Comparative transcriptome profiling of pomegranate genotypes having resistance and susceptible reaction to Xanthomonas axonopodis pv. punicae

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Abstract
Pomegranate (Punica granatum L.) is an important fruit crop, rich in fiber, vitamins, antioxidants, minerals and source of different biologically active compounds. The bacterial blight caused by Xanthomonas axonopodis pv. punicae is a serious threat to the crop leading to 60–80% yield loss under epiphytotic conditions. In this work, we have generated comparative transcriptome profile to mark the gene expression signatures during resistance and susceptible interactions. We analyzed leaf and fruits samples of moderately resistant genotype (IC 524207) and susceptible variety (Bhagawa) of pomegranate at three progressive infection stages upon inoculation with the pathogen. RNA-Seq with the Illumina HiSeq 2500 platform revealed 1,88,337 non-redundant (nr) transcript sequences from raw sequencing data, for a total of 34,626 unigenes with size >2 kb. Moreover, 85.3% unigenes were annotated in at least one of the seven databases examined. Comparative analysis of gene-expression signatures in resistant and susceptible varieties showed that the genes known to be involved in defense mechanism in plants were up-regulated in resistant variety. Gene Ontology (GO) analysis successfully annotated 90,485 pomegranate unigenes, of which 68,464 were assigned to biological, 78,107 unigenes molecular function and 44,414 to cellular components. Significantly enriched GO terms in DEGs were related to oxidations reduction biological process, protein binding and oxidoreductase activity. This transcriptome data on pomegranate could help in understanding resistance and susceptibility nature of cultivars and further detailed fine mapping and functional validation of identified candidate gene would provide scope for resistance breeding programme in pomegranate.

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1. Introduction

Pomegranate (Punica granatum L.) is an important fruit crop in countries such as India, Iran, China, and Turkey (Soloklui et al., 2012). It is a perennial fruit tree that is native from Iran to the Himalayan Mountains in northern India (Morton, 1987, Soloklui et al., 2012). India and Iran collectively produce more than half the world’s pomegranate (Petersen et al., 2010, Sharma et al., 2010). Pomegranate aril, seed, rind, flower, bark and root produce different types of biologically active compounds and phytochemicals, including galloflavonoids, ellagic acid, flavonoids, antioxidants,
terpenoids and alkaloids, and are used in treating diseases such as atherosclerosis, breast cancer, skin cancer, and prostate cancer (Bayazit and Caliskan, 2018, MayouniKirschbaum and Porat, 2014, Syed et al., 2013, Ophir et al., 2014). With the characteristics of medicinal properties, sustainability in diversified environment and soil conditions, less irrigation water requirement, high returns on investment and huge export and domestic demand, pomegranate is a good choice for cultivation. Thus, it is an important fruit crop for ensuring the livelihood of farmers in water-scarce regions (Bhandari, 2012). However, during last one and half decades, pomegranate growers in India have been in dire straights because of the severe outbreak of bacterial blight disease caused by Xanthomonas axonopodis pv. punicae, which has resulted in heavy yield losses (Sharma et al., 2017).

The bacterial blight-causing pathogen in pomegranate, X. axonopodis pv. punicae, was identified in 1952 (Hingorani and Mehta, 1952) but was not considered a serious threat. Gradually, the disease has become a serious threat and outbreaks have reached an epiphytotic level in several parts of central India, resulting in yield loss both in terms of quality and quantity (Kumar et al., 2011, Sharma et al., 2017, Sharma et al., 2010). Bacterial blight disease in pomegranate has been reported in countries such as Pakistan (Akhtar and Bhatti, 1992), South Africa (Petersen et al., 2010), and Turkey (Icoz et al., 2014).

X. axonopodis pv. punicae is a gram-negative rod-shaped and non-sporing bacterium. In general, the mode of infection of this pathogen is via rain, irrigation water, infected planting material, insect vectors, farm implements and humans. This pathogen also spreads via air and enters the host through natural openings or physical injuries to plants, the daily average temperature between 25 and 35 °C and RH > 30% favours the rapid blight spread. (Sharma et al., 2017). The blight affects all above ground parts of the plant of which the fruits are the most vulnerable (Sharma et al., 2010, Singh et al., 2015). After inoculation, X. axonopodis pv. punicae infection has three prominent stages in the host. The stage 1 is associated with the appearance of water soaked lesions on leaves and fruit, the stage 2 is associated with necrotic dark brown/blackish brown spots with a yellow halo against light on leaf and necrotic dark brown/blackish depressed lesions on fruit, and stage 3 is characterized by large blighted areas with dried silvery bacterial ooze on leaves and enlarged necrotic lesions with cracks and dried white encrustation of bacterial ooze on fruit (Sharma et al., 2017). PCR based early and reliable diagnostic techniques are available for detection and confirmation of X. axonopodis pv. punicae infection in pomegranate (Sharma et al., 2017, Doddaraju et al., 2019).

Previously, transcriptome analysis based on two phenotypically different pomegranate cultivars led to the identification of simple sequence repeats (SSRs) and also single nucleotide polymorphisms (SNPs) in pomegranate (Ophir et al., 2014). A few attempts have been made to develop a genetic map of economically important traits in pomegranate based on the transcript markers enriched with quantitative trait loci (Hareb-Beja et al., 2015). Whole genome sequencing and limited transcriptome analysis using fruit peel, leaves, roots, flowers along with their parts and fruit are available to add to the large scale comparative transcriptome data generated in the current investigation, all these can result in a tremendous increase in genomics resources on pomegranate (Ono et al., 2011, Ophir et al., 2014, Qin et al., 2017, Yuan et al., 2018).

Although these resources provide good information about the biology of pomegranate, host–pathogen interactions with special reference to X. axonopodis pv. punicae via RNA-Seq is not available. To the best of our knowledge, this is the first report describing the identification of the differentially expressed candidate genes responsible for resistance against pathogen infection by using NGS technologies such as RNA-Seq analysis. Here, we report results from high-throughput Illumina sequencing, de novo assembly and functional annotation of the pomegranate transcriptome under Xanthomonas infection and the identification of differentially expressed genes (DEGs) in susceptible and moderately resistant pomegranate genotypes. The transcriptome data generated could help in understanding resistance and susceptibility nature of cultivars and further detailed fine mapping and functional validation of identified candidate gene would provide scope for resistance breeding programme in pomegranate.

2. Material and methods
2.1. Experimental site and climatic conditions

The study was carried out at Kegaon Research Farm, ICAR-National Research Centre on Pomegranate, Solapur, India, having 17°43’ N latitude, 75°50’ E longitude and 486 m altitude from mean sea level. During the experimental period, the minimum average temperature and maximum average temperature of the month ranged from 18.19 °C to 33.16 °C and the mean relative humidity during the period was 66.87% with average wind velocity 6.37 ms⁻¹.

2.2. Plant material and challenge inoculation

‘Bhagawa’ is India’s most commercial but blight susceptible pomegranate cultivar having red rind and sweet soft red arils where as IC 524207 is a wild type pomegranate genotype having small acidic fruits but moderately resistant to blight. These pomegranate plants were inoculated by spraying pure culture of X. axonopodis pv. punicae under conditions favourable for blight infection (average daily temperature 25-35 °C and RH > 30%). The culture was maintained at the Plant Pathology Laboratory of ICAR-NRCP. The pathogen was isolated from infected samples on NGA medium.

Briefly, samples were surface-sterilized in sodium hypochlorite (2%) for 2–3 min, then washed with sterilized deionized water three times and blot dried. The samples were macerated on a sterilized glass slide and streaked on the NGA. The culture plates were incubated at 28 ± 1 °C. The colonies that were developed after 48–72 h of incubation were chosen for further confirmation. The colonies of X. axonopodis pv. punicae were identified by colour, texture, morphology and characteristic brown pigmented fuscan production. The bacterial identity was further confirmed by gyrB-specific PCR amplification that is known to yield a 491-bp ampli-

con in X. axonopodis pv. punicae (Mondal et al., 2012, Sharma et al., 2017). To prepare the inoculum, a loop full of bacterium was inoculated in nutrient glucose broth with constant shaking at 100 rpm. Bacterial suspension was diluted to 10⁻² with shaking at 100 rpm. The spray inoculation method was used for challenge inoculation under field conditions (Fig. 1B) and symptoms on leaf and fruits were confirmed as bacterial blight infection by visual identification (Fig. 1C–K) and in vitro culture characteristics of X. axonopodis pv. punicae (Sharma et al., 2017).

2.3. Preparation of samples for RNA-Seq analysis

We used 12 samples (pooled total RNA samples from three technical replicates) in this study: three leaves and fruit samples of Bhagawa (pooled sample of three technical replicates) with progressive stages of blight infection symptoms from stage 1 to 3 were collected, LS_1 i.e., first stage of infection on leaf of Bhagawa appeared on 5th day after challenge inoculation (days to post-inoculation-dpi), second stage- LS_2 (8 dpi), third stage- LS_3 (11 dpi) and first stage on fruit of ‘Bhagawa’-FS_1 (11dpi), second stage-FS_2 (15 dpi), third stage-FS_3 (21 dpi), respectively; along with non-inoculated control leaf and fruit samples of Bhagawa.
(LS_C, FS_C); three pooled samples each of leaf and fruit of IC 524204 at stage 1 of blight infection was taken (LT_1: 7 dpi and FT_1: 14 dpi) along with control samples of IC 524204 (LT_C, FT_C). The samples of 2nd and 3rd stages of infection on both leaf and fruit of IC 524204 were ignored as symptom progression was very slow and overlapping infection symptoms were observed at stage 1 and 2, however infection stage 3 (cracking of fruits) was not observed on fruits of tolerant genotype. Total RNA was extracted from all 12 samples by using the Nucleospin RNA isolation kit. RNA integrity was tested by separating an aliquot of the RNA sample on 1% agarose gel electrophoresis. Quality was assessed by visualizing and checking the appearance of ribosomal RNA bands and by lack of degradation products. The integrity of RNA was re-confirmed by using Bioanalyzer 2100 (Agilent, Folsom, CA). Total RNA from three technical replicates were pooled for each sample and samples were enriched for mRNA by using oligo (dT) beads followed by removal of rRNA by using a Ribo-Zero kit. Then, mRNA was fragmented randomly into small pieces by adding the fragmentation buffer provided with the Illumina mRNA-seq kit (Illumina, San Diego, CA). The fragmented mRNA was reverse-transcribed by using random hexamers and further amplified to produce double-stranded DNA. Repair of the fragment ends and 3′-end adenylation were implemented with the NEBNext™ DNA Sample Prep Reagent Set 1 (New England BioLabs, Ipswich, MA). The paired end adaptors were linked to the ends of the DNA fragments. The resulting cDNA templates were gel-purified and PCR-enriched. Quality of the mRNA-Seq library was verified by using Bioanalyzer.

2.4. Illumina sequencing, quality control and transcriptome de novo assembly

The quantified mRNA-Seq libraries were sequenced by using Illumina HiSeq 2500. We used DrSeq, an automated RNA Seq pipeline developed by Nucleome Informatics, for data analysis. FastQC was used to check the quality of the raw data and to avoid low-quality bases that result in mis-assemblies at the time of assembly. FastQC checks the quality of the data by considering sequencing error rate (e) and sequencing base quality (Qphred) value or quality score (QC). The filtered high-quality reads were used for downstream analyses. The clean transcriptome reads were assembled de novo by using Trinity software (Grabherr et al., 2011).

2.5. Functional annotation and classification of genes

Unigenes were searched against seven public databases: the NCBI non-redundant protein sequence database (Nr database) (https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/); the NCBI nucleotide sequence database (Nt database) (https://www.ncbi.nlm.nih.gov/nucleotide/?cmd=search); the KO database (https://www.genome.jp/kegg/ko.html); SwissProt (Boeckmann et al., 2003); Proteinfamily (Pfam) database (Bateman et al., 2004); Geneontology (GO) database ( Consortium, 2004) and Eukaryotic orthologous groups (KOG) database by using BLASTX. The BLASTX results were import to Blast2GO v2.5 (Conesa and Götz, 2008) for functional classification. We used in house scripts for mapping, retrieving and allocation of GO terms to genes. These genes were also annotated with unique enzyme codes (ECs) and KEGG maps (http://www.genome.jp/kegg) by using KAAS software (Kanehisa and Goto, 2000, Kanehisa et al., 2011). The genes present in the transcriptome of bacterial blight-challenged tissues were classified into three GO categories: biological process, cellular component and molecular function.

2.6. Estimation of transcript abundance and analysis of differential expression

The quantification of transcripts involved RNA-Seq with Expectation Maximization RSEM 1.2.7 (https://www.encodeproject.org/software/rsem/). To estimate the abundance of each samples at various stages, the expression-normalizing fragments per kilobase of exon fragments per million mapped (FPKM) were calculated by using RSEM 1.2.7. P-values for differential expression of genes among two samples was calculated by using the likelihood ratio test in the R package, DEGseq (Wang et al., 2009; Marioni et al., 2008). The P-values were adjusted by using the Q-value (Storey and Tibshirani, 2003). Q-value < 0.05 was used to identify the significantly differentially expressed genes. Finally, the combination of Q-value (<0.05 and fold change (>2 and < -2) was used to identify up- and down regulated genes.

2.7. Prediction of R genes among the upregulated genes of different sample combinations

We used all upregulated gene IDs for all combinations of samples as described earlier. We downloaded the R gene protein sequences of different plant species from (http://prgdb.crg.eu/)
The pomegranate variety Bhagawa is cultivated type and highly susceptible to *X. axonopodis pv. punicae* infection and the genotype IC 524207 wild type collected from hills of Himalayas, is moderately resistant to *X. axonopodis pv. punicae* infection. With a high-throughput IlluminaHiSeq 2500 sequencing platform, we obtained 50,10,95,768 raw transcriptome reads from both susceptible Bhagawa and moderately-resistant IC 524207 from *X. axonopodis pv. punicae* infected and control fruit and leaf samples, which accounted for approximately 75.16 GB of sequenced data (NCBI Accession No. PRJNA361285). Furthermore, raw reads were subjected to quality control (Fast QC) to obtain clean transcriptome reads of 48,58,02,868, which represented about 72.87 GB (96.95%) of high-quality sequence data with a >92% quality check score for all samples and 94% for most samples at Q30 with high GC content (>47.50). The data on sequencing, quality of data and error rates are in Table 1. All these filtered reads were used in transcriptome assembly. The minimum, mean, median, and max length as well as N50 and N90 value and total nucleotides sequenced were 201, 1184, 642, 23,878, 2081, 471, and 22,29,23,528, respectively (Table 2). The transcriptome assembly in our study resulted from 188,337 unigenes with maximum and minimum read lengths of 23,878 and 200 bp, respectively, with an average size of assembled unigene of 1184 bp and 34,626 unigenes >2 kb in size (Table 3, Fig. 2A and B), which indicated increased coverage as well as the depth and high quality assembly of the sequenced transcriptome data.

### 3. Results

#### 3.1. De novo assembly and clustering of transcriptome from infected tissues of pomegranate

Table 1

| Sample | Raw Reads | Clean Reads | Clean Bases Size (GB) | Error % | Q20% | Q30% | GC Content |
|--------|-----------|-------------|-----------------------|---------|------|------|------------|
| LS_1   | 58,279,956| 56,911,978  | 8.54                  | 0.01    | 98.21| 95.57| 50.07      |
| LS_2   | 64,644,180| 63,436,302  | 9.52                  | 0.01    | 98.23| 95.59| 49.32      |
| LS_3   | 57,946,290| 56,346,058  | 8.45                  | 0.01    | 97.77| 94.31| 49.85      |
| FS_1   | 20,630,104| 19,691,646  | 2.95                  | 0.01    | 97.35| 93.47| 54.63      |
| FS_2   | 19,578,544| 18,764,108  | 2.81                  | 0.02    | 97.06| 92.7 | 54.39      |
| FS_3   | 21,913,568| 21,063,454  | 3.16                  | 0.02    | 97.01| 92.57| 54.29      |
| LS_C   | 49,579,848| 48,131,131  | 7.22                  | 0.02    | 96.83| 92.01| 50.84      |
| FS_C   | 49,733,888| 47,679,996  | 7.15                  | 0.01    | 97.94| 94.85| 49.9       |
| LT_1   | 59,916,394| 58,049,980  | 8.71                  | 0.01    | 97.8 | 94.69| 49.99      |
| FT_1   | 23,247,992| 22,516,058  | 3.38                  | 0.01    | 97.56| 93.84| 47.5       |
| LT_C   | 55,469,742| 53,602,820  | 8.04                  | 0.01    | 97.91| 94.53| 51.03      |
| FT_C   | 20,155,262| 19,607,290  | 2.94                  | 0.01    | 97.83| 94.21| 48.23      |

Table 2

| Minimum length of Transcript | Mean Length of Transcript | Median Length of Transcript | Maximum Length of Transcript | N50 value | N90 value | Total nucleotides |
|------------------------------|--------------------------|-----------------------------|------------------------------|-----------|-----------|-------------------|
| 201                          | 1184                     | 642                         | 23,878                       | 2081      | 471       | 222,923,628       |
is shown in Table 4. BLASTX searches showed that about 51.6% of annotated sequences belong to other species (many different species of plants mixed together), followed by 29.3% in *Eucalyptus grandis* (Fig. 3B), which shows the evolutionary relationship of pomegranate with *Eucalyptus*. However, very small proportions of sequences were annotated with reference to *Vitis* (5.8%), *Theobroma cacao* (5.2%), *Ricinus communis* (3.9%), *Jatropha curcas* (3.2%) and *Gossypium raimondii* (2.6%) (Fig. 3B).

Table 3

| Unigene interval length | 200–500 bp | 500–1 Kbp | 1–2 kbp | >2 kbp | Maximum length | Total |
|-------------------------|------------|-----------|---------|--------|----------------|-------|
| Total no. of unigenes   | 71,157     | 49,611    | 32,943  | 34,626 | 23,878         | 188,337 |

Fig. 2. (A) Distribution of unigenes based on length frequency. (B) Unigenes length distribution and their number.
3.3. Functional classification of pomegranate unigenes based on GO, KEGG and KOG

Pomegranate unigenes with non-redundant annotations were functionally annotated by Gene Ontology (GO) analysis for biological process, molecular function and cellular component. From a total of 90,485 pomegranate unigenes, 68,464 were assigned to biological functions. In total, 78,107 unigenes were assigned to molecular function and 44,414 to cellular components (Fig. 4A). Functional classification of transcripts with respect to Kyoto Encyclopedia of Genes and Genomes (KEGG) showed most were under the metabolism category and gene processing category (Fig. 4B). The transcripts we obtained were aligned to the Eukaryotic orthologous groups (KOG) database to predict and classify their possible functions. Transcripts falling under categories “post translational modification”, “protein turnover”, and “chaperones” were highly clustered (Fig. 4C).

3.4. Analysis of differentially expressed genes

In order to compare gene expression levels under different experimental conditions, an FPKM frequency distribution graph and box plot analysis were performed. Both these graphs display overall gene expression levels in test samples. The significant differences for mean gene expression levels with wide range between FT_1 and FT_C was noticed in comparison to all other conditions as reflected from both the FPKM density distribution and box plot graphs (Fig. 5A and B). It was also interesting to note that fruit tissues of tolerant genotypes displayed large number of genes expressed at stage 1 compared to susceptible plant tissues at different stages. Further, FT_1 has higher expression compared to control FT_C indicating induced activation of defense related genes after infection at stage 1. In-order to find genes with similar expression patterns under various experimental conditions hierarchical FPKM clustering analysis was performed using the log10 (FPKM + 1) values. By clustering genes with similar expression pattern, it is possible to discern unknown functions of previously characterized genes or the functions of unknown genes. Genes within the same cluster exhibit the same trends in expression under different conditions. Heatmap showed many genes which were up or down regulated among different conditions. We found higher differential up and down regulation of genes in fruit tissues FT_1 and FT_C. However, higher differential down regulation of genes in FS_1, 2 and 3 as compared to FS_C (Fig. 5C).

3.5. Identification of DEGs between control and infected fruit and leaf samples of IC 524207 and Bhagawa

To understand the transcriptional response of the host at the time of infection, we identified the DEGs between control and infected samples. We selected the combinations of control and infected samples shown in Table 5. In our analysis, we ignored infection stage 2nd and 3rd of leaf and fruits in IC 524507 as symptom progression was very slow and overlapping of infection symptoms was observed at stage 1 and 2, however infection stage 3 was not observed on fruits and leaves of tolerant genotype.

3.6. Identification of DEGs in leaf and fruit samples of control and infected tissues of IC 524207

We identified DEGs between the control and infected fruit samples (FT_1 vs FT_C) of IC 524207 at stage 1 (14 dpi). In infected fruit samples (FT_1 vs FT_C), about 937 and 1047 genes were up- and downregulated, respectively (Table 6) (Supplementary dataset 1 & 2). Similarly, a total of 622 and 593 genes were up- and downregulated in infected leaf samples versus the control (LT_1 vs LT_C) at 7 dpi (Table 6) (Supplementary dataset 3 & 4).
3.7. Identification of DEGs between leaf and fruit samples of control and infected Bhagawa tissues

In the first stage of infection of fruit in Bhagawa, 717 and 2735 genes were up- and downregulated, respectively at 11 dpi (FS_1 vs FS_C) (Table 6) (Supplementary dataset 5 & 6). In leaf infected samples, a total of 595 and 705 genes were up- and downregulated at 5 dpi (Table 6) (Supplementary dataset 7 & 8). In the second stage of pathogen infection, 795 genes were upregulated and 2740 downregulated with respect to the control at 15 dpi (Table 6) (Supplementary dataset 9 & 10). Similarly, a total of 1101 and 1185 genes were up- and downregulated in the infected samples of leaf with respect to the control at 8 dpi (Table 6) (Supplementary dataset 11 & 12). In the third stage of pathogen infection, 795 genes were upregulated and 2860 downregulated with respect to the control at 18 dpi (Table 6) (Supplementary dataset 13 & 14).
and 2656 genes were up- and downregulated in infected fruit samples at 21 dpi (Table 6) (Supplementary dataset 13 & 14) and 952 and 1143 were up- and downregulated in infected leaf samples with respect to the control at 11 dpi (Table 6) (Supplementary dataset 15 & 16).

### Table 5
| Sample No. | Stage of infection | Combination of samples | Total no. of upregulated genes | Total no. of downregulated genes |
|------------|--------------------|------------------------|-------------------------------|---------------------------------|
| 1          | First stage        | FT_1 and FT_C          | 1011                          | 1077                            |
| 2          | Second stage       | LS_1 and LS_C          | 771                           | 705                             |
| 3          | Third stage        | FS_1 and FS_C          | 2102                          | 4221                            |
| 4          | First stage        | LT_1 and LT_C          | 622                           | 593                             |

### Table 6
Combinations of samples considered for understanding the differential expression of the genes between the control samples and infected samples. FT and LT represent the infected fruit and leaf samples of IC 524207, respectively. FS and LS represent the infected fruit and leaf samples of Bhagawa, respectively. C represents the control.

### Table 7
Combinations of samples considered for understanding the differential expression of the genes at each stage. IC 524207 represents the moderately resistant pomegranate variety, L represents leaf sample, F represents fruit sample, S represents susceptible and T represents tolerant.

### Table 8
Combination of the samples compared and total number of up- and downregulated genes in infected fruit and leaf samples of IC 524207 and Bhagawa pomegranate between each stage of infection.

### 3.8. Comparative study of the identification of up and down-regulated genes among infected samples of IC 524207 and Bhagawa

We compared infected fruit and leaf samples at different stages (dpi) in IC 524207 and Bhagawa along with control to retrieve the DEGs. The combinations for this analysis are shown in Table 7. Table 8 represents the comprehensive information for the compared samples and total number of DEGs at each stage of infection. At the first stage of fruit infection in Bhagawa and IC 524207, 2102 and 4221 genes were up- and downregulated, respectively. In comparing the second stage of fruit of Bhagawa and the first stage of IC 524207, 2305 and 4447 genes were up- and downregulated. Similarly, in comparing the Bhagawa third stage and IC 524207 first stage for fruit, 2347 and 4455 genes were up- and downregulated, respectively. Furthermore, 771 and 768 genes were up- and downregulated in leaf samples at stage 2 of Bhagawa and stage 1 of IC 524207. Moreover, 1302 and 1240 genes were up- and downregulated in comparing stage 3 of Bhagawa and stage 1 of IC 524207.

### Table 9
Identification of up and down-regulated genes among infected leaf and fruits samples of Bhagawa.

While comparing leaf and fruit tissues of different progressive blight infection stages, the maximum number of differentially expressed genes were found in FS_1 vs FS_3 (399 downregulated and 358 upregulated) (Table 9 and 10). However, GO enrichment was higher in LS_1 vs LS_3. The most significantly enriched GO term was related to catalytic activity and metabolic process (Fig. 6). Major KEGG enriched pathways for DEGs were related to ribosome, photosynthesis, plant pathogen interaction, phenylpropanoid biosynthesis and plant hormone signal transduction.

### 3.10. Identification of R genes among the upregulated genes of all combinations

We identified the R genes among upregulated genes of different combinations (Table 11). The comparison of FS_C vs FT_C and FS_2 vs FT_1 had more numbers (about 175 and 140, respectively) of R genes.

### Table 10
Identification of up and down-regulated genes among infected leaf and fruits samples of Bhagawa.

We identified the R genes among upregulated genes of different combinations (Table 11). The comparison of FS_C vs FT_C and FS_2 vs FT_1 had more numbers (about 175 and 140, respectively) of R genes.
Table 10
Gene Ontology classification of significantly enriched up- and down-regulated genes among the different diseased leaf and fruit samples of ‘Bhagawa’

| Samples compared | Gene Ontology classification of upregulated genes | Gene Ontology classification of major downregulated genes |
|------------------|-----------------------------------------------|----------------------------------------------------------|
|                  | Biological Processes | Cellular Processes | Molecular Functions | Biological Processes | Cellular Processes | Molecular Functions |
| FS_1 vs FS_2     | 73                 | 43             | 83             | 85                        | 45                      | 98                        |
| FS_1 vs FS_3     | 68                 | 37             | 76             | 96                        | 49                      | 108                       |
| FS_2 vs FS_3     | 67                 | 33             | 75             | 75                        | 38                      | 84                        |
| LS_1 vs LS_2     | 122                | 71             | 129            | 145                       | 69                      | 147                       |
| LS_1 vs LS_3     | 126                | 61             | 132            | 178                       | 89                      | 188                       |
| LS_2 vs LS_3     | 103                | 50             | 114            | 125                       | 81                      | 143                       |

Fig. 6. GO enrichment of up and downregulated genes of infected leaf and fruit transcriptome of Bhagawa.
To validate the results of RNA-Seq, we used RT-qPCR to validate the expression of three genes coding for xyloglucan endotransglycosylase, superoxide dismutase, and alcohol dehydrogenase. We selected these genes randomly from the data generated from DEG-seq analysis. We designed the forward and reverse primers for the genes and amplified the genes in all 12 samples (Table 12). The gene that codes for xyloglucan endotransglycosylase was slightly over-expressed in infected leaf samples of Bhagawa at stage 1 and 3 of infection as compared with the control (Fig. 7A). Similarly, the gene that codes for xyloglucan endotransglycosylase was under-expressed in infected leaf samples of IC 524207 at stage 1 of infection versus the control. Xyloglucan endotransglycosylase showed higher expression in infected fruit samples of Bhagawa at stage 2 of infection versus the control. We did not obtain any data for infected fruit samples of Bhagawa at stage 1 and stage 3 or IC 524207 at stage 2 (Fig. 7A).

For the gene coding for alcohol dehydrogenase, the control sample of infected leaf of Bhagawa had higher expression than at any of the stages (Fig. 7B). We did not obtain any data for the control infected leaf samples of IC 425207. Similarly, we did not obtain any data from infected fruit samples of Bhagawa at stage 1 and 2 (11 and 15 dpi), but the expression of alcohol dehydrogenase was similar at stage 3 of Bhagawa and its control in infected fruit samples.

For the gene coding for superoxide dismutase, we did not obtain any data for infected fruit samples of Bhagawa at 11 dpi and 21 dpi, but we found over-expression of the gene in infected fruit samples.

### Table 11
The predicted upregulated genes in each combination. Among the compared samples, FS_C vs FT_C had the highest number of genes among the upregulated genes.

| Sample Combination | Total number of upregulated R genes |
|--------------------|-------------------------------------|
| FS_1 vs FS_C       | 72                                  |
| FS_1 vs FT_1       | 115                                 |
| FS_2 vs FS_C       | 70                                  |
| FS_2 vs FT_1       | 140                                 |
| FS_3 vs FS_C       | 72                                  |
| FS_3 vs FT_1       | 126                                 |
| FS_C vs FT_1       | 175                                 |
| FT_1 vs FT_C       | 83                                  |
| LS_1 vs LS_C       | 54                                  |
| LS_1 vs LT_1       | 93                                  |
| LS_2 vs LS_C       | 114                                 |
| LS_2 vs LT_1       | 76                                  |
| LS_3 vs LS_C       | 75                                  |
| LS_3 vs LT_1       | 110                                 |
| LS_C vs LT_C       | 73                                  |
| LT_1 vs LT_C       | 49                                  |

### Table 12
List of forward primer and reverse primers used for RT-qPCR of the selected genes.

| S.No. | Name of the gene           | Forward Primer | Reverse Primer |
|-------|----------------------------|----------------|----------------|
| 1     | Xyloglucan endo- transglycosylase | CCTCCCACAGGGTAGAGTAG | GAAAGGGTGACAGAGAACAGAG |
| 2     | Alcohol dehydrogenase     | GTGGTTTGTAGTTCCCGAAGA | AAGAGGTGATTGCCGAGATG |
| 3     | Superoxide dismutase       | AATTTCAGCCGTACACAGACC | CACTGGACAAAATCCCATATA |
of Bhagawa at stage 2 versus the control (15 dpi). However, we did not obtain any data for IC 524207 at stage 1 of fruit infection (Fig. 7C).

4. Discussion

In 1952, *X. axonopodis pv. punicae* was identified in India and was found to be highly evolved and a host-specific pathogen (Hingorani and Mehta, 1952). At the time of infection, the infected leaves show early water-soaked lesions that later convert to necrotic blighting. The infected fruits show coalesced water-soaked lesions leading to necrosis and development of small cracks (Petersen et al., 2010). As an immunological response towards infection, the plant infected with the pathogen at different stages of infection is expected to have a definite transcriptional response (Song et al., 2019). Disease progression and appearance of symptoms on leaf and fruit tissues of tolerant type was slow which confirm moderate resistance in *X. axonopodis* against *Xap* and similar disease reaction pattern was obtained in resistant and susceptible kiwifruit genotypes upon *Pseudomonas syringae* infection in kiwifruit by Song, et al. (2019). Using RNA-Seq, we analyzed the transcriptome of these pomegranate samples infected with *X. axonopodis* pv. punicae at a large scale. To the best of our knowledge, these data are the first with high quality in pomegranate as compared with previous reports (Ophir et al., 2014). From the preliminary analysis, before considering the comparative analysis, we found a change in signal transduction genes during pathogenesis (Fig. 4A). Similar observations were found in soybean during *Pseudomonas syringae* infection (Dong et al., 2018), which may be due to stronger signals as a result of bacterial invasion. Also, Fig. 4B shows high expression of genes involved in chaperone activities, which are required for degradation of misfolded proteins during bacterial stress and also to increase other protein turnover to cope with the stress condition.

After preliminary analysis, in-depth data analysis was performed in three different ways: comparing the transcriptome data of the infected fruit and leaf samples with the controls, by comparison of progressive disease transcriptome data of Bhagawa leaf and fruits and a comparative study among the infected samples of IC 524207 and Bhagawa.

GO terms obtained by the comparison of the IC 524207 infected fruit samples with their controls (FT_1 vs FT_C) showed that the genes involved in single organism transport and single organism localization biological processes were upregulated and those involved in cell, cell part, intracellular processes were downregulated with infection (Table 13) (Fig. 8A, B).

Comparison of infected leaf samples of IC 524207 and its control (LT_1 vs LT_C) showed upregulation of genes involved in biological process including oxidation-reduction and molecular functions such as oxidoreductase (Table 13) (Fig. 9A, B). At the time of infection, there may be a biostatic stimulus to cope with the oxidative stress caused by pathogen infection, so genes involved in oxidation-reduction were upregulated (Li et al., 2016, Liu et al., 2017). We found no significant KEGG pathways in both comparisons.

The comparison of infected fruit samples of Bhagawa and its control (FS_1 vs FS_C) showed upregulation of genes involved in oxidation and reduction processes, which predominantly represents the biological process. Under the molecular functions category, oxidoreductase was upregulated, which may be due to the oxidative stress described earlier (Table 13). Under the downregulated category, we did not find significant GO-enriched terms except in FS_1 vs FS_C and FS_2 and FS_C, which showed downregulated genes in the binding and protein binding category. KEGG annotations in all the comparisons showed that metabolic processes such as photosynthesis, oxidative
phosphorylation, and protein processing in endoplasmic reticulum were predominantly enriched and most of the upregulated genes were under these categories (Table 14). In general, processes such as photosynthesis in plants were downregulated, which is a defense strategy adopted by the infected plant (Rojas et al., 2014). However, in citrus, during infection with Candidatus liberibacter asiaticus, which results in huanglongbing or citrus green disease, (Martinelli et al., 2012) transcriptome analysis showed upregulation of photosynthesis genes. Similar to citrus, when comparing infected fruit samples of Bhagawa to controls, photosynthesis genes were upregulated. A similar consideration was made in analyzing citrus green disease (Martinelli et al., 2012). More number of both up and down regulated genes were observed during first stage of fruit infection in tolerant type (FT_1 vs. FT_C) as compared to number of up regulated genes in FS_1 vs. FS_C, FS_2 and FS_C vs. FS_3 vs. FS_C results suggest that more upregulated DEGs are involved in tolerant type and there is rapid response of tolerant type to pathogen infection as compared to susceptible type. Higher number of downregulated genes in control compared susceptible leaf and fruit samples at different stages of blight infection as compared to leaf and fruit transcriptome of tolerant types suggest downregulation of genes involved in pathways important for disease resistance like phenylpropanoid biosynthesis, which mainly carry out synthesis of lignin and flavonoids responsible for resisting pathogen infection. Similar results were obtained by Song, et al. (2019) while carrying DEG analysis of Pseudomonas syringae.

![Enriched GO Terms](image)

**Fig. 8.** (A) GO annotation of upregulated genes between the sample FT_1 vs FT_C. (B) GO annotations of downregulated genes between FT_1 vs FT_C. Y-axis represents the total number of genes in each category. X-axis represents different GO classifications of up- and downregulated genes.
pv. *actinidiae* resistant and susceptible kiwifruit genotypes upon challenge inoculation.

The most enriched GO terms were related to oxidation reduction and protein binding in differentially expressed genes (Table 13). The downregulated genes were found to be under the spliceosome and RNA transport category (FS_1 vs FS_C, FS_2 vs FS_C, FS_3 vs FS_C) (Table 14). When the infected leaf samples of Bhagawa were compared with the control (LS_1 vs LS_C) (LC_2 vs LS_C) (LS_3 vs LS_C), most of the upregulated genes were in metabolic processes on GO analysis and downregulated genes were predominantly involved in catalytic activity (Table 13). The KEGG annotation showed that processes such as photosynthesis, phenyl propanoid biosynthesis and amino sugar/nucleotide metabolism were downregulated (Table 14). This finding agrees with previously published literature in that for the invocation of the plant defense system toward the invading pathogen, energy plays a critical role for the differential expression of several genes and pathways (Scheideler et al., 2002). The genes involved in photosynthesis and chrolophyll biosynthesis were downregulated at the time of invasion of virulent and avirulent pathogens (Denoux et al., 2008, Rojas et al., 2014, Swarbrick et al., 2006, Truman et al., 2006).

We found that when comparing LS_2 vs LS_C, along with the pathways that were downregulated in stage 1, starch sugar metabolism was downregulated. In sweet orange, *Citrus sinensis*, the starch levels were increased in leaves infected with *C. liberibacter asiaticus*, which causes citrus huanglongbing, by about 3 to 7 folds as compared with controls. The cross-talk between the pathogen and the plant may lead to the differential expression of starch metabolism genes and their downregulation (Fan et al., 2010). Similar phenomena might be exhibited by infected pomegranate, which needs further investigation.

As described in Table 8, combinations were selected for the comparative transcriptome analysis between infected leaf and fruit...
samples between IC 524207 and Bhagawa. While comparing FS_1 vs FT_1 and FS_2 vs FT_1, KEGG annotation showed that the carbon metabolism pathway was upregulated (Supplementary dataset 18). At the time of pathogen infection, generally carbon metabolism is high because of the upregulation of associated genes, which turns on the defense mechanisms by the activation of defense genes and finally leads to the accumulation of H₂O₂ and salicylic acid, etc. There is down regulation of pathways which play critical role in defense response against pathogen like protein processing in endoplasmic reticulum, flavonoid biosynthesis, phenylpropanoid biosynthesis, etc, when transcriptome of infected fruit samples Bhagawa was compared with infected fruit transcriptome of IC 524207, this might be one of the reasons for fast progression of blight infection in susceptible tissues (Korner et al., 2015, Song et al., 2019). The downregulation of the carbon metabolism pathway in infected leaf samples of Bhagawa when compared with infected leaf samples of IC 524207 and (LS_1 vs LT_1 and LS_2 vs LT_1) (Supplementary dataset 17 and 18) may occur because of downregulation of genes involved in photosynthesis, which alter the carbohydrate metabolism and was also observed in Potato infected with Rhizoctonia solani (Aliferis and Jabaji, 2012). Finally, some of the important genes that code for xyloglucan endotransglycosylase, superoxide dismutase, and alcohol dehydrogenase were validated by RT-qPCR.

5. Conclusions

We aimed to study and understand the defense response of pomegranate against the X. axonopodis pv. punicae infection, which is the sole cause of blight in pomegranate. The data from this large-scale transcriptome study by using Illumina sequencing was of high quality and the largest pomegranate transcriptome data available to date. Moreover, in-depth comparative analysis of RNA-Seq data, especially the comparison between infected leaf samples of Bhagawa with the control provides insights into the biological significance of downregulation of genes involved in photosynthesis. Similarly, comparison of infected fruit samples of Bhagawa with infected fruit samples of IC 524207 showed how the upregulation of the carbon metabolism pathway would provide resistance against the infection. This report gives information on the defense strategies adopted by the pomegranate against X. axonopodis pv. punicae and also provides the preliminary information about the differentially expressed genes between two pomegranate varieties and their possible role in tolerance/susceptibility to infection.

Table 14

| Samples compared       | KEGG classification of upregulated genes and their number | KEGG classification of downregulated genes and their number |
|------------------------|----------------------------------------------------------|----------------------------------------------------------|
| LT_1 vs LT_C           | Photosynthesis (15), terpenoid biosynthesis (11)         | Thiamine metabolism and gap junction (9)                  |
| FT_1 vs FT_C           | Carbon metabolism (24), biosynthesis of aminoacid (20), protein processing in endoplasmic reticulum (38) | Ribosome (60)                                               |
| FS_1 vs FS_C           | Photosynthesis (35), oxidative phosphorylation (35), protein processing in endoplasmic reticulum (26) | Spliceosome (59)                                           |
| FS_2 vs FS_C           | Photosynthesis (36), oxidative phosphorylation (29), protein processing in endoplasmic reticulum (28) | Spliceosome (63), RNA transport (40)                       |
| FS_3 vs FS_C           | Photosynthesis (36), oxidative phosphorylation (31), protein processing in endoplasmic reticulum (28) | Spliceosome (63)                                           |
| LS_1 vs LS_C           | Photosynthesis (12), oxidative phosphorylation (14)      | Photosynthesis (16), amino sugar and nucleotide sugar metabolism (15) |
| LS_2 vs LS_C           | Plant pathogen interaction (18)                          | Starch and sucrose metabolism (27), photosynthesis (24), amino sugar and nucleotide sugar metabolism (24), phenylpropanoid biosynthesis (18) |
| LS_3 vs LS_C           | Phenylpropanoid biosynthesis (14), phenyl alanine metabolism (13) | Amino sugar and nucleotide sugar metabolism (26), starch and sucrose metabolism (23), phenylpropanoid biosynthesis (14), biosynthesis of aminoacid (20) |

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Consent for publication

All the authors have approved the final article.

Availability of data and material

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Authors’ contributions

Design of the study by NVS, RKP, SP and UKR. Drafting of the manuscript and manuscript preparation by NVS, RS, DBK, RKP, PP, BK, PVPSA, UKR, and DSB. RNA sequencing and data analysis by NVRM, AT, PVPSA, HK and DSB. Sample preparation and collection of experimental materials by NVS, SP, DMM and VRS. Preparation of pure cultures of pathogen, challenge inoculation, confirmation of Xanthomonas axonopodis pv. punicae infection by JS, SP, NVS. DNA isolation, purification and quantification and qPCR validation by SKP. RNA isolation and cDNA synthesis by NVS, SKP.
