Transcriptome Analysis of Capsicum Chlorosis Virus-Induced Hypersensitive Resistance Response in Bell Capsicum

Shirani M. K. Widana Gamage1, Desmond J. McGrath2, Denis M. Persley3, Ralf G. Dietzgen1*

1 Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Queensland, Australia, 2 Queensland Department of Agriculture and Fisheries, AgriScience Queensland, Gatton, Queensland, Australia, 3 Queensland Department of Agriculture and Fisheries, AgriScience Queensland, EcoSciences Precinct, Dutton Park, Queensland, Australia

* r.dietzgen@uq.edu.au

Abstract

Background
Capsicum chlorosis virus (CaCV) is an emerging pathogen of capsicum, tomato and peanut crops in Australia and South-East Asia. Commercial capsicum cultivars with CaCV resistance are not yet available, but CaCV resistance identified in Capsicum chinense is being introgressed into commercial Bell capsicum. However, our knowledge of the molecular mechanisms leading to the resistance response to CaCV infection is limited. Therefore, transcriptome and expression profiling data provide an important resource to better understand CaCV resistance mechanisms.

Methodology/Principal Findings
We assembled capsicum transcriptomes and analysed gene expression using Illumina HiSeq platform combined with a tag-based digital gene expression system. Total RNA extracted from CaCV/mock inoculated CaCV resistant (R) and susceptible (S) capsicum at the time point when R line showed a strong hypersensitive response to CaCV infection was used in transcriptome assembly. Gene expression profiles of R and S capsicum in CaCV- and buffer-inoculated conditions were compared. None of the genes were differentially expressed (DE) between R and S cultivars when mock-inoculated, while 2484 genes were DE when inoculated with CaCV. Functional classification revealed that the most highly up-regulated DE genes in R capsicum included pathogenesis-related genes, cell death-associated genes, genes associated with hormone-mediated signalling pathways and genes encoding enzymes involved in synthesis of defense-related secondary metabolites. We selected 15 genes to confirm DE expression levels by real-time quantitative PCR.
Conclusion/Significance

DE transcript profiling data provided comprehensive gene expression information to gain an understanding of the underlying CaCV resistance mechanisms. Further, we identified candidate CaCV resistance genes in the CaCV-resistant *C. annuum x C. chinense* breeding line. This knowledge will be useful in future for fine mapping of the CaCV resistance locus and potential genetic engineering of resistance into CaCV-susceptible crops.

Introduction

Capsicum (*Capsicum* spp.) is a genus in Solanaceae, a family that contains economically important crops including potato and tomato. Capsicum is native to Central and South America comprising 30 species but only 5 species, *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. pubescens* have been domesticated [1]. *Capsicum* spp. are grown worldwide for their nutritional and condimental value. Pungent capsicum, such as paprika, chilies and hot pepper are used as spices and sweet capsicum such as Bell capsicum is consumed as vegetable. In 2012, world fresh capsicum production reached 31 million tons with highest contribution from China (www.fao.org). As with other crops, capsicum is attacked by various pathogens and pests that can cause extensive losses in production. Pathogens, such as *Xanthomonas* spp., *Phytophthora* spp, *Colletotrichum* spp., chili leaf curl virus, tomato spotted wilt virus (TSWV) and groundnut bud necrosis virus (GBNV) have been reported to cause major diseases in capsicum [2–4]. Recently, capsicum chlorosis virus (CaCV) emerged as a serious pathogen of capsicum and chili in Australia and India, respectively [5, 6]. In addition, CaCV infects tomato and peanut crops in Australia, Thailand and China [5, 7, 8]. Disease symptoms on capsicum leaves include marginal and interveinal chlorosis with narrow strap-like appearance, mottling, ring spots, chlorotic spots and tissue necrosis on the top leaves while infected capsicum fruits often become distorted with necrotic lesions [5, 9, 10]. CaCV is known to be transmitted by three species of thrips, *Ceratothripoides claratris*, *Frankliniella schultzei* and *Thrips palmi* in a circulative and propagative manner [5, 7]. Taxonomically, CaCV is a tentative species in the genus *Tospovirus*, family *Bunyaviridae* [11]. It contains a tripartite single strand RNA genome with negative and ambisense polarity which codes for RNA-dependent RNA polymerase, two structural proteins, nucleocapsid and glycoproteins and two non-structural proteins with proposed suppressor of RNA silencing and movement protein functions [12]. CaCV protein that triggers susceptibility or resistance responses in host plants remains unresolved.

Commercial cultivars carrying CaCV resistance are presently unavailable. As a result, breeding programmes’ search for natural CaCV resistance has become increasingly urgent. In Australia, CaCV resistance has been identified in several *C. chinense* Plant Introduction (PI) accessions. A sub-line obtained through self-pollination of *C. chinense* PI290972 showed uniform resistance to field isolates of both TSWV and CaCV [5]. Previously, TSWV resistance (*Tsw*) had been identified in *C. chinense* PI 152225 and PI 159236 [13]. However, *Tsw* resistance is not effective against CaCV [11]. TSWV resistance identified in *C. chinense* PI290972 appeared to be due to *Tsw* since resistance was overcome by a *Tsw* resistance-breaking strain [5]. CaCV resistance in *C. chinense* PI290972 is thought to be governed by a single dominant gene that segregates independently from TSWV resistance (DJ McGrath and DM Persley, unpublished). A current breeding program by the Queensland Department of Agriculture and Fisheries (DAF) transferred CaCV resistance identified in PI 290972 into commercial Bell
capsicum cultivars. Genomes of *C. annuum* and *C. chinense* PI159236 [14, 15] were recently sequenced. These genomes laid the foundation for further studies on genetics and genomics combined with Next Generation Sequencing (NGS) including RNA-Seq. RNA-Seq provides a powerful tool to identify differential expression of genes [16] and has been previously used to investigate the molecular basis of host plant responses to pathogens including viruses [17–22]. The objective of the present study is to investigate molecular responses of resistant Bell capsicum line to CaCV infection and to identify candidate CaCV resistance genes. We used comparative transcriptome profiling of CaCV-susceptible and -resistant Bell capsicum cultivars using Illumina NGS platform to compare transcriptional changes in response to CaCV infection. Detailed analysis of the most highly up-regulated genes in the resistant line revealed at least 45 annotated genes associated with plant defense response at different levels, such as localized cell death, cell signalling and synthesis of defense-related secondary metabolites. Furthermore, we identified candidate CaCV resistance genes and predicted their chromosomal location.

**Materials and Methods**

**CaCV inoculation and RNA extraction**

Bell capsicum (*Capsicum annuum* L.) seedlings from a CaCV-resistant (R) and a CaCV-susceptible (S) line were grown at 25°C and 16 h light/8 h dark conditions. CaCV-resistant line was derived from the DAF breeding program aimed to introgress CaCV resistance from *C. chinense* (PI 290972) into commercial Bell capsicum. The R seedlings used were from the third backcross generation of PI 290972 x *C. annuum* cv. Mazurka and cv. Warlock inbred lines. Commercial cultivar *C. annuum* cv. Yolo Wonder, an open pollinated inbred line, was used as CaCV-susceptible line. Seedlings aged 4–5 weeks were mechanically inoculated with CaCV isolate QLD 3432 [23]. Inoculum was prepared by grinding fresh CaCV-infected symptomatic capsicum leaves of cv. Yolo Wonder in 10 mM phosphate buffer, pH 7.6 with freshly added 20 mM sodium sulphite. Inoculum was rub-inoculated onto Carborundum-dusted leaves. Control plants were rub-inoculated with buffer (mock). Inoculated leaves were harvested from all treatments at the time point when CaCV-challenged R capsicum plants had developed necrotic lesions, which for this experiment was 4 days post inoculation (dpi) and were stored at -80°C until RNA extraction. To minimize biological variability, total RNA was extracted from three biological replicates for each treatment that consisted of at least 3 plants each. Briefly, pools of leaves of at least 3 plants were ground in liquid nitrogen and RNA was extracted from 100 mg of leaf powder using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol. RNA was eluted in 30 μl of RNase-free water. RNA preparations were treated with DNase to remove genomic DNA using a Turbo DNA-free kit (Ambion) following the manufacturer’s protocol. Presence or absence of CaCV in RNA preparations was confirmed by RT-PCR using CaCV N gene-specific primers forward 5’ –ATGTCTAACGTCAGGCAACTT-3’ and reverse 5’ –CACTTCTATAGAAGTACTAGG-3’.

**Illumina sequencing and bioinformatics**

Illumina sequencing, quality control, alignment, transcript assembly, quantification, normalization and expression profiling comparisons were outsourced to the Australian Genome Research Facility (AGRF, Melbourne, Australia). Briefly, rRNA in total RNA preparations was depleted as the first step to enrich non-rRNA species including pre-mature mRNA and long non-coding RNA using Ribo-Zero Plant rRNA Removal Kit (Epicentre, Illumina). Next, RNA quality and concentration were determined prior to library preparation using Agilent 2100 Bioanalyzer (Agilent Technologies). In total 12 cDNA libraries were prepared using poly (A) enrichment from 3 replicates of 4 treatments consisting of samples from CaCV- or mock-inoculated R and
S lines. One lane of an Illumina HiSeq 2000 sequencer was used to generate 100-bp paired-end reads. High quality reads obtained through FastQC filter were further cleaned to remove sequences of cross species contamination including CaCV [23]. Clean reads were aligned against C. annuum L. Zunla-1 complete genome [15] using Tophat aligner [16]. Transcript abundance and differential gene expression were calculated using Cufflinks with reference based assembly option (RABT) which generates known and potentially novel transcripts [16]. Differential expression of genes was compared between R and S lines using CaCV- and mock-inoculated conditions. Cufflinks generated normalized read counts per gene per sample with fragments per kilobase of exon per million mapped reads (FPKM) values. Significantly differentially expressed genes were determined by using a binary statistical assessment. Briefly, a p-value was calculated for each gene in each sample and comparison. Then p-values were corrected for multiple tests and comparisons (q-value) using false discovery rate (FDR). In this experiment, a FDR 0.05 cut off was used to determine p-value threshold.

Functional annotation of differentially expressed genes

We applied stringent filtering criteria to select highly significant differentially expressed genes (DE) at least two-fold between two conditions by setting cut off q-value < 0.01 and log2 fold change > 1. Selected DE genes were classified into functional categories using Blast2GO with default parameters [24]. Annotations were fine-tuned by using Annex-based gene ontology (GO) term augmentation followed by removal of First Level GO terms and mapped to GO Slim (plant). KEGG enzyme codes were assigned for annotations using Blast2GO to identify which biological pathways are catalyzed by DE enzymes. Enzyme codes were mapped into KEGG database [25].

Validation of DE genes by real-time quantitative PCR

RNA-Seq gene expression profiles for 15 selected genes were validated using real-time quantitative PCR (qPCR). Four genes, actin, TATA box-binding, nuclear cap-binding and galactono-lacton dehydrogenase (GLDH), were selected from non-DE genes in the RNA-Seq dataset as internal reference genes. Primers for target and reference genes were designed using Primer3 [26, 27]. Primer sequences are listed in S1 Table. Complementary DNA was synthesized with oligo dT primers using Superscript III First-strand cDNA synthesis kit (Life Technologies) using the same total RNA preparations (DNase-treated) used for Illumina sequencing. SensiFAST SYBR No-ROX Kit (Bioline) was used in a Rotor-Gene Q real-time PCR cycler (Qiagen) with 20 μl of final volumes containing 1 μl (20 ng) cDNA, 0.8 μl of each primer (10 μM), 7.4 μl of DNase- and RNase-free water and 10 μl of 2x SYBR No-ROX mix. Reaction conditions were 2 min at 95°C followed by 40 cycles of 95°C for 5s, 60°C for 10s and 72°C for 20s. Experimental design and subsequent data analysis methods applied were in accordance with a previously described protocol [28]. Three biological replicates and two technical replicates were used per sample. Since all reference genes showed stable gene expression across all samples and treatments, we arbitrarily chose actin for all future experiments. Real-time PCR efficiencies of target genes and actin were determined by standard curve method using a ten-fold dilution series of cDNA (from 100 ng to 0.001 ng). PCR efficiencies were calculated from the slopes of standard curves. Threshold cycle (Ct) number was determined from log scale amplification curves. Reaction efficiencies for target and reference genes showed 95–100% efficiency for 1–100 ng of cDNA template input. Hence, it was decided to use 20 ng of cDNA template for further reactions. For each reaction, no-template and no-RT control samples were included. Relative expression levels of target genes were calculated as $2^{-\Delta\Delta Ct}$ method; $2^{-(\Delta Ct \text{ of treatment} - \Delta Ct \text{ of control})}$.
[29]. For validation, qPCR derived log2-fold changes were compared with log2-fold values obtained through RNA-Seq analysis.

Results and Discussion
Transcriptome sequencing and identification of differentially expressed genes

To determine genome-wide CaCV resistance responses in *C. annuum*, we sequenced and compared transcriptome of a CaCV-resistant breeding line (R) and a susceptible (S) commercial cultivar using Illumina next generation sequencing. This is the first such analysis for CaCV molecular interactions with capsicum. Previously, molecular responses of host plants to impatiens necrotic spot virus (INSV) and TSWV tospovirus infections were reported using microarrays derived from expressed sequence tags. In *N. benthamiana*, INSV infection led to the up-regulation of genes involved in plant defense, transcription, signal transduction, protein synthesis and metabolism in comparison to mock plants while expression of histones was noticeably down-regulated [30]. In chrysanthemum, TSWV infection led to the up-regulation of chitin response genes and down-regulation of genes involved in DNA replication, chromatin biogenesis and cytokinesis compared to mock plants [31]. However, these studies targeted molecular responses to tospovirus infections in susceptible host plants. This report is the first study to determine molecular host plant resistance responses to infection by a tospovirus.

We compared the transcriptome response of CaCV-resistant and susceptible capsicum in the presence and absence of CaCV infection. Due to a lack of CaCV resistance in commercial cultivars, in this study we used a resistant backcross line from our breeding program. R and S lines were mechanically inoculated with a Queensland isolate of CaCV, whereas control plants were mock-inoculated with buffer. When challenged with CaCV, plants carrying CaCV resistance gene showed strong hypersensitive response (HR) on inoculated leaves with necrotic lesions and premature leaf abscission at 4 dpi while susceptible plants did not show any symptom at this early time point (Fig 1). We extracted total RNA from symptomatic inoculated leaves at 4 dpi to determine resistance response against CaCV. As controls, total RNA was extracted from CaCV-inoculated leaves of S line and mock-inoculated R and S line plants at
the same time point. RT-PCR of total RNA extracts using CaCV N gene-specific primers confirmed that all CaCV-inoculated plants were indeed infected and mock-inoculated plants did not contain any detectable CaCV. Illumina HiSeq generated 79,562,608 paired-end (100-bp) clean reads distributed across 12 cDNA libraries. Sequence reads were mapped against complete genome of a Chinese inbred line C. annuum L. Zunla-1. In total 32,939,994 (41.4%) reads mapped to this reference genome with 10–30 minimum to maximum exon coverage. This unexpectedly low percentage may be due to the fact that for this analysis only unique, paired reads were counted and the available reference genome is only a draft assembly that still contains gaps.

Reads that mapped to genes of the capsicum reference genome were calculated in two pairwise comparisons; R/CaCV vs S/CaCV and R/mock vs S/mock. Mapping reads to the reference genome identified 37,502 and 38,417 transcripts for R/CaCV vs S/CaCV and R/mock vs S/mock comparisons, respectively. Of these transcripts, 34,905 (93.1%) and 34,986 (91.1%) transcripts, respectively, corresponded to predicted protein coding regions (> 300bp) in the reference capsicum genome. Excluding transcripts smaller than 300 bp, 2270 and 3035 transcripts in R/CaCV vs S/CaCV and R/mock vs S/mock comparisons respectively could not be linked to protein coding regions in the reference genome. These transcripts are likely either non-coding RNAs or transcriptionally active regions in Zunla-1 genome which were not annotated during reference genome assembly or transcripts of genes that may have been introduced to Bell capsicum from C. chinense PI line during introgression. Bell capsicum RNA-seq transcriptome contained 34,905–34,986 protein-coding regions, similar to those of other capsicum genomes and transcriptomes suggesting sufficient coverage (S2 Table).

Normalized read counts (FPKM values) between two samples were statistically compared to determine whether a gene was significantly differentially expressed (DE) or not. A gene was considered significantly DE when the q-value was <0.01 and log2-fold >1.0. None of the genes in mock-inoculated plants were DE between S and R lines, indicating a common response to rub-inoculation treatment. In contrast, when R and S lines were CaCV-inoculated, 2484 DE genes were identified. Hence, we assumed that these genes were DE in response to CaCV infection.

Functional classification of DE genes in response to CaCV

To determine putative functions of DE genes that appear to be involved in resistance responses against CaCV infection, DE genes were subjected to gene ontology (GO) analysis using Blast2GO software [24]. Of the 2484 DE genes, 1316 (53%) were found to be up-regulated in R line, while 1168 (47%) were down-regulated. Only 273 (11%) genes were uniquely expressed in R line and 70 (2.8%) in S line. Such uniquely expressed genes may play a critical role in plant resistance and susceptibility to CaCV. However, expression of majority of genes in common in R and S lines, but at different levels, suggests resistance and susceptibility to CaCV is underpinned by broad up-regulated general defence responses rather than expression of different sets of genes. Up- or down-regulated genes were separately classified into functional categories. In total 2603 up-regulated and 1754 down-regulated transcript sequences were uploaded into Blast2GO. Transcript counts were higher than corresponding gene counts due to the presence of transcript isoforms, which may arise due to alternative splicing events in multi-exon genes [32]. Blast2GO annotated 2042 (78.4%) up-regulated transcripts that code for 1019 predicted protein-coding genes and 1490 (84.9%) down-regulated transcripts that code for 766 predicted-protein coding genes. DE genes were classified into 3 major functional domains, Biological Process (BP), Molecular Function (MF) and Cellular Component (CC). GO assignments in up-regulated genes included MF; 571, BP; 310 and CC; 139, and in down-regulated genes BP;
334, MF; 323 and CC; 127. GO annotation results were plotted using Web Gene Ontology (WEGO) Annotation tool to compare proportions of up- and down-regulated GO terms (Fig 2) [33].

Within BP domain, a large number of DE genes were placed in the categories of ‘metabolic process’ (80.2%) and ‘cellular process’ (71.6%) irrespective of being up- or down-regulated. Genes involved in ‘immune system process’, ‘cellular component biogenesis’ and ‘anatomical structure formation’ were found restricted to up-regulated genes or in other words specific to R line response. Within MF domain, a large number of DE genes fell into ‘binding’ (66.4%) and ‘catalytic activity’ (67.8%). Genes involved in ‘electron carrier activity’ and ‘antioxidant activity’ were uniquely expressed in R line. Within CC domain, majority of DE genes were found to be involved in producing proteins localized in ‘cell’, ‘cell part’ and ‘organelle’. Proteins that are localized in ‘symplast’ and ‘envelope’, including cellular membrane associated protein genes were found to be R line specific.

Assuming that genes that were up-regulated in R line are linked to disease resistance, functional annotations were searched for GO categories that showed direct or indirect links with plant resistance response and compared with known functions of homologues. Previously reported transcriptional responses to viral infection in resistant host plants showed up-regulation or overexpression of genes assigned to several GO categories. For instance, in tomato, genes related to defense response, cell wall reorganization, transcriptional regulation and secondary metabolite synthesis were up-regulated in response to tomato yellow leaf curl virus [17]. In Cassava, genes related to hormone signalling and synthesis of secondary metabolites were overexpressed in response to ipomovirus infection [18]. During an early infection of potato Y virus in potato, transcripts classified in GO terms ‘photosynthesis’, ‘light harvesting’, ‘protein-chromophore linkage’, ‘response to auxin stimulus’ and ‘negative regulation of peptidase activity’ were specifically up-regulated [19]. In this report, we specifically focus on the
most highly up-regulated genes with GO terms, ‘defense response’, response to stress’, ‘signal transduction’ and GOs related to hormone mediated signalling and secondary metabolism. Potential roles of such genes during capsicum-CaCV hypersensitive defense response are discussed below. Further, putative disease resistance genes identified in the dataset are also discussed.

**Defense-related genes**

GO analysis showed 60 genes were associated with ‘defense response’ category and 15 genes with ‘response to stress’ (S3 Table). Of these groups of genes, 16 were among the most highly up-regulated (log2-fold > 3.0, FPKM >300) 45 genes listed in Table 1 and include genes, such as those coding for pathogenesis-related (PR) proteins. PR proteins are known to be induced upon pathogen invasion and restrict pathogen development and spread [34]. The most highly up-regulated PR protein gene was PR leaf protein 4-like, which showed close relation to PR-1 type proteins. Interestingly, the most highly up-regulated R line specific (only expressed in R line) PR protein gene was also a precursor of PR-1. Although PR-1 represents a highly conserved family of plant proteins, mechanism by which PR-1 proteins affect pathogens remain to be elucidated [34, 35]. According to Blast2GO analysis, GO assignments for PR leaf protein 4-like and PR-1 precursor proteins were ‘defense response’ and ‘extracellular region’. These GO assignments and higher expression levels are temptation to speculate that PR-1 type proteins of capsicum may play specific roles in defense against CaCV. Defensin-like PR proteins were also identified among the up-regulated genes. Plant defensins are known to possess broad antimicrobial activities and are induced by ethylene [34, 36, 37]. Another class of PR proteins induced in capsicum transcriptome was thionin-like proteins which are classified into PR-13 family [34]. Thionins are toxic secondary metabolites that act against fungi, bacteria and insects through membrane degradation [38, 39]. However, their role in plant-virus interactions remains unknown. It is likely that significant higher expression of thionins in this plant-pathogen interaction may contribute to the establishment of systemic acquired resistance [40]. In addition to PR-1 precursor, osmotin-like PR-5 protein was found specially expressed in R line. Other preferentially highly expressed PR protein genes in capsicum transcriptome include those encoding class II chitinase (PR-3) and lignin-forming anionic peroxidase (PR-9). We identified a highly expressed germin-like protein (GLP) gene that shared 99% identity to a previously characterized GLP1 in hot pepper (C. annuum cv. Bugang). Hot pepper GLP1 (CaGLP1) has been classified into PR-16 family and is thought to be involved in defense against viral and bacterial pathogens [41]. CaGLP1 plant defense effect is assumed to occur through cell wall remodeling. Other GLPs function as enzymes, structural proteins and receptors [42]. The GLP in our dataset was classified in the ‘extracellular region’ suggesting localization outside cell wall. Accordingly, we speculate that this GLP may not be a receptor, but may function similar to CaGLP1 that is involved in cell wall remodeling or possess an enzymatic activity. Significant up-regulation of PR family proteins appears to play important roles in capsicum defense against CaCV.

Proteinase inhibitors (PIs) are another kind of defense-related proteins known to provide defense against insects and pathogens in plants that induce formation of abscission zone and thereby sacrifice tissues to prevent further invasion of pathogens [34, 73, 74]. We identified several highly up-regulated PI proteins including proteinase inhibitor PSI-1.2 [50]. PSI-1.2 was the most highly expressed R line specific gene with 4030 FPKM. Proteinase inhibitor 1-B was another highly expressed PI, which was classified with ‘response to wounding’ GO term indicating direct involvement in stress response. Other than the above-mentioned PIs, a Kunitz-like protease inhibitor gene was expressed with 6.67 log2-fold change. Kunitz family PIs are
Table 1. List of most highly up-regulated DE (log2-fold > 3.0, FPKM >300) putative defense-related genes in CaCV-resistant C. annuum x C. chinense in comparison to CaCV-susceptible C. annuum.

| Transcript Seq. ID | Seq. description (E-value) | FPKM (R line) | FPKM (S line) | log2-fold | GO term | Function |
|-------------------|---------------------------|---------------|---------------|-----------|---------|----------|
| 22352             | Pepper esterase (0.0E0)   | 2132.7        | 3.0           | 9.46      | Hydrolase activity | Defense [67] |
| 50328             | 5-epi-aristolochene synthase (0.0E0) | 1465.9        | 2.3           | 9.33      | Defense response | Defense [43] |
| 30661             | Lipoxgenase (0.0E0)       | 1187.8        | 1.9           | 9.22      | Biosynthetic process | Defense and cell death [44] |
| 39531             | PR leaf protein-4-like (6.3E-112) | 3045.1        | 5.8           | 9.02      | Defense response | Defense [34] |
| 03349             | 1-aminoacyclopropane-1-carboxylate oxidase (ACC oxidase) (0.0E0) | 1760.4        | 4.3           | 8.67      | Defense response | Defense [45, 46] |
| 19897             | Cytochrome P450 (0.0E0)   | 777.6         | 2.7           | 8.15      | Oxidation and reduction | Defense [47] |
| 42568             | Apoplastic invertase (0.0E0) | 434.9         | 1.6           | 8.09      | Metabolic process | Defense [48] |
| 11987             | Cannabidiolic acid synthase-like (0.0E0) | 938.5         | 3.6           | 8.03      | Oxidation and reduction | Cell death [49] |
| 35659             | Proteinase inhibitor psi-1.2-like (1.97E-22) | 1612.9        | 7.4           | 7.77      | Serine-type inhibitor activity | Defense [50] |
| 04833             | Defensin-like (3.3E-39)   | 5268.4        | 26.3          | 7.64      | Defense response | Defense [34] |
| 03989             | 1-aminoacyclopropane-1-carboxylate oxidase (ACC oxidase) (0.0E0) | 1894.2        | 12.0          | 7.30      | Defense response | Defense [45, 46] |
| 39790             | Omega-6 fatty acid desaturase endoplasmic reticulum isozyme-2 (0.0E0) | 937.3         | 6.0           | 7.28      | Lipid metabolic process | Defense [51, 52] |
| 31825             | Thionin-like protein (1.1E-40) | 7903.6        | 52.0          | 7.24      | Defense response | Defense [53] |
| 21202             | Pleiotropic drug resistance protein (PDR) 1 (0.0E0) | 438.9         | 3.7           | 6.88      | ATP binding | Defense [54] |
| 16797             | Kunitz-like protease inhibitor partial (0.4E-77) | 726.9         | 6.7           | 6.76      | Endopeptidase inhibitor activity | Defense [55] |
| 11123             | Hydroxymethylglutaryl-synthase-like (0.0E0) | 345.1         | 3.8           | 6.50      | Biosynthetic process | Defense [56] |
| 12646             | Class II chitinase (2.9E-170) | 1875.7        | 22.3          | 6.39      | Defense response | Defense [34] |
| 37429             | Uncharacterized protein LOC102581412 (0.0E0) | 1183.6        | 14.4          | 6.36      | Defense response | Defense [54] |
| 08215             | UDP-glucosyltransferase 1 (0.0E0) | 431.6         | 5.5           | 6.30      | Transferase activity | Defense [57] |
| 09442             | Lignin-forming anionic peroxidase (0.0E0) | 1403.7        | 20.4          | 6.10      | Response to stress | Defense [34] |
| 49199             | Probable aminotransferase TAT2 (0.0E0) | 643.9         | 11.5          | 5.81      | Biosynthetic process | Defense [58] |
| 03028             | Peroxidase 21 (3.1E-74) | 475.9         | 8.6           | 5.78      | Defense response | Defense [59] |
| 15678             | Alpha-dioxygenase 1-like (0.0E0) | 683.4         | 15.1          | 5.50      | Response to stress | Defense [60] |
| 11195             | Hydroxymethylglutaryl coenzyme A reductase 2 (0.0E0) | 498.9         | 11.8          | 5.39      | Biosynthetic process | Defense [61] |
| 01189             | Glutathione S-transferase (4.2E-135) | 336.4         | 8.6           | 5.29      | Auxin activated signalling pathway | Multiple functions [62] |
| 43744             | Trypsin inhibitor 1-like (1.2E-33) | 1034.8        | 26.9          | 5.26      | Response to wounding | Defense [63] |
| 22442             | Abscisic stress-ripening protein 2 (2.0E-25) | 891.2         | 25.2          | 5.14      | Response to stress | Defense [64] |
| 16174             | Stellacyanin-like protein (5.1E-69) | 870.6         | 28.4          | 4.94      | Electron transport | Defense [65] |
| 15700             | Formate dehydrogenase mitochondrial (0.0E0) | 896.6         | 39.1          | 4.52      | Oxidation-reduction process | Defense [66] |
| 37151             | Tyramine n-hydroxycinnamoyl transferase (1.4E-180) | 305.9         | 14.1          | 4.43      | Biosynthetic process | Response to stress [67] |
| 00644             | Defensin-like protein (5.4E-21) | 842.4         | 41.3          | 4.35      | Defense response | Defense [34] |
| 35935             | Germin-like protein (1.1E-145) | 409.6         | 22.8          | 4.16      | Extracellular region | Defense [41] |
| 04723             | Stress induced protein 11 (6.7E-64) | 815.1         | 55.0          | 3.88      | Response to stress | Response to stress accession AHI85716 |
| 21689             | Ornithine decarboxylase (0.0E0) | 631.0         | 45.1          | 3.80      | Biosynthetic process | Defense [68] |
| 31824             | Thionin-like protein (1.5E-19) | 660.3         | 47.7          | 3.78      | Defense response | Defense [53] |
| 10398             | Citrate synthase (0.0E0) | 316.7         | 24.9          | 3.66      | Metabolic process | Interact with plant hormone signalling pathways [69] |

(Continued)
involved in regulation of programmed cell death or apoptosis during HR of plant response to pathogens [75]. The role of these proteins in plant defense is further supported by a Kunitz family trypsin and protease inhibitor gene that was reported up-regulated in lethal systemic HR in soybean in response to soybean mosaic virus infection [20]. It is likely that the highly up-regulated PI protein genes, especially those specific for R line, are involved in HR and induction of premature leaf abscission of necrotic leaves that was observed as early as 4 dpi.

Apart from PR proteins and proteinase inhibitors, we noticed a significant up-regulation of genes associated with cell death such as lipoxygenase (LOX) [44], cannabidiolic synthase [49], peroxidases [59], alpha-dioxygenase 1-like enzyme [60], ornithine decarboxylase [68], patatin-like phospholipase [71] and non-symbiotic haemoglobin 1 [72]. Among these, LOX was one of the highest up-regulated genes with 9.2 log2-fold change. This gene was 99% identical in sequence to a previously characterized pepper LOX gene which induced cell death phenotype upon transient expression in leaves [44]. Additionally, LOX is a key enzyme in jasmonic acid (JA) biosynthesis suggesting a role in cell signalling [76].

Another up-regulated defence related gene was pleotrophic drug resistant (PDR) protein. According to studies on a previously reported homologue, this gene may effect fungal pathogens through transport of an antifungal compound scareol [54]. The most highly up-regulated gene (9.46 log2-fold) in the entire dataset was pepper esterase (PepEST), which was 99% identical to PepEST of Capsicum annuum [77]. However, its mode of action in defence against viral pathogens is not known.

Cell signalling-related genes
We have also identified genes that may be involved in hormone-mediated signalling pathways and signal transduction induced upon CaCV infection (S3 Table). Plant hormones play a vital role in disease resistance through regulation of expression of gene networks associated with defense responses [78]. Involvement of salicylic acid (SA), JA and ethylene (ET) have been extensively studied in several plant-pathogen interactions and has been covered in many reviews [78–80]. As discussed above, PR proteins are highly represented among up-regulated genes in our data set. Most PR proteins are induced following the activation of signalling
molecules such as SA, JA and ET [34]. Expression of PR proteins such as PR-1, -2 and -5 are promoted through increased SA levels, while PR-3, -4 and -12 defensin expression are promoted through JA and ET [34, 80]. One of the significantly up-regulated genes thought to be involved in cell signalling was 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase). ACC oxidases catalyze oxidative cleavage of ACC to form ET and induces leaf senescence [45]. In addition, ET-responsive transcription factors, ET receptor and sensor genes were also found up-regulated. All these up-regulated genes suggest increased levels of ET production and activity. Another cell-signaling related gene was cytochrome P450, which was 99% identical to a putative cytochrome P450 gene (CaCYP1) of chili pepper (C. annuum L. Bukang). CaCYP1 is thought to play a defense role through SA and abscisic acid (ABA) signalling pathways [47]. UDP-glucosyltransferase was another SA-associated up-regulated gene which is thought to be involved in the biosynthesis of SA [81]. A previously reported homologue in capsicum was also shown to be involved in tobacco mosaic virus resistance through controlling SA accumulation [57]. Auxin and ABA have also been implemented in plant-pathogen interactions [80]. Auxin has been shown to increase virulence of viral pathogens and thereby increase susceptibility. By contrast, we annotated several genes for auxin-responsive proteins among up-regulated genes in this study. Effects of these genes during disease resistance warrant future research.

In addition to genes involved in hormone-mediated signalling, we found several other highly up-regulated genes which may function as signal molecules such as patatin-like phospholipase and apoplastic invertase [48, 71]. Invertase is a key hydrolytic enzyme in sucrose metabolism and is known to produce a metabolic signal which induces defence-related genes and down-regulates photosynthesis [48]. Another set of up-regulated genes was several genes coding for glutathione-S-transferases (GST), which were classified with ‘auxin-activated signalling pathway’ and ‘protein binding’ GO terms. A previous study reported that different auxins activate and bind to GST and may have different functions [82]. Hence, functions of GST genes in our dataset could not be predicted without further study. We observed a 7.28 log2-fold increase in omega-6 fatty acid desaturase (FAD). Various FADs that are induced during biotic and abiotic stress conditions have been reviewed [52]. In parsley (Petroselinum crispum L.) a FAD with high sequence similarity to Omega-6 FAD has been shown to be induced in response to a fungal elicitor and thought to function as signalling molecule in the early defense response [51].

Secondary metabolism-related genes

Several genes involved in the biosynthesis of secondary metabolic compounds such as antibiotics and phytoalexins were also up-regulated in CaCV-resistant line upon virus infection. Mapping enzymes into KEGG pathways revealed the most enriched pathway was ‘biosynthesis of antibiotics’ with 60 up-regulated enzymes involved in biosynthesis of antibiotics and links with other biosynthetic pathways of defense-related secondary metabolites such as phenylalanine, sesquiterpenoids and mevalonate. In addition, phenylalanine, tyrosine and tryptophan biosynthesis (20 enzymes), phenylalanine metabolism (11 enzymes), phenylpropanoid biosynthesis (10 enzymes) and terpenoid backbone synthesis pathway (9 enzymes) were also found enriched. These pathways lead to the synthesis of secondary metabolites involved in plant defense against pathogens including phytoalexins, phytoanticipins, phenolic compounds such as alkaloids, terpenoids and lectins. One of the compounds synthesized through phenylalanine metabolism was capsaicin. Capsaicin is one of the most abundant capsaicinoids in capsicum [83]. Accumulation of capsaicinoids cause pungency in fruits [84]. Interestingly, these compounds also possess antimicrobial and antifungal properties and induce expression of defense-related genes such as chitinases suggesting potential role in plant defense [85, 86]. Up-regulation of enzymes involved in
capsaicin synthesis implies accumulated capsaicin may be involved in defense responses against CaCV. Capsaicin may affect integrity of the virus’ lipid envelope but this is difficult to validate due to fast HR response in the R line. Previously, capsaicin has been shown to interact with phospholipids and alter membrane function [87]. The 5-epi-aristolochene synthase (EAS) was the second most highly up-regulated gene in our dataset. EAS catalyzes the production of 5-epi-aristolochene, a precursor of capsidiol, the main phytoalexin produced by capsicum which has been previously shown to be induced by elicitors [43, 88]. We identified two candidate genes involved in biosynthesis of mevalonate, which is the precursor of a class of plant defense metabolites, sesquiterpene phytoalexins. The 3-hydroxy-3-methylglutaryl-CoA synthase (HMGA) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) are key enzymes that regulate mevalonate pathway [89].

Putative disease resistance genes

The most enriched GO term of the entire dataset was ‘nucleotide binding’ in the MF domain (S3 Table). Annotation showed 214 up-regulated nucleotide binding protein genes. Of those, receptor-like protein kinases and abc transporter family proteins were the most prominent. In addition, endonuclease dicer homolog 2, a component of the RNA silencing defense showed 2.50 log2-fold increased gene expression. Among the other nucleotide binding protein genes, we were most interested in candidate disease resistance genes.

Pathogen resistance mediated through specific disease resistance proteins is often associated with localized cell death at the site of infection and downstream signal transduction for systemic resistance [90]. Majority of known disease resistance genes (Rg) in plants belong to the nucleotide-binding (NB) leucine-rich repeat (LRR) gene family. The NB region is part of a complex NB-ARC domain [91]. Based on the domains present in the N-terminus, disease resistance genes are classified into two groups. Genes containing Toll interleukin-1 receptor (TIR) are referred to as TIR-NB-LRR (TNL) while the other group without TIR is referred to as CNL [92]. Some members of CNL contain a coiled-coil (CC) structure at the N-terminus [93]. In our dataset, we identified 12 putative NB-LRR family disease resistance genes that were up-regulated in R capsicum line. Rg-3 appeared to encode a complete TIR-NB-LRR gene model while other genes were of the CNL type. CNLs were found as partial genes that lacked specific domains and contained only, for example CC-, NB- and CC-NB. Partial NB-LRR genes have been previously reported in genome annotations of Arabidopsis, Brassica rapa and potato [94–96]. Two genes, Rg-2 and -3 contained more than one transcript sequence per gene. We considered those sequences as transcript isoforms of the same gene. Rg-2 gene contained 4 transcript isoforms with NB- and CC-NB- of varying sizes. One of the Rg-3 transcript isoforms contained complete TIR-NB-LRR gene model while the other transcript isoforms contained NB- and partial LRR and lacked TIR domain. Transcript isoforms may arise due to alternative splicing (AS) events, which are thought to play an important role in regulation of gene expression and are considered as a critical regulatory mechanism in plants to defend against pathogens [97–99]. To determine potential mechanisms of AS events, transcript isoforms were aligned using Geneious software and manually searched for exon-exon splice junctions and transcription start sites (TSS) [100]. TSSs were further confirmed through Tophat aligned files generated during transcript assembly. Transcript isoforms in Rg-2 seemed to be generated through intron retention, alternative splicing and selection of alternative 5’ TSS while in Rg-3, they appear to have occurred through alternative TSSs and an alternative splicing event. Interestingly, alternative splicing in Rg-3 has utilized a non-canonical splice donor/acceptor (GT/CA instead of GT/AG). Whatever the mechanism used, AS appears to lead to truncated
proteins. Functional consequences of AS derived transcript isoforms in our dataset remain to be evaluated in future research. Previously characterized AS disease resistance genes such as Arabidopsis RPS4, Medicago trunculata RCT1 and tobacco N revealed truncated proteins are essential to exhibit complete resistance, while AS transcript isoforms alone showed partial or no resistance [98, 99, 101]. On the other hand, disease resistance genes such as flax L6 and tomato BS4 do not require alternatively spliced transcript isoforms to lead to complete resistance [102, 103].

Of the 12 putative disease resistance genes in our dataset, only 8 mapped to predicted protein-coding regions in the C. annuum cv. Zunla-1 reference genome. Rg10 expression was limited to the R line but it mapped with 100% sequence identity to a protein-coding region of the C. annuum reference genome. Therefore, we consider it unlikely that this is the gene responsible for triggering the resistance response to CaCV, which was introgressed from C. chinense. However, Rg10 is likely involved downstream as part of the response cascade leading to HR in the R line. It is possible that the other genes could not be aligned to predicted protein-coding regions in the capsicum reference genome due to incomplete annotation or novelty of genes that are not present in C. annuum-Zunla inbred line. We assumed that these four unmapped genes may originate from CaCV-resistant C. chinense. All four candidate genes contained a single transcript sequence per gene with partial NB-LRR gene models such as Rg-7, NB-, Rg-8, RX-CC-NB-, Rg-9, partial NB- and Rg-11, RX-CC-. To determine whether Rg-7, -8, -9 and -11 genes originated from C. chinense, transcript sequences were BLAST searched against C. chinense (v0.5) scaffolds (Pepper Genome Platform). Scaffolds with hits were selected based on E-value and percentage of sequence coverage. Selected scaffolds were aligned with corresponding transcript sequences using Geneious software and manually annotated [100]. We were able to predict NB-LRR gene models for Rg-7 and Rg-8.

Rg-8 transcript sequence contained a complete RX-CC-NB-LRR model and could easily be aligned to C. chinense scaffold 14663 containing a sequence with 100% identity and coverage, implying that it originated from C. chinense. Rg-8 protein was encoded by a single open reading frame (ORF) of 3792 nucleotides (nt) without introns. In a subsequent GenBank search Rg-8 protein had 78% identity with 95% sequence coverage to a C. annuum root-knot nematode resistance protein (accession ACI43068, unpublished) that was not annotated in the cv. Zunla reference genome. Rg-7 contained a partial gene model (NB-) and aligned with several C. chinense scaffolds (hereafter referred to as candidate scaffolds) with 87–95% identity due to the highly conserved NB domain. To obtain a complete gene model for Rg-7, paired-end Illumina reads were mapped to each candidate scaffold. A complete NB-LRR gene model could be predicted from the sequences aligned to C. chinense scaffold 35219. However, this gene model lacked CC domain. A consensus sequence of 3870 nt containing a single ORF without introns was derived for Rg-7. Blast searches of the deduced amino acid sequence showed Rg-7 was 82% identical to previously characterized CC-NB-LRR type proteins of C. chinense, C. chacoense, C. baccatum, C. frutescens and C. annuum [104]. We could not annotate Rg-9 and -11 transcripts aligned scaffolds and predict gene models.

Since the chromosomal location of CaCV resistance gene in R breeding line is unknown, we searched for possible associations of candidate resistance genes based on genome assemblies. Rg-7 and -8 ORFs were BLAST searched against C. annuum Zunla-1 and Chiltepin (C. annuum var. glabriusculum) and hot pepper (C. annuum cv. CM334) assemblies (Pepper Genome Platform) to determine chromosomal location. BLAST revealed Rg-7 is located on chromosome 11 and Rg-8 on chromosome 6. RNA-Seq and qPCR log2-fold expression of Rg-7 was 3.19 and 4.63 respectively. Fold expression of Rg-8 was less compared to Rg-7, which was 1.64 in RNA-Seq and 1.48 in qPCR. Since a dominant CaCV resistance gene would likely
express with higher fold change, it is possible that Rg-7 may be the CaCV resistance gene in R line that has been introgressed from *C. chinense*. However, this hypothesis needs to be confirmed experimentally by phenotyping using gene silencing or by transgenic approaches and molecular fine mapping of segregating populations.

Fig 3. Validation of RNA-Seq gene expression by real-time quantitative PCR (qPCR). FPKM (fragments per kilobase of exon per million fragments mapped) values obtained by RNA-Seq analysis and relative expression levels obtained by qPCR for 15 selected genes in CaCV resistant (R) and susceptible (S) *Capsicum annuum* plants inoculated with capsicum chlorosis virus are shown. Error bars represent the standard error for three biological replicates. Putative resistance genes are consecutively numbered 1–12; PR, pathogenesis-related.

doi:10.1371/journal.pone.0159085.g003
Validation of RNA-Seq gene expression profiles by real-time qPCR

RNA-Seq based gene expression levels of 15 selected genes of interest were validated by real-time qPCR using the same total RNA preparations used for RNA-Seq library preparation. We selected 12 candidate disease resistance genes, two PR protein genes and a lipoxygenase gene to validate gene expression data. Initially, expression patterns of 4 housekeeping genes; actin, TATA box-binding, nuclear cap-binding and GLDH selected from RNA-Seq dataset were tested as potential internal reference genes. Since all these genes showed stable gene expression patterns across all treatments (data not shown), the widely used actin gene was selected as internal reference for qPCR experiments. Expression of target genes was normalized to actin and relative expression levels were calculated for each target gene and compared between R and S lines (Fig 3). Expression levels of disease resistance genes were always less than the highly expressed actin in both R and S lines. Since the calculated qPCR relative expression values were smaller than 1, we multiplied them by a factor of 1000 for plotting data in graphs. Fold changes in target gene expression in R line as compared to S line showed similar trends in qPCR and RNA-Seq (Table 2). However, genes that had zero FPKM values in S line showed a low level of expression in qPCR analysis, indicating higher sensitivity of qPCR and/or insufficient depth of coverage in RNA-Seq.

Conclusions

CaCV-induced resistance response was evaluated in a comparative transcriptome analysis of CaCV-resistant and susceptible C. annuum using Illumina HiSeq sequencing platform and subsequent bioinformatics analysis. CaCV induced strong HR on inoculated leaves of the resistant line as early as 4 days after CaCV infection. We identified a range of defense-related genes involved in this HR and genes that may induce resistance in systemic tissues such as signalling molecules. Genes involved in localized cell death, cell signalling, synthesis of antimicrobial compounds and PR proteins were found highly up-regulated. Two potential CaCV resistance candidate genes were identified in the resistant C. annuum x C. chinense breeding line. These genes were further validated by qPCR.

Table 2. Comparison of transcript fold change as detected by RNA-Seq and qPCR.

| Transcript Seq. ID | Seq. description         | FPKM R line | FPKM S line | log2-fold | qPCR log2-fold | validated |
|-------------------|--------------------------|-------------|-------------|-----------|----------------|-----------|
| 21345             | Rg1                      | 15.72       | 0.79        | 4.29      | 3.76 ± 0.526   | yes       |
| 10552             | Rg2                      | 37.12       | 10.76       | 3.38      | 1.61 ± 0.368   | yes       |
| 48569             | Rg3                      | 20.39       | 2.97        | 2.77      | 3.68 ± 0.328   | yes       |
| 26003             | Rg4                      | 16.09       | 0.0         |           | 0.65 ± 0.201   | yes       |
| 26004             | Rg5                      | 15.78       | 0.0         |           | 2.3 ± 0.312    | yes       |
| 26005             | Rg6                      | 13.67       | 0.0         |           | 4.89 ± 0.472   | yes       |
| 27596             | Rg7                      | 14.38       | 1.56        | 2.79      | 4.63 ± 0.853   | yes       |
| 31761             | Rg8                      | 12.70       | 4.05        | 1.64      | 1.48 ± 0.345   | yes       |
| 38700             | Rg9                      | 12.65       | 0.0         |           | 2.75 ± 0.209   | yes       |
| 40213             | Rg10                     | 11.06       | 0.0         |           | Only in R line | yes       |
| 40214             | Rg11                     | 11.86       | 0.0         |           | 2.74 ± 0.671   | yes       |
| 40215             | Rg12                     | 13.21       | 0.0         |           | 6.81 ± 0.263   | yes       |
| 18520             | PR sth-2-like            | 170.77      | 3.42        | 5.63      | 8.59 ± 0.364   | yes       |
| 39531             | PR leaf protein 4-like   | 3045.13     | 5.85        | 9.02      | 11.76 ± 0.206  | yes       |
| 22353             | LOX                      | 2132.67     | 3.02        | 9.46      | 11.30 ± 0.081  | yes       |

qPCR fold changes represent mean of three biological replicates.

doi:10.1371/journal.pone.0159085.t002
genes were manually annotated in *C. chinense* genome with a NB-LRR gene model, which belongs to CNL type resistance genes.

**Supporting Information**

S1 Table. Sequences of oligonucleotide primers used in qPCR.

(SDOCX)

S2 Table. Statistics of predicted-protein coding genes of capsicum genomes and transcriptomes in comparison to Bell capsicum transcriptome sequenced in this study.

(SDOCX)

S3 Table. Differentially expressed putative resistance response-related genes and respective gene ontology (GO) assignments in response to CaCV infection in CaCV-resistant *Capsicum annuum* in comparison to CaCV-susceptible *C. annuum* after 4 days of CaCV inoculation. GO assignments for all transcript variants per gene is indicated. C: Cellular Component, F: Molecular Function, P: Biological Process.

(SXLSX)

**Acknowledgments**

We thank Dr Sonika Tyagi (Australian Genome Research Facility Ltd) for exon coverage calculations and helpful comments and Dr Van Hoang (Translational Research Institute) for advice in qPCR.

**Author Contributions**

Conceived and designed the experiments: SMKWG DJM DMP RGD. Performed the experiments: SMKWG. Analyzed the data: SMKWG RGD. Contributed reagents/materials/analysis tools: DJM DMP RGD. Wrote the paper: SMKWG RGD.

**References**

1. Moscone EA, Scaldaferro MA, Grabiele M, Cecchini NM, Sánchez García Y, Jarret R, et al., editors. The evolution of chili peppers (Capsicum-Solanaceae): a cytogenetic perspective. VI International Solanaceae Conference: Genomics Meets Biodiversity 745; 2006.
2. Stall RE, Jones JB, Minsavage GV. Durability of resistance in tomato and pepper to xanthomonads causing bacterial spot. Annual review of phytopathology. 2009; 47:265–84. doi:10.1146/annurev-phyto-080508-081752 PMID: 19400644
3. Persley D, Cooke T, House S. Diseases of vegetable crops in Australia: CSIRO PUBLISHING; 2010.
4. Reddy M, Srivastava A, Kumar S, Kumar R, Chawda N, Ebert A, et al. Chilli (Capsicum annuum L.) breeding in India: an overview. SABRAO Journal of Breeding and Genetics. 2014; 46(2):160–73.
5. Persley D, Thomas J, Sharman M. Tospoviruses—an Australian perspective. Australasian Plant Pathology. 2006; 35(2):161–80.
6. Mandal B, Jain R, Krishnareddy M, Krishna Kumar N, Ravi K, Pappu H. Emerging problems of Tospoviruses (Bunyaviridae) and their management in the Indian subcontinent. Plant Disease. 2012; 96(4):468–79.
7. Premachandra W, Borgemeister C, Maiss E, Knierim D, Poething H-M. Ceratothripoides claratris, a new vector of a Capsicum chlorosis virus isolate infecting tomato in Thailand. Phytopathology. 2005; 95(6):659–63. doi:10.1094/PHYTO-95-0659 PMID: 18943782
8. Chen K, Xu Z, Yan L, Wang G. Characterization of a new strain of Capsicum chlorosis virus from peanut (Arachis hypogaea L.) in China. Journal of Phytopathology. 2007; 155(3):178–81.
9. Krishnareddy M, Rani RU, Kumar KA, Reddy KM, Pappu H. Capsicum chlorosis virus (Genus Tospovirus) infecting Chili pepper (Capsicum annuum) in India. Plant Disease. 2008; 92(10):1469-.
10. Kunkalikar S, Sudarsana P, Rajagopalan P, Zehr UB, Ravi K. Biological and molecular characterization of Capsicum chlorosis virus infecting chilli and tomato in India. Archives of virology. 2010; 155(7):1047–57. doi: 10.1007/s00705-010-0681-5 PMID: 20443030

11. McMichael LA, Persley DM, Thomas JE. A new tospovirus serogroup IV species infecting capsicum and tomato in Queensland, Australia. Australasian Plant Pathology. 2002; 31(3):231–9.

12. Knierim D, Blawid R, Maissa E. The complete nucleotide sequence of a capsicum chlorosis virus isolate from Lycopersicum esculentum in Thailand. Archives of virology. 2006; 151(9):1761–82. PMID: 16601925

13. Black L, Hobbs H, Gatti J Jr. Tomato spotted wilt virus resistance in Capsicum chinense PI 152225 and 159236. Plant Disease. 1991; 75(8):.

14. Kim S, Park M, Yeom S-I, Kim Y-M, Lee JM, Lee H-A, et al. Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. Nature Genetics. 2014; 46(3):270–8. doi: 10.1038/ng.2877 PMID: 24441736

15. Qin C, Yu C, Shen Y, Fang X, Chen L, Min J, et al. Whole-genome sequencing of cultivated and wild peppers provides insights into Capsicum domestication and specialization. Proceedings of the National Academy of Sciences. 2014; 111(14):5135–40.

16. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols. 2012; 7 (3):562–78. doi: 10.1038/nprot.2012.016 PMID: 22383036

17. Chen T, Lv Y, Zhao T, Li N, Yang Y, Yu W, et al. Comparative transcriptome profiling of a resistant vs. susceptible tomato (Solanum lycopersicum) cultivar in response to infection by tomato yellow leaf curl virus. PLoS One. 2013; 8(1):e80816. doi: 10.1371/journal.pone.0080816 PMID: 24260487

18. Maruthi M, Bouvaine S, Tufan HA, Mohammed IU, Hillocks RJ. Transcriptional response of virus-infected cassava and identification of putative sources of resistance for cassava brown streak disease. PloS one. 2014; 9(5):e96642. doi: 10.1371/journal.pone.0096642 PMID: 24846209

19. Goyer A, Hamlin L, Crosslin JM, Buchanan A, Chang JH. RNA-Seq analysis of resistant and susceptible potato varieties during the early stages of potato virus Y infection. BMC genomics. 2015; 16(1):472.

20. Chen H, Arsovski AA, Yu K, Wang A. Deep sequencing leads to the identification of eukaryotic translation initiation factor 5a as a key element in Rsv1-mediated lethal systemic hypersensitive response to Soybean mosaic virus infection in Soybean. Molecular Plant Pathology. 2016.

21. Lu J, Du Z-X, Kong J, Chen L-N, Qiu Y-H, Li G-F, et al. Transcriptome analysis of Nicotiana tabacum infected by Cucumber mosaic virus during systemic symptom development. PLoS One. 2012; 7(8):e43447. doi: 10.1371/journal.pone.0043447 PMID: 22952684

22. Rubio M, Rodríguez-Moreno L, Ballester AR, Moura MC, Bonghi C, Cândresse T, et al. Analysis of gene expression changes in peach leaves in response to Plum pox virus infection using RNA-Seq. Molecular plant pathology. 2015; 16(2):164–76. doi: 10.1111/mpp.12169 PMID: 24989162

23. Widana Gamage S, Persley DM, Higgins CM, Dietzgen RG. First complete genome sequence of a capsicum chlorosis tospovirus isolate from Australia with an unusually large S RNA intergenic region. Archives of virology. 2015;1–4.

24. Conesa A, Gótz S, García-Gómez JM, Terol J, Talón M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 2005; 21(18):3674–6. PMID: 16081474

25. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research. 2000; 28(1):27–30. PMID: 10592173

26. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3–new capabilities and interfaces. Nucleic acids research. 2012; 40(15):e115–e. PMID: 22730293

27. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007; 23(10):1289–91. PMID: 17379693

28. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008; 3(6):1101–8. PMID: 18546601

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the ΔΔCT method. methods. 2001; 25(4):402–8. PMID: 11846609

30. Senthil G, Liu H, Puram V, Clark A, Stromberg A, Goodin M. Specific and common changes in Nicotiana benthamiana gene expression in response to infection by enveloped viruses. Journal of General Virology. 2006; 86(9):2615–25.

31. Choi H, Jo Y, Lian S, Jo K-M, Chu H, Yoon J-Y, et al. Comparative analysis of chrysanthemum transcriptome in response to three RNA viruses: Cucumber mosaic virus, Tomato spotted wilt virus and Tomato spotted wilt virus.
Potato virus X. Plant molecular biology. 2015; 88(3):233–48. doi: 10.1007/s11103-015-0317-y PMID: 25904110

32. Nilsen TW, Graveley BR. Expansion of the eukaryotic proteome by alternative splicing. Nature. 2010; 463(7280):457–63. doi: 10.1038/nature08909 PMID: 20110989

33. Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, et al. WEGO: a web tool for plotting GO annotations. Nucleic acids research. 2006; 34(suppl 2):W293–W7

34. Van Loon LC, Rep M, Peterse C. Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol. 2006; 44:135–62. PMID: 16602946

35. Van Loon L, Van Strien E. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and molecular plant pathology. 1999; 55(2):85–97.

36. Lay F, Anderson M. Defensins-components of the innate immune system in plants. Current Protein and Peptide Science. 2005; 6(1):85–101. PMID: 15638771

37. Thomma BP, Penninckx IA, Cammue BP, Broekaert WF. The complexity of disease signaling in Arabidopsis. Current opinion in immunology. 2001; 13(1):63–8. PMID: 1154919

38. Bohmann H, Broekaert W. The role of thionins in plant protection. Critical reviews in plant sciences. 1994; 13(1):1–16.

39. Epple P, Apel K, Bohmann H. Overexpression of an endogenous thionin enhances resistance of Arabidopsis against Fusarium oxysporum. The Plant Cell. 1997; 9(4):509–20. PMID: 9144959

40. Lee SC, Hwang BK. Induction of some defense-related genes and oxidative burst is required for the establishment of systemic acquired resistance in Capsicum annuum. Planta. 2005; 221(6):790–800. PMID: 15729568

41. Park C-J, An J-M, Shin Y-C, Kim K-J, Lee B-J, Paek K-H. Molecular characterization of pepper germin-like protein as the novel PR-16 family of pathogenesis-related proteins isolated during the resistance response to viral and bacterial infection. Planta. 2004; 219(5):797–806. PMID: 15185078

42. Bernier F, Berna A. Germins and germin-like proteins: plant do-all proteins. But what do they do exactly? Plant Physiology and Biochemistry. 2001; 39(7):545–54.

43. Zavala-Páramo G, Chávez-Moctezuma MP, García-Pineda E, Yin S, Chappell J, Lozoya-Gloria E. Isolation of an elicitor-stimulated 5-epi-aristolochene synthase gene (gPEAS1) from chili pepper (Capsicum annuum). Physiologia Plantarum. 2000; 110(3):410–8.

44. Hwang IS, Hwang BK. The pepper 9-lipoxygenase gene CaLOX1 functions in defense and cell death responses to microbial pathogens. Plant physiology. 2010; 152(2):948–67. doi: 10.1104/pp.109.147827 PMID: 19939946

45. Nie X, Singh RP, Tai GC. Molecular characterization and expression analysis of 1-aminoacyclopropane-1-carboxylate oxidase homologs from potato under abiotic and biotic stresses. Genome. 2002; 45(5):905–13. PMID: 12416623

46. Morgan PW, Drew MC. Ethylene and plant responses to stress. Physiologia Plantarum. 1997; 100(3):620–30.

47. Kim Y-C, Kim S-Y, Paek K-H, Choi D, Park JM. Suppression of CaCYP1, a novel cytochrome P450 gene, compromises the basal pathogen defense response of pepper plants. Biochemical and biophysical research communications. 2006; 345(2):638–45. PMID: 16696948

48. Roitsch T, Balibrea M, Hofmann M, Proels R, Sinha A. Extracellular invertase: key metabolic enzyme and PR protein. Journal of Experimental Botany. 2003; 54(382):513–24. PMID: 12508062

49. Morimoto S, Tanaka Y, Sasaki K, Tanaka H, Fukamizu T, Shoyama Y, et al. Identification and characterization of cannabinoids that induce cell death through mitochondrial permeability transition in Cannabis leaf cells. Journal of Biological Chemistry. 2007; 282(28):20739–51. PMID: 17513301

50. Antcheva N, Pintar A, Patthry A, Simonscts A, Barta E, Torchbanov B, et al. Proteins of circularly permuted sequence present within the same organism: the major serine proteinase inhibitor from Capsicum annuum seeds. Protein Science. 2001; 10(11):2280–90. PMID: 11604534

51. Kirsch C, Hahlbrock K, Somssich IE. Rapid and transient induction of a parsley microsomal [delta] 12 fatty acid desaturase mRNA by fungal elicitor. Plant physiology. 1997; 115(1):283–9. PMID: 9306702

52. Upchurch RG. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnology letters. 2008; 30(6):967–77. doi: 10.1007/s10529-008-9639-z PMID: 18227974

53. Oh B-J, Ko MK, Kostenyuk I, Shin B, Kim KS. Coexpression of a defensin gene and a thionin-like gene via different signal transduction pathways in pepper and Colletotrichum gloeosporioides interactions. Plant molecular biology. 1999; 41(3):313–9. PMID: 10598099

54. Stukrens Y, Bultrye A, Greer S, Trombik T, Vanham D, Boutry M. NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from Nicotiana plumbaginifolia, plays a major role in plant pathogen defense. Plant Physiology. 2005; 139(1):341–52. PMID: 16126865
55. Hermosa M, Turra D, Fogliano V, Monte E, Lorito M. Identification and characterization of potato protease inhibitors able to inhibit pathogenicity and growth of Botrytis cinerea. Physiological and molecular plant pathology. 2006; 68(4):138–48.

56. Alex D, Bach TJ, Chye ML. Expression of Brassica juncea 3-hydroxy-3-methylglutaryl CoA synthase is developmentally regulated and stress-responsive. The Plant Journal. 2000; 22(5):415–26. PMID: 10849357

57. Lee B-J, Kim S-K, Choi SB, Bae J, Kim K-J, Kim Y-J, et al. Pathogen-inducible CaUGT1 is involved in resistance response against TMV infection by controlling salicylic acid accumulation. FEBS letters. 2009; 583(13):2315–20. doi: 10.1016/j.febslet.2009.06.028 PMID: 19540833

58. Lopukhina A, Dettenberg M, Weiler EW, Holländer-Czytko H. Cloning and characterization of a corrinoid-regulated tyrosine aminotransferase from Arabidopsis. Plant biology. 2001; 126(4):1678–87. PMID: 11500565

59. Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, et al. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. The Plant Journal. 2006; 47(6):851–63. PMID: 16889645

60. De León IP, Sanz A, Hamberg M, Castresana C. Involvement of the Arabidopsis DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. The Plant Journal. 2002; 29(1):61–72. PMID: 12060227

61. Choi D, Bostock RM, Avdiushko S, Hildebrand DF. Lipid-derived signals that discriminate wound-and pathogen-responsive isoprenoid pathways in plants: methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in Solanum tuberosum L. Proceedings of the National Academy of Sciences. 1994; 91(6):2329–33.

62. Dixon DP, Lapthorn A, Edwards R. Plant glutathione transferases. Genome Biol. 2002; 3(3):3004.1–10.

63. Linthorst HJ, Brederode FT, van der Does C, Bol JF. Tobacco proteinase inhibitor I genes are locally, but not systemically induced by stress. Plant molecular biology. 1993; 21(6):985–92. PMID: 8490144

64. Liu HY, Dai JR, Feng DR, Liu B, Wang HB, Wang JF. Characterization of a novel plantain Asr gene, MpAsr, that is regulated in response to infection of Fusarium oxysporum f. sp. cubense and abiotic stresses. Journal of integrative plant biology. 2010; 52(3):315–23. doi: 10.1111/j.1744-7909.2010.00912.x PMID: 20377692

65. Kong HY, Jung HW, Lee SC, Choi D, Hwang BK. A gene encoding stellacyanin is induced in Capsicum annuum by pathogens, methyl jasmonate, abscisic acid, wounding, drought and salt stress. Physiologia plantarum. 2002; 115(4):550–62. PMID: 12121461

66. David P, des Francs-Small CC, Sévignac M, Thareau V, Macadré C, Langin T, et al. Three highly similar formate dehydrogenase genes located in the vicinity of the B4 resistance gene cluster are differentially expressed under biotic and abiotic stresses in Phaseolus vulgaris. Theoretical and Applied Genetos. 2010; 121(1):87–103. doi: 10.1007/s00122-010-1293-x PMID: 20182695

67. Back K, Jang SM, Lee B-C, Schmidt A, Strack D, Kim K-M. Cloning and characterization of a hydroxycinnamoyl-CoA: tyramine N-(hydroxycinnamoyl) transferase induced in response to UV-C and wounding from Capsicum annuum. Plant and Cell Physiology. 2001; 42(5):475–81. PMID: 11982813

68. Walters D. Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism. New Phytologist. 2003; 159(1):109–15.

69. Finkemeier I, König A-C, Heard W, Nunes-Nesi A, Pham PA, Leister D, et al. Transcriptomic analysis of the role of carboxylic acids in metabolite signaling in Arabidopsis leaves. Plant physiology. 2013; 162(1):239–53. doi: 10.1104/pp.113.214114 PMID: 23487434

70. Edwards R, Dixon DP, Walbot V. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends in plant science. 2000; 5(5):193–8. PMID: 10785664

71. Kim DS, Jeun Y, Hwang BK. The pepper patatin-like phospholipase CaPLP1 functions in plant cell death and defense signaling. Plant molecular biology. 2014; 84(3):329–44. doi: 10.1007/s11103-013-0137-x PMID: 24085708

72. Ou Z-L, Zhong N-Q, Wang H-Y, Chen A-P, Jian G-L, Xia G-X. Ectopic expression of the cotton nonsymbiotic hemoglobin gene GhHbd1 triggers defense responses and increases disease tolerance in Arabidopsis. Plant and Cell Physiology. 2006; 47(8):1058–68. PMID: 16854938

73. Ryan CA. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annual review of phytopathology. 1990; 28(1):425–49.

74. Roberts JA, Whitelaw CA, Gonzalez-Carranza ZH, McManus MT. Cell separation processes in plants—models, mechanisms and manipulation. Annals of Botany. 2000; 86(2):223–35.
75. Li J, Brader G, Palva ET. Kunitz trypsin inhibitor: an antagonist of cell death triggered by phytopathogens and fumonisins b1 in Arabidopsis. Molecular plant. 2008; 1(3):482–95. doi: 10.1093/mp/ssn013 PMID: 19825555

76. Vick BA, Zimmerman DC. The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. Biochemical and biophysical research communications. 1983; 111(2):470–7. PMID: 6404266

77. Ko MK, Jeon WB, Kim KS, Lee HH, Seo HH, Kim YS, et al. A Colletotrichum gloeosporioides-induced esterase gene of nonclimacteric pepper (Capsicum annuum) fruit during ripening plays a role in resistance against fungal infection. Plant molecular biology. 2005; 58(4):529–41. PMID: 16021337

78. Bari R, Jones JD. Role of plant hormones in plant defense responses. Plant molecular biology. 2009; 69(4):473–88. doi: 10.1007/s11103-008-9435-0 PMID: 19083153

79. Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC. Networking by small-molecule hormones in plant immunity. Nature chemical biology. 2009; 5(5):308–16. doi: 10.1038/nchembio.164 PMID: 19377457

80. Grant M, Lamb C. Systemic immunity. Current opinion in plant biology. 2006; 9(4):414–20. PMID: 16753329

81. Lee H-I, Raskin I. Purification, cloning, and expression of a pathogen inducible UDP-glucose: salicylic acid glucosyltransferase from tobacco. Journal of Biological Chemistry. 1999; 274(51):36637–42. PMID: 10593966

82. Aza-González C, Núñez-Palenius HG, Ochoa-Alejo N. Molecular biology of capsaicinoid biosynthesis in chili pepper (Capsicum spp.). Plant cell reports. 2011; 30(5):695–706. doi: 10.1007/s00299-010-0968-8 PMID: 21161234

83. Díaz J, Pomar F, Bernal A, Merino F. Peroxidases and the metabolism of capsaicin in Capsicum annuum L. Phytochemistry Reviews. 2004; 3(1–2):141–57.

84. Kraikruan W, Sangchote S, Sukprakarn S. Effect of capsaicin on germination of Colletotrichum capsici conidia. 2008.

85. Veloso J, Prego C, Varela M, Carballeira R, Bernal A, Merino F, et al. Properties of capsaicinoids for the control of fungi and oomycetes pathogenic to pepper. Plant Biology. 2014; 16(1):177–85. doi: 10.1111/j.1438-8677.2012.00717.x PMID: 23452049

86. Aranda FJ, Villalain J, Gómez-Fernández JC. Capsaicin affects the structure and phase organization of phospholipid membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1995; 1234(2):225–34.

87. Whitehead IM, Threlfall DR, Ewing DF. 5-Epi-aristolochene is a common precursor of the sesquiterpenoid phytoalexins capsidiol and debneyol. Phytochemistry. 1989; 28(3):775–9.

88. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990; 343(6257):425–30. PMID: 1967820

89. van Ooijen G, van den Burg HA, Cornelissen BJ, Takken FL. Structure and function of resistance proteins in solanaceous plants. Annu Rev Phytopathol. 2007; 45:43–72. PMID: 17367271

90. van der Biezen EA, Jones JD. The NB-ARC domain: a novel signalling motif shared by plant resistance genes and regulators of cell death in animals. Current Biology. 1998; 8(7):R226–R8. PMID: 9545207

91. Hammond-Kosack KE, Jones JD. Plant disease resistance genes. Annual review of plant biology. 1997; 48(1):575–607.

92. McHale L, Tan X, Koehl P, Michelmore RW. Plant NBS-LRR proteins: adaptable guards. Genome Biol. 2006; 7(4):212. PMID: 16677430

93. Jupe F, Pritchard L, Etherington GJ, MacKenzie K, Cock PJ, Wright F, et al. Identification and localisation of the NB-LRR gene family within the potato genome. BMC genomics. 2012; 13(1):75.

94. Guo Y-L, Fitz J, Schneeberger K, Ossowski S, Cao J, Weigel D. Genome-wide comparison of nucleotide-binding site-leucine-rich repeat-encoding genes in Arabidopsis. Plant physiology. 2011; 157(2):757–69. doi: 10.1104/pp.111.181990 PMID: 21810963

95. Mun J-H, Yu H-J, Park S, Park B-S. Genome-wide identification of NBS-encoding resistance genes in Brassica rapa. Molecular Genetics and Genomics. 2009; 282(6):617–31. doi: 10.1007/s00438-009-0492-0 PMID: 19838736

96. Yang S, Zhu H. Alternative splicing in plant immunity. International journal of molecular sciences. 2014; 15(6):10424–45. doi: 10.3390/ijms150610424 PMID: 24918296
98. Zhang X-C, Gassmann W. RPS4-mediated disease resistance requires the combined presence of RPS4 transcripts with full-length and truncated open reading frames. The Plant Cell. 2003; 15(10):2333–42. PMID: 14523247

99. Dinesh-Kumar S, Baker BJ. Alternatively spliced N resistance gene transcripts: their possible role in tobacco mosaic virus resistance. Proceedings of the National Academy of Sciences. 2000; 97(4):1908–13.

100. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28(12):1647–9. doi: 10.1093/bioinformatics/bts199 PMID: 22543367

101. Tang F, Yang S, Gao M, Zhu H. Alternative splicing is required for RCT1-mediated disease resistance in Medicago truncatula. Plant molecular biology. 2013; 82(4–5):367–74. doi: 10.1007/s11103-013-0068-6 PMID: 23657790

102. Ayliffe MA, Frost DV, Finnegan EJ, Lawrence GJ, Anderson PA, Ellis JG. Analysis of alternative transcripts of the flaxL6rust resistance gene. The Plant Journal. 1999; 17(3):287–92. PMID: 10097386

103. Schornack S, Baltvora A, Gürlebeck D, Peart J, Ganal M, Baker B, et al. The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. The Plant Journal. 2004; 37(1):46–60. PMID: 14675431

104. Tomita R, Sekine K-T, Mizumoto H, Sakamoto M, Murai J, Kiba A, et al. Genetic basis for the hierarchical interaction between Tobamovirus spp. and L resistance gene alleles from different pepper species. Molecular plant-microbe interactions. 2011; 24(1):108–17. doi: 10.1094/MPMI-06-10-0127 PMID: 20879841