Plant Gene 23 (2020) 100243

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plantgene

Peanut (*Arachis hypogaea*) transcriptome revealed the molecular interactions of the defense mechanism in response to early leaf spot fungi (*Cercospora arachidicola)*

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ARTICLE INFO

Keywords:
Cercospora arachidicola
DEGs
RT-qPCR
RNA-Seq
Arachis hypogaea
EST-SSRs
Gene ontology

ABSTRACT

Cercospora arachidicola fungus is a causative agent of early leaf spot disease in peanut (*Arachis hypogaea*). However, little has been reported about the molecular interactions for defense mechanisms in the peanut plant against the fungal attack. In this study, we used RNA-Seq to analyse the expression profile of resistant (GPBD-4) and susceptible (JL-24) genotypes of peanut against *C. arachidicola* infection. A total of 91,734,735 raw reads were generated from RNA-Seq data. Differentially expressed genes (DEGs) were analysed for Gene Ontology to find essential genes responsible for defense mechanisms and to study their pathways by KEGG analysis. The resistant variety was able to withstand biotic stress due to exclusive up-regulation of defense-related genes (thaumatin, glutathione peroxidase, and cinnamyl alcohol dehydrogenase), while the susceptible variety was more prone to damage by infection due to down-regulation of genes including (F-box, cytochrome *p*450, Leucine-rich repeat protein kinase and terpene synthase) associated with a majority of biological function. RNA-Seq profile was validated by Reverse Transcriptase qPCR (RT-qPCR) technique, which showed a high correlation (R^2 = 0.92) with the gene expression profile of RNA-Seq analysis. A total of 8591 EST-SSR markers were developed from the transcriptome library and 15 PCR primers based on microsatellite markers were used to validate SSR amplification. This study provides insight into the understanding of molecular interactions between plant and pathogen, and will also help to generate molecular markers for genetic mapping of peanut.

1. Introduction

Peanut (*A. hypogaea* L.) is self-pollinated, allotetraploid plant (2n = 4× = 40), with genome size of 2891 Mbp (Janila et al., 2016). Peanut is the sixth major oil-yielding cash crop and grown in semi-arid tropical to semi-tropical regions across the world (Ansah et al., 2017). India is the second-largest producer, accounting for 6.8 million tons of pods of peanut during 2017 (faostat.fao.org). A major biotic constraint for peanut production is the foliar diseases like leaf spot and rust (Subrahmanyam et al., 1984). The early leaf spot disease in peanut is caused by *Cercospora arachidicola,* characterized by the development of brown-coloured leaf spot with a yellow halo, followed by lesion formation and leaflet abscission leading to a reduction in active photosynthetic area (McDonald et al., 1985). A loss in production, of about 10 to 50%, has been reported due to this disease (Subrahmanyam et al., 1985). It is also known as 'tikka' disease. The disease generally occurs in 3–4 weeks old plants and symptoms appear on leaflets 10 days after infection. Conidia that are produced directly from mycelium in crop debris, act as inoculums. The pathogen is favoured by high humidity and temperature in the range of 25 to 30 °C for causing infection in *A. hypogaea*.

Traditionally, plant breeding with resistant variety was used to protect crop against such fungal infections. The use of advance technology like RNA-Seq provides a better estimate of total gene expression level without any prior knowledge of gene sequence (Kawahara et al., 2012). Molecular interaction between resistance genes (R) of plant and

Abbreviations: RC, Resistant control; RI, Resistant Infected; SC, Susceptible control; SI, Susceptible Infected; FDR, False Discovery Rate; GO, Gene Ontology; DEG, Differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; NGS, Next generation Sequencing

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https://doi.org/10.1016/j.plgene.2020.100243
Received 31 March 2020; Received in revised form 20 June 2020; Accepted 2 July 2020
Available online 08 July 2020

avirulence genes (Avr) of a pathogen is involved in the defense mechanism elicited by the plant, during biotic stress. Such gene to gene interaction leads to the activation of metabolic pathways and the production of phytoalexins related to plant immunity (Kumar and Kirti, 2015). Not much is known about the defense mechanism of peanut against *C. arachidicola*. In this study, we used RNA-Seq to perform transcriptome profiling on the leaf samples of peanut using two genotypes viz. resistant (R) (GPBD-4) and susceptible (S) (JL-24), subjected to *C. arachidicola* infection.

Identification of DEGs responsible for plant defense mechanism can be used to develop molecular markers. Such molecular markers can be used to evaluate genetic diversity, QTL mapping and genetic improvement of the crop through marker assisted selection (Janila et al., 2016). Transcriptomics-based approach has been used to study different plant defense-mechanism against pathogen. Rust infection in the wheat plant was studied to identify candidate effector genes involved in defense mechanisms (Cantu et al., 2013). Molecular defense mechanism against *Rhizoctonia solani* infection, which causes banded leaf and sheath blight (BLSB), was similarly studied in maize (Gao et al., 2014). Transcriptome analysis of peanut revealed genes involved in responses to *Cercosporidium personatum* (fungi) infection and drought stress (Guimarães et al., 2012). Expression profile of peanut plant was studied for the identification of drought-responsive candidate genes under water limited environment (Brasileiro et al., 2015). RNA-Seq analysis of *A. hypogaea* has been conducted during seed development (Zhang et al., 2012). Transcriptomics-based approach has been reported to identify candidate gene for peanut defense against other pathogens like *Ralstonia solanacearum* (Chen et al., 2014) as well as *Meloidogyne arenaria* (Guimaraes et al., 2015).

This analysis involves the identification of DEGs from all four treatments viz. resistant infected (RI), resistant control (RC), susceptible infected (SI) and susceptible control (SC). The differential expression profiling through RNA-Seq data between control and infected condition was compared in both the genotypes. Also, the study identifies SSR markers, Gene Ontology (GO), gene expression validation done by RT-qPCR of selected genes, different pathway analysis by KEGG for better understanding of peanut resistance to *Cercospora arachidicola*.

2. Materials and methods

2.1. Plant materials and inoculation of the pathogen

Peanut plants seed of two varieties GPBD-4 (resistant) and JL-24 (susceptible) were collected from ICAR-Directorate of Groundnut Research, Junagadh, Gujarat, India. The selection of genotypes for RNA-Seq was done based on their resistance against fungal infection (Mondal et al., 2008). The inoculum suspension was prepared using *C. arachidicola* spores with a concentration of 10^5 spores/ml in 0.02% tween-80 (Sigma-Aldrich, MO, USA) and applied on the abaxial surface of plant leaves with the help of paintbrush (Ramegowda and Senthil-Kumar, 2015). Forty days old plants of resistant as well as susceptible genotypes of peanut were inoculated with pathogen suspension during evening time and maintained in a greenhouse with high humidity (McDonald et al., 1985). The control plants were kept in the similar conditions without any inoculation. Peanut leaves of both the genotypes were collected in three biological replicates at two different stages, i.e. control stage (without inoculation) and infected stage (24 h post-inoculation). All the samples were collected and quickly frozen in liquid nitrogen and stored at −80 °C in deep freeze for carrying out further experiments. The cDNA libraries of each biological replicates were pooled in equivalent molar concentration for sequencing process. Further disease development on the plant was observed for 15 days and simultaneously pathogen morphology was studied by using a microscopic technique (Fig. 1). The workflow of the experiment is displayed in Fig. 2.

2.2. Isolation of mRNA and cDNA synthesis for sequencing

Total RNA isolation was carried out with approximately 200 mg of the preserved leaf tissue using the Qiagen RNeasy Plant Mini kit (Qiagen, CA, USA). Isolated RNA was treated with DNase I (Fermentas, MA, USA) to remove DNA contamination and RNA was eluted by using RNeasy Plant Mini kit (Qiagen, CA, USA). RNA quantity and quality were checked by using Bioanalyzer 2100 (Agilent Technologies, CA, USA). Further Dynabeads mRNA DIRECT Purification Kit (Invitrogen, CA, USA) was used for the isolation of mRNA from total RNA. Isolated mRNA was used to synthesize cDNA library using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, MA, USA). AMPure XP beads (Beckman Coulter, CA, USA) were used for library size selection as well as for the removal of contamination. cDNA library was analysed by Bio-analyser 2100 equipment and diluted to 100 pmol for sequencing in the Ion Torrent S5 sequencer system (Thermo Fisher Scientific, MA, USA).

2.3. Quality control, read mapping to the reference genome and differential gene expression analysis

Quality of RNA-Seq data was first checked by the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Brown et al., 2017). Low-quality data such as adapter dimers, sequence reads of < 50 bp in length, poly-N and nucleotide bases with $Q < 25$ were removed by the PRINSEQ tool for downstream analysis (Schmieder and Edwards, 2011). High-quality RNA-Seq data were mapped to a reference genome of peanut (https://www.peanutbase.org) by using STAR 2.5.1a software (https://github.com/alexdobin/STAR/releases/) (Dobin and Gingeras, 2015). DEGs were identified from control and infected samples of both peanut genotypes by using the Cuffdiff tool in the Cufflinks package (http://sihua.us/Cufflinks.htm) (Trapnell et al., 2010), (Ghosh and Chan, 2016). An abundance of the normalized transcript was estimated with the same tool and represented as fragments per kilo-base per million reads (FPKM) (Trapnell et al., 2009). During analysis, DEGs with a false discovery rate (FDR) of < 0.05 and P-value < 0.01 with at least a log2-fold change ≥ 1 were considered to produce high-quality data (Li et al., 2015). Venn diagram was used to compare DEGs shared by different samples and was prepared by VENNY software (Oliveros, 2007).

2.4. Gene ontology and KEGG pathway analysis

DEGs were analysed using “REVIGO” software (http://revigo.irb.hr) (Supek et al., 2011) for GO. Comparative GO analysis of RI/RC and SI/SC was performed by using WEGO software (http://wego.genomics.org.cn/) (Ye et al., 2006). Different GO terms were analysed and classified into: cellular component, biological process, and molecular functions (Supek et al., 2011). DEGs involvement in different metabolic pathways related to defense mechanisms was studied by KEGG analysis (http://www.genome.jp/kegg/kass) (Liu et al., 2013). This provides an understanding of gene function and their utility in a biological system (Kanehisa et al., 2007).

2.5. Validation of RNA-Seq data through RT-qPCR

RNA-Seq data of the experiment were validated by performing RT-qPCR for genes corresponding to 15 randomly selected transcripts (Koringa et al., 2013; Rajkumar et al., 2015). The 15 primers were designed using primer3 software (http://primer3.sourceforge.net/) with the following parameters: primer size 20-–24 bp, melting temperature 60 °C, product size 90–200 bp with the rest being default parameters (Untergasser et al., 2007). The same three biological replicates of RNA samples were used for both RT-qPCR as well as RNA-Seq analysis. RNA samples of all biological replicates for each sample were pooled before cDNA synthesis. RT-qPCR was performed in three replicates for each sample. The cDNA was synthesized using the

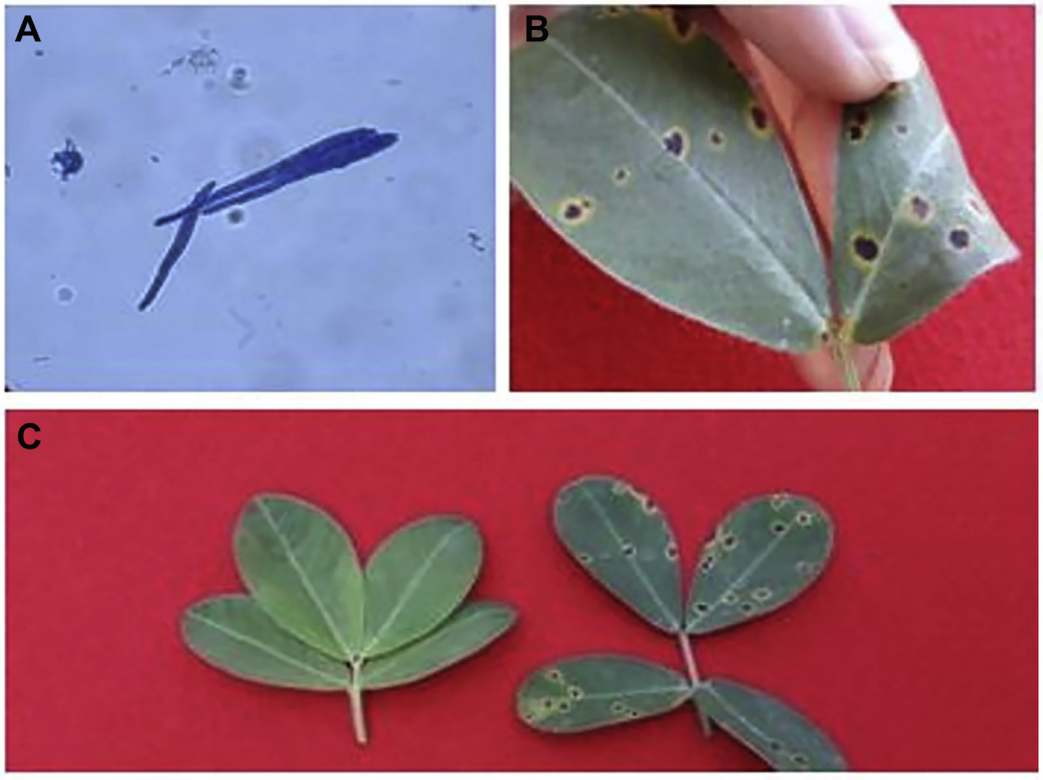

Fig. 1. Disease symptoms and fungal spore morphological study by microscopy. A. Conidia of *Cercospora arachidicola*, B. Disease development on peanut leaves, C. JL-24 (susceptible) (control) v/s (infected).

QuantiTech Reverse Transcription kit (Qiagen, CA, USA). RT-qPCR was performed in three replications using the QuantiFast SYBR Green PCR Kit (Qiagen, CA, USA). RT-qPCR was performed in ABI 7500 Fast Real-Time PCR (Thermo Fisher Scientific, MA, USA) with following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 30 s at 60 °C. Actin gene was used as control for RT-qPCR (Jiang et al., 2011). The reaction mixture contained 1 µl of template cDNA, 1 µl of forward and reverse primer (concentration of each primer: 10 pM), 10 µl SYBR Green Master Mix (2×) and the final reaction volume was made up to 20 µl by addition of nuclease-free water (Qiagen, CA, USA). ABI 7500 Software v2.3 was used to calculate Cq values of primer and gene expression analysis was done by Livak's $-\Delta\Delta$ CT method (Livak and Schmittgen, 2001).

2.6. Expressed sequence tag- simple sequence repeats (EST-SSR) marker development, primer designing and validation by PCR

Sequencing reads were assembled with the help of genome-guided Trinity de novo transcriptome assembly for SSR identification (Grabherr et al., 2011). EST-SSRs were identified using the Microsatellite identification tool (MISA) with the following criteria: removal of redundant transcripts and a minimum number of repeats (2–6, 3–5, 4–4, 5–3 and 6–3) (Beier et al., 2017). Different primers for SSR were designed by primer3 online tool (http://primer3.sourceforge.net/) (Untergasser et al., 2007) with following criteria: product size 100–300 bp, primer length 19–22 bp, temperature (57 to 63 °C) and GC content of 50%. For validation of identified SSR, 15 random primer sets were used for PCR amplification with DNA of both the varieties (GPBD-4 and JL-24) of peanut. PCR products produced using SSR primers were observed on 2.5% agarose gel.

3. Results

3.1. RNA-Seq with alignment and analysis

Morphological study of ELS infected peanut leaves showed brown coloured spots with a yellow halo on the upper leaf surface. Susceptible variety (JL-24) showed symptoms of Early leaf spot disease after infection of *C. arachidicola*, while there was no symptom found in control plants. Conidia of fungi are the most important primary source of inoculum, which were observed under the microscopic field for its morphology and release of hair-like conidia, which is required for infection (Fig. 1). The plants were analysed by RNA-Seq, after 24 h of pathogen encounter. RNA-Seq was used to generate transcriptome profile data of resistant (GPBD-4) (RI and RC) and susceptible (JL-24) (SI and SC) varieties of peanut under biotic stress *of C. arachidicola* infection as well as for control plants. The sequencing of cDNA libraries generated 35,507,858 and 56,226,877 raw reads of susceptible and resistant varieties, respectively. The Ion S5 sequencer generated 150–250 bp RNA-Seq library with an average read length of 156 bp. RNA-Seq data was checked for quality control by various tools to remove low quality and undesired data. The summary of obtained data with quality filtering and sequence alignment results are shown in Table 1. Sequence alignment and mapping displayed reads with an average matching rate of 68.92%.

3.2. Profiling of differentially expressed genes

Comprehensive detail of molecular mechanisms involved in plant-pathogen interaction has not been reported so far in the case of peanut. DEGs were identified during biotic stress using the cuffdiff tool. We obtained deferentially expressed transcripts in RI/RC as well as SI/SC with FDR < 0.05, P-value < 0.01, and Log2 fold change of ≥ 1. Assembled sequences were analysed to check their differential

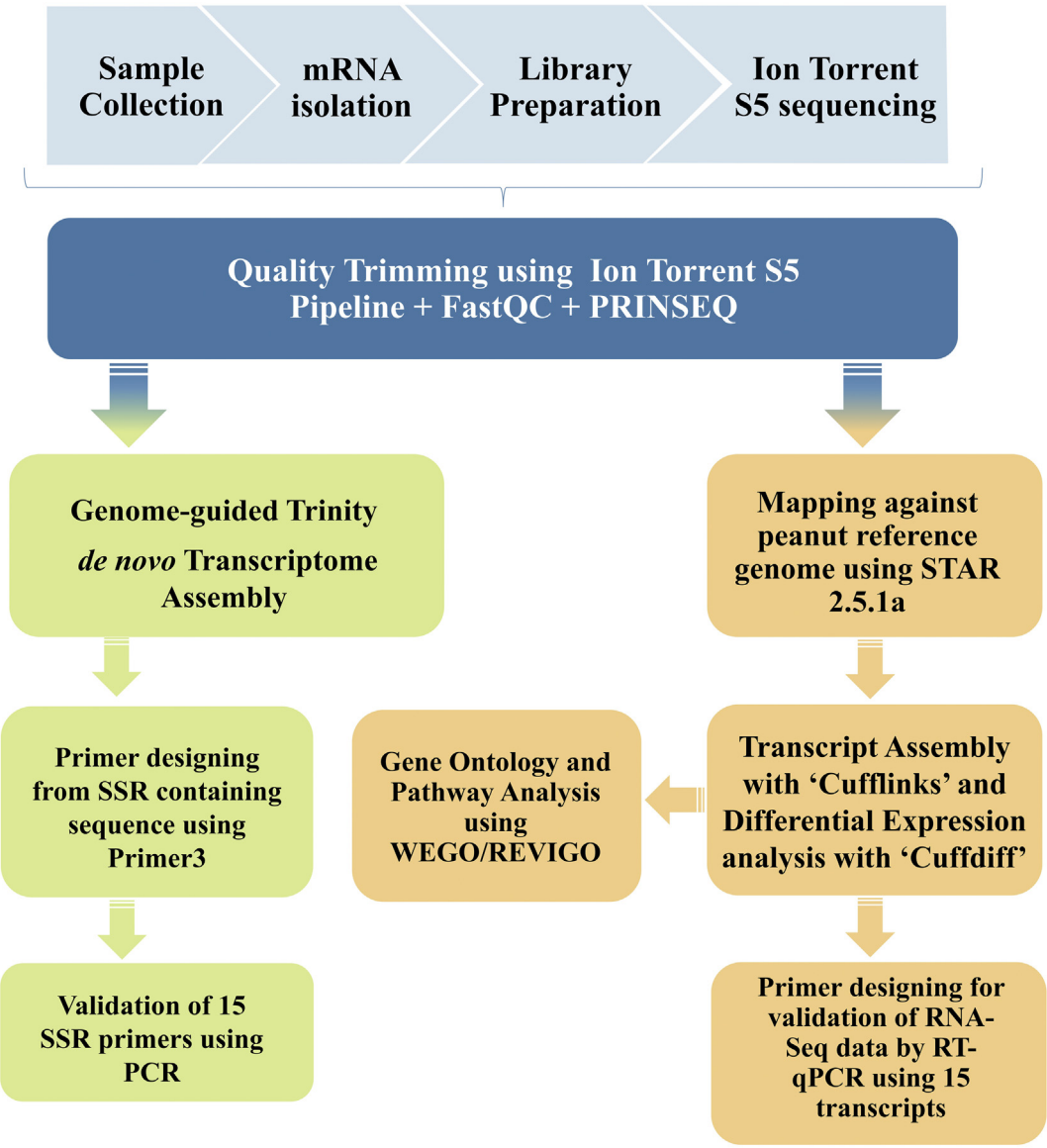

Fig. 2. Overview of the workflow implemented in transcriptome profiling and designing EST-SSR in *Arachis hypogaea* during *Cercospora arachidicola* infection.

expression levels and measures as FPKM values (Hao et al., 2017). In the case of RI/RC library comparison, 127 DEGs were identified, where 126 genes were up-regulated in RI, while only one gene was down-regulated (Supplementary Table 1). During DEGs analysis of SI/SC library samples, a total of 164 DEGs were identified among which 6 genes were up-regulated and 158 genes were down-regulated in SI (Supplementary Table 2). This indicated that resistance-related genes were highly expressed during infection in the resistant variety (GPBD-4) as compared to that in the susceptible variety (JL-24).

Gene expression of all the four samples with different fold change corresponding to gene numbers is presented in Fig. 3A. Genes displaying more than fivefold difference in expression between RI/RC mainly coded for cell wall protein, alcohol dehydrogenase, 1-amino cyclopropane-1-carboxylate synthase, heavy metal transport protein and pathogenesis-related thaumatin protein (Supplementary Table 1). Such a high fold-change in DEGs indicates their involvement in defense mechanisms against *C. arachidicola* infection. The distribution of DEGs among the four samples was compared using Venn-diagram. In total 36

Table 1
Details of transcripts assembled based on the reference genome from RNA-Seq of the susceptible and resistant varieties of *Arachis hypogea* at the control and infected stages.

	SI	RI	SC	RC
Number of transcripts	18,171,618	19,601,567	17,336,240	36,625,310
RNA-Seq length (bp)	3,065,130,119	3,262,971,039	2,928,728,266	5,127,297,546
Average transcript length (bp)	168.68	166.46	168.94	139.99
Total read counts after quality filtration	15,313,301 (84.27%)	16,746,581 (85.43%)	15,038,010 (86.74%)	31,262,640 (85.36%)
Uniquely mapped reads number	10,876,920 (71.03%)	12,022,136 (71.79%)	10,728,674 (71.34%)	19,233,708 (61.52%)
Number of reads mapped to multiple loci	3,581,533 (23.39%)	4,080,494 (24.37%)	3,757,635 (24.99%)	9,796,877 (31.34%)
Average contig length after mapping (bp)	153.41	150.26	151.93	125.17

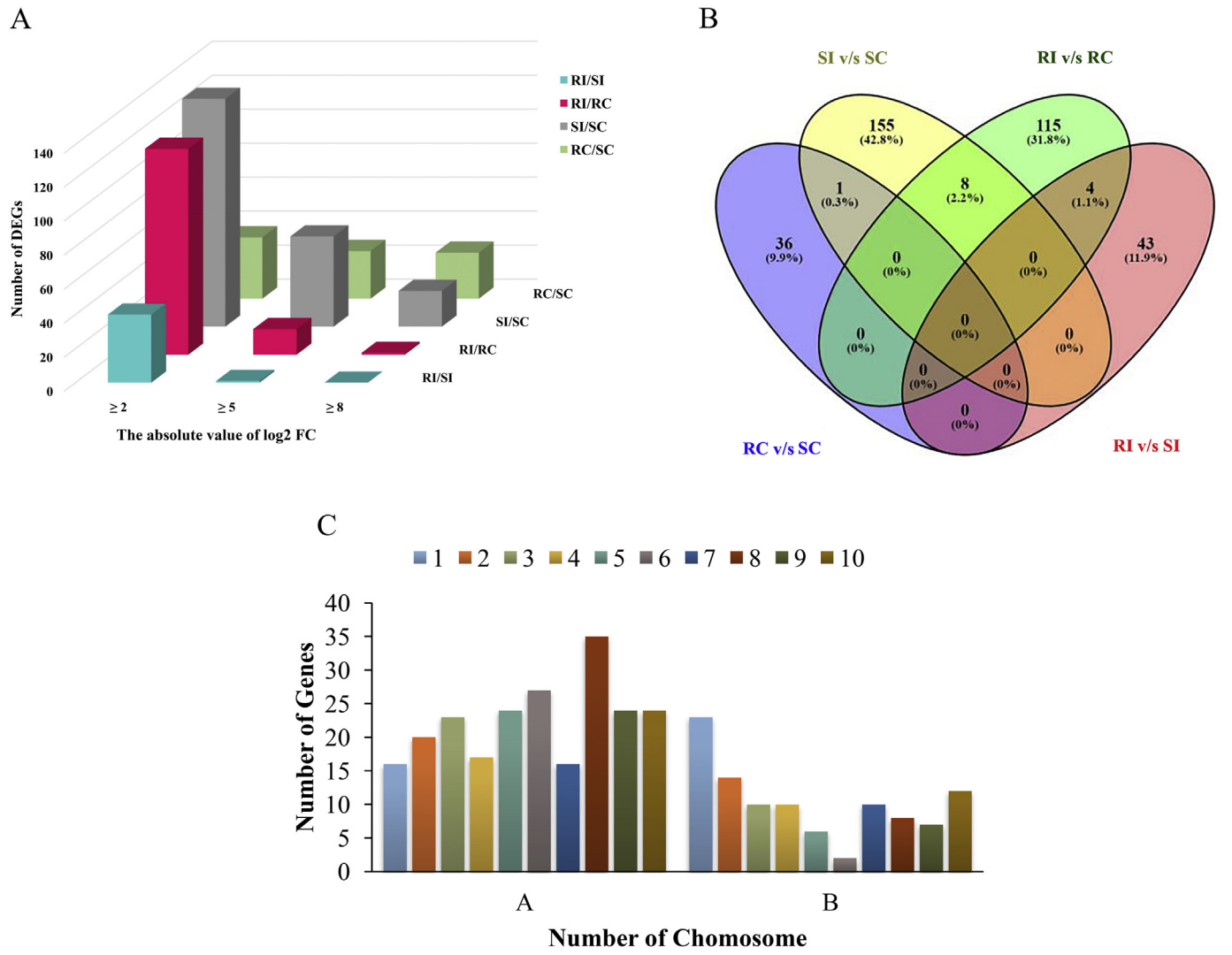

Fig. 3. Differentially expressed genes compared between control and infected peanut plants. A. The Fold change wise distribution of DEGs under the comparison of four different samples. B. Venn diagram showing the distribution of unique and common DEGs among control and infected stages of susceptible and resistant variety. C. The total number of DEGs present on each chromosome of the peanut plant. A total number of DEGs on genome-A refers to *A. duranensis* and genome-B referes to *A. ipaensis*. The X-axis represents different chromosomes while the Y-axis represents number of genes on each chromosome.

(9.9%), 155 (42.8%), 115 (31.8%) and 43 (11.9%) DEGs were expressed uniquely in RC v/s SC, SI v/s SC, RI v/s RC and RI v/s SI respectively. We also found 8 (2.2%) genes common between SI v/s SC and RI v/s RC, 1 (0.3%) gene common between SI v/s SC and RC v/s SC, whereas 4 (1.1%) genes common between RI v/s RC and RI v/s SI (Fig. 3B). Eight DEGs associated with the GO terms 'disease-resistance response protein', 'U-box protein', '1-aminocyclopropane-1-carboxylate synthase', and 'caffeate O-methyltransferase (COMT)' were common between RI v/s RC and SI v/s SC pair.

These DEGs represented around 330 non-redundant genes, which were distributed across the ten chromosomes of peanut (Fig. 3C). A mapping approach was adopted to develop closely linked markers for resistance genes in peanut for foliar disease, where QTL was found to be on the A03 chromosome (Mondal and Badigannavar, 2018). It is interesting to note that in our study as well, Chr_A03 carried possible defense-related protein-coding genes (Aradu.Z6I4Q, Aradu.GP6IB, Aradu.E03Z4 and Aradu.B1C6F) that were differentially expressed between RI/RC.

Differential expression of genes such as pathogenesis-related (PR) genes, defense-related R genes, genes involved in signal transduction, secondary metabolite related genes and transcription factors (TF) as gene regulatory elements play important roles in plant defense mechanism against pathogens (Fig. 4, Table 2) (www.peanutbase.org). Results related to the differential expression of these genes are briefly mentioned in the following subsections.

3.2.1. *Differentially expressed PR genes affecting cell wall modification*

PR genes are differentially expressed in legumes during the biotic or abiotic stressed condition (van Loon et al., 2006). In this experiment, expression of PR genes like chitinase family protein (Aradu.118NW) was up-regulated in the RI genotype and down-regulated (Aradu.NF8QC, −5.55-fold) in SI genotype as compared to their respective control samples (Supplementary Tables 1 and 2). Cell wall of the fungal pathogen is degraded by different hydrolytic enzymes like, pectate lyase and beta-galactosidase, which were up-regulated in RI plant genotype as compared to susceptible infected (SI) genotype (Supplementary Table 3). DEGs encoding (PR-5) thaumatin protein (Aradu.D14Q2) were significantly up-regulated in RI genotype as compared to the RC genotype (Table 2). Differential expression of PR genes can be explained by their important role in degradation of fungal cell wall and osmotic regulation of cell during defense mechanism. Plant cell-wall synthesis related genes such as cellulose synthase (Aradu.BZH82, −2.0-fold) were down-regulated in susceptible genotype during infection. Gene responsible for cell wall hydrolase affecting fungal pathogen

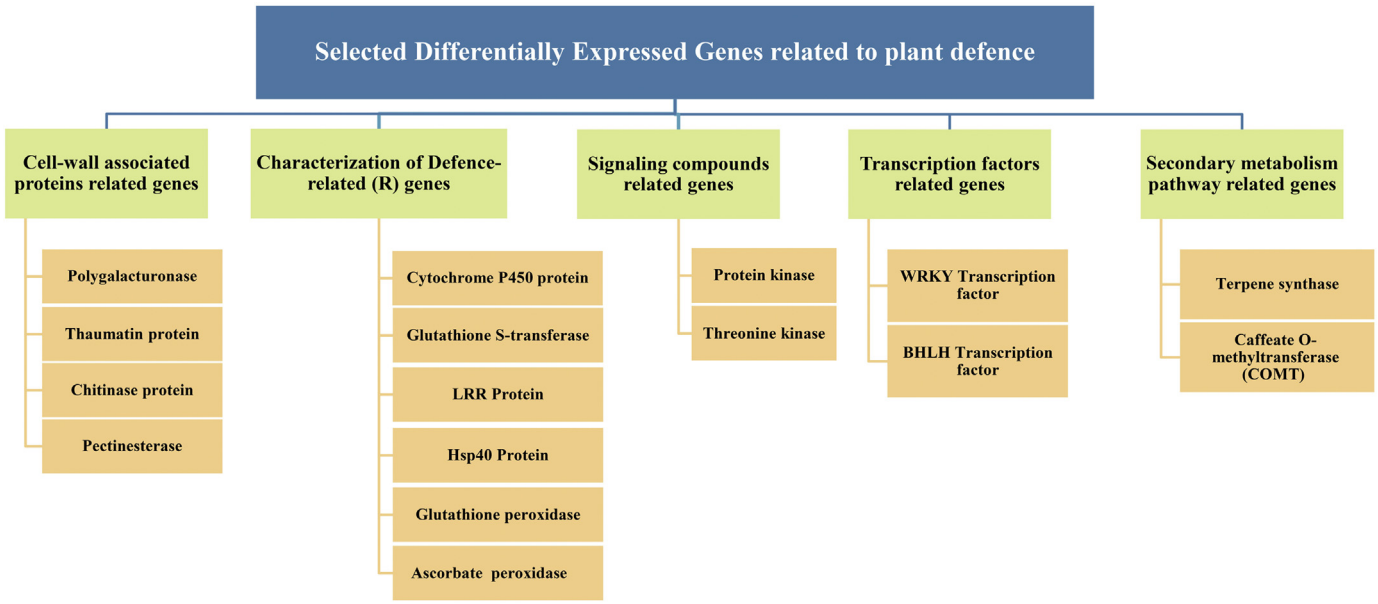

Fig. 4. Classification of DEGs related to plant defense mechanism in resistant (GPBD-4) genotype.

adversely was highly expressed in resistant genotype (RI/RC) as compared to susceptible (SI/SC) genotype of peanut.

3.2.2. Expression analysis of R genes related to defense mechanism

The transcripts related to cytochrome P450 involved in the defense mechanism via oxidation of many substrates by molecular oxygen were up-regulated in the resistant genotype (RI/RC) (Aradu.B4JF5, 4-fold), while cytochrome P450 (Aradu.P0VF2) was down-regulated in SI sample as compared to SC sample (Supplementary Table 2). DEGs encoding peroxidase protein (POD) (Aradu.8I3T0), glutathione peroxidase (Aradu.M4TPQ) and ascorbate peroxidase (Aradu.A61Z4) were up-regulated in the resistant genotype (RI/RC), while peroxidase enzymes (Aradu.8I3T0, −7.38-fold) were down-regulated in case of susceptible genotypes (SI/SC). Other defense-related genes such as the one coding for glutathione S-transferase (GST) (Aradu.TMM2A, 3.9-fold) was up-regulated in resistant (RI) sample as compared to control (RC) sample, while another similar gene such as GST protein (Aradu.6PF06) was down-regulated in SI sample as compared to SC sample (Supplementary Table 2). R gene products play important roles in plant defense mechanisms by interacting with avirulence (Avr) effector proteins through effector-triggered immunity (ETI) and prevent the growth of pathogen by a hypersensitive response (HR) (Lee and Yeom, 2015). Genes related to defense mechanism, such as Cytochrome P450 (12.2-fold) and GST were found to be up-regulated in RC/SC (Supplementary Table 4). Nucleotide binding-Leucine rich repeat (NB-LRR) protein (Aradu.3S3UE, 4.58 fold) was up-regulated in RI as compared to RC, which helps in the activation of signal transduction required for defense mechanism (Chandra et al., 2016).

3.2.3. DEGs related to TFs as gene regulatory elements and signal transduction

Different TFs play important roles in plant immunity by their involvement in the regulation of expression of various genes. Genes for different TFs like, NAC, bZIP, WRKY, MYB, and basic-Helix-Loop-Helix (bHLH) are involved in activation of MAPK cascade for defense mechanism (Qiu et al., 2008). Transcripts of the WRKY TF family were differentially up-regulated in the resistant genotype (Aradu.XE5AY, 4.05-fold) (RI/RC) as compared to the susceptible (SI/SC) genotype (Araip.761TD) (Supplementary Table 2). Transcripts of bHLH protein were differentially up-regulated in the RI/RC genotype (Aradu.Z6I4Q) while being down-regulated in the SI/SC (Aradu.Z6I4Q).

DEGs related to signal transductions such as, protein kinase and serine threonine kinase may lead to activation of defense-related pathways. Signal transduction related DEGs such as protein kinase (Aradu.2717A) and Serine/threonine kinase protein (Aradu.ZBZ36) were found to be up-regulated in resistant genotype (RI/RC) of peanut (Supplementary Table 1). On the other hand, these signal transduction related genes including, protein kinase (Araip.GE7ZX, −3.67-fold) and Serine/threonine kinase protein (Aradu.CED81) were exclusively down-regulated in susceptible genotypes (SI/SC) (Supplementary Table 2).

3.2.4. Expression analysis of genes involved in secondary metabolite production

In the present study, DEGs of secondary metabolite production such as those for terpenoid (act as phytoalexin) and flavonoid were found to play a role in defense against *C. arachidicola*. Transcripts related to terpene synthase were highly expressed in case of resistant (RI/RC) (Aradu.I338M) (Table 2) as compared to susceptible (SI/SC) (Aradu.YEB6U) genotype of peanut. The expression level of COMT gene (Aradu.BM2KZ, 4.03-fold) was higher in RI as compared to RC. Transcripts for enzymes involved in the flavonoid biosynthesis pathway including cinnamyl alcohol dehydrogenase (CAD) (Aradu.GP6IB, 5.26-fold) were exclusively up-regulated in RI//RC genotype. CADs are associated with biosynthesis of lignin polymers, which is involved in defense against biotic stresses and mechanical damage (Eom et al., 2016).

3.3. Enriched comparative GO analysis of resistant and susceptible genotype by WEGO

Comparative GO analysis between RI/RC and SI/SC was accomplished by WEGO software (Ye et al., 2006). During GO analysis, GO terms associated with the DEGs identified in two cDNA libraries (RI/RC and SI/SC) were classified into three different classes viz. cellular components, molecular functions and biological processes. Comparative GO analysis between resistant and susceptible genotypes is displayed as a histogram by WEGO software (Fig. 5). In total, 270 genes of RI/RC and 446 genes of SI/SC were classified as GO terms in three different classes (Supplementary Table 5). Cell (GO: 0005623), cell part (GO: 0044464) and membrane (GO: 0016020) are the top three groups of cellular components category as well as, they were higher in number in RI/RC compared to SI/SC. The catalytic and binding activity (GO:

Table 2
Selected DEGs with their probable function in plant defense against pathogen.

Gene name	Gene Length	Log2FC (RI/RC)	Description
Aradu.P0G7F	2111	4.340	Polygalacturonase
Aradu.118NW	2499	4.878	Chitinase protein
Aradu.D14Q2	9705	6.119	Thaumatin protein
Aradu.WLE0A	1633	5.215	Cell wall protein
Aradu.V8XMS	2116	4.0592	Cell wall protein
Aradu.R7CTG	1712	4.039	Hsp40 cysteine-rich protein
Aradu.3S3UE	3805	4.589	Leucine-rich repeat protein
Aradu.Y31BQ	4941	4.020	Leucine-rich repeat protein
Aradu.M4TPQ	1911	4.505	Glutathione peroxidase
Aradu.A61Z4	4880	4.303	Ascorbate peroxidase
Aradu.8I3T0	1957	4.100	Peroxidase
Aradu.ZH6BL	868	4.300	Disease-resistance response protein
Araip.Z24SH	921	3.915	Disease-resistance response protein
Aradu.KI3UZ	877	3.860	Disease-resistance response protein
Aradu.B4JF5	7940	4.003	Cytochrome P450 protein
Aradu.P0VF2	2574	3.997	Cytochrome P450 protein
Aradu.TMM2A	1539	3.994	Glutathione S-transferase protein
Aradu.XE5AY	1842	4.056	WRKY family transcription factor
Aradu.U1ZNR	770	4.043	WRKY family transcription factor
Aradu.1C3SG	8845	3.972	WRKY family transcription factor
Aradu.Z6I4Q	2108	4.670	bHLH-transcription factor (myc)
Aradu.ZBZ36	2191	4.723	Threonine synthase
Aradu.2717A	4265	4.280	Protein kinase
Aradu.S6IQK	3868	4.099	Protein kinase
Aradu.K34WH	6329	4.075	Protein kinase
Aradu.TG162	5098	4.013	Protein kinase
Aradu.I338M	4942	4.245	Terpene synthase
Aradu.8JG2E	3560	4.171	Terpene synthase
Aradu.L1M29	1963	4.106	Terpene synthase
Aradu.EF5K9	3367	4.098	Terpene synthase
Aradu.IVA52	4591	4.037	Terpene synthase
Aradu.EC244	3733	3.956	Terpene synthase
Aradu.BM2KZ	8741	4.032	Caffeate *O*-methyltransferase
Aradu.C09GA	2754	4.009	Caffeate *O*-methyltransferase
Aradu.B1CT2	978	4.004	Thiamine thiazole synthase

Note: P-value for Log2FC (RI/RC) ≤0.0001. Source for gene description data: www.peanutbase.org

0003824 and GO: 0005488, respectively) are the two major groups of molecular functions category. The metabolic and cellular process (GO: 0008152 and GO: 0009987, respectively) and localization (GO: 0051179) are the top three groups of metabolic processes, while localization (GO: 0051179) and response to a stimulus (GO: 0050896) were higher in number in RI/RC as compared to SI/SC.

3.4. GO analysis of DEGs by REVIGO and KEGG pathway analysis

REVIGO software was used for enriched GO analysis of DEGs of all four samples (Supek et al., 2011). Enriched GO analyses of DEGs of both genotypes were classified into three different groups viz. Biological Processes (BP), Cellular Component (CC) and Molecular Function (MF) (Supplementary Table 6). Approximately 231 GO terms were assigned and their percent distribution for biological process (87 GO terms), cell component (31 GO terms) and molecular function (113 GO terms) were 37.66%, 13.42%, and 48.92% respectively (Supplementary Fig. 1). They mainly include BP-like defense response to fungus (GO:0006952), response to biotic stimulus (GO:0009607), oxidation-reduction process (GO:0055114), and terpenoid biosynthetic process (GO:0016114). This shows the acceleration of the plant's defense response towards fungi by the production of secondary metabolites such as terpene during plant-pathogen interaction. Among the biological process category, based on the abundance of transcripts, transport (GO:0006810), oxidation-reduction process and transmembrane transport (GO:0055085) were found to be the most represented GO terms, indicating their role in the different oxidation-reduction process involved in different metabolic pathways. Within the cellular component group, based on gene expression, GO terms related to a membrane (GO:0016020) and integral component of the plasma membrane (GO:0016021) were the most mentioned GO terms, while in case of MF group, protein kinase activity (GO:0004672) followed by methyltransferase activity (GO:0008168) and signal transducer activity (GO:0004871) were found as the most represented GO terms, which indicate their role in important signalling pathways, such as MAPK signalling pathways, which is involved in the production of defense enzymes against a pathogen.

Identification of different pathways responsible for a defense mechanism was performed using the KEGG database (Kanehisa and Goto, 2000). During KEGG analysis of the four samples viz. RI, RC, SI and SC, it was found that 686 genes were assigned to 26 pathways (Supplementary Table 7). The majority of the transcripts were assigned to plant-pathogen interaction pathways (75 genes; 10.93%), followed by metabolic pathways (69 genes; 10.06%) and biosynthesis of secondary metabolites (60 genes; 8.75%) (Supplementary Fig. 2). This may lead to the annotation of genes and the identification of the pathway in response to pathogen infection. Few important pathways such as plant-pathogen interaction, MAPK signalling pathway, and biosynthesis of secondary metabolites were found during KEGG analysis of DEGs. These pathways have been reported to be important for the defense mechanism in plants (Hulbert et al., 2007).

3.5. RNA-Seq data ***validation by RT-qPCR***

The DEGs were selected for validation through RT-qPCR. It was conducted using 15 randomly selected primers (Supplementary Table 8). There were slight differences in fold change of DEGs between RNA-Seq and RT-qPCR analyses data. Out of the 15 primers, 14 primers gave results that correlated well with RNA-Seq results. Such differences in DEGs expression levels between the two methods have been reported in several studies (Soria-Guerra et al., 2010; Hamid et al., 2018). RNA-Seq data showed a higher fold change in DEGs expression as compared to RT-qPCR data (Supplementary Table 8). For example, Chitinase family protein was up-regulated in RI plants. RNA-Seq data showed 4.87-fold changes in transcript abundance in RI/RC genotypes, while RT-qPCR analysis yielded around 2.98-fold changes in RI when compared to RC genotype. The actin gene transcript was used as an endogenous control for normalizing the relative gene expression of transcripts. The majority of transcripts except the MYB showed similar results in RT-qPCR and RNA-Seq data. Thus, a comparison of transcript expression levels between RNA-Seq data and RT-qPCR could be represented by the positive correlation ($R^2 = 0.92$) as shown in Fig. 6.

3.6. Identification of EST-SSRs in Arachis hypogaea

After filtering the RNA-Seq data for quality, > 78.36 million raw reads were assembled into 257,823 contigs, which ranged from 201 to 4253 bp. A total of 5776 primer sets were designed from 8591 EST-SSRs by primer3 online tool (Table 3). For validation of designed SSR, 15 random primers were selected for amplification with both peanut genotypes viz. resistant (GPBD-4) and susceptible (JL-24) (Supplementary Table 9). The use of these primers resulted in successful amplification and the amplicons were visualized on 2.5% agarose gel (Supplementary Fig. 3). It is important to identify allelic variants responsible for defense responsive mechanisms in groundnut; however, it was difficult to discriminate them on 2.5% agarose gel. Therefore, further confirmation is essential to identify the allelic variants between the two varieties (i.e. resistant and susceptible), responsible for defense responsive mechanisms in groundnut. EST-SSRs size was distributed in different proportions viz.; tri-nucleotide repeats (67.33%), dinucleotide

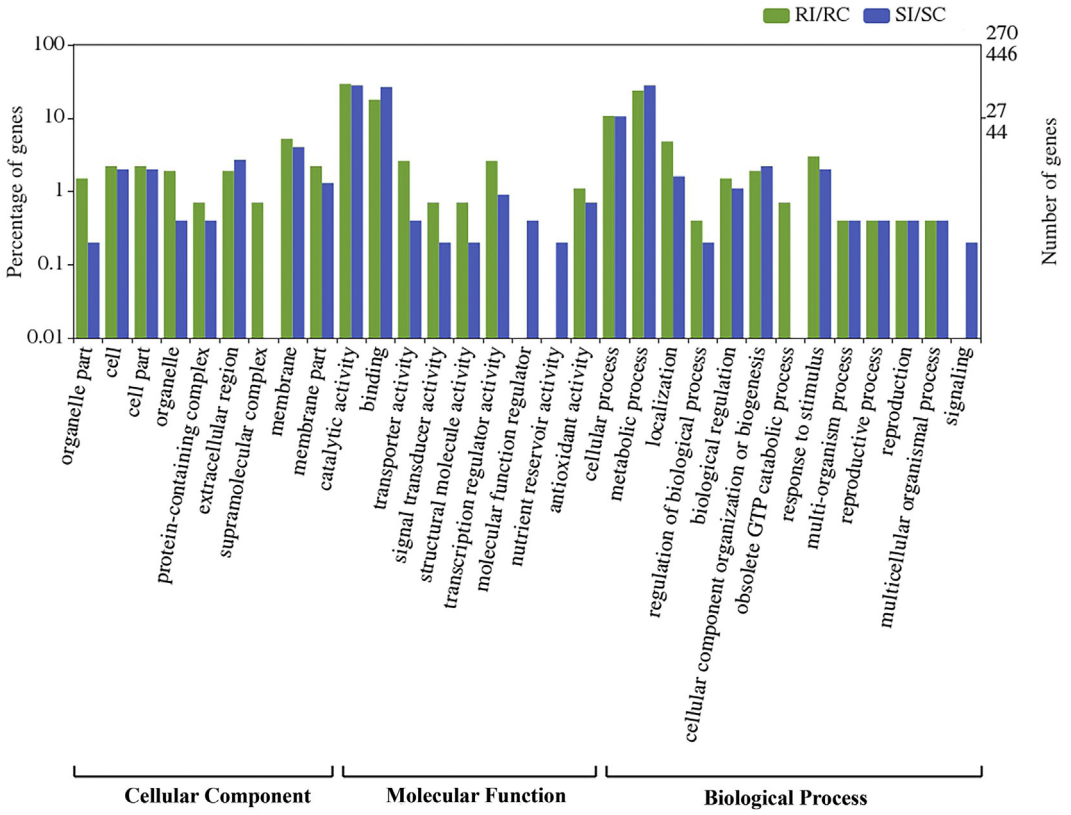

Fig. 5. GO classification of non-redundantly expressed genes among RI/RC and SI/SC. Bars show the percentages of genes matches to each GO term using a web-based tool, WEGO. Results are grouped by three main functional categories; biological process, cellular component, and molecular function.

(23.63%), tetranucleotide (2.04%), hexanucleotide (0.47%) and pentanucleotide (0.35%) (Fig. 7). The frequency of identified SSRs was found to be 3.4 kb in the peanut genome. Also, the (AAG/CTT)n showed the highest frequency among all the types of repeat motifs (21.42%), followed by (AG/CT)n and (ATC/ATG)n with a frequency of 16.82% and 11.65% respectively.

3.7. Exclusively expressed genes in resistant and susceptible varieties of Arachis hypogaea

DEGs analysis indicated that different defense-related genes; thaumatin (Aradu.D14Q2, 6.11 fold), glutathione peroxidase (Aradu.M4TPQ, 4.5 fold), cinnamyl alcohol dehydrogenase (CAD) (Aradu.GP6IB, 5.26 fold) and photosynthesis-related gene such as

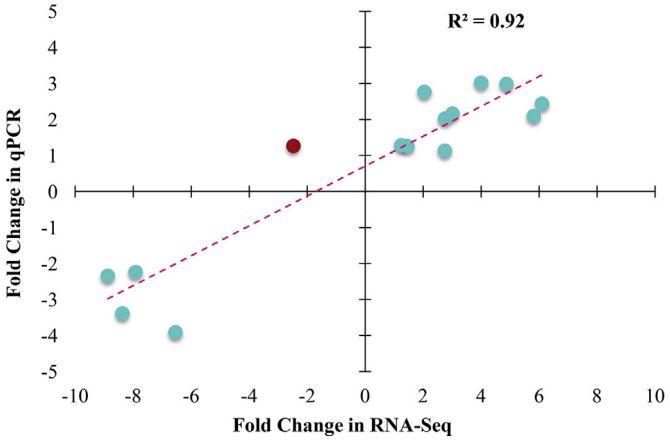

Fig. 6. Correlation of transcripts expressions between RNA-seq and RT-qPCR.

thiamine thiazole synthase (Aradu.B1CT2, 4.0 fold) were exclusively expressed in the resistant variety during plant-pathogen interaction. Glutathione peroxidase is a major reactive oxygen species (ROS) scavenging enzyme for the reduction of H_2O_2 to prevent cellular damage. Increased mRNA levels of glutathione peroxidase were reported earlier during biotic stress responses (Ozyigit et al., 2016). Thaumatin protein causes transmembrane pores in fungal cell membranes, which acts as a permatin to inhibit fungal growth (Abad et al., 1996). CADs are involved in flavonoid and lignin polymers biosynthesis, which is involved in defense against biotic stresses and mechanical damage (Eom et al., 2016). Some important defense-related genes, such as F-box protein (Aradu.SAQ03, −6.05-fold), MYB TF (Aradu.GCV2U, −2.46-fold) and beta-glucosidase (Aradu.E03Z4, −6.49-fold), were found to be exclusively down-regulated in a susceptible variety during infection. F-box genes are involved in the regulation of plant hormone signalling pathways such as, those for auxin, gibberellins, ethylene, and jasmonic acid (JA) and disease resistance (Yu et al., 2007). Transcription factors like NAC, bZIP and MYB play a major role in different signalling pathways such as MAPK cascade for defense mechanism through activation of protein kinase (Qiu et al., 2008). Down-regulation of such defense-related genes and photosynthesis-related genes such as chlorophyll A/B binding protein (Araip.MTL36, −3.46-fold) in susceptible genotype could be the reason for rapid disease development.

4. Discussion

In this study, we focused on the identification of DEGs responsible for the early phase of *Cercospora arachidicola* infection in peanut. Plants have developed the immune system to defend against pathogen infection in form of physical barrier, production of fungal cell-wall degrading enzymes and production of antimicrobial compounds as secondary metabolites (Zhang et al., 2017). Major defense mechanisms observed in peanut plants as a result of fungal infection and the analysis

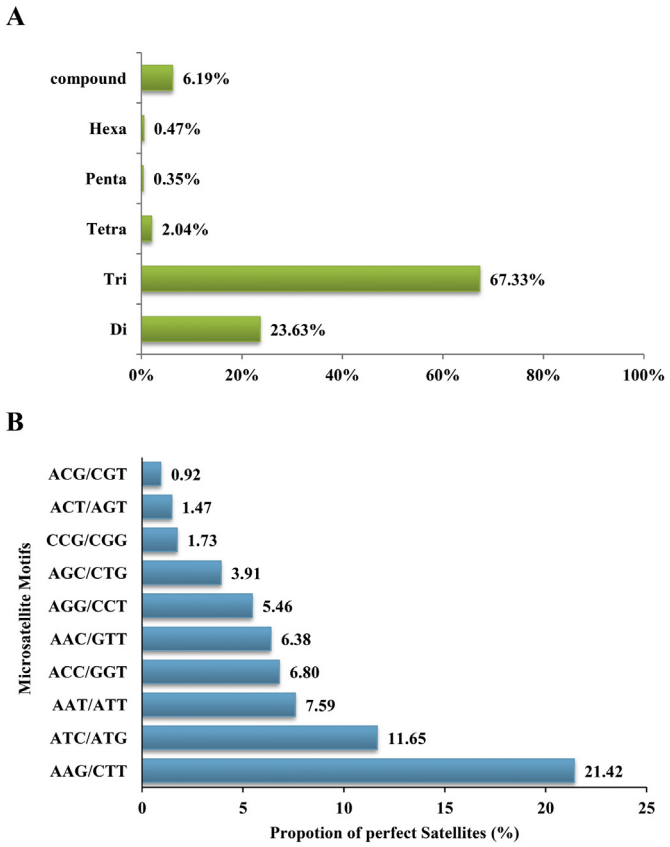

Fig. 7. Distribution and classification of microsatellites identified in the peanut transcriptome data. (A) Distribution of the total microsatellites with different motif types. (Di-, tri-, tetra-, penta-, hexa- and compound nucleotide repeats. (B) Proportion distribution of selected motifs of trinucleotide repeats.

Table 3
Summary and statistics of identification of non-redundant SSR in *Arachis hypogaea* using Trinity and MISA tool.

Parameters	Value
Analysed RNA-Seq sequences for SSR	118,864
Analysed sequences size (bp)	57,150,256
Identified total SSRs from sequences	8591
Total sequences containing SSR	7516
Total sequences with more than 1 SSR	900
Total SSR found in a compound formation	598
Primers designed from SSR	5776

carried out in the present study can be summarised in a schematic diagram, as shown in Fig. 8. Recent advancements in molecular tools such as RNA-Seq technology have enabled us to identify important genes and pathways responsible for defense mechanisms against a pathogen.

During the pathogen attack, PR proteins get induced by systemic acquired resistance (SAR) at the infection site and help to reduce the infection (Van Loon and Van Strien, 1999). Different cell-wall hydrolase enzymes such as chitinase, ß-1, 3-glucanases and pectin esterase degrade fungal cell-wall components (chitin, ß-1,3-glucans and pectin) to prevent fungal growth in a plant (Minic, 2008). Thaumatin (PR-5) protein acts as permatins and causes transmembrane pores into fungal cell membranes leading to their lysis (Abad et al., 1996). Such activity of permeabilization of fungal cell membrane have been reported against *Alternaria solani, Phytophthora infestans* and *Fusarium oxysporum* pathogen (Kitajima and Sato, 1999). In the present study, chitinase was up-regulated in inoculated samples of R genotype and was down-regulated in inoculated S genotype as compared to their respective controls, indicating its role in the potential defense mechanism of *A. hypogaea* resistant variety against *C. arachidicola*.

In this experiment, we found up-regulated peroxidase genes in resistant infected genotype as compared to susceptible infected genotype (Supplementary Table 3). Peroxidases are involved in the prevention of the growth of pathogens by the production of ROS. This cytochrome P450 is involved in the production of phyto-oxylipins, which plays a critical role in plant defense response (Soria-Guerra et al., 2010). Cytochrome P450 gene expression was found higher in rice cultivar during *Fusarium fujikuroi* pathogen infection (Matić et al., 2016). GST is involved in various cellular processes as an ROS scavenging enzyme during any biotic stress (Liao et al., 2014). GST enzyme was reported to be induced during *Pseudomonas syringae* bacterial infection in *A. thaliana* plant for detoxification as well as to help in stress response (Liao et al., 2014). In our study, GST gene was (2.76-fold) up-regulated in RI/SI. We found that NB-LRR receptor, which has a major role in recognizing specific effectors to activate signal transduction pathways against pathogen infection (Jelenska et al., 2010; Chandra et al., 2016), was down-regulated in SI/SC (Supplementary Table 2). R gene products play important roles in plant defense mechanisms by interacting with avirulence (Avr) effector proteins through effector-triggered immunity (ETI) and prevent the growth of pathogen by a hypersensitive response (HR) (Lee and Yeom, 2015).

The transcription factors such as WRKY, MYB and bHLH are well known for their involvement in the pathogen defense mechanism in plants. The bHLH TF and the ERF transcripts are the two major components of the JA signalling pathway, which were up-regulated in RI genotype as compared to SI genotype (Supplementary Table 3). A genome-wide analysis of *Solanumlyco persicum* revealed the up-regulation of bHLH TF when it was challenged by a yellow leaf curl virus of tomato plant (Wang et al., 2015). Genes related to WRKY TF were found to be up-regulated in RI genotype as compared to SI genotype. A similar kind of higher level of gene expression of the WRKY gene was found in the case of a wheat plant during *Puccinia triticina* infection (causative agent of leaf rust) (Chandra et al., 2016). In our study, the MYB TF was down-regulated in SI as compared to SC genotype (Supplementary Table 2). An MYB TF is involved in various cellular processes related to stress tolerance and disease resistance (Alves et al., 2014).

Genes related to signal transduction components such as protein kinase were down-regulated in susceptible genotype (SI) during biotic stress as compared to before infection (SC) (Supplementary Table 2), while this gene was up-regulated in RI/SI genotype (Supplementary Table 3). Down-regulation of such genes may affect signalling pathways involved in the defense mechanism (Goring and Walker, 2004). In the case of plants, serine/threonine kinase is involved in the activation of mitogen-activated protein kinase (MAPKs) pathway, which leads to the expression of defense-related genes (Afzal et al., 2008). Secondary metabolites such as COMT and terpenoids were up-regulated in resistant variety, which may serve as important markers for screening of resistant variety (Naqvi et al., 2017). The COMT gene was down-regulated in susceptible variety during pathogen infection, while terpene synthase was (2.56-fold) up-regulated in RI/SI genotype (Supplementary Table 3). The terpenoid (which acts as phytoalexin) and flavonoid are the primary components of essential oils, which function as toxins against fungal attack (Freeman and Beattie, 2008).

Although levels of expression were slightly different between RNA-Seq and RT-qPCR analyses, the fold change was generally higher in the RNA-Seq analysis than that observed for RT-qPCR, and this was observed for all genes analysed (Supplementary Table 8). Differences at the level of expression between the two methods have been reported in several other studies. However, the fact that quantities were not highly consistent suggests sensitivity differences between the two methods (Soria-Guerra et al., 2010; Hamid et al., 2019). Serine/threonine phosphatase and WRKY TF were strongly up-regulated in resistant

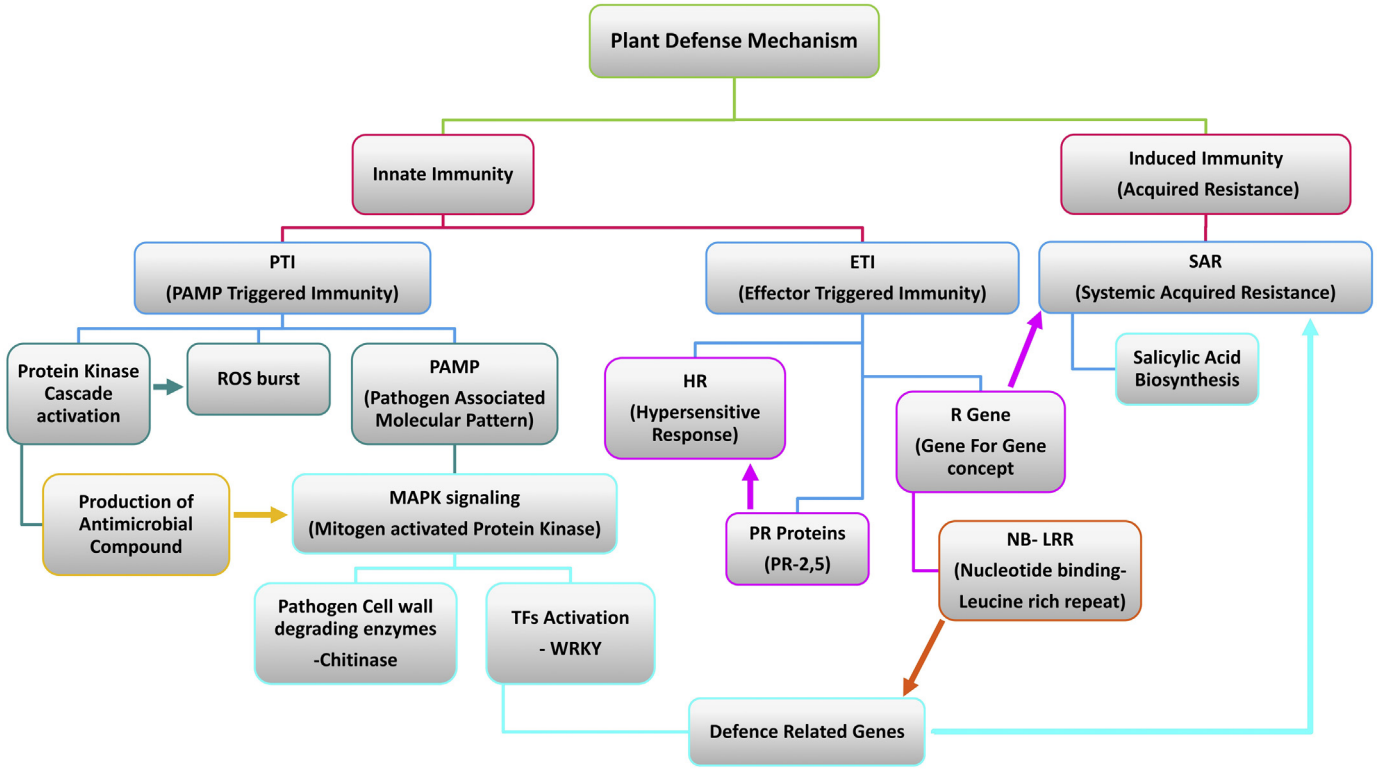

Fig. 8. Innate and induced immunity (acquired immunity) mechanisms of peanut plants against fungal pathogen infection. Innate immunity functions in two ways; PTI (PAMP-triggered immunity) and ETI. PTI includes the activation of multiple signalling pathways involving the production of ROS, activation of MAPK leading to the production of antimicrobial compounds and cell wall degrading enzymes for pathogens. MAPK signalling pathway also activates TF like WRKY and defense-related genes. R genes encode NB-LRR proteins which are responsible for their specific binding interactions, which activate ETI and often result in a HR to restrict water and nutrient access to a pathogen. Another aspect of resistance is the systemic acquired resistance which involves the production of defense-related plant hormones such as, salicylic acid.

plants compared to susceptible plants. Also, the RT-qPCR results positively correlated with the RNA-Seq data validating the sequencing results.

The EST-SSRs markers developed in this study not only provide a better understanding of SSR in the peanut genome but also provided a useful source for conducting additional genetic and genomic studies that may lead to improved varieties of peanut. However, in the case of peanut seed transcriptome data, 5,883 SSR were detected in 4,993 unigenes, out of which, 728 sequences contained more than 1 SSR (Zhang et al., 2012). Another group studying stress response in *A. hypogaea* found a total of 2456 EST-SSR novel primers related to various stresses and were validated by PCR technique with 11 diverse peanut genotypes (Bosamia et al., 2015). Identification of genome-wide markers through RNA-Seq technology can play an important role to develop new varieties through marker-assisted selection (Onaga and Wydra, 2016). The sequence resources generated throughout the present study may be used to develop novel molecular markers in the future. Such molecular markers and gene discovery may help in developing *C. arachidicola* pathogen-resistant peanut varieties.

5. Conclusions

RNA-Seq technology was used to study the transcriptome profile of peanut resistance against *P. arachidis*. GO and KEGG analysis of DEGs revealed essential genes and their pathways responsible for the defense response to fungus. DEGs involved in defense mechanisms such as PR genes, R genes, TFs and secondary metabolite related genes were up-regulated in resistant variety during pathogen infection. RNA-Seq data were validated by using the RT-qPCR technique. The development of EST-SSRs is very useful for evaluating genetic diversity among genotypes, QTL mapping, and marker-assisted breeding.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2020.100243.

Funding details

We are thankful to the Department of Agricultural Biotechnology and Biochemistry, Junagadh Agricultural University, Gujarat, India for providing the resources, laboratory facilities and infrastructure required for the experiments. Also, we are thankful to ICAR-Directorate of Groundnut Research Station for providing peanut resistant and susceptible germplasms for the experiments.

Data availability statement

The datasets generated during and/or analysed during the current study are available in the GenBank repository. For ELS infected samples BioProject ID: PRJNA490413: *Arachis hypogaea* (Resistant GPBD-4 Infected) SRA ID: SRX4806915 and *Arachis hypogaea* (susceptible JL-24 Infected) SRA ID: SRX4806916 while for control samples BioProject ID: PRJNA490412: *Arachis hypogaea* (Resistant GPBD-4 control) SRA ID: SRX4782027 and *Arachis hypogaea* (susceptible JL-24 control) SRA ID: SRX4782028.

https://www.ncbi.nlm.nih.gov/sra/PRJNA490413
https://www.ncbi.nlm.nih.gov/sra/PRJNA490412

Declaration of Competing Interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the research paper.

Acknowledgments

The authors would like to acknowledge the contribution of Dr. B. A. Golakiya and Mr. Manoj Parakhia at the Department of Agricultural Biotechnology and Biochemistry, Junagadh for extending their support for the research work.

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