Molecular characterization and expression analysis of pitaya (*Hylocereus polyrhizus*) HpLRR genes in response to *Neoscytalidium dimidiatum* infection

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Abstract

Background: Canker disease caused by *Neoscytalidium dimidiatum* is a devastating disease resulting in a major loss to the pitaya industry. However, resistance proteins in plants play crucial roles to against pathogen infection. Among resistance proteins, the leucine-rich repeat (LRR) proteins are a major family that play crucial roles in plant growth, development, and biotic and abiotic stress responses, especially in disease defence.

Results: In the present study, a transcriptomics analysis identified a total of 272 LRR genes, 233 of which had coding sequences (CDSs), in the plant pitaya (*Hylocereus polyrhizus*) in response to fungal *Neoscytalidium dimidiatum* infection. These genes were divided into various subgroups based on specific domains and phylogenetic analysis. Molecular characterization, functional annotation of proteins, and an expression analysis of the LRR genes were conducted. Additionally, four LRR genes (CL445.Contig4_All, Unigene28_All, CL28.Contig2_All, and Unigene2712_All, which were selected because they had the four longest CDSs were further assessed using quantitative reverse transcription PCR (qRT-PCR) at different fungal infection stages in different pitaya species (*Hylocereus polyrhizus* and *Hylocereus undatus*), in different pitaya tissues, and after treatment with salicylic acid (SA), methyl jasmonate (MeJA), and abscisic acid (ABA) hormones. The associated protein functions and roles in signaling pathways were identified.

Conclusions: This study provides a comprehensive overview of the Hp LRR family genes at transcriptional level in pitaya in response to *N. dimidiatum* infection and provides a basis for further in-depth functional studies.

Background

Plants suffer from various biotic and abiotic stresses during growth and development, and one of the most important stresses is disease caused by pathogens. Once attacked by a pathogen, plants perceive and recognize the pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) via cell surface receptors and trigger an immune response, which is known as PAMP or MAMP-triggered immunity (PTI/MTI) [1]. In pathogenic microorganism, flagellin (flg22), elongation factor Tu (EF-Tu), peptidoglycan (PGN) and lipopolysaccharide (LPS) from bacterial, with chitin, chitosan from fungal and β -glucans from oomycetes are typical PAMPs [2]. Once attacked by pathogens, plant first to activate defenses by plant pattern recognition receptors (PRRs) which located in cell surface. PRRs which mainly including TM-LRR are either surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) containing various ligand-binding ectodomains that detect widely conserved PAMPs of the pathogen infection and to activate plant PTI/MTI [3]. The PRR-RKs have a ligand-binding ectodomains, a single-pass transmembrane domain, and an intracellular kinase domain. While, the PRR-RLPs share the same overall structure but lack an intracellular kinase domain.

In plants, two different types of transmembrane receptor kinases are well-known which including receptor-like serine/threonine (Ser/Thr) kinases (also known as receptor-like kinases RLKs) and receptor histidine kinases of PRR-RKs. The RLKs are a large family of well-studied kinases with an extracellular domain, a transmembrane domain and an intracellular kinase domain in generally [4]. RLKs are sub-classed to leucine-rich repeat (LRR), lysine motifs receptor kinase (LYK) and *Catharanthus roseus* RLK1-like (CrRLK1L) according to its N-terminal extracellular domain and the leucine-rich repeat receptor like kinases (LRR-RLKs) were the best studied [5]. RLKs are cell surface localized and can recognize distinct ligands of microbial origin. For example, the flg22 and EF-Tu elf18 are detected by flagellin sensitive 2 (FLS2) and elongation factor Tu receptor (EFR) respectively which belong to LRR-RLKs. The chitin elicitor receptor kinases (CERK1) through homodimerization and phosphorylation to mediate chitin-induced signaling [6]. The LysM-RK lysine motif receptor kinase5 (LYK5) bind fungal chitin to form a chitin inducible complex with CERK1 to induce plant immunity in *Arabidopsis* [7].

However, there also have kinases named receptor-like cytoplasmic protein kinases (RLCKs) based on intracellular localization which with catalytic domain similar to RLKs but without extracellular or transmembrane domains in the RLK family [4]. Botrytis-induced kinase1 (BIK1), a plasma membrane-localized RLCK which defined as a serine/threonine kinase interact with FLS2 and brassinosteroid (BR) insensitive 1-associated kinase 1 (BAK1) to initiate plant immune responses to bacterial flagellin [8]. Another RLCK which named BR-signaling kinase 1 (BSK1) played as a substrate of the brassinosteroid receptor BR insensitive 1 (BRI1) to physically associates with the PAMP receptor FLS2 and positively regulates the PTI [9]. These results investigated that PRRs including receptor kinases (RKs), receptor-like proteins (RLPs) and their interacted kinases or proteins play important roles in plant defense of PTI process.

If PAMP-triggered immunity response is considerable, intracellular immunity is activated. Resistance (R) proteins in plants directly or indirectly recognize pathogen effectors and activate effector-triggered intracellular immunity (ETI). In plants, there is a large leucine-rich repeat (LRR) family of R proteins with the consensus amino acid (aa) sequence motif LxxLxLxxN/CxL (x denotes any aa) [10]. Most LRRs are immune receptors and mainly participate in growth, development, the ETI process of plant–pathogen interactions, and stress defense [11]. The ETI employs intracellular immune receptors which most are nucleotide binding site leucine repeat receptors (NBS-LRRs) proteins to perceive secreted virulence effectors directly or indirectly [3]. The NBS-LRR type proteins are classified to toll/interleukin receptor (TIR)-NBS-LRR (TNL) and coiled-coil (CC)-NBS-LRR (CNL), and these are the majority resistance (R) proteins in response to pathogen infection in plants [12, 13].

The LRR family genes are mainly referenced to encode proteins with a leucine-rich repeat (LRR) conserved domain which including leucine-rich repeat receptor/receptor-like kinases (LRR-RKs/RLKs) subfamily, leucine-rich repeat receptor/receptor-like proteins (LRR-RPs/LRR-RLPs) subfamily, F-box/LRR-repeat protein (FBXL) subfamily and nucleotide binding site leucine rich repeat (NBS-LRR) subfamily that generally participated in plants immunity. In the aspect of structure, the main features of the LRR genes are their structural domain of 20–30 aa and their transmembrane domain containing 2–42 LRRs [14]. Previous studies showed that the LRR domain provides a versatile structural framework for protein–protein interactions and allows proteins to act as molecular switches in relation to pathogen recognition and defense activation [15, 16].

A typical LRR-RLK contains three functional domains: an N-terminal extracellular receptor domain that perceives signals, a single-pass transmembrane domain that anchors the protein within the membrane, and a C-terminal intracellular kinase domain that transduces signals downstream via autophosphorylation, followed by subsequent phosphorylation of specific substrates [17]. In *Arabidopsis thaliana*, the LRR transmembrane receptor kinase

FLS2 is a pattern-recognition receptor that determines the specificity of flagellin 22 (flg22) perception [18]. A *Capsicum annuum* (pepper) LRR protein (CaLRR1) was reported to regulate plant cell death and defense by interacting with two pathogenesis-related proteins (CaPR10 and CaPR4b) and hypersensitive induced reaction 1 protein (CaHIR1) [19]. Another *C. annuum* plasma membrane LRR protein (CaLRR51) acts as a positive regulator in response to bacterial *Ralstonia solanacearum* infection [20].

In the LRR-RLK family, the LRR receptor-like serine/threonine-protein kinase (LRR-STK) subfamily is the largest subfamily. The members play crucial roles in pathogen recognition signaling, the subsequent activation of plant defense mechanisms, and developmental control [21]. BRI1 and BAK1 are a pair of RLKs involved in BR signaling, which regulates plant growth, development, and stress responses [22, 23]. BAK1 is also an LRR-STK that acts as a signaling regulator by interacting with BRI1 in vitro and in vivo in *Arabidopsis* [22]. In contrast to LRR-RLKs, LRR-RPs lack a cytoplasmic kinase domain for initiation of downstream signal transduction in plants [24].

The FBXL subfamily is widely found in animals and plants, and there are approximately 40–50 aa in the N-terminus of the conserved F-box domain [25]. It was reported that F-box proteins are the substrate-recognition subunits of Skp1-Cullin-F-box protein (SCF) ubiquitin ligase complexes [26]. Genome-wide analyses showed that F-box genes respond to salt stress, heavy metals, and drought in soybean and the legume *Medicago truncatula* [27, 28]. Auxin-signaling F-box protein 4 (AFB4), which is similar to the auxin receptor known as Toll/interleukin-1 receptor (TIR1), plays a pivotal role in plant growth, development, and innate immunity, as shown by a combination of physiological, molecular, and genetic approaches [29]. An interesting study by Angot *et al.* (2006) showed that seven type III secretion system (T3SS) effectors that contain both an LRR domain and an F-box domain promote disease in several host plants. The authors stated that this may be because they hijack their host SCF-type E3 ubiquitin ligases to interfere with the host ubiquitin/proteasome pathway to promote disease [30].

In mammals, FBXL2 was reported to interact with the pool of p85 beta subunits to control the phosphatidylinositol-3-kinase (PI3K) signaling cascade by harnessing the proteasomal degradation process [26]. Immunoprecipitation and tandem mass spectrometry (IP-MS) analysis showed that the forkhead box M1 (FoxM1) transcription factor interacted with the FBXL2 protein in gastric cancer [31]. Meanwhile, Chen *et al.* showed that FBXL2 ubiquitinates Aurora B to inhibit tumorigenesis [32]. Furthermore, research by Tosto *et al.* showed that FBXL7 overexpression was associated with Alzheimer's disease in a study that compared Alzheimer's disease-like transgenic mice to wild-type littermates [33]. These results show that FBXL proteins not only participate in growth, development, and disease in plants but also in mammals.

In plants, the NBS-LRR subfamily can directly associate with pathogen-derived effectors as receptors and indirectly sense effector-mediated modification of other host proteins [34]. Functioning as a molecular switch, NBS is a part of the NB-ARC ("nucleotide binding adaptor shared by APAF-1, resistance proteins, and CED-4") domain [35]. Activated plant NBS-LRRs trigger a range of immune responses, such as the hypersensitive response (HR), which culminates in death of infected cells [36]. There are two major classes of NBS-LRRs in plants: the coiled-coil motif (CC)-NBS-LRR (CNL) subfamily and the TIR-NBS-LRR (TNL) subfamily [37]. The CC-NBS-LRR subfamily have an N-terminal CC domain, while the TIR-NBS-LRR subfamily have a TIR domain [38]. A wheat CC-NBS-LRR (*TaRCR1*) was reported to positively contribute to the defence response to the fungal pathogen *Rhizoctonia cerealis* by maintaining reactive oxygen species (ROS) homeostasis [39]. Another CC-NBS-LRR protein *Pm21* in wheat was proved to confer powdery mildew resistance [40]. In the plant *Nicotiana benthamiana*, overexpression of the novel fungal *Plasmopara viticola*-induced TIR-NBS-LRR gene (*VaRGA1*) enhanced disease resistance and drought and salt tolerance [41]. In soybean, overexpression of the TIR-NBS-LRR type R gene GmKR3 enhanced the plant's resistance to several strains of soybean mosaic virus (SMV), the closely related potyviruses bean common mosaic virus (BCMV) and watermelon mosaic virus (WMV), and the secovirus bean pod mottle virus (BPMV) [42]. Similar to other LRR genes, NBS-LRR genes play important roles in plant disease defense signaling.

Pitaya (*Hylocereus polyrhizus* and *Hylocereus undatus*) is an important tropical-subtropical fruit tree found in Central America, East Asia and Southeast Asia. Canker disease caused by the fungi *Neoscytalidium dimidiatum* is one of the most destructive and economically important diseases of in the pitaya industry [43, 44]. At present, due to the lack of reports on the pitaya genome, it is economic and efficient to study biotic and abiotic stress response genes using a high-throughput approach.

The fungal which named *Neoscytalidium dimidiatum* caused canker disease of pitaya has been isolated and identified in our previous work in Hainan Province, China [44]. Transcriptome-wide high-throughput RNA-Sequencing (RNA-Seq) about the expression profiles of resistant genes related to *Neoscytalidium dimidiatum* defense in normal and diseased stem tissues were made ensue [45]. Here, we analyzed and studied the pitaya (*Hylocereus polyrhizus*) LRR receptor-like (*HpLRR*) family resistant genes in response to *N. dimidiatum* infection based on previous RNA-Seq data detailly. Transcriptomics, molecular characterization, and expression results regarding LRR subfamilies in pitaya after *N. dimidiatum* infection have not been previously reported. This study provides an overview of the subfamily classification of LRR genes in pitaya and provides a basis for future functional studies. The evolutionary history and functions of each LRR subfamily in pitaya remains to be understood in future research.

Results

Identification and phylogenetic analysis of pitaya *HpLRR* transcriptional genes

A total of 272 *HpLRR* transcriptional genes were identified based on *de novo* transcriptomic analysis. The heatmap analysis indicated that most LRR genes were up-regulated in diseased pitaya tissues. Among these genes, 12 (Unigene19327_All, Unigene21125_All, Unigene13635_All, CL1260.Contig2_All, CL2218.Contig1_All, Unigene19955_All, Unigene13405_All, Unigene18881_All, Unigene9087_All, Unigene13867_All, Unigene15298_All, and Unigene12636_All) were significantly up-regulated based on a log2FoldChange (D/N) ≥ 1.0 (FDR ≤ 0.001) (Supplemental Material 1). The 272 *HpLRR* transcriptional genes underwent a BLAST analysis (Supplemental Material 1) involving six protein databases (nr/nt, SwissProt, KEGG, COG, InterPro, and GO). Based on the BLAST analysis, these genes were annotated as belonging to the LRR-STK subfamily (135), FBXL subfamily (49), NBS-LRR subfamily (29), LRR-RLK subfamily (26),

plant intracellular Ras group-related LRR (PIRL) subfamily (8), LRR transmembrane protein kinase subfamily (5), brassinosteroid LRR receptor kinase subfamily (3), and other LRR genes (17) (Table 1). Heatmaps of the 272 *Hp*LRR gene expression levels in D1, D3, N2 and N3 samples are presented in Fig 1 and 2. Although LRR-STK genes belong to the LRR-RLK subfamily, in order to distinguish them from common LRR-RLK genes, separate heatmaps were constructed.

Among the 272 *Hp*LRR genes, 39 have no CDS, based on base sequence analysis using A plasmid Editor (ApE) software (<http://jorgensen.biology.utah.edu/wayned/appe/>). In contrast, some *Hp*LRR genes had multiple CDSs. The 33 *Hp*LRR genes with CDSs >1.0 kb were selected for gene annotation and gene structure analysis, while the four *Hp*LRR genes with the longest CDSs were selected for further expression analysis (at different fungal infection stages, in different pitaya tissues, and after plant hormone treatment). The aa sequence and molecular weight of the 233 *Hp*LRR genes with CDSs varied greatly, from 30 aa/3.382 kDa to 1138 aa/128.777 kDa (Supplementary Material 2). The aa sequences of the 233 *Hp*LRR genes were used for phylogenetic analysis using MEGA 6.0 [46], and the nine significantly up-regulated genes with CDSs are marked in red (Fig 3). The phylogenetic analysis showed that the 233 genes could be divided into eight subfamilies (Fig 3 and Table 2). This indicated that these genes have some degree of aa-level similarity, suggesting evolutionary relationships. Nevertheless, the base and aa sequence evolutionary results were not consistent (Table 2), due to the degeneracy of codons.

Gene structure of the 33 *Hp*LRR transcriptional genes with CDSs >1.0 kb

It was hard to conduct further research on all the LRR genes as there were many of them. Hence, we selected genes with CDSs >1.0 kb for gene structure analysis and conserved motifs analysis (Fig 4) for further study. The detailed informations of these 33 genes with CDSs >1.0 kb was showed in Table 3. All 33 *Hp*LRR transcriptional genes have upstream and downstream sequences. CL1599.Contig2_All and CL1599.Contig1_All; CL971.Contig1_All and CL971.Contig2_All; CL1165.Contig6_All and CL1165.Contig3_All; Unigene11462_All, Unigene8930_All, and Unigene7537_All; and Unigene2712_All and CL332.Contig2_All have 99–100% similarity, indicating that each set of two or three genes have close evolutionary relationships.

Verification of 12 differentially expressed LRR genes (DEGs) by qRT-PCR

Based on a PoissonDis analysis, 12 genes were significantly up-regulated ($\log_2\text{FoldChange (D/N)} \geq 1.0$, $\text{FDR} \leq 0.001$) among the total set of 272 LRR genes. Among these 12 genes, seven (Unigene15298_All, Unigene21125_All, Unigene13635_All, CL1260.Contig2_All, Unigene13867_All, Unigene19327_All, and Unigene9087_All) were annotated as belonging to the LRR-STK subfamily, three (CL2218.Contig1_All, Unigene19955_All and Unigene13405_All) as belonging to the FBXL subfamily, one (Unigene18881_All) as a disease resistance gene, and one (Unigene12636_All) as belonging to the PIRL subfamily.

To verify the RNA-Seq results, qRT-PCR assays were performed. The qRT-PCR expression level trends of 11 of the 12 genes (not Unigene19955_All) were consistent with the RNA-Seq results (Fig 5). In addition, three of the 12 (Unigene15298_All, Unigene21125_All, and CL2218.Contig1_All) have no CDS, and the number of aa of the remaining nine genes ranged from 35–290 (Supplementary Material 2).

Expression profiles of four *Hp*LRR genes under different stages of *N. dimidiatum* infection in different pitaya species

Four *Hp*LRR genes (CL445.Contig4_All, Unigene28_All, CL28.Contig2_All, and Unigene2712_All) were selected for the subsequent expression analysis as they had the four longest CDSs (Supplementary Material 2). The conserved domain analysis indicated that CL445.Contig4_All and Unigene2712_All were NBS-LRR type resistance proteins, while Unigene28_All and CL28.Contig2_All were probable LRR-STKs (Fig 6). The four genes have 3–9 LRR motifs (Fig 6). In addition, CL445.Contig4_All and Unigene2712_All belong to the RX-CC_like family ("Coiled-coil domain of the potato virus X resistance protein and similar proteins") and the NB-ARC family. Unigene28_All and CL28.Contig2_All have specific hits regarding the STKc_IRAK family ("Catalytic domain of the serine/threonine kinases, interleukin–1 receptor associated kinases, and related STKs") and a common LRR-RLK (the PLN00113 superfamily). CL28.Contig2_All has a specific malectin-like domain; malectin is a novel endoplasmic reticulum carbohydrate-binding protein and a candidate player in the early steps of protein N-glycosylation [47].

The pitaya stems presented obvious symptoms after 3 days of *N. dimidiatum* infection and rotted by day 15 (Fig 7). However, compared with red-fleshed pitaya (Fig 7A), the white-fleshed pitaya (Fig 7B) possessed strong resistance. Due to the protective hard wax coat on the pitaya stems, the expression levels of the four LRR genes peaked after 3/4 days of infection in red-fleshed pitaya while 4/6 days in white-fleshed pitaya. The expression results (Fig 8A) suggested that, in red fleshed pitaya *Hylocereus polyrhizus*), compared with control level (0 h), CL445.Contig4_All expression first decreased and then suddenly increased on day 3, then decreased, and finally exhibited an increasing trend but remained lower than the control level. Unigene28_All and CL28.Contig2_All expression levels were increased after *N. dimidiatum* infection, but Unigene28_All reached a peak on day 3 while CL28.Contig2_All reached a peak on day 4. In contrast to the other three genes, Unigene2712_All expression always decreased compared with the control level. These results indicated that the four genes participated in the fungal infection response of pitaya, especially CL28.Contig2_All, which was significantly up-regulated on day 4. In contrast to the other three genes, Unigene2712_All may act as a negative regulator in plant–pathogen interactions. In the white-fleshed pitaya (*Hylocereus undatus*), the genes expression has the similarity trend. However, the infection time of the genes reached a peak in white-fleshed pitaya was later one day compared with red-fleshed pitaya (Fig 8B). The four *Hp*LRR genes expression files results were consistent with the infection symptoms of the two different pitaya species (Fig 7).

Tissue-specific expression profiles of the four *HpLRR* genes

Tissue-specific genes (also known as luxury genes) are genes whose products have specific functions in specific cell types. To investigate whether the four *HpLRR* genes we selected have specific functions in specific cells, tissue-specific expression profiles were obtained by qRT-PCR for 14 pitaya tissues (Fig 9). CL445.Contig4_All was mainly expressed in the pericarp of a young green fruit; Unigene28_All was mainly expressed in the stamen, petal, and fruit pulp of both a young green fruit and a red fruit; CL28.Contig2_All was significantly expressed in the pericarp of both a young green fruit and a red fruit, and Unigene2712_All was mainly expressed in the flower bud. These results showed that Unigene28_All was significantly up-regulated and may play pivotal roles in pitaya flower and fruit growth and development.

Expression profiles of the four *HpLRR* genes in response to SA, ABA, and MeJA treatments

In plants, hormones play important roles in response to a wide range of biotic and abiotic stress signaling networks. SA, ABA, jasmonates (JAs), and ethylene have crucial well-known roles in plant disease and pest resistance [48]. To better understand the four *HpLRR* genes' responses to hormonal regulation of the plant-pathology interaction pathways, the expression patterns of these genes in response to SA, ABA, and MeJA treatments were assessed by qRT-PCR (Fig 10). All four genes responded to the three hormones to some degree. Unigene28_All expression was significantly changed after 2 h of ABA treatment. CL28.Contig2_All was prominently expressed at 48 h of SA treatment. Unigene2712_All was significantly down-regulated by ABA, but significantly up-regulated by SA, reaching a peak at 24 h. The results showed that Unigene28_All and CL28.Contig2_All may play pivotal roles in the hormone-mediated disease resistance response.

Discussion

In the present study, we identified 272 LRR genes in pitaya in the *de novo* transcriptome assembly analysis, and 233 of them have CDSs. Most of these genes belonged to the LRR-RLK, NBS-LRR, or FBXL subfamilies and, within the LRR-RLK subfamily, the LRR-STK subfamily had the largest number of genes. Despite having distinct evolutionary origins, most animals and plants need cell-surface or intracellular immune receptors with an LRR domain to detect pathogens and trigger defence responses [49]. In mammals, LRR kinases play key roles in disease, especially Parkinson's and Crohn's diseases [50, 51]. In plants, most of these LRRs are resistance proteins and can directly associate with pathogen-derived effectors or indirectly sense effector-mediated modification of other host proteins [52]. Diverse classes of cell-surface immune LRR receptors share important features for the initiation of resistance. The LRR genes identified in this study may play crucial roles in the pitaya immune response. The phylogenetic analysis showed that the base and aa sequence results were not consistent (Table 2) due to the degeneracy of codons. Additionally, genes with similar base sequences do not necessarily have similar functions.

Besides the three large LRR subfamilies, we also found eight PIRL genes, five LRR transmembrane protein kinase genes, three brassinosteroid related LRR receptor kinase genes, and 17 other LRR genes, such as genes for CLAVATA (CLV)-like LRR receptor kinases, the LRR protein SHOC-2-like, and the LRR receptor-like protein FASCIATED EAR2 (Fig 2). PIRLs are a plant-specific class of LRR proteins that take part in developmental cell signaling and gene regulation, for example, in *A. thaliana*, PIRL1 and PIRL9 have redundant roles that are essential at a key transition point early in pollen development [53].

Most LRR-RLKs are transmembrane kinase and phosphorylate serine/threonine residues (also known as LRR-STK), and most LRR-RLKs is recognition of an extracellular ligand, which leads to activation of the intracellular kinase domain and subsequent transduction of downstream signaling pathways [54]. The five LRR transmembrane protein kinases genes that we identified may belong to the LRR-RLKs subfamily.

It has been reported that the LRR-RLK gene BAK1/SERK3 is involved in brassinosteroid signaling and is a major modulator of PAMP-triggered immunity in *A. thaliana* and *N. benthamiana* [55]. The three brassinosteroid LRR receptor kinase genes in this study may play critical roles in the brassinosteroid signaling pathway in response to *N. dimidiatum* infection of pitaya.

It has been reported that CLV1 is an RLK composed of an LRR-containing extracellular domain (with putative receptor function) and a cytoplasmic STK domain (linked via a transmembrane domain); it is thought to play an important role in meristem maintenance and flower development [54, 56]. Compared with CLV1, CLV2 is structurally similar but with a very short predicted cytoplasmic tail [57]. CLV3 is a stem cell-specific protein that activates the *Arabidopsis* CLAVATA stem cell signaling pathway [58]. These reports indicate that CLV proteins mainly participate in the growth and development of plants.

Four genes (CL445.Contig4_All, Unigene28_All, CL28.Contig2_All, and Unigene2712_All) were selected (because they had the four longest CDSs) for a further expression analysis. Among them, CL445.Contig4_All and Unigene2712_All were NBS-LRR resistance proteins, while Unigene28_All and CL28.Contig2_All were probable LRR receptor-like STK genes (Fig 6). CL28.Contig2_All was significantly up-regulated on day 4 after *N. dimidiatum* infection while Unigene2712_All was significantly down-regulated in all infection stages. These two genes may play positive or negative roles in plant disease responses. Unigene28_All was significantly expressed on day 3 after *N. dimidiatum* infection and, based on the tissue-specific expression profiles, also significantly expressed in the stamen, petal, and fruit pulp of both a young green fruit and a red fruit. These results showed that Unigene28_All not only participates in pitaya flower and fruit growth and development but also in the disease resistance response.

Plant hormones, which include SA, JAs, ABA, brassinosteroid, ethylene, and auxin, act as signals to trigger and mediate plant immune responses [59]. In plants, SA is a secondary metabolite with a central role as a signaling molecule in the activation of plant resistance to pathogens [60, 61]. Research has shown that SA accumulation was increased in infected leaves and uninfected leaves that mediated systemic acquired resistance (SAR) [62]. SA accumulation can parallel the increase in pathogenesis-related protein gene expression [61]. For example, BAK1 overexpression caused SA accumulation and deregulation of cell death control genes [63].

JAs, including jasmonic acid and the MeJA derivatives, have proved effective at improving plant stress tolerance [64]. It was reported that JAs are key signaling compounds in hypersensitive response cell death and accumulate in response to bacterial flg22 in the grape *Vitis rupestris* [65].

Similar to SA and JAs, ABA (a weak acid that contains 15 carbon atoms) is an important stress hormone and is involved in physiological processes [66]. ABA participates in embryogenesis, seed germination, floral transition, and so on [67, 68].

However, plant growth, development, and stress responses need multiple phytohormones involved in complex signaling network crosstalk [69]. In this study, pitaya stems were treated with SA, MeJA, and ABA. Although all four genes responded to the three hormones, CL28.Contig2_All expression reached a peak at 48 h after SA treatment and Unigene28_All was clearly regulated by ABA at 2 h. Taking all the expression results together, we selected these two genes for further functional research. The subsequent analysis of conserved functional motifs in Unigene28_All and CL28.Contig2_All indicated that these two genes both have the typical LxxLxLxxNxL domain and the STRVGTIGYMAPE or SSVAXGTL/VGYLDPE conserved sites of STKc-IRAK (x denotes any aa) (Fig 11). These domains or conserved sites may play important roles in protein function and signaling. However, in some cases, an LRR protein requires another helper or partner protein for functionality, so a complete functional study (including screening for interacting proteins) regarding Unigene28_All and CL28.Contig2_All is being carried out and the results will be published in the future.

Canker disease caused by *N. dimidiatum* is one of the most serious diseases of pitaya in the main growing regions [44]. The *N. dimidiatum* is a fungal which has a strong specificity infection of its host pitaya. Currently, there is a very hard technological bottleneck to break through in building tissues culture system in pitaya. So, it is very difficult to investigate the genes functions according to transgene methods in pitaya limit to the technology of genetic transformation system. The fungal also can't infect the model plants like *Arabidopsis thaliana*, tobacco and tomato. So, it is a very hard work to identify the genes function with really deeply experiments. At present, there are no reports on transcriptomics, molecular characterization, and expression analyses of LRR genes in pitaya in response to *N. dimidiatum* infection. From the expression profiles, we can identify the *HpLRR* genes in response to pitaya canker disease preliminary. These results provide important research information for a relatively poor studied pathosystem at this moment and provide a framework for further functional investigation of the pitaya LRR subfamilies and contribute to a better understanding of the complexity of LRR genes in higher plants.

Conclusions

Canker disease caused by the fungus *N. dimidiatum* is one of the most important diseases because of its rapid spread and strong specificity infection in its host pitaya. The occurrence of canker disease not only poses a significant threat in yields but also affects the quality of pitaya. Currently, it is an important job to excavate the resistant genes and explain the functions of them. This study mainly analyzed the structures, molecular characterizations and expression profiles in different infected stages and plant hormone treatments of *HpLRR* family resistant genes. Results of this study provided a comprehensive overview of the *HpLRR* family genes at transcriptional level in pitaya in response to *N. dimidiatum* infection and provided a basis for further in-depth functional studies.

Methods

Plant materials and growth conditions

The normal (N1, N2, and N3) and diseased (D1, D2, and D3) tissues of pitaya (*Hylocereus polyrhizus*) stems used for RNA-seq were collected from Ledong County, Hainan Province, China that had been grown for about 5 years under natural conditions on a plantation. *De novo* transcriptome assembly and RNA-Seq were conducted using an Illumina HiSeq System by Shenzhen BGI Tech Company (Shenzhen, China). After analysis, the N1 and D2 samples were abandoned due to poor repeatability. Therefore, the bioinformatics analysis and follow-up experiments were carried out based on four samples (N2, N3, D1, and D3). The clean RNA-Seq data and processed files were submitted to the Gene Expression Omnibus (GEO) database via the GenBank website (accession number GSE119976). In addition, the pitaya stem tissues for *N. dimidiatum* fungal infection, hormone treatment and tissues specific expression were collected in a plantation of Hainan University not same with those used for RNAseq. Five pitaya plants were used in each condition and displayed similar symptoms. All five pitaya plants were merged together to use for qRT-PCR experiments.

Identification and phylogenetic analysis of pitaya *HpLRR* transcriptional genes

A *de novo* transcriptome assembly analysis was used to identify LRR genes and a Basic Local Alignment Search Tool (BLAST) analysis in the non-redundant protein sequences (nr/nt), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Cluster of Orthologous Groups of proteins (COG), InterPro, and SwissProt public protein databases was used to annotate and analyze these genes. The LRR transcripts were selected from the all-unigene database (nr, GO, KEGG, COG, InterPro and SwissProt). The expression levels of all *HpLRR* genes were analyzed by hierarchical clustering and heatmap analysis using the free online data analysis platform OmicShare tools (<http://www.omicshare.com/tools/>, Fig 1 and 2). Thereafter, 233 LRR genes with coding sequences (CDSs) were used to construct a phylogenetic tree based on 1000 bootstrap replicates and the neighbor-joining (NJ) method using MEGA 6.0 software [46].

Gene structure and phylogenetic analysis of 33 *HpLRR* transcriptional genes with CDSs >1.0 kb

There were 33 *HpLRR* transcriptional genes with CDSs >1.0 kb. Gene structure analysis of these genes was performed using the free online platform Gene Structure Display Server (GSDS v2.0, <http://gsds.cbi.pku.edu.cn>) by uploading the genes' CDSs and assembly sequences. Conserved motifs analysis was carried on MEME program based on the amino acid sequences of the CDS. The phylogenetic analysis was carried out based on 1000 bootstrap replicates and the neighbor-joining (NJ) method using MEGA 6.0 software [46].

Verification of 12 significantly up-regulated differentially expressed LRR genes (DEGs) by quantitative reverse transcription (qRT)-PCR

In order to verify the reliability and consistency of the up or down regulated genes, all of the 12 differentially expressed LRR genes based on a PossionDis analysis were selected to perform qRT-PCR assay. The total RNA extraction of N2, N3, D1 and D3 pitaya samples were used improved cetyltrimethylammonium bromide (CTAB) method [70]. After treated with DNase I (Thermo Fisher Scientific, USA), first strand cDNA synthesis was conducted according to the manufacturer's protocols using the PrimeScript™ 1st strand cDNA synthesis kit (code No.6210A, Takara, Japan). The cDNA template of N2 and N3 samples were balanced to mixed as total N sample, same treatment with the cDNA of D1 and D3 to total D sample. qRT-PCR was performed using a 20-μL reaction system (ChamQ™ Universal SYBR® qPCR Master Mix, Q711–02/03, Vazyme Biotech Co., Ltd, Beijing, China), containing 10 μL of ChamQ Universal SYBRqPCR Master Mix (2×), 2 μL cDNA templates, 0.4 μL PCR forward primer (10 μM), 0.4 μL PCR reverse primer (10 μM) and 7.2 μL of double distilled water. For 12 significantly up-regulated LRR genes with log2FoldChange (D/N) ≥ 1.0 (false discovery rate [FDR] ≤ 0.001), qRT-PCR assays (with three technical replicates) were carried out using a 7500 Applied Biosystems qRT-PCR System (Life Tech, 81 Wyman Street, Waltham, MA, 02454, USA). The primers for the 12 LRR genes were designed using Primer Premier 6 software (www.premierbiosoft.com). The pitaya ubiquitin gene (*UBQ*) was used as an internal reference for data normalization [71]. The primer information for the 12 genes and *UBQ* is presented in Supplementary Information 3. The PCR conditions were performed as following: 50°C for 3 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Output data were generated using the Applied Biosystems 7500 software version 2.0.6 (ABI, America). The relative quantification method (Delta-Delta cycle threshold, $2^{-\Delta\Delta CT}$) was conducted to evaluate quantitative variation based on three technical replicates.

Expression profiles of four *Hpl*LRR transcriptional genes under different stages of *N. dimidiatum* infection in different pitaya species

Among the total set of LRR genes, four *Hpl*LRR transcriptional genes (CL445.Contig4_All, Unigene 28_All, CL28.Contig2_All, and Unigene 2712_All) were selected for further expression research as they had the four longest CDSs. Red-fleshed pitaya (*Hylocereus polyrhizus*) “Jinduyihao” and white-fleshed pitaya (*Hylocereus undatus*) “Yuenanbairou” tender stems were used for *N. dimidiatum* infection. *N. dimidiatum* hypha were collected after growing them in potato dextrose agar medium for 7–10 days. Before inoculation, the stems were sterilized twice using 75% ethyl alcohol. The hyphae were diluted, with 20–40 conidia in suspension, using 400X microscope magnification (Olympus Corporation, Tokyo, Japan). Inoculation was conducted by spraying the conidial suspension onto the pitaya stems. After spraying, stems were wrapped in absorbent wool with distilled water to retain moisture. The plants were then placed in an illuminated incubator at 28°C under a 16/8 h light/dark cycle for 15 days. The pitaya stem tissues were collected for total RNA extraction after 0 h, 4 h, 12 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, and 15 days of *N. dimidiatum* infection. The total RNA was reversed transcribed into cDNA and the cDNA was used for qRT-PCR (as in the previous steps). The qRT-PCR primers for the four LRR genes were designed using Primer Premier 6.0 software (www.premierbiosoft.com). The primer details for the four genes are presented in Supplementary Information 3.

Expression profiles of the four *Hpl*LRR genes in different tissues of pitaya

The following 14 pitaya tissues were studied: (1) flower bud, (2) stamen, (3) pistil, (4) simple style, (5) petal, (6) calyx, (7–12) pericarp and fruit pulp of a young green, mature green, and red fruit, (13) tender stems, and (14) older stems were collected in pitaya plantation, Hainan province. The total RNA of the 14 tissues was extracted, reversed transcribed into cDNA, and used for qRT-PCR to obtain the expression profiles of the four selected genes.

Response of the four *Hpl*LRR genes to salicylic acid (SA), methyl jasmonate (MeJA), and abscisic acid (ABA) treatments

Pitaya stems were collected from the plantation and sterilized twice using 75% ethyl alcohol before being treated with 5 mM SA solution (pH 5.7), 1 mM MeJA solution, and 100 μM ABA solution. The three solutions were all added with 0.01% Silwet–77 (Code: DE0025, Beijing BioDee Biotechnology Co. Ltd, China) to reduce the water's surface tension. The stem tissues were collected after 0 h, 0.5 h, 1 h, 2 h, 4 h, 12 h, 24 h, and 48 h of hormone treatment. RNA extraction and qRT-PCR assays were performed as in the previous steps.

Abbreviations

LRRs: leucine-rich repeat genes; LRR-RLKs: leucine-rich repeats receptor-like kinases; NBS-LRRs: nucleotide binding site leucine-rich repeats; LRR-STKs: leucine-rich repeats receptor-like serine / threonine-protein kinases; BRI1: Brassinosteroid-insensitive 1; BAK1: BRI1-associated receptor kinase 1; NB-ARC: nucleotide binding adaptor shared by Apaf–1 resistance proteins, CED–4 domain; CC-NBS-LRR: coiled-coil motif nucleotide-binding site leucine-rich repeat; SA: Salicylic acid; JA: jasmonates; MeJA: acid methyl ester; ABA: abscisic acid; SAR: systemic acquired resistance; PR: pathogenesis related protein.

Declarations

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Author contributions

TH* conceived the study and participated in the design of all experiments. HJQ* as co-corresponding author provided funding supports for the experiments and edited the language of the manuscript. XM performed the qRT-PCR analysis, statistical analysis and wrote the manuscript. LCL carried out RNA extraction, qRT-PCR validation and statistical analysis. FY, LZW and GPY conducted the bioinformatics studies. XR, CY and WSS participated in data analysis and discussion about the experiments. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The orchard of Ledong Country, Hainan Province clarified the permissions granted to collect *H. polyrhizus* plant materials for research in this article.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Classification of LRRs family genes based on six protein databases

Kinds of LRR	Description	number	
I	LRR-STK subfamily	135	CL340.Contig1_AIII CL340.Contig2_AIII CL340.Contig7_AIII Unigene8225_AIII Unigene14565_AIII Unigene18117_AIII Unigene6414_AIII CL13.Contig1_AIII CL13.Contig2_AIII CL1417.Contig2_AIII Unigene24242_AIII Unigene1372_AIII CL
II	LRR-RLK subfamily	26	
III	NBS-LRR subfamily	29	
IIII	FBXL subfamily	49	
V	plant intracellular Ras group-related LRR (PIRL) subfamily	8	
VI	LRR transmembrane protein kinase	5	
VII	Brassinosteroid LRR receptor kinase	3	
VIII	Other LRR genes	17	
Total Number LRR Family Genes			

Table 2. Sequence characteristics of 233 HpLRR genes with CDSs.

Phylogenetic analysis classification	Number of genes	aa length	Molecular weight (kDa)	Gene annotation and number
I	16	31–465	3.723–50.518	LRR receptor-like/receptor-like STK (9)
				NBS-LRR type resistance protein (3)
				FBXL (2)
				Brassinosteroid LRR receptor kinase (1)
II	32	31–927	3.382–103.312	LRR transmembrane protein kinase (1)
				LRR receptor-like/receptor-like STK (16)
				FBXL (6)
				NBS-LRR type resistance protein (6)
				LRR transmembrane protein kinase (1)
III	24	32–604	3.393–66.517	Brassinosteroid LRR receptor kinase (1)
				LRR receptor-like/receptor-like STK (15)
				FBXL (3)
				NBS-LRR type resistance protein (3)
				PIRL (2)
IV	30	31–1138	3.451–128.777	Brassinosteroid LRR receptor kinase-like (1)
				LRR receptor-like/receptor-like STK (16)
				NBS-LRR type resistance protein (6)
V	26	34–364	3.652–40.136	FBXL (5)
				LRR transmembrane protein kinase (3)
				LRR receptor-like/receptor-like STK (24)
VI	27	36–501	4.176–55.805	FBXL (2)
				LRR receptor-like/receptor-like STK (16)
				FBXL (5)
				PIRL (4)
VII	24	32–515	3.610–57.478	NBS-LRR type resistance protein (1)
				Disease resistance protein RGA4 (1)
				LRR receptor-like/receptor-like STK (15)
VIII	54	30–504	3.501–54.341	FBXL (7)
				NBS-LRR type resistance protein (2)
				LRR receptor-like/receptor-like STK (36)
				FBXL (11)
				NBS-LRR type resistance protein (5)
				PIRL (1)
				Disease resistance RPP13-like protein (1)

FBXL: F-box/LRR-repeat protein; LRR: leucine-rich repeat; NBS: nucleotide-binding site; PIRL: plant intracellular Ras group-related LRR; RLK: receptor-like kinase; STK: serine/threonine-protein kinase.

Table 3. Information of the 33 pitaya *Hp*LRR transcriptional genes with CDSs >1.0 kb

Unigene	Length	CDS	N-FCPM	D-FCPM	log2FoldChange(D/N)	Annotation (nr, SwissProt, KEGG, COG, InterPro, GO)
CL445.Contig4_All	3716	3417	0.49	1.77	1.85	NBS-LRR type resistance protein
Unigene28_All	3386	3006	0.88	3.82	2.13	Probable LRR receptor-like STK RFK1 isoform X1
CL28.Contig2_All	3487	2784	0.65	2.89	2.15	Probable LRR receptor-like STK At1g67720-like
Unigene2712_All	2825	2636	1.39	3.10	1.15	NBS-LRR type resistance protein
CL218.Contig5_All	4019	2529	0.62	0.82	0.42	NBS-LRR type resistance protein
CL218.Contig2_All	4089	2529	0.78	1.41	0.85	NBS-LRR type resistance protein
CL1599.Contig2_All	2057	1938	0.81	1.82	1.17	FBXL 10-like
CL1599.Contig1_All	2093	1938	0.26	0.64	1.29	FBXL 10-like
Unigene12546_All	2161	1815	0.47	2.1	2.16	Probable LRR receptor-like protein kinase At5g63930-like
CL332.Contig2_All	1749	1714	1.05	2.34	1.16	NBS-LRR type resistance protein
CL1165.Contig6_All	1953	1566	0.12	0.12	0.00	Probable LRR receptor-like STK At5g10290-like isoform X1
CL1260.Contig3_All	1971	1548	0.09	2.45	4.76	Probable LRR receptor-like STK At3g47570-like
Unigene4411_All	1802	1515	0.23	1.15	2.35	FBXL At5g63520-like
Unigene6828_All	2223	1506	0.79	1.7	1.11	PIRL 1-like
Unigene9555_All	1769	1449	0.87	1.56	0.85	Probable LRR receptor-like STK At1g06840-like
CL218.Contig3_All	2429	1428	0.48	0.64	0.43	NBS-LRR type resistance protein
Unigene8646_All	1712	1398	3.05	22.85	2.91	LRR receptor-like STK FLS2
CL9.Contig5_All	3183	1389	1.18	4.88	2.05	NBS-LRR gene cluster, complete sequence
CL1165.Contig3_All	2675	1281	0.33	1.15	1.80	Probable LRR receptor-like STK At5g10290-like isoform X1
CL332.Contig1_All	1245	1219	0.96	1.33	0.48	NBS-LRR type resistance protein
CL971.Contig1_All	1708	1203	0.05	0.71	3.97	Probable LRR receptor-like STK At5g45840-like
CL971.Contig2_All	1828	1203	0.48	0.50	0.04	Probable LRR receptor-like STK At5g45840-like
CL218.Contig4_All	2640	1194	0.82	1.70	1.05	NBS-LRR type resistance protein
Unigene8930_All	1465	1188	0.26	2.41	3.24	Probable LRR receptor-like STK At1g56130-like
Unigene7537_All	1389	1182	0.22	1.84	3.09	Probable LRR receptor-like STK At1g53430
CL6.Contig2_All	1344	1179	0.11	0.86	2.96	Probable LRR receptor-like STK At1g53430-like
Unigene19708_All	1682	1179	0.05	1.78	5.15	Putative LRR receptor-like STK
Unigene8268_All	1469	1167	0.74	0.76	0.04	Probable LRR receptor-like STK At4g36180
Unigene8383_All	1373	1113	0.25	1.03	2.07	Putative FBXL
Unigene11462_All	1447	1095	0.26	1.89	2.86	Probable LRR receptor-like STK At1g56140
CL1259.Contig3_All	1145	1091	1.09	4.54	2.06	NBS-LRR type resistance protein
CL1240.Contig2_All	1174	1049	0.10	0.68	2.75	Probable LRR receptor-like STK At1g06840 isoform X3
Unigene179_All	2179	1005	1.16	1.79	0.63	Probable LRR receptor-like STK At1g14390

F-box/LRR-repeat protein; LRR: leucine-rich repeat; NBS: nucleotide-binding site; PIRL: plant intracellular Ras group-related LRR; RLK: receptor-like kinase; STK: serine/threonine-protein kinase.

Figures

D1	D3	N2	N3	Gene ID and Description	D1	D3	N2	N3	Gene ID and Description
2	1	0	0	CL1340.Contig1_All LRR-STK, BAM1	0	0	0	0	CL128.Contig1_All LRR-STK, Atlg67720 isoform X1
3	2	0	0	CL1340.Contig2_All LRR-STK, BAM1	0	1	0	0	Unigene04431_All LRR-STK, Atlg67720-like
2	5	1	1	CL1340.Contig7_All LRR-STK, BAM1	2	4	1	1	CL128.Contig2_All LRR-STK, Atlg67720-like
1	3	1	1	Unigene2252_All LRR-STK	1	0	0	0	Unigene251245_All LRR-STK, Atlg74360
0	1	0	0	Unigene14565_All LRR-STK, EFR	0	1	0	0	Unigene17869_All LRR-STK, Atg216250
0	1	0	0	Unigene18117_All LRR-STK, EFR	1	0	0	0	Unigene10557_All LRR-STK, Atg216250
1	0	0	0	Unigene4144_All LRR-STK, EFR-like	1	1	0	0	Unigene10791_All LRR-STK, Atg216250
1	0	0	0	CL131.Contig1_All LRR-STK, EFR-like	1	1	0	0	Unigene47114_All LRR-STK, Atg216250-like
1	0	0	0	CL131.Contig2_All LRR-STK, EFR-like	0	0	0	0	Unigene14537_All LRR-STK, Atg242130-like
0	2	0	0	CL1417.Contig2_All LRR-STK, ERF1A-like isoform X2	0	1	0	0	Unigene16909_All LRR-STK, Atg242130-like
1	0	0	0	Unigene24242_All LRR-STK, FFI	0	1	0	0	Unigene18179_All LRR-STK, Atg47570
3	1	1	1	Unigene1372_All LRR-STK, FFI	0	1	0	0	Unigene19845_All LRR-STK, Atg47570
2	2	2	2	CL1877.Contig2_All LRR-STK, FFI1	0	1	0	0	Unigene19994_All LRR-STK, Atg47570
0	0	0	0	Unigene5090_All LRR-STK, FFI1	0	1	0	0	Unigene13782_All LRR-STK, Atg47570
1	1	0	0	CL1877.Contig4_All LRR-STK, FFI1	0	3	0	0	Unigene13635_All LRR-STK, Atg47570
1	3	1	1	CL1877.Contig1_All LRR-STK, FFI1-like	1	3	0	0	CL1260.Contig2_All LRR-STK, Atg47570
0	1	0	0	CL1877.Contig3_All LRR-STK, FFI1-like isoform 1	0	3	0	0	CL1586.Contig2_All LRR-STK, Atg47570
0	0	0	0	Unigene26481_All LRR-STK, F1S2	1	1	0	1	Unigene5348_All LRR-STK, Atg47570
0	0	0	0	Unigene21861_All LRR-STK, F1S2	0	2	0	0	CL292.Contig1_All LRR-STK, Atg47570
1	0	0	0	Unigene26736_All LRR-STK, F1S2	3	2	1	1	Unigene17164_All LRR-STK, Atg47570
0	0	1	1	Unigene13319_All LRR-STK, F1S2	0	1	0	0	Unigene7827_All LRR-STK, Atg47570
0	1	0	0	Unigene16887_All LRR-STK, F1S2	0	0	0	0	Unigene13612_All LRR-STK, Atg47570 isoform X2
0	1	0	0	Unigene1318_All LRR-STK, F1S2	0	2	0	0	Unigene19327_All LRR-STK, Atg47570-like
0	0	1	0	Unigene11109_All LRR-STK, F1S2	1	0	0	0	CL1260.Contig1_All LRR-STK, Atg47570-like
0	0	0	0	Unigene1644_All LRR-STK, F1S2	2	2	0	0	Unigene13867_All LRR-STK, Atg47570-like
0	1	0	0	Unigene18552_All LRR-STK, F1S2-like	0	2	0	0	CL471.Contig2_All LRR-STK, Atg47570-like
0	2	0	0	Unigene2001_All LRR-STK, F1S2-like	1	4	0	0	CL1260.Contig3_All LRR-STK, Atg47570-like
4	1	0	0	Unigene8244_All LRR-STK, F1S2-like	1	0	0	0	Unigene26202_All LRR-STK, Atg08850
0	0	0	0	Unigene18671_All LRR-STK, GSO1	1	1	1	1	Unigene1367_All LRR-STK, Atg08850
1	1	0	0	Unigene13266_All LRR-STK, GSO1-like	1	1	0	0	CL2283.Contig2_All LRR-STK, Atg08850-like
1	2	0	0	Unigene9087_All LRR-STK, GSO1-like	0	2	0	0	Unigene13618_All LRR-STK, Atg20940
0	1	0	0	Unigene20408_All LRR-STK, GSO2	1	0	0	0	Unigene24779_All LRR-STK, Atg16180
0	1	0	0	Unigene21250_All LRR-STK, GSO2 isoform X2	1	0	0	0	Unigene25652_All LRR-STK, Atg16180
2	4	0	0	CL1426.Contig1_All LRR-STK, GSO2-like isoform X7	0	2	0	1	Unigene14631_All LRR-STK, Atg16180
1	1	0	0	CL649.Contig6_All LRR-STK, HSL2	0	0	0	0	Unigene14344_All LRR-STK, Atg16180
0	2	0	0	Unigene21125_All LRR-STK, RCH1	0	1	0	0	Unigene13989_All LRR-STK, Atg16180
0	2	0	0	Unigene2429_All LRR-STK, RCH1	1	2	0	0	CL1426.Contig2_All LRR-STK, Atg16180
7	0	0	0	Unigene1969_All LRR-STK, RCH1	0	1	1	1	Unigene2568_All LRR-STK, Atg16180
1	1	0	0	Unigene12595_All LRR-STK, RPK2-like	0	0	0	0	Unigene21504_All LRR-STK, Atg16180 isoform X2
0	3	0	0	Unigene19708_All LRR-STK	0	1	0	0	CL1510.Contig1_All LRR-STK, Atg16180-like
0	12	0	0	Unigene1812_All LRR-STK	0	0	0	0	CL1510.Contig2_All LRR-STK, Atg16180-like
0	1	0	0	Unigene19023_All LRR-STK, Atlg67720	1	1	0	0	CL1510.Contig3_All LRR-STK, Atg16180-like
0	1	0	0	Unigene19111_All LRR-STK, Atlg67720	1	3	0	0	Unigene11953_All LRR-STK, Atg10290 isoform X2
2	1	0	0	CL1168.Contig1_All LRR-STK, Atlg67720	1	3	1	1	CL1165.Contig5_All LRR-STK, Atg10290-like isoform X1
1	1	0	0	Unigene1068_All LRR-STK	0	0	0	0	CL1165.Contig6_All LRR-STK, Atg10290-like isoform X1
0	3	1	1	Unigene9555_All LRR-STK, Atlg06840-like	1	3	0	0	CL1165.Contig2_All LRR-STK, Atg10290-like isoform X1
0	0	1	0	Unigene15766_All LRR-STK, Atlg12460	0	2	0	0	CL1165.Contig3_All LRR-STK, Atg10290-like isoform X1
0	1	0	0	Unigene20363_All LRR-STK, Atlg14390	1	0	0	0	CL1165.Contig4_All LRR-STK, Atg10290-like isoform X1
0	1	0	0	Unigene7662_All LRR-STK, Atlg14390	2	0	2	1	CL1165.Contig1_All LRR-STK, Atg10290-like isoform X1
7	3	0	0	Unigene179_All LRR-STK, Atlg14900	0	1	0	0	Unigene12018_All LRR-STK, Atg45840-like
2	3	0	0	CL1966.Contig1_All LRR-STK, Atlg14110	0	0	0	0	CL1971.Contig1_All LRR-STK, Atg45840-like
1	0	0	0	Unigene24276_All LRR-STK, Atlg34110	0	1	1	4	CL1971.Contig2_All LRR-STK, Atg45840-like
0	1	0	0	Unigene15670_All LRR-STK, Atlg34110	1	3	1	1	Unigene6823_All LRR-STK, Atg45840-like
0	1	0	0	Unigene17878_All LRR-STK, Atlg51860	1	0	0	0	Unigene29_All LRR-STK, RPK1
1	0	1	0	Unigene2355_All LRR-STK, Atlg53880-like	4	3	1	1	Unigene28_All LRR-STK, RPK1 isoform X1
2	1	0	0	Unigene7537_All LRR-STK, Atlg53430	1	1	0	0	Unigene7824_All LRR-STK, RPK3
1	0	0	0	Unigene9123_All LRR-STK, Atlg53430-like	1	1	0	0	Unigene799_All LRR-STK, RPK3
1	1	0	0	Unigene8034_All LRR-STK, Atlg53430-like	2	2	1	1	Unigene705_All LRR-STK, RPK3
1	1	0	0	Unigene162_All LRR-STK, Atlg53430-like	0	1	0	0	CL292.Contig2_All LRR-STK
1	1	0	0	CL6.Contig2_All LRR-STK, Atlg53430-like	1	2	0	0	Unigene8930_All LRR-STK, Atlg56130-like
0	1	0	0	Unigene20264_All LRR-STK, Atlg53440-like	1	1	0	0	Unigene308_All LRR-STK, Atlg56140
7	0	0	0	Unigene275_All LRR-STK, Atlg53440-like	2	2	0	0	Unigene11462_All LRR-STK, Atlg56140
0	1	0	0	Unigene1550_All LRR-STK, Atlg53440-like	0	5	0	0	Unigene15791_All LRR-STK, Atlg53430
0	0	0	0	Unigene26043_All Probable LRR-STK, Atlg56130	0	1	0	0	CL2032.Contig2_All LRR-STK, Atlg63430 isoform X3
2	0	0	0	CL10.Contig1_All Probable LRR-STK, Atlg56130	1	0	0	0	Unigene26099_All LRR-STK, Atlg66840
0	1	0	0	Unigene12553_All Probable LRR-STK, Atlg56130-like	0	0	0	0	CL1240.Contig1_All LRR-STK, Atlg66840
0	1	0	0	Unigene17845_All Probable LRR-STK, Atlg56130-like	0	1	0	0	CL1240.Contig2_All LRR-STK, Atlg66840 isoform X3
1	1	0	0	Unigene10245_All Probable LRR-STK, Atlg56130-like					

Figure 1

Expression profiles of LRR-STK genes in four samples. N2: normal sample 2; N3: normal sample 3; D1: diseased sample 1; D3: diseased sample 3. LRR-STK: leucine-rich repeat receptor-like serine/threonine protein kinase

Figure 4

Gene structure and conserved motifs analysis of the 33 pitaya HpLRR transcriptional genes with coding sequences (CDSs) >1.0 kb. (A) Gene structures: Yellow represents the gene CDS and blue represents the upstream or downstream gene sequences. (B) Conserved motifs of the CDS. The conserved motifs were using the MEME program. Different motifs were highlighted with different color boxes.

Figure 5

Verification of 12 differentially expressed LRR genes (DEGs) by qRT-PCR assays. Gene expression levels were measured by qRT-PCR and compared with RNA-Seq results. The histogram represents the fold changes of genes (diseased [D]/normal [N]) according to qRT-PCR, and the line chart represents gene expression according to RNA-Seq. All genes selected for qRT-PCR analysis were analyzed using three biological replicates. Error bars represent $\pm$ SD ($2^{-\Delta\Delta Ct}$) based on three experiments.

Figure 6

Conserved domains of the four LRR genes used in further research. Annotations: CL445.contig4_All and Unigene2712_All: NBS-LRR; Unigene28_All and CL28.contig2_All: LRR-STK. LRR: leucine-rich repeat; NBS: nucleotide-binding site; STK: serine/threonine-protein kinase.

Figure 7

Symptoms of different stages of *N. dimidiatum* infection of pitaya. dpi: days post infection.

Figure 8

Expression profiles of four HpLRR transcriptional genes at different stages of *N. dimidiatum* infection. The X-axis represents different stages of *N. dimidiatum* infection and the Y-axis represents the fold change ($2^{-\Delta\Delta C_t}$) according to qRT-PCR. Annotations: CL445.Contig4_All and Unigene2712_All: NBS-LRR; Unigene28_All and CL28.Contig2_All: LRR-STK. LRR: leucine-rich repeat; NBS: nucleotide-binding site; STK: serine/threonine-protein kinase.

Figure 9

Expression profiles of four HpLRR genes in different pitaya tissues. The X-axis represents different pitaya tissues and the Y-axis represents the fold change ($2^{-\Delta\Delta C_t}$) according to qRT-PCR. Annotations: CL445.contig4_All and Unigene2712_All: NBS-LRR; Unigene28_All and CL28.contig2_All: LRR-STK. LRR: leucine-rich repeat; NBS: nucleotide-binding site; STK: serine/threonine-protein kinase.

Figure 10

Expression profiles of four HpLRR genes in response to SA, MeJA and ABA treatment. Annotations: CL445.contig4_All and Unigene2712_All: NBS-LRR; Unigene28_All and CL28.contig2_All: LRR-STK. LRR: leucine-rich repeat; NBS: nucleotide-binding site; STK: serine/threonine-protein kinase. Different letters (a, b, c) indicate significant difference of the expression of the target gene based on three technological replications [$P < 0.05$, Single factor ANOVA test (F test), $n=3$].

Figure 11

Conservation of functional motifs in non-angiosperm species of two genes (Unigene28_All and CL28.Contig2_All). Red arrows represent the typical conservation of LRR, while green arrows represent the typical conservation of the STKc_IRAK conserved sites. The representation of the domains is approximate and not to scale.

Supplementary Files

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