5,14,16 and 24 genes with two fold change while CaRLK 9,7,10,12 genes with 1.79 to 0.56 fold change in their expression. While only six genes showed a significant down regulation of 2.18 to 0.63 dropped fold change in the expression. Nevertheless, a total of sixteen genes showed upregulation from 0.4 to 2.6 fold change in their expression and ten genes showed decrease in their expression levels, from 1.6 to 0.3 fold change was observed in Pusa Jwala with respect to control. At mycelial aggregation stage of 72 Hai, a greater number of genes were upregulated in both genotypes. Nineteen genes from PBC-80 within LRR, CRK and WAK family members displayed their strength of expression from 5.0 to 0.5 fold change, whereas five genes from same families showed significant decrease in their expression with 0.5 to 2.5 fold change. Subsequently eleven genes exhibited increase in their expression with 11 a fold change value from 1.67 to 0.2 fold, while thirteen genes showed significant down regulation from 1.6 to 0.2 fold change respectively in Pusa Jwala. At acervuli formation stage of infection (148 hai), a total of 22 CaRLK genes showed upregulation varying from 5.34 to 0.23 fold change increase in PBC-80 while only two genes CaRLK 5,10,11,13 from LRR family showed a down regulation of 3.42 to 0.34 fold. In susceptible genotype while 21 CaRLK genes showed a nominal decrease of 1.6 to 0.3 fold change in their expression while particularly five CaRLK genes 7,16,15, 23 and 24 showed an increase of 0.6 to 1.8 fold change respectively. During conidial dispersion stage of infection (216 hai), in PBC-80 CaRLK 10 showed highest level of expression with fivefold change followed by CaRLK 3,4,14 with four fold, CaRLK 1,6,15,18, 21, 23 with three fold,CaRLK2,5,16,24, 26 with twofold and CaRLK 12,17 with one fold increase when compared to that of control, while in susceptible variety highest expression was shown by gene CaRLK 23 with one fold increase followed by CaRLK 16 with 0.72 folds while CaRLK 17,4 with 0.4 folds and CaRLK 15 with 0.3 folds of lowest expression. Moreover, among twenty-six genes only eight genes showed significant reduction from 2.03 to 0.63 fold change in their expression of PBC-80 with respect to control. Contrarily twenty-one genes showed lowest fold change ranging from 2.5 to 0.3 fold decrease in Pusa Jwala. Detailed information

**Fig. 6** Hierarchical clustering and heat map representation displaying the expression profiles of twenty-eight non-RD Receptor like kinase genes under *Colletotrichum truncatum* stress conditions during a time course of infection. a Genotype PBC 80 (Resistant). b Genotype Pusa Jwala (Susceptible). The color scale represents Log2 expression. Yellow, blue and white boxes indicate high, medium and low level of expression.
of twenty-six genes relative level expressions were given in detail for resistant variety in Fig. 7a, b and susceptible variety in Fig. 8a, b respectively.

**Discussion**

Receptor Like Kinases play a vital role in signal transduction, defense responses and plant development (Afzal et al. 2008). Availability of sequenced genomes had facilitated the researchers to study various functional roles of RLK family genes in various stress adaptation procedures in many model plants like rice, arabidopsis, tobacco, wheat, tomato, soybean etc., (Passricha et al. 2019). Most of the candidate RLK genes involved in primary immune responses associated with disease resistance belong to non-RD class of Receptor Like Kinases. Present investigation was aimed at genome wide identification of non-RD kinases in hot pepper a vegetable crop with global cultivated and economic importance, whose production has been hindered by several biotic stresses (Saxena et al. 2016). A total of 8 and 35% of non-RD motifs were identified among IRAK family members in rice and Arabidopsis (Dardick et al. 2012). This huge difference may be due to monocot-dicot diversification. Comparative phylogeny of hot pepper with model plants revealed the evolutionary existence of five subfamilies from non-RD class of RLK gene family. Moreover, CaRLK genes were more strictly linked to genes from Arabidopsis and tomato, recollecting the truth that Arabidopsis, pepper and tomato were eudicots and diverged more recently on or after a common ancestor (Li et al. 2013).

Gene duplication events majorly include segmental, tandem and whole-genome duplications with substantial roles occurred in the evolution (Xu et al. 2012). In hot pepper among 26 genes, two pairs from chromosome 2 and 11 unveiled tandem duplications within LRR and WAK families. These two pairs of genes were evolved from common ancestor LRRXII family. Tandem duplication may signify LRRXII family lineage specific expansion with novel gene expression. Whereas Hofberger et al. (2015) reported lineage specific expansion of L-type lectin receptor like kinase gene family by tandem duplication event in brassicaceae. While
only one pair from stress-antifung subfamily of G-type lectin showed segmental duplication in second chromosome. Segmental duplication majorly contributes with gene expression and play a significant role in immunity, growth and defense responses to external stimuli (Feng et al. 2017). Results were in accordance with Cannon et al. (2004) who reported negative correlation between tandem and segmental duplication within Arabidopsis gene families. Furthermore, non-synonymous (Ka) and synonymous (Ks) mutations substitution rate was assessed to evaluate the mode of selection and divergence time succeeded among the duplicated CaRLK gene pairs (Table S1). LRR and WAK gene families shaped by natural selection in hot pepper may hold extra functional members allied with speciation or adaptation. While single gene pair G-type lectin family showed positive selection and may be involved in functional diversification as described by Huang et al. (2016).

Introns execute a major role in developmental and cellular processes through alternate splicing otherwise gene expression regulation (Wang et al. 2015). Five genes devoid of introns were found in hot pepper, likewise Yang et al. (2009) also reported presence of intron less genes in taxonomic species like Arabidopsis thaliana, Populus deltoides and Oryza sativa representing their lineage specific expansion with specific function in the evolution. Eleven non-RD genes were observed with single intron in Capsicum annuum. Xu et al. (2017) also reported the presence of fifteen single intron genes in Populus deltoides with multiple functions. Genes present in a family usually contain same structural organization but conversely some genes from SD2b, LRK10L2, and LRRXII in hot pepper showed varied intron exon organization. These variations may be due to substituted residues in conserved positions depicting the evolutionary changes occurring within a family (Liu et al. 2018).

---

**Fig. 8** Expression levels of CaRLK genes in response to Colletotrichum truncatum stress within various time intervals. The data a, b represents the mean ± SD each from three biologically independent experiments conducted in susceptible genotype. Statistical significance was analyzed using one-way ANOVA. The asterisks represent significance difference (*p* < 0.05)
positive regulators and OsWAK112d as negative regulator three genes (OsWAK91, OsWAK14 and OsWAK92) act as infection. Congruently in rice among four WAK members, upregulation in PBC-80 genotype at necrotrophic phase of regulation, whereas gene CaRLK 22, 25 had showed significant down regulation whereas gene CaRLK 4, 17 and 23 remained up regulated while rest of twenty-one genes, thirteen from LRR family (CaRLK 1,2,3,5,6,7,8,9,10,11,12,13,14), four from G-type lectin (18,19,20,21) and four from WAK and its associated family members (CaRLK-22,24,25,26) exhibited downregulation in all stages of infection from hyphal penetration to acervulli formation.

Presence of cis regulatory elements like TGACG (MeJA responsive), ERE (Ethylene stress responses) STRE, MYB and MYC (defense responsive) in promoter region which are majorly involved in JA-ET pathway may be responsible for providing resistance. Chowdhury et al. (2017) reported an increase in ethylene (ET), Jasmonic Acid (JA) hormone mediated signaling pathways during biotrophic and necrotrophic phase of Colletotrichum infection was responsible for governing disease resistance in sesame. Consistently two genes CaRLK-15, 16 belonging to CRK family has shown upregulation in both resistant and susceptible genotypes under severe stress conditions. CaRLK 1 gene from LRRXII subfamily showed significant upregulation of four folds higher expression levels than that of susceptible genotype. In susceptible genotype, among 26 genes only three genes CaRLK 4, 17 and 23 remained up regulated while rest of twenty-one genes, thirteen from LRR family (CaRLK 1,2,3,5,6,7,8,9,10,11,12,13,14), four from G-type lectin (18,19,20,21) and four from WAK and its associated family members (CaRLK-22,24,25,26) exhibited downregulation in all stages of infection from hyphal penetration to acervulli formation.

Presence of cis regulatory elements like TGACG (MeJA responsive), ERE (Ethylene stress responses) STRE, MYB and MYC (defense responsive) in promoter region which are majorly involved in JA-ET pathway may be responsible for providing resistance. Chowdhury et al. (2017) reported an increase in ethylene (ET), Jasmonic Acid (JA) hormone mediated signaling pathways during biotrophic and necrotrophic phase of Colletotrichum infection was responsible for governing disease resistance in sesame. Consistently two genes CaRLK-17, 18 were confined with more cis regulatory elements like TCA element involved in salicylic acid regulation had showed significant up regulation during hyphal colonization and penetration. Qi et al. (2012) also described the role of SA in hyphal growth and basal defense response. In present investigation among five non-RD WAK members two genes CaRLK 22, 25 had showed significant down regulation, whereas gene CaRLK 23, 24, 26 had showed upregulation in PBC-80 genotype at necrotrophic phase of infection. Congruently in rice among four WAK members, three genes (OsWAK91, OsWAK14 and OsWAK92) act as positive regulators and OsWAK112d as negative regulator while providing quantitative resistance against Magnaporthe oryzae Expression studies by RT-qPCR were used to identify candidate genes / functional markers majorly involved in crop improvement programs.

Conclusion

Classification of non-RD kinase gene family in hot pepper would facilitate the identification of crucial genes tangled in the disease resistance. As per our knowledge, this is the primary report on genome-wide identification, characterization and expression profiling of non-RD kinase gene family in hot pepper. The current work was organised systematically to identify and characterize 26 CaRLK genes, by means of bioinformatics tools and deliberate expression analyses in response to Colletotrichum truncatum stress conditions. After nine days of infection one gene (CaRLK1) from LRRXII subfamily out of 26 non-RD genes was found to be expressed more in resistant genotype (PBC-80) than the susceptible genotype (Pusa Jwala). In addition, two genes (CaRLK-15, CaRLK 16) from stress-antifungal subfamily revealed their potential ability with significant upregulation in both the genotypes under stress conditions. Moreover, documentation of cis-regulatory elements and their role in conferring resistance enabled us to understand the role of activation of JA-ET signaling pathway in PBC-80 Genotype. Therefore, this comprehensive analysis serves as a central platform to understand various physiological and biochemical functions executed by the CaRLK1, CaRLK15 and CaRLK 16 genes in providing resistance. Further characterization of these genes and their validation might be used for the development of functional markers associated with disease resistance in hot pepper.

Acknowledgement Authors were thankful to Head of the department and Co-scholars of bioinformatics lab for providing facilities to carry out the work.

Compliance with ethical standards

Conflict of interest Authors do not have any conflict of interest to declare.

References

Afzal AJ, Wood AJ, Lightfoot DA (2008) Plant receptor-like serine threonine kinases: roles in signaling and plant defense. Mol Plant-Microbe Interact 21:507–517. https://doi.org/10.1094/MPMI-21-5-0507

Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, Wishart DS (2016) Heatmapper: web-enabled heat mapping for
Hofberger JA, Nsibo DL, Govers F, Bouwmeester K, Schranz ME (2015) A complex interplay of tandem-and whole-genome duplication drives expansion of the L-type lectin receptor kinase gene family in the brassicaceae. Genome Biol Evol 7:720–734. https://doi.org/10.1093/gbe/evv020

Huang BH, Chen YW, Huang CL, Gao J, Liao PC (2016) Imbalanced positive selection maintains the functional divergence of duplicated DIHYDROKAEMPFEROL 4-REDUCTASE genes. Sci Rep 6:39031. https://doi.org/10.1038/srep39031

He S (2015) Genome-wide identification and transcriptional expression analysis of mitogen-activated protein kinase and mitogen-activated protein kinase genes in Capsicum annuum. Front Plant Sci 6:780. https://doi.org/10.3389/fpls.2015.00780

Jin B, Sheng Z, Muhammad I, Chen J, Yang H (2019) Cloning and functional analysis of the promoter of a stress-inducible gene (Zmap) in maize. PLoS ONE 14:e0211941. https://doi.org/10.1371/journal.pone.0211941

Kishimoto K, Kozunai Y, Kaku H, Shibuya N, Nishizawa Y (2010) Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus Magnaporthe oryzae in rice. Plant J 64:343–354. https://doi.org/10.1111/j.1365-313X.2010.04328.x

Koch MA, Haubold B, Mitchell-Olds T (2000) Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in Arabidopsis, Arabis, and related genera (Brassicaceae). Mol Biol Evol 17:1263–1249. https://doi.org/10.1093/oxfordjournals.molbev.a026248

Kong W, Ding L, Cheng J, Wang B (2018) Identification and expression analysis of genes with pathogen-inducible cis-regulatory elements in the promoter regions in Orzya sativa. Rice 11:52. https://doi.org/10.1186/s12284-018-0243-0

Letunic I, Doerks T, Bork P (2011) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res 40:302–305. https://doi.org/10.1093/nar/gkr931

Passricha N, Saiﬁ SK, Singh R, Kharb P, Tuteja N (2019) Receptor-like kinases control the development, stress response, and senescence in plants. Senescence signalling and control in plants. Academic Press, New York, pp 199–210

Li J, Hou H, Li X, Xiang J, Yin X, Gao H, Zheng Y, Bassett CL, Wang X (2013) Genome-wide identiﬁcation and analysis of the SBP-box family genes in apple (Malus×domestica Borkh). Plant Physiol Biochem 70:100–114. https://doi.org/10.1016/j.plaphy.2013.05.021

Liu PL, Huang Y, Shi PH, Yu M, Xie JB, Xie L (2018) Duplication and diversiﬁcation of lectin receptor-like genes (LecRLK) genes in soybean. Sci Rep 8:5861. https://doi.org/10.1038/s41598-018-24266-6

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−DDCT method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Mistra R, Nanda S, Rout E, Chand SK, Mohanty JN, Joshi RK (2017) Differential expression of defense-related genes in chilli pepper infected with anthracnose pathogen Colletotrichum truncatum. Physiol Mol Plant Pathol 97:1–10. https://doi.org/10.1016/j.pmpp.2016.11.001

Petersen TN, Brunak S, Von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785. https://doi.org/10.1038/nmeth.1701

Qi PF, Johnston A, Balcerzak M, Rocheleau H, Harris LJ, Long XY, Wei YM, Zheng YL, Ouellet T (2012) Effect of salicylic acid on Fusarium graminearum, the major causal agent of fusarium head blight in wheat. Fungal Biol 116:413–426. https://doi.org/10.1016/j.funbio.2012.01.001

Qin L, Mo N, Muhammad T, Liang Y (2018) Genome-wide analysis of DCL, AGO, and RDR gene families in Hot pepper (Capsicum
annuum L.). Int. J. Mol Sci 19:1038. https://doi.org/10.3390/ijms19041038

Ridzuan R, Rafii M, Ismail S, Mohammad Yusoff M, Miah G, Usman M (2018) Breeding for anthracnose disease resistance in chili: progress and prospects. Int J Mol Sci 19:3122. https://doi.org/10.3390/ijms19103122

Roy CB, Liu H, Rajamani A, Saha T (2019) Transcriptome profiling reveals genetic basis of disease resistance against Corynespora cassicola in rubber tree (Hevea brasiliensis). Curr Plant Biol 17:2–16

Raj TS, Christopher DJ (2009) Effect of bio-control agents and fungicides against Colletotrichum capsici causing fruit rot of chilli. Ann Plant Protect Sci 17:143–145. https://doi.org/10.5897/AJP2013.13558

Saijo Y, Loo EPI, Yasuda S (2018) Pattern recognition receptors and signaling in plant–microbe interactions. Plant J 39:592–613. https://doi.org/10.1111/tpj.13808

Sakamoto T, Deguchi M, Brustolini OJ, Santos AA, Silva FF, Fontes EP (2012) The tomato RLK superfamily: phylogeny and functional predictions about the role of the LRR-RLK subfamily in antiviral defense. BMC Plant Biol 12(1):229

Saxena A, Raghuwanshi R, Singh HB (2018) Molecular, phenotypic and pathogenic variability in Colletotrichum isolates of sub-tropical region in north-eastern India, causing fruit rot of chilies. J Appl Microbiol 117:1422–1434. https://doi.org/10.1111/jam.12607

Saxena A, Raghuwanshi R, Gupta VK, Singh HB (2016) Chilli anthracnose: the epidemiology and management. Front Microbiol 7:1527. https://doi.org/10.3389/fmicb.2016.01527

Singh P, Kuo YC, Mishra S, Tsai CH, Chien CC, Chen CW, Deschlos-Theveniau M, Chu PW, Schulze B, Chinchilla D, Boller T (2012) The lectin receptor kinase-VL2 is required for priming and positively regulates Arabidopsis pattern-triggered immunity. Plant Cell 24:12561270. https://doi.org/10.1105/tpc.112.095778

Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: a fast-online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res 15(44):W232–235. https://doi.org/10.1093/nar/gkw256

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197

Wang Y, Liu J, Huang BO, Xu YM, Li J, Huang LF, Lin J, Zhang J, Min QH, Wang YWM (2015) Mechanism of alternative splicing and its regulation. Biomed Rep 3:152–158. https://doi.org/10.3892/br.2014.407

Xu G, Guo C, Shan H, Kong H (2012) Divergence of duplicate genes in exon–intron structure. PNAS 109:1187–1192

Xu Z, Gao L, Tang M, Qu C, Huang J, Wang Q, Yang C, Liu G, Yang C (2017) Genome-wide identification and expression profile analysis of CCH gene family in populus. PeerJ 5:e3962. https://doi.org/10.7717/peerj.3962

Yang X, Jawdy S, Tschaplinski TJ, Tuskan GA (2009) Genome-wide identification of lineage-specific genes in Arabidopsis, Oryza and Populus. Genomics 93:473–480. https://doi.org/10.1016/j.ygeno.2009.01.002

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125:749–760. https://doi.org/10.1016/j.cell.2006.03.037

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.