

Genome-wide Identification, characterization and expression analysis of the effector-triggered immunity (ETI) pathway-mediated defense responsive genes in grapes against powdery and downy mildew infections

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Abstract

Grapevine productivity is severely affected by fungal diseases worldwide and for the diseases control in eco-friendly way, it is essential to understand the molecular mechanisms of fungal resistance in grapes. Therefore, we performed genome-wide identification of various *Resistance (R)* genes expressed during PM and DM infection in grapevine. Consequently, we identified 6, 21, 2, 5, 3 and 48 *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR* and *PR* genes respectively in the grapevine genome. Further, differential expression analysis resulted in identification of 2, 4, 7, 2, 4, 1 and 7 differentially expressed PM-responsive *Resistance (R)* genes (*NBS-LRR*, *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR*) and 28, 2, 5, 4, 1 and 19 differentially expressed DM-responsive *Resistance (R)* genes (*NBS-LRR*, *EDS1*, *NDR1*, *NPR*, *RAR1* and *PR*) in *V. vinifera*. These genes are involved in salicylic acid mediated Effector-triggered immunity (ETI) pathway, therefore, we examined their co-expression to determine the sequence of events that occurs during a signaling cascade in order to respond against PM and DM-infection. Altogether, the PM and DM responsive *R genes* of ETI pathway found in this study can be used in future to produce new and improved grape varieties that are immune to biotic stresses.

Introduction

Grape is one of the most commercially valuable fruit crops grown in the world. Nevertheless, its growth is significantly hampered by the climate of major-producing areas, which allows the development of various fungal diseases. Powdery mildew (PM) and downy mildew (DM) are two of those destructive diseases that propagate in areas with colder temperatures higher relative humidity that infects grapevine [1]. The epidemic of PM and DM acutely diminishes both the productivity as well as quality by infecting all green tissues of the grapevine. Hence, as the majority of the cultivated varieties belong to *Vitis vinifera*, which is susceptible to PM and DM, it is vital to understand the molecular processes involved in grapevine resistance to powdery and downy mildew infection to manage these fungal diseases in an environmentally friendly manner. Under abiotic and biotic stress environments, different defensive mechanisms are triggered in plants as part of their innate immune response to defend themselves. Essentially, three main steps are involved in host defense against pathogenic attack, i.e. pathogens identification, signal transduction and gene induction, contributing to the production of molecules with antimicrobial activity. Once the emergence of PM and DM infection happens in grapes, under optimal environmental conditions, the conidiospores of *Erysiphe necator* (PM fungus) and *Plasmopara viticola* (DM fungus) germinate on the grapevine leaf surface. This resulted in initial association of pathogen-associated molecular patterns (PAMPs) and grapevine defense molecules known as pattern recognition receptors (PRRs). thereby activating the first defense layer i.e. Pathogen-triggered immunity (PTI) [2] [3]. However, the activated defense is not strong enough to deter plant fungal proliferation. In addition, fungus delivers other effector proteins within the cell through different mechanisms such as Type 3 effector proteins, haustoria etc. to intensify its invasion in plants. During this time, another defense mechanism known as effector-triggered immunity (ETI) is activated in plants that triggers the expression

of resistance (R) proteins often leading to apoptosis of the infected cell, thus, blocking any further pathogenic proliferation [4] [5].

During plant immune response, numerous signal transduction pathways are triggered which lead to the host plant transcriptome re-programming and the activation of defense-responsive genes. The plant's resistance response to biotrophic pathogens is regulated by salicylic acid (SA) signaling, in which SA serves as secondary messenger [6]. Since *Erysiphe necator* and *Plasmopara viticola* are both biotrophic pathogens, the defensive mechanism triggered in the plant in response to infection with powdery and downy mildew is therefore signaled by SA. During this signaling, there is a cascade of events occurring, and different genes are activated that function upstream and downstream of SA accumulation.

In the present research we have focused on *R-gene* mediated defense signaling. The *R-gene* mediated resistance has been previously documented to be triggered via 2 main proteins i.e. Enhanced Disease Susceptibility 1 (EDS1) and Non-Race-specific Disease Resistance (NDR1), both interacting with different types of R proteins. EDS1 interacts with Toll/interleukin-1 receptor (TNL) type R proteins and NDR1 interacts with Coiled coil (CNL) type R proteins [7].

Recent studies have demonstrated that any perturbation in the interaction between EDS1, NDR1 and R proteins can affect the activation of signaling pathway thereby indicating the importance of these two main plant defense regulators [8] [9] [10] [11]. Several other major molecules of SA mediated defense signaling includes *Phytoalexin-deficient 4 (PAD4)*, *avrPphB susceptible 2 (PBS2)*, *Senescence associated gene 101 SAG101*, *required for Mla-specified resistance (RAR1)*. All of these stated proteins are functionally activated upstream of SA and induces the accumulation of SA.

In addition, one major protein class known as *Nonexpressor of PR Gene* (comprising of *NPR1*, *NPR2*, *NPR3* and *NPR4*) is also activated during SA signaling. This class is a pivotal SA-mediated signal transduction regulator that is triggered after accumulation of SA [12]. The *NPR* genes interact with various TGA and WRKY transcription factors (TFs) in the nucleus and eventually contribute to the expression of *Pathogenesis Related (PR)* genes, the final players of plant defense response [6] [13] [14]. The accumulation of PR proteins is induced in plants during conditions of pathogenic infection, thereby these proteins are playing an important role in improving the defensive ability of plant [15]. Taken together, the accumulation of SA and expression of *PR* genes leads to development of Systemic Acquired Resistance (SAR) i.e. a broad spectrum resistance in plants [16].

Previously, we identified 386 *NBS-LRR* genes in the grape genome, of which 63 such genes were found that were responsive to PM stress [17]. In the present study, we have carried out the genome-wide identification of various defense-responsive *Resistance (R)* gene families (*EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR*) involved in ETI pathway. *In silico* characterization of the identified gene families was conducted on the basis of gene structure and domain analysis, chromosomal distribution and physicochemical characteristics. Further, we identified various PM and DM-responsive *Resistance (R)* genes of above mentioned gene families (*EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR*) by conducting differential expression analysis. Their co-interaction study was performed to identify highly associating *Resistance (R)* genes

responsive to PM and DM infection. The different genes obtained through co-expression analysis were functionally characterized by the Blast2GO results. Moreover, various *cis*-acting regulatory elements (CARE) for the identified genes have also been predicted against PM and DM stress.

Materials And Methods

2.1 Genome-wide identification of various defense-responsive *Resistance (R)* gene families in *Vitis vinifera*

For the identification of *V. vinifera* *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* gene families, firstly, all possible protein coding sequences were procured from NCBI genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/Vitis_vinifera/protein/) and constructed their local sequence database.

Next, the previously documented protein sequences of above listed gene families from different plants namely *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Oryza sativa*, *Populus trichocarpa* and *Vitis vinifera* were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/protein>) and their FASTA file was made which was considered as query file.

To identify the putative hits in the grape genome, standalone protein BLAST (Basic Local Alignment Search Tool) was performed between the database and query file created above and the e-value used was 1e-05, respectively. The candidate gene sequences were verified by assessing the occurrence of respective domains of various gene families at different servers such as Grape Genome Browser (12X) (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>), conserved domains database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [18], InterProScan (<https://www.ebi.ac.uk/interpro/search/sequence-search>) [19] and SMART (<http://smart.embl-heidelberg.de/>) [20].

2.2 *In silico* characterization of defense-responsive *Resistance (R)* gene families

2.2.1 Gene and protein structure analysis

The gene structure was determined on the basis of the exons and introns found in gene sequences. An online tool 'Splign' was used to detect exons and introns (<http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>). The genomic and coding sequence information for the gene was provided, and the algorithm performs an alignment to determine the introns and exons location. The alignment results were confirmed by using Ensembl Plants portal (http://plants.ensembl.org/Vitis_vinifera/Gene/). Visualization of exons and introns location was done by using an online GSDS2.0 (<http://gsds.cbi.pku.edu.cn/>) [21]. In addition, the same tool was used to map

the intron phases for our gene sequences. We also determined the presence of various conserved motifs in order to better understand the structure and function of different protein classes discussed in our study. The tools used were Multiple Expectation Maximization for Motif Elicitation (MEME) Suite (<http://meme-suite.org/>) and Pfam database (<https://pfam.xfam.org/>) [22] [23].

2.2.2 Chromosomal distribution

To determine the locations of *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* gene families on the respective chromosomes, the gene sequences were searched by running BLASTN against non-redundant (NR) database. The hits which showed maximum identity were subsequently short-listed for placement on respective chromosomes. The confirmation of respective gene location on a particular chromosome was done through Grape Genome Browser at Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) and Ensembl Plants portal (https://plants.ensembl.org/Vitis_vinifera/Location/Genome/).

2.2.3 Physicochemical characteristics

The physicochemical properties of *Resistance (R)* gene families were depicted by using ProtParam bioinformatics tool (<https://web.expasy.org/protparam/>) with default parameters. This tool provides information about various important protein properties such as the amino acid number, molecular weight, isoelectric point, instability index etc. [24].

2.3 Identification of PM and DM responsive *Resistance (R)* gene families in *V. vinifera*

To identify PM and DM responsive *Resistance (R)* genes, we made a FASTA file of protein coding sequences of the above mentioned *Resistance (R)* gene families (*EDS1*, *NDR1*, *NPR1*, *RAR1*, *PR*) and *NBS-LRR* genes identified in our previous research [17]. In addition, as transcription factors (TFs) are also presumed to participate in *R-gene* mediated defense pathway, various transcription factors (TFs) of *V. vinifera* belonging to 3 distinct families i.e. TGA, WRKY and NAC were retrieved from Plant Transcription Factor Database (TFDB) [25]. We then derived the RNA-seq data of two *V. vinifera* varieties i.e. Thompson Seedless and Pinot Noir from NCBI's Sequence Read Archive (SRA) database and the project I.Ds for these studies were SRP116308 and PRJEB24540 (<http://www.ncbi.nlm.nih.gov/sra/>). The transcriptomic data of Thompson Seedless was responsive to PM and was accessible at 1 time point i.e. 36 hours post inoculation (hpi) (Northwest A&F University). Similarly, the RNA-seq data of Pinot Noir was DM-responsive and retrieved at 3 time points i.e. 1dpi, 2dpi and 3dpi [26]. Each of the derived SRA data consisted of three biological replicates. The identification of PM and DM-responsive *Resistance (R)* genes is based on the digital expression analysis of different group of proteins identified in our study. The Trinity-V2.4 package's RSEM (RNA-Seq by Expectation-Maximization) program was used to quantify the abundance of all

fungal responsive *Resistance (R)* genes as fragments per kilobase of transcript per million fragments mapped (FPKM) [27]. All the 3 biological replicates of every condition were analyzed individually. Thereafter, EdgeR was used to calculate the differential gene expression by assigning a cut-off value of 4-fold change and P-value of 0.001. The visualization of differential gene expressions was done by constructing heat maps with Hierarchical Clustering Explorer 3.5 (<http://www.cs.umd.edu/hcil/hce/>) [28].

2.4 Co-expression analysis of PM and DM responsive *Resistance (R)* genes in *V. vinifera*

Next, we conducted co-expression (CE) analysis of differentially expressed *Resistance (R)* genes to determine the sequence of events that occurs during a signaling cascade in reaction to PM and DM-infection. The software used for detecting the co-expression was CoExpress 1.5.2. This is a stand-alone software tool that is focused on the Pearson correlation coefficient (R). The parameters were set to default while running this software. (<http://Bioinformatics.lu/CoExpress/>). The expression values of two sets of genes was loaded at a time and a linear correlation was measured amongst them. Finally, we screened genes that are highly co-expressing with the R value of 1 or close to 1. The visualization of co-expression networks for genes was achieved using the software Cytoscape 3.7.1 (<https://cytoscape.org/>). Based on the study of co-expression, we indicated a pathway of SA mediated defense mechanism activated during PM and DM infection.

2.5 Plant material and treatment

As SA is playing a pivotal role in *R-gene* mediated defense signaling against fungal infections, we studied its effect on Thompson Seedless variety of *V. vinifera*. The stem cuttings were collected from National Research Centre for Grapes (NRCG), Pune. The Centre has the National Active Germplasm Site for grapes, where the grape germplasm is being maintained. The institute code number for Thompson seedless variety of *V. vinifera* is A37-3. The collected stem cuttings were grown in pots containing soil:soil-rite in the ratio of 2:1 in growth chamber in Department of Biotechnology, Panjab University, Chandigarh. The experiment was performed at 5 different time intervals i.e. 1hpi, 2hpi, 1dpi, 2dpi and 3dpi in the sets of 3 biological replicates at each time point. The SA treatment was given at the final concentration of 100 mg/litre. The control and treated leaves were collected, snap frozen and stored in -80°C till further use. Additionally, we also studied the effects of PM and DM infection on the activity of defense-responsive *Resistance (R)* genes identified in our study in grapevine. Subsequently, we collected the healthy and fungal infected leaf samples of Thompson Seedless variety of *V. vinifera* from National Research Centre for Grapes (NRCG), Pune. The time points for PM and DM infected leaf samples collected were 2dpi for PM infected samples and 1dpi and 3dpi for DM infected samples. This experiment was also performed in the sets of three biological replicates at respective time points.

2.6 RNA isolation, cDNA synthesis and quantitative real time PCR analysis

Based on the genes identified in the co-expression analysis, we performed quantitative real time PCR (qPCR) of PM and DM-responsive differentially expressed *Resistance (R)* genes to measure the relative expression. Leaf samples were used to extract the total RNA by following Ghawana et al. 2011 protocol and the cDNA was subsequently prepared using the Superscript III first strand cDNA synthesis kit (Invitrogen USA) [29].

We used two housekeeping genes namely *actin (ACT)* and *elongation factor 1 (EF1)* as internal reference genes for the normalization of qPCR results. The real time primers were designed by using Primer 3 software (<http://primer3.ut.ee/>). The qPCR experiment was performed with three replicates of healthy, fungal and SA treated leaf samples by employing Bio-Rad CFX96 Real-Time PCR detection system. The following conditions were used while performing an experiment: 95°C for 7 min, followed by 40 cycles of 95°C for 20 sec, T_m for 20 sec and 72°C for 20 sec. In order to interpret the results of qPCR, we used the REST 2009 algorithm (Qiagen) and 2(-ΔΔCT) method to obtain relative gene expression ratios of target genes with respect to control genes (<http://www.REST.de.com/>) [30] [31].

2.7 Functional annotation of PM and DM responsive *Resistance (R)* genes in *V. vinifera*

For assigning biological functions to PM and DM-responsive *Resistance (R)* gene sequences, Blast2GO (<https://www.blast2go.com/blast2go-pro/>) tool was used [32]. Essentially, Blast2GO annotation requires 3 steps: BLAST to locate homologous sequences, Mapping to allocate GO terms to each hit obtained, Annotation to assign role to query sequences on the basis of their cellular positions, molecular functions and biological processes. Furthermore, knowledge regarding various protein domains or motifs may also be obtained from Blast2GO by running InterProScan.

2.8 Promoter study of PM and DM responsive *Resistance (R)* genes in *V. vinifera*

We also predicted various *cis*-acting regulatory elements of PM and DM-responsive *Resistance (R)* genes by using PlantCare database with default parameters (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [33]. To conduct the analysis, an upstream sequence from transcription start site was needed. Nucleotide BLAST was then done between genomic sequence and coding sequence of respective gene. Eventually, different regulatory elements were predicted that might be playing an important role in the regulation of PM and DM-responsive genes of defense pathway.

Results

3.1 Genome-wide identification of various defense-responsive *R* gene families in *Vitis vinifera*

The genome-wide identification of various classes of *Resistance (R)* genes in *V. vinifera*, namely *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* in *V. vinifera* was conducted by running standalone BLASTP between protein coding sequences of *V. vinifera* and *Resistance (R)* gene families. Firstly, we collected 38,120 *V. vinifera* protein coding sequences from the NCBI genome database and created their local sequence database. Then, we retrieved 41, 127, 17, 34, 9 and 92 *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* protein coding sequences of *A. thaliana*, *A. lyrata*, *O. sativa*, *P. trichocarpa* and *V. vinifera* from NCBI and made their FASTA file. The sequence alignments were done and consequently, 27, 47, 29, 928, 3 and 148 putative hits from *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* gene families were detected in *V. vinifera*. The confirmation of the presence of respective domains was done by performing BLASTP of putative hits against non-redundant (NR) database. Finally, 6, 21, 2, 5, 3 and 48 such sequences were obtained in *V. vinifera* that were considered as *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* proteins (Fig. 1). The validation of respective protein was further done by using bioinformatics tools and databases such as Genoscope, CDD, Pfam, InterProScan and SMART. The information regarding protein IDs and transcript IDs of all gene families is given in Supplementary File 1.

3.2 *In silico* characterization of defense-responsive gene families

The prediction of gene structure, motif analysis, chromosomal mapping and physicochemical properties was done by using various Bioinformatics tools.. Analysis of the gene arrangement was done using GSDS server in terms of exons and introns. The number of introns in the *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* gene families ranged from 1–2, 1–2, 1–3, 1–4, 1–4, 1–4 and 1–2 respectively. Amongst all, most of the *NDR1* genes were found to be intron-less (Fig. 2).

Next, the intron phases were depicted depending on the intron's localization in relation to reading frame of gene. Three types of intron phases namely phase 0, phase 1 and phase 2 have been tested for the *Resistance (R)* gene sequences. The findings indicated that all *EDS1* gene sequences were present in phase 0; *NDR1* gene sequences in phase 0 and phase 1; *PAD4* gene sequences in phase 1 and phase 2; *NPR*, *RAR1* and *PR* gene sequences were obtained in each of the three intron phases respectively (Fig. 2).

Next, the protein sequences of all gene families were assessed with the use of 'MEME' server for the occurrence of different types of motifs. The results for the presence of desired motif were verified through Pfam server and InterProScan. Consequently, a total of 16 different conserved motifs were identified in various *Resistance (R)* gene families studied (Fig. 2). The various conserved domains identified amongst different classes of *Resistance (R)* proteins includes Lipase 3 and EDS1 EP in *EDS1* proteins; LEA2

domain in NDR1 proteins; EDS1 EP in PAD4 proteins; Ank2, Ank5, NPR1_like_C; NPR1_interact in NPR proteins; CHORD domain in RAR1 proteins; CAP, Thaumatin, Glycohydro 17, Glycohydro 18, Glycohydro 19, Betv1, Barwin and Chitin_bind_1 in different classes of PR proteins. The block diagrams and the logos of the conserved motifs are shown in Fig. 2.

Further, we characterized the identified genes on the basis of chromosomal distribution and physicochemical properties. The information regarding chromosomal distribution and physicochemical properties is provided in Supplementary File 1. Amongst all, the *EDS1* genes were shown to be located on chromosome 17; *NDR1* on chromosomes 4, 6, 8, 9, 10, 15, 16, 18 and 19; *PAD4* on chromosome 7; *NPR* on chromosomes 7, 8, 10 and 11; *RAR1* on chromosome 16 and *PR* genes were distributed on chromosomes 1, 3, 4, 5, 6, 7, 8, 11, 13, 14, 15 and 18 respectively (Supplementary File 1).

Altogether, the findings of chromosomal distribution showed that the defense-responsive *Resistance (R)* genes identified in our study are dispersed extensively in the grape genome.

The various parameters computed to determine physicochemical characteristics of defense-responsive Resistance (R) proteins included amino acid length, molecular weight, isoelectric point (pI) and instability index.

The range of amino acid length for the Resistance (R) proteins is 112–629 and the molecular weight range is between 12–71.3 kDa. Based on pI prediction, it was depicted that EDS1 proteins were acidic, basic and neutral in nature; all the NDR1 proteins were basic in nature; PAD4 and NPR proteins were acidic; RAR1 proteins were neutral and basic in nature; PR were mostly acidic and some are basic in nature. The results of instability index prediction suggested that most of the defense-responsive Resistance (R) proteins occurred in unstable state under in-vitro conditions with the exception of some NDR1 and PR proteins that were found to be present in stable state.

3.3 Identification of fungal responsive *Resistance (R)* genes in *V. vinifera*

To better understand the *R-gene* mediated molecular mechanism of powdery and downy mildew resistance in grapevine, the identification of PM and DM-responsive *Resistance (R)* gene families (*NBS-LRR*, *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PAD4*) and transcription factors (TF) was done on the basis of differential expression analysis. The transcriptomic data of one PM-susceptible (Thompson seedless) and one DM-susceptible (Pinot Noir) *V. vinifera* varieties were collected from NCBI and the normalized expression analysis (FPKM) of gene families and TFs described above was conducted. Our analysis showed that the FPKM values obtained for the triplicates of each condition were nearly same. Therefore, we took the cumulative mean of FPKM values of triplicates for the representation of heat map.

We reported the occurrence of 386 NBS-LRR gene sequences in the grape genome in our previous research, including 63 such sequences that were sensitive to PM stress [17]. In this study, by conducting

differential expression of the above-mentioned 386 NBS-LRR gene sequences, we found 2 new highly expressing PM-responsive and 28 DM-responsive NBS-LRR genes (Fig. 3). Also, from the result it was noticed that most of the fungal responsive *NBS-LRR* genes were up-regulated in later stages of fungal infection relative to initial phase.

Next, the differential expression analysis (DEA) of several other *Resistance (R)* gene families described above was carried out in response to fungal stress. As a consequence, of the 6, 21, 2, 5, 3 and 48 *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR* and *PR* genes identified genome-wide, we found 4, 7, 2, 4, 1 and 7 *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR* genes expressed differentially in reaction to PM infection and 2, 5, 4, 1 and 19 differentially expressed DM-responsive *Resistance (R)* genes (*EDS1*, *NDR1*, *NPR*, *RAR1* and *PR*) in *V. vinifera*.

The FPKM findings of the *EDS1* and *NDR1* gene families showed that all *EDS1* genes were up-regulated during PM-infection relative to control condition and out of 7, 5 *NDR1* genes were up-regulated during PM-infection. During DM-infection, all genes got up-regulated during later stages (3 dpi) of infection. Likewise, the analysis of *PAD4* gene family revealed that 2 *PAD4* genes were detected analysis in PM infected samples by FPKM analysis, one of which is up-regulated but surprisingly, we did not get any *PAD4* gene expressed DM-infected sample (Fig. 3). We also found 1 PM-responsive and 1 DM-responsive differentially expressed transcript encoding *RAR1* in both PM and DM infection and both transcripts demonstrated up-regulation during fungal stress (Fig. 3). The DEA of *NPR* proteins indicated that 4 PM-responsive and 4 DM-responsive *NPR* (comprised of *NPR1* and *NPR2*) genes were expressed differentially during fungal infection and all *NPR* genes were shown to be steadily up-regulated from initial to later stages of both powdery and downy mildew infection (Fig. 3).

Additionally, as *NPR* proteins are known to interact with various TF types, specific TF families were tested for differential expression. By using Plant Transcription Factor Database (TFDB), the retrieval of 177 transcription factors (TFs) was done that belongs to 3 different families i.e. TGA, WRKY and NAC. The total number of TGA, WRKY and NAC TFs retrieved was 47, 59 and 71. Their differential expression was conducted and consequently, 20 PM-responsive and 39 DM-responsive TFs were identified. The interaction of *NPR* proteins with several TFs eventually triggers numerous *PR* proteins that provide SAR to plant. In our research, we found 7 PM-responsive and 19 DM-responsive differentially expressed *PR* proteins that belongs to 5 different classes i.e. *PR1* (Antifungal), *PR2* (β -1,3-Glucanase), *PR5* (Thaumatococcus-like), *PR10.1* (Ribonuclease-like) and *PR10.7* (Ribonuclease-like) (Fig. 3).

Overall in our research, we identified 47 PM-responsive and 98 DM-responsive differentially expressed *Resistance (R)* genes in *V. vinifera* accessions used in the current study (Fig. 3). The findings indicated that most genes were up-regulated during PM and DM infection relative to control ones.

3.4 Co-expression analysis of PM and DM responsive *Resistance (R)* genes in *V. vinifera*

Next, we conducted co-expression (CE) analysis of differentially expressed PM and DM-responsive *Resistance (R)* genes using the software tool CoExpress 1.5.2. Subsequently, we screened some highly co-expressing genes in *V. vinifera* that could regulate the powdery and downy mildew infection through SA signaling cascade. Visualization of the co-expressed genes was attained using Cytoscape 3.7.1 software with default parameters (Fig. 4). Amongst all, there were 6 PM-responsive and 7 DM-responsive *NBS-LRR* genes which were found to exhibit strong co-interaction with *EDS1* and *NDR1* genes residing downstream of *NBS-LRR* genes. In case of PM-responsive genes, out of 6 *NBS-LRR* genes, 4 genes consisting of CC domain were interacting with *NDR1* genes, while 2 genes comprising the TIR domain associated with the *EDS1* genes (Fig. 4A). Similarly, we got 7 *NBS-LRR* genes in response to DM infection, 4 genes with CC domains were interacting with *NDR1* and 3 TIR domain carrying genes were associated with *EDS1* genes (Fig. 4B).

Next, we studied the interaction of *EDS1* and *PAD4* genes and consequently, we got one PM-responsive *PAD4* gene i.e. XP_010652916.1 that interacted strongly with the *EDS1* gene (NP_001267967.1) (Fig. 4A). We also find a strong interaction of *EDS1* and *NDR1* genes with the *RAR1* gene. This protein stabilizes the *NBS-LRR* proteins and thus plays a vital role in the ETI [34] [35] [36] [37] [38] [39].

All these proteins interacting thus far contribute to an accumulation of SA in cells that further promotes the activation of NPR1 and NPR2 proteins. These proteins are initially present in cytoplasm, when activated with SA accumulation, it got translocated to nucleus where they interact with various transcription factors which eventually lead to the activation of various PR proteins [40]. Through co-expression analysis, we identified 2 NPR1 proteins and 1 NPR2 protein against PM-infection and 1 NPR2 protein against DM-infection (Fig. 4). The co-expression results also showed that the NPR1 and NPR2 proteins interacted with TGA, WRKY and NAC transcription factors in case of both powdery and downy mildew infection. The interaction of NPR proteins with several TFs finally activates various PR proteins that provide SAR to plant. Amongst all, we chose 5 each highly interacting (co-expression value 1) TFs from both PM and DM-responsive data that were shown to be interacted with various classes of PR proteins. Finally, we got 6 PM-responsive and 4 DM-responsive highly co-expressing PR proteins that belonged to 5 different PR protein families namely PR1, PR2, PR5, PR10.1 and PR10.7 (Fig. 4).

Altogether, we screened 24 PM-responsive and 22 DM-responsive differentially expressed genes in *V. vinifera* accessions, which were found to be highly interacting amongst each other during powdery and downy mildew stress condition (Fig. 4). Additionally, on the basis of co-expression analysis, we reconfigure a pathway of SA mediated defense response that could occur in grapevine during powdery and downy mildew infection (Fig. 5). Whenever the pathogenic avirulent proteins suppress PTI to enter inside cell, another plant defense mechanism known as ETI is triggered that is functional within the cell. This pathway depicted a series of defense-responsive Resistance (R) proteins that are activated in plant cell during pathogenic attack.

3.5 qPCR expression analysis

On the basis of FPKM and co-expression analysis, we picked some highly interacting *Resistance (R)* genes that were activated against PM and DM infections during a signaling cascade for validation by qPCR. We chose 14 PM-responsive and 13 DM-responsive genes for real time expression analysis under various stress conditions (Fig. 6). The primer sequences, amplicon length and annealing temperature of PM and DM-responsive *Resistance (R)* genes are listed in Supplementary Tables S1 and S2.

The exogenous application of SA or SA analogs induces SAR and restores resistance in numerous mutants compromised in signaling steps upstream of SA production [41] [42] [43] [44]. To study the effects of SA on resistance response, we had applied SA exogenously on leaves of Thompson seedless variety at 5 different time intervals i.e. 1hpi, 2hpi, 1dpi, 2dpi and 3dpi to test the expression pattern changes of various defense-responsive *Resistance (R)* genes. In addition, we also collected PM and DM infected leaf samples at various time intervals of 1dpi for PM and 1dpi and 3dpi for DM infection.

The results of qPCR indicated that most genes were up-regulated during 2dpi PM infection, 2 hpi and 3 dpi SA treatment (Fig. 6A). Likewise, for DM, genes were mainly up-regulated during late fungal infection i.e. 3dpi DM infection, 1hpi and 3 dpi SA treatment (Fig. 6B).

3.6 Functional annotation of fungal responsive *Resistance (R)* genes

In order to recognize the diverse roles of proteins at the molecular level, we performed Blast2GO of highly interacting *Resistance (R)* genes found in the co-expression study. From this analysis, the depiction of biological process, cellular component and molecular function, the 3 key characteristics of genes was done. Various GO terms were assigned to our gene sequences to determine the possible functions of PM and DM-responsive *Resistance (R)* genes. For PM-responsive genes, in the biological process category, we obtained 16% sequences that were participating in stimulus response (GO:0050896); 10% each in biological process regulation (GO:0065007) and cellular process regulation (GO:0009987); 11% sequences in metabolic process regulation (GO:0019222); 7% each in immune system process (GO:0002376) and signaling (GO: 0023052) etc. [Supplementary Fig. 1A(a)]. Likewise, the GO term annotations in cellular component category suggested that most of our gene sequences performed their respective functions in cell (23%), cell part (23%), organelle (20%), membrane (7%), extracellular region (7%), membrane part (6%) etc. [Supplementary Fig. 1A (b)] and the GO terms assigned to these components included GO:0005623, GO:0044464, GO:0043226, GO:0016020, GO:0005576 and GO:0044425. The findings of molecular process category revealed that maximum number of sequences (52%) were involved in binding activity (GO:0005488), accompanied by 19% in transcription regulator activity (GO:0140110), 15% in catalytic activity (GO:0003824), 7% each in molecular function regulator (GO:0098772) and molecular transducer activity (

GO:0060089) [Supplementary Fig. 1A(c)].

Similarly, for DM-responsive genes, in biological process annotations, the percentage of gene sequences involved in various processes was 11% for metabolic process, 9% for cellular process, 7% for immune system process, 10% for biological regulation, 15% in response to stimulus, 5% for signaling etc. [Supplementary Fig. 1B(a)]. In cellular component category, 24% of sequences each were found to be executing their role in cell and cell part; 19% in organelle, 10% in membrane, 5% each in membrane part and extracellular region [Supplementary Fig. 1B (b)]. The annotations of molecular process category depicted most of the genes to be involved in binding activity (60%); 20% of the sequences were included in transcription regulator activity; 12% in catalytic activity, 4% each in molecular function regulator and molecular transducer activity [Supplementary Fig. 1B(c)].

Altogether, our results depicted that the PM and DM-responsive *Resistance (R)* genes found in our study may play an immense role in biological, cellular and molecular processes.

3.7 Prediction of *cis*-acting regulatory elements in PM and DM-responsive *Resistance (R)* genes

Predictions of *cis*-regulatory elements in PM and DM-responsive *Resistance (R)* genes derived from a co-expression study revealed the existence of two key *cis*-acting elements i.e. TATA and CAAT boxes in the promoter and enhancer parts of all gene sequences. In addition, 5 classes of PM and DM-responsive regulatory elements were identified, namely defense and stress responsive element, salicylic acid responsive element, wound and pathogen responsive element, stress responsive element, As-1 and TGA element. Among all, the defense and stress responsive element was located in 12 PM-responsive and 10 DM-responsive promoter sequences, salicylic acid responsive element in 15 each PM-responsive and DM-responsive sequences, wound and pathogen responsive element in 17 PM-responsive and 16 DM-responsive sequences, As-1 in 10 each PM-responsive and DM-responsive sequences and TGA motif in 6 PM-responsive and 9 DM-responsive promoter sequences (Fig. 7).

Discussion

As the cultivation of grapes is vulnerable to the emergence of fungal diseases like PM and DM, efforts are increasingly directed towards the understanding of defense mechanisms of disease resistance at molecular level. Various signaling pathways are triggered in plants at molecular level during pathogenic infection that provide plant defense. It has been reported in literature that during invasion by biotrophic organisms, the active defense response in plants is signaled by SA [6]. The importance of the role of SA or its derivatives in triggering defense-responsive proteins is well stated in literature. For instance, various PR proteins known to provide SAR got expressed when the plant is treated with SA or its derivatives [45][46][47]. Plant susceptibility to pathogen increases when the plant becomes deficient in SA synthesis or accumulation [48][49]. It is evident in literature through experiments performed in tobacco and Arabidopsis plants that basal and SAR signaled through *R genes* will be compromised if SA is not available [48][45][50]. Similarly, the Arabidopsis *eds5* and *eds16* mutants that are lacking SA

accumulation are compromised in some *R-gene* pathways as well as SAR and basal resistance [51][52][49]. This suggested the importance of SA in providing immunity to plants whenever the plant gets pathogenic infection.

Since powdery mildew and downy mildew fungi are both biotrophic in nature, we have identified various defense-responsive Resistance (*R*) proteins of SA signaling pathway that are expressed differentially in the grape genome during fungal infections. We have studied the interaction of these proteins in order to elucidate the sequence of events that may occur during the defense reaction at molecular level.

To accomplish our goal, we have conducted genome-wide identification of various defense-responsive *Resistance (R)* genes (*EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR1* and *PR*) that are known to participate in plant resistance response mechanism. The members of these gene families have also been identified previously in grapevine and other plants, but there is no report of genome-wide recognition study of such gene families. The changes in their expression patterns under pathogenic and non-pathogenic conditions are studied by conducting differential expression. Our analysis depicted that during fungal infection, most of the defense-responsive *Resistance (R)* genes were up-regulated relative to non-pathogenic conditions, indicating the active involvement of SA-mediated defense responsive pathway during infection with PM and DM.

To confirm our FPKM analysis findings, we have performed real time PCR of PM and DM infected leaf samples to test differences in relative expression between healthy and infected samples. In addition, since SA plays a prominent role in activating the defense mechanism, we gave SA treatment to leaf samples at different intervals of time. Consequently, the results of real time study revealed that during SA treatment, PM infection and DM infection, most of the defense-responsive *Resistance (R)* genes get up-regulated relative to control condition. This meant that anytime the plant have an outbreak, the innate immune response of the plant gets activated through which various signaling pathways comes into action. The expression levels of defense-responsive *Resistance (R)* genes get enhanced during infection as compared to non-pathogenic condition that eventually induces SAR in plant. We found that in case of PM-responsive genes, initially, the expression level of most genes increases from 1 hpi to 2hpi of SA treatment; however, a sudden decrease in expression levels was witnessed after 2 hpi till 2 dpi. After that, ultimately, at 3dpi, a tremendous increase in most of the genes was visualized. In case of DM-responsive genes, initially, the defense-responsive *Resistance (R)* genes got highly expressed at 1hpi of SA treatment; after that there was a decline in expression levels; and finally at 3dpi, most defense-responsive *Resistance (R)* genes were up-regulated. The possible assumption would be that following a fungal infection, SA levels will rise in plant, leading to defense reaction. Thus, when we gave SA treatment to our samples, initially genes got up-regulated in 1hr or 2 hr; and later on to provide SAR to plant, the expression levels of both PM and DM-responsive *Resistance (R)* genes peaks up at 3dpi. Additionally, our results of powdery and downy mildew infection through quantitative real time PCR are in accordance with the findings of the FPKM study. Most of the genes got up-regulated during PM and DM infection as compared to control condition in both FPKM and real time study.

Past studies have shown that over-expression of these genes confers broad spectrum resistance to infection in plants. For instance, one analysis in Arabidopsis showed that over-expression of *EDS1* enhances accumulation of PR1 protein that ultimately provides broad spectrum resistance to plant [53]. Similarly, another group also demonstrated in Arabidopsis study that over-expression of *PAD4* gene positively regulated the SA signaling pathway to activate the defense response in plant [54]. Previously, NPR proteins have been reported to engage in SA signal transduction in various plants. Over-expression of the *NPR1* gene in plants like Arabidopsis, wheat, tomato and apple provides broad spectrum resistance against fungal and bacterial pathogens [55][56][57][58]. In one study conducted in Fuji apples, it has been shown that over-expression of *NPR1* genes enhances PM resistance through SA mediated pathway [59]. All these studies supported our results of expression analysis of various defense responsive *Resistance (R)* genes conducted in grapes.

Next, we have examined the interaction between the identified defense-responsive *Resistance (R)* genes by conducting co-expression study and subsequently reconfigured the pathway of SA mediated defense response that might occur in grapevine during PM and DM infections. The foremost proteins of the ETI pathway that recognize avirulent proteins are NBS-LRR proteins that are located intracellularly. The interaction of NBS-LRR and avirulent proteins generates energy that is further used in activating downstream signaling pathway of plant defense responses. Various fungal responsive *NBS-LRR* genes have also been described previously in many plants such as apple, Arabidopsis, wheat, grapes, Cucumber etc. [60] [61] [62] [63] [64] [65] [66]. In one study conducted by Coleman et al in 2009, a cluster of *NBS-LRR* genes was identified in *Resistance to Erysiphe necator 1 (REN1)* locus responsive to PM in Kishmish vatkana' and 'Dzhandzhal kara' grapevine varieties [64]. Likewise, in *V. amurensis* and *V. riparia* grapevine varieties, the *NBS-LRR* gene cluster was identified in response to DM infection [65].

It has been reported in literature that the activation of NBS-LRR proteins is mediated through EDS1 and NDR1 proteins that are interacting with different R proteins subsets. The EDS1 interacts with TIR domain containing *R genes* and NDR1 associates with CC containing *R genes* [7]. Interestingly, we also achieve the similar results of EDS1 and NDR1 interactions with different subsets of *NBS-LRR* genes through co-expression analysis. Further from one study conducted in Arabidopsis, it was known that EDS1 interacts with PAD4 present downstream and both these proteins positively regulate the accumulation of SA to provide *R-gene* mediated resistance in plant [67]. We also find a strong interaction between EDS1 and PAD4 genes in our analysis.

There is one another class of defense-responsive *Resistance (R)* genes known as *NPR* that acts downstream of SA. This gene class is considered as master regulator of SA-mediated immune response. We have identified 2 types of NPR proteins i.e. NPR1 and NPR2 in co-expression analysis. Both NPR1 and NPR2 proteins interact with different classes of transcription factors. Through differential expression analysis, we have identified TFs of 3 different classes (bZIP, WRKY and NAC), that participates in activation of PR proteins by interacting with NPR1 and NPR2 proteins. There have been several reports that showed that *NPR* genes regulate the expression of *PR* genes by interacting with various TFs. Previously, it has been shown in Arabidopsis that the *PR-1* gene expression is regulated by *NPR1* gene by

binding to TGA transcription factors [68]. Similarly WRKY and NAC transcription factors were also found to interact with *NPR1* gene in previous studies [69] [70].

The ultimate players of plant defense mediated through SA are PR proteins that were described firstly in tobacco leaves infected with the tobacco mosaic virus [71]. Since then, these proteins have been identified in many monocot and dicot plants. In our study, we find various classes of PR proteins to be expressed differentially in reaction to PM and DM infection. We have identified, amongst others, 5 different PR families i.e. PR-1 (Antifungal), PR-2 (β -1,3-Glucanase), PR-5 (Thaumatin-like), PR-10.1 (Ribonuclease-like) and PR-10.7 (Ribonuclease-like) that are showing strong co-interaction with upstream genes of SA mediated signaling pathway. The role of all the PR proteins identified in our study has been previously identified in various grape varieties as documented in literature.

Previously, PR-1 proteins exhibiting anti-fungal property have been identified and cloned in *V. vinifera* [72]. Nevertheless, its role in the signaling pathway has not been determined in grapes to date. Our study suggested that the PR-1 protein is triggered during the SA induced signal cascade against PM and DM infection in grapes. PR-2 proteins have the capacity of hydrolyzing the components of fungal cell wall, thus exhibiting antimicrobial activity. In 1999, Jacobs et al. showed that the hydrolytic activity in grape has a direct impact on the development of powdery mildew at the pathogen infection site [73]. Similarly, PR-5 proteins enhance fungal membrane permeability and causing osmotic breakup of fungal plasma membrane providing immunity against fungal attack [74]. PR-10 proteins acts directly against pathogens or can contribute to plant defense through programmed cell death during hypersensitive response (HR) [75]. Previously, the *PR-10* gene was isolated from *V. pseudoreticulata* which indicates tolerance to *P. viticola* infection [76]. All these PR proteins studies supported our findings of PR proteins triggered in response to SA in grapes.

We also performed the bioinformatics characterization of the identified defense-responsive *Resistance (R)* genes on the basis of chromosomal distribution, gene structure analysis, physicochemical properties and motif study. The physicochemical properties depicted that most Resistance (R) proteins identified have length approximately between 200–600 amino acids. Such genes could be easily cloned due to such a short polypeptide length and would subsequently be available for performing various transgenic studies. The majority of the detected proteins are unstable in vitro environments. Yet certain NDR1 and PR proteins belonging to the protein families PR1, PR2, PR10.1 and PR10.7 are found stable in vitro conditions.

From the gene structural studies, it is depicted that the exon/intron configurations of *Resistance (R)* genes are diverged extensively. Three processes are considered to be responsible for complex exons and introns structures: insertion/ deletion, exonization/pseudo-exonization and gain/loss of exon/intron [77] [78] [79]. The most prominent intron number observed in maximum defense-responsive *Resistance (R)* genes are 1 or 2; prominent intron phase is 0 which suggests that maximum introns reside in between 2 codons. There are small number of introns find in phase 1; the codon of these introns is disrupted between base 1 and base 2. The minimal number of introns are in phase 2, in which the codon disruption

happens between base 2 and base 3 [80]. The domain study illustrates the presence in Resistance (R) protein sequences of different conserved motifs and in order to conduct different essential activities, Resistance (R) proteins require these motifs.

Next, to allocate roles to fungal responsive *Resistance (R)* genes identified in the present research, Blast2GO was done. The study of GO annotations has showed that the maximum defense-responsive *Resistance (R)* gene sequences are engaged in biological processes, accompanied by sequences in cellular component and molecular function category. The GO terms in biological process category are directly implicated in defense response which supported the functioning of proteins identified in our study in resistance mechanism. The proteins in cellular component class are found to perform their role inside cell which clearly indicated that the defensive pathway we are targeting is functional inside the cell. In the category of molecular processes, many of the proteins are involved in binding activity which supported the interacting nature of defense-responsive proteins.

In our research, we have recognized multiple regulatory elements that react to fungal stress, namely defense and stress responsive element (TC-rich repeats), salicylic acid responsive element (TCA element), wound and pathogen responsive element (WUN-motif and W-box), stress responsive element (GT1 Box), As-1 and TGA; they provide an important information regarding upstream control of PM and DM-responsive Resistance (R) proteins. The function of the TFs in response to PM and DM-stress has been well elucidated in previous studies. For example; the NAC TFs associate with GT1 box, bZIP bind to TGA elements and WRKY TFs associates with W-box in reaction to fungal infection [81] [82] [83]. In the present analysis, we have observed the involvement of these elements in Resistance (R) proteins, which clearly confirmed that these genes were up-regulated as a defense mechanism in the situation of PM and DM stress. Altogether, numerous regulatory elements were identified in the present study that could play an important role in regulating the expression of stress-responsive genes.

Conclusion

In our research, grapevine genome was examined to identify various classes of *Resistance (R)* genes that were directly implicated in *R-gene* mediated defense signaling during powdery and downy mildew infection. In total, we identified 6, 21, 2, 5, 3 and 48 *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR* and *PR* genes in the grape genome. Additionally, 47 PM-responsive and 98 DM-responsive differentially expressed *Resistance (R)* genes (*NBS-LRR*, *EDS1*, *NDR1*, *PAD4*, *NPR*, *RAR* and *PR*) were identified in 2 different *V. vinifera* accessions amongst which 24 PM-responsive and 22 DM-responsive *Resistance (R)* genes were found to be highly interacting with each other. Taken together, we can summarize that *Resistance (R)* genes identified in the current research could be beneficial in improving grapevine resistance to powdery and downy mildew.

Abbreviations

ACT: Actin

CARE: Cis-acting Regulatory Elements

CE: Co-Expression

CNL: coiled-coil NLRs

DEA: Differential Expression Analysis

DM: Downy Mildew

DPI: days post inoculation

EDS1: Enhanced Disease Susceptibility

EF1: Elongation Factor 1

ETI: Effector Triggered Immunity

FPKM: Fragments per kilobase of Transcript per million Fragments Mapped

GO: Gene Ontology

GSDS: Gene Structure Display Server

HPI: hours post inoculation

HR: Hypersensitive Response

LEA2: Late embryogenesis abundant

MEME: Multiple Expectation Maximization for Motif Elicitation

NBS-LRR: Nucleotide Binding Site Leucine Rich Repeat

NDR1: Non-Race-specific Disease Resistance

NPR: Nonexpressor of PR

nr: Non Redundant

PAD4: Phytoalexin-deficient 4

PAMP: Pathogen-associated Molecular Patterns

PBS2: avrPphB susceptible 2

pI: Isoelectric point

PM: Powdery Mildew

PR: Pathogenesis Related

PRR: Pattern Recognition Receptors

PTI: Pathogen Triggered Immunity

qPCR: quantitative real-time PCR

R: Resistance

RAR1: Required for Mla-specified Resistance

RSEM: RNA-Seq by Expectation-Maximization

SA: Salicylic Acid

SAG101: Senescence associated gene 101

SAR: Systemic Acquired Resistance

SRA: Sequence Read Archive

TF: Transcription Factor

TNL: TIR-type NLRs

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE: Not Applicable

CONSENT FOR PUBLICATION: Not Applicable

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Supplementary Data

Supplementary File 1. Chromosomal locations and physicochemical properties of *EDS1* (Sheet 1), *NDR1* (Sheet 1), *PAD4* (Sheet 1), *NPR* (Sheet 1), *RAR1* (Sheet 1) and *PR* (Sheet 1) *Resistance (R)* genes. The table describes the protein ID, transcript ID, chromosomal position, polypeptide length, molecular weight, isoelectric point and instability index of various *Resistance (R)* gene families.

Supplementary Table S1: List of primers used for qRT-PCR analysis of selected PM-responsive *Resistance (R)* genes as well as Internal control genes.

Supplementary Table S2: List of primers used for qRT-PCR analysis of selected DM-responsive *Resistance (R)* genes as well as Internal control genes.

Supplementary Fig. 1. Functional characterization of *Resistance (R)* genes by assigning Gene Ontology (GO) terms using Blast2GO tool. (a) Representation of (a) PM-responsive (b) DM-responsive *Resistance (R)* genes classified on the basis of GO terms enrichment in biological process, cellular component and molecular function categories.

Figures

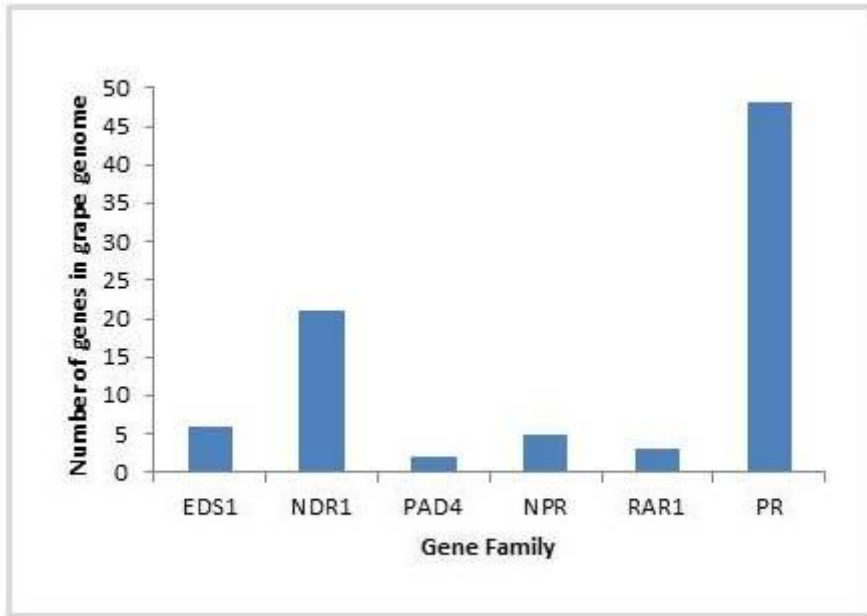


Figure 1

Representation of various defense-responsive Resistance R gene families (EDS1, NDR1, PAD4, NPR, RAR1 and PR) identified in the grape genome. The bar length indicates the number of particular class of Resistance (R) genes.

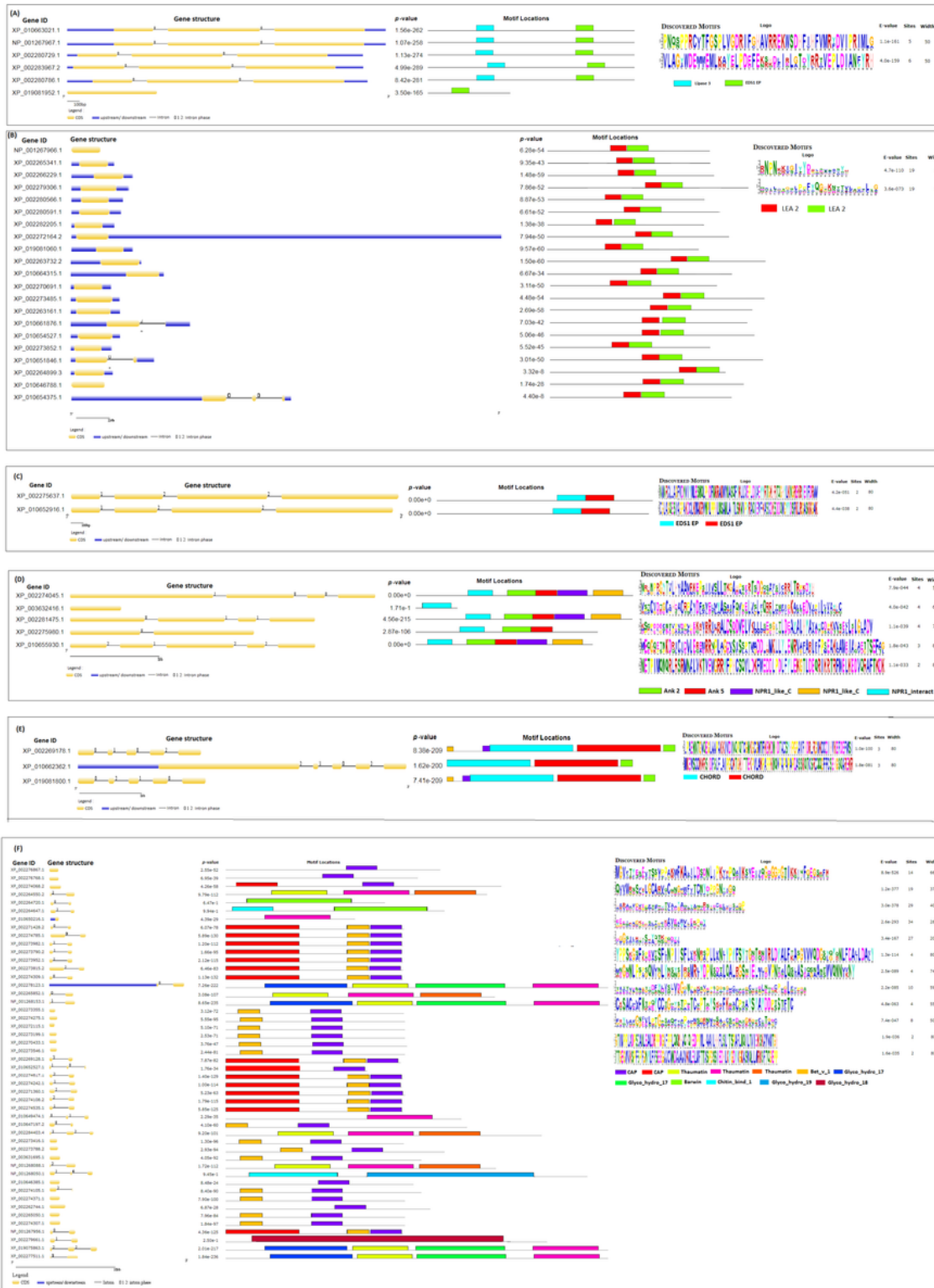


Figure 2

Gene configuration and motif study of various classes of Resistance (R) genes. The arrangement of introns and exons is defined by GSDS 2.0 server and motif analysis was conducted with Pfam database and MEME tool (A) Gene structure and conserved domains of EDS1 class of Resistance (R) genes. (B) Gene structure and conserved domains of NDR1 class of Resistance (R) genes. (C) Gene structure and conserved domains of NPR class of Resistance (R) genes. (D) Gene structure and conserved domains of

PAD4 class of Resistance (R) genes. (E) Gene structure and conserved domains of PR class of Resistance (R) genes. (F) Gene structure and conserved domains of RAR1 class of Resistance (R) genes. Exons are represented by yellow boxes partitioned by thin intron lines and blue boxes reflect UTRs. Different types of conserved motifs are shown with different colored boxes.

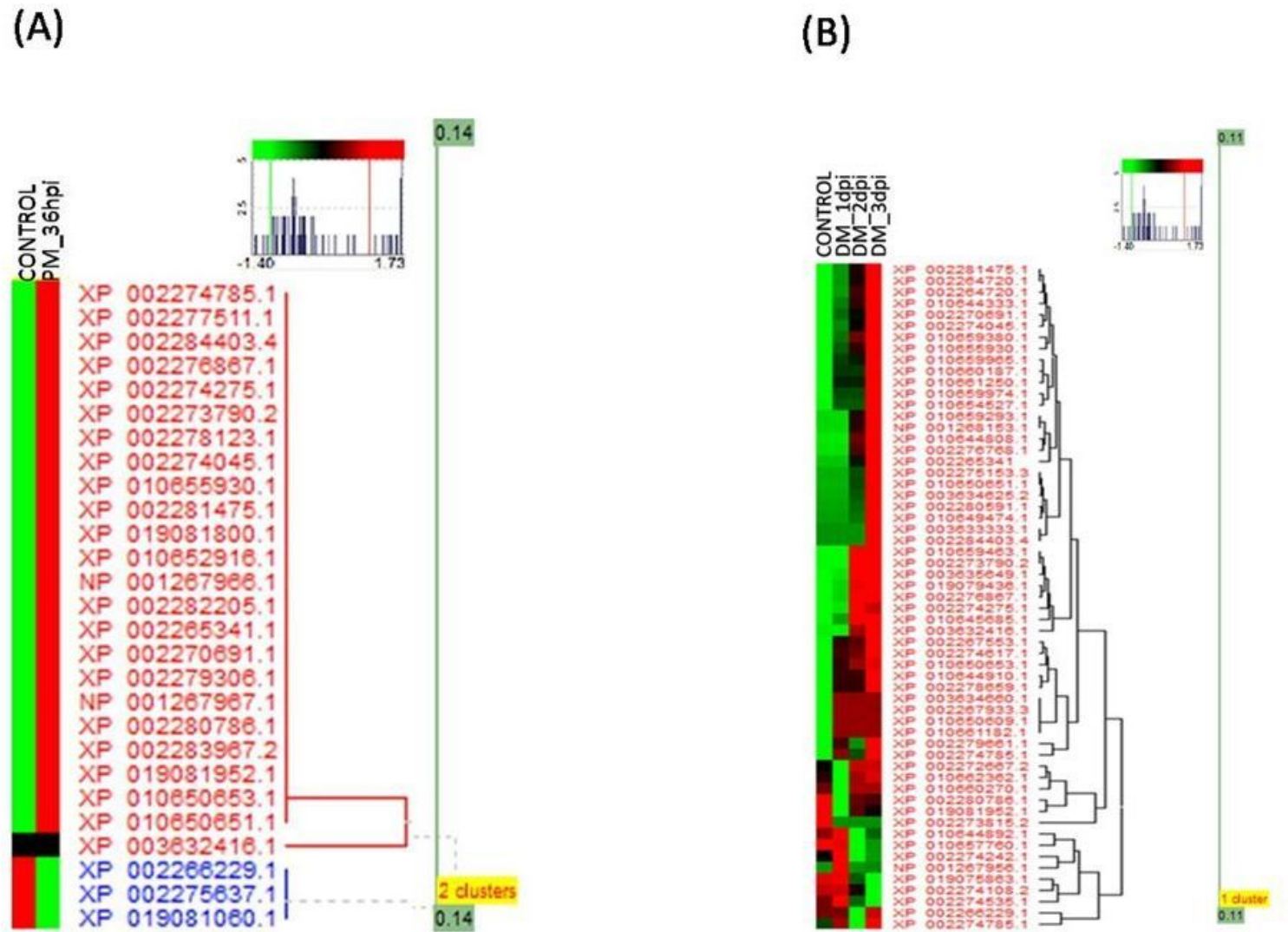


Figure 3

Heat maps illustrating the differential expression pattern of Resistance (R) genes in *Vitis vinifera*. (a) PM-responsive Resistance (R) genes at time points of 0 hr and 36 hpi. (b) DM-responsive Resistance (R) genes in at time points of 0hr, 1dpi, 2dpi and 3dpi. By enhancing the intensity of green and red colours, the declined and elevated expression levels were exhibited.

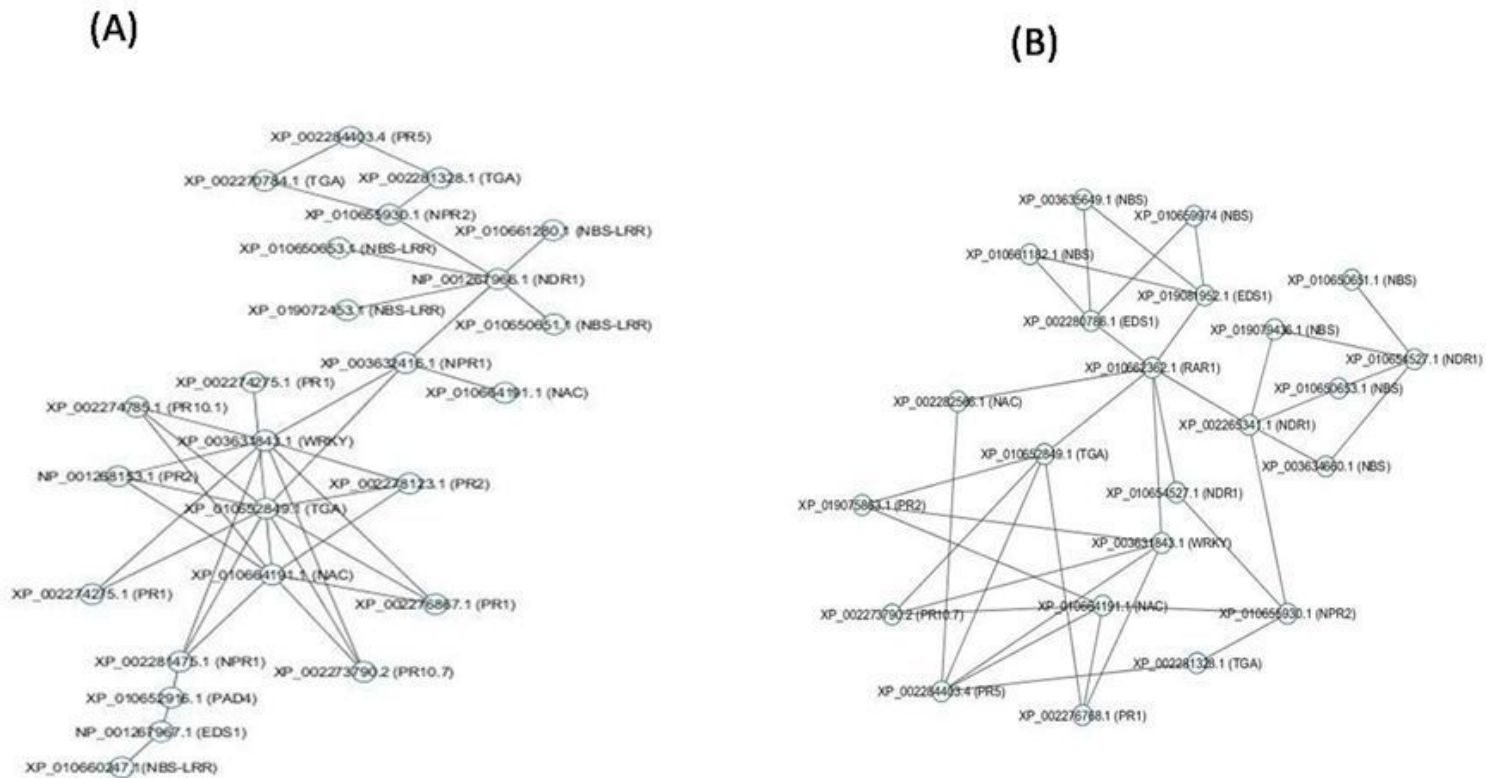


Figure 4

Co-expression analysis of Resistance (R) genes showing interaction of different types of proteins (NBS-LRR, EDS1, PAD4, NPR1, WRKY, TGA, PR) involved in signaling cascade using CoExpress 1.5.2 software. (a) Interaction amongst PM-responsive Resistance (R) genes visualized by cytoscape 3.7.1. (b) Interaction amongst DM-responsive Resistance (R) genes was shown.

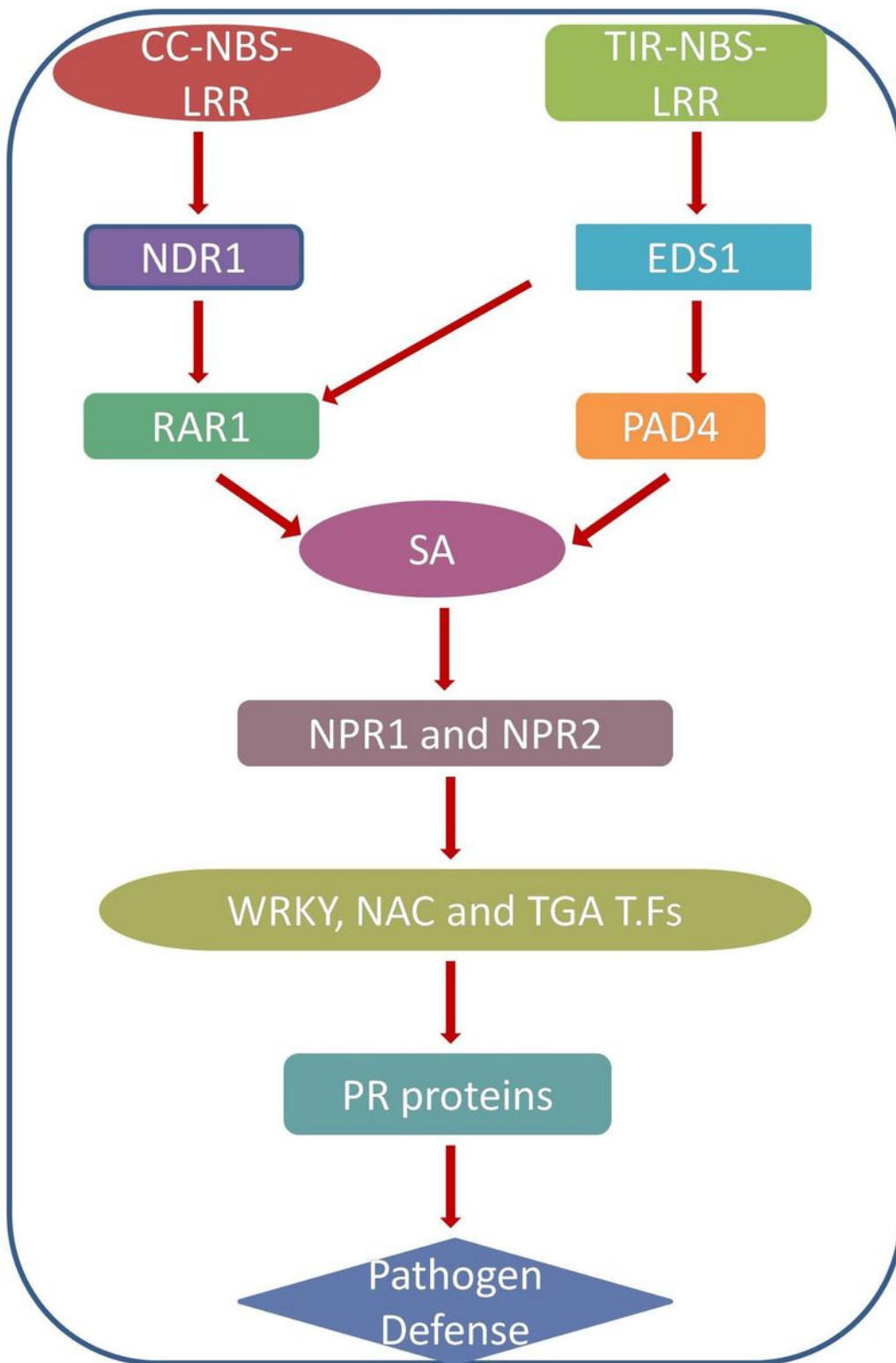


Figure 5

SA signaling cascade pathway reconfigured in *Vitis vinifera*. A pathogen activated signal stimulates R-gene mediated defense signaling that stimulates SA production in plant. Various proteins got activated during this signaling that are functioning upstream and downstream of SA. Ultimately, the PR proteins got expressed in cell that provides SAR to plant.

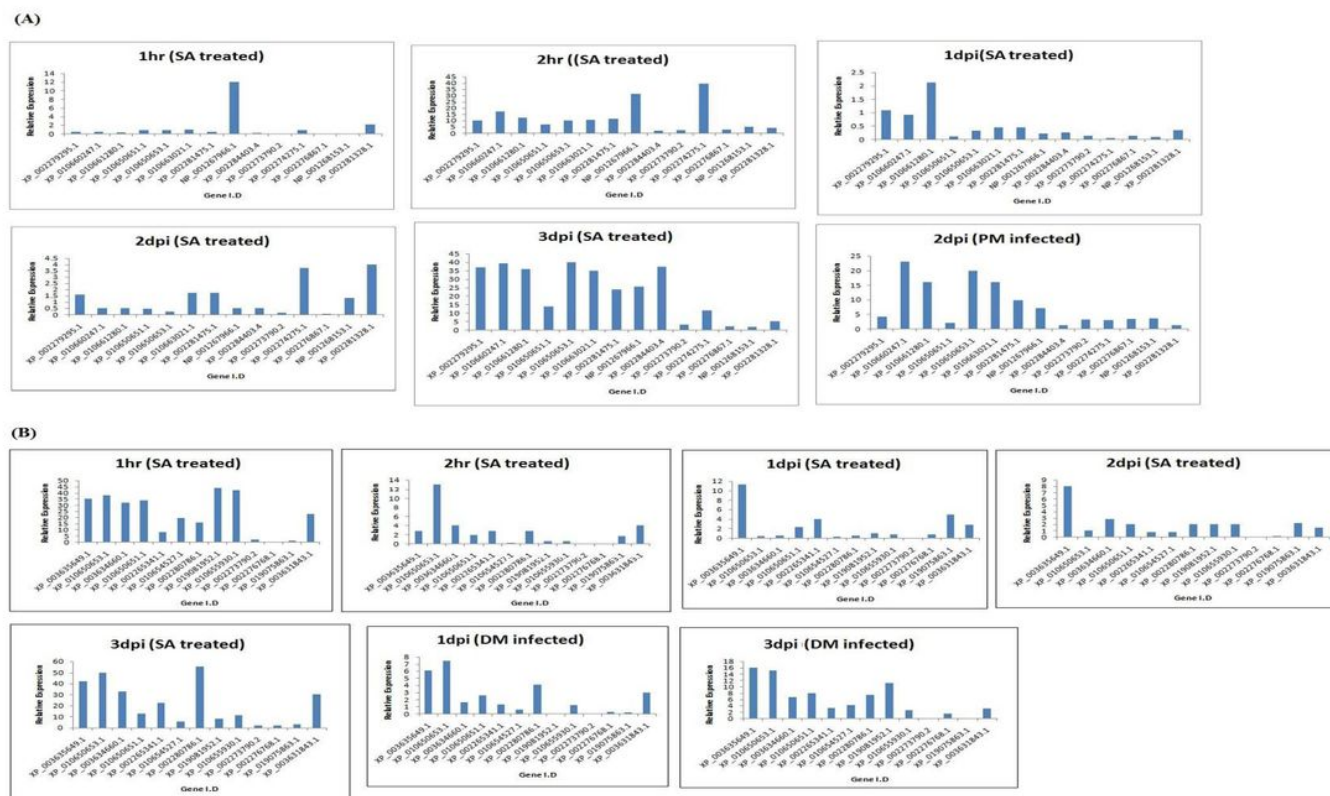


Figure 6

Relative expression levels of Resistance (R) genes through quantitative real time PCR under various stress conditions of PM and DM infection and salicylic acid (SA) treatment. (a) Real time graph of PM-responsive Resistance (R) genes during PM infection and salicylic acid (SA) treatment. (b) Real time graph of DM-responsive Resistance (R) genes during DM infection and salicylic acid (SA) treatment.

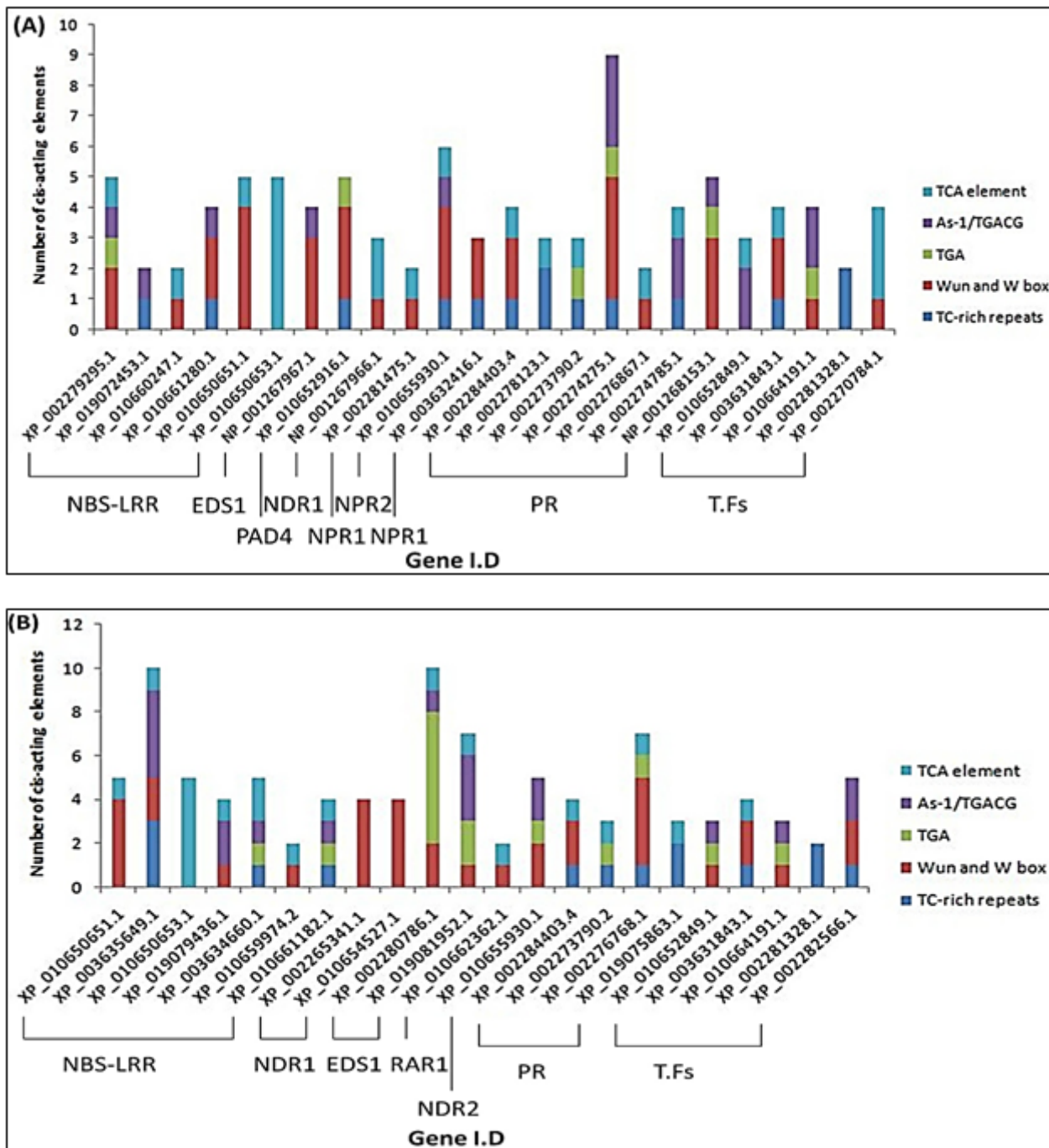


Figure 7

Assessment of promoter sequences of fungal stress responsive Resistance (R) genes. (a) PM-stress responsive (b) DM-stress responsive cis-elements were shown in different colors. Each color represents different cis-element.

Supplementary Files

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