

Powdery and Downy mildew infections regulates a cascade of defense-responsive genes in *Vitis Vinifera*

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Abstract

Background: Grape cultivation is severely affected by powdery mildew and downy mildew worldwide leading to significant losses in productivity and quality. To control such diseases in an environment friendly way, it is essential to understand the molecular mechanisms of fungal resistance in grapes. When the plant is infected with pathogen, various defense mechanisms are activated in plants at molecular level, the ultimate aim of all is to provide immunity against infection.

Results: In our study, we tried to understand the Salicylic acid (SA) mediated pathway of ETI through a cascade of defense molecules ultimately leading to activation and expression of PR proteins that provides SAR in plants. We performed genome-wide identification of defensive molecules expressed during PM and DM infection in grapevine. Consequently, we identified 2, 4, 7, 4, 2, 1, 20 and 7 differentially expressed PM-responsive defensive genes (NBS-LRR, EDS1, NDR1, NPR, PAD4, RAR1, TFs and PR) and 28, 2, 5, 4, 1, 39 and 19 differentially expressed DM-responsive defensive genes (NBS-LRR, EDS1, NDR1, NPR, RAR1, TFs and PR) in *V. vinifera*. Next, the co-expression between the identified defensive genes was performed that tells us about the sequence of events that occurs during a signaling cascade in response to PM and DM-infection. Further, we characterized the identified genes on the basis of physico-chemical properties, chromosomal positions, gene and protein structure analysis and functional annotation.

Conclusion: The powdery and downy mildew responsive defensive genes identified in this study can be used to develop new and improved PM and DM-resistant grape varieties in future.

Introduction

Grape is one of the commercially important fruit crop cultivated in India. However, the production of grapes is severely hampered due to the climate of major grape producing areas in India that allows the development of various fungal diseases. Powdery mildew (PM) and downy mildew (DM) are two such devastating diseases that require cooler temperatures and higher relative humidity to propagate and infect the grapevine [1]. The epidemic of PM and DM acutely diminishes the quality as well as productivity by infecting all green tissues of the grapevine. As most of the cultivated varieties in India belong to *Vitis vinifera*, which is susceptible to PM and DM, therefore, it is necessary to understand the molecular biology of powdery and downy mildew resistance mechanism in grapevine for controlling such fungal diseases.

During conditions of abiotic and biotic stresses, various defense mechanisms are activated in plant as a part of their natural immune response to protect itself. Basically, 3 major steps are involved in host defense against pathogenic attack i.e recognition of pathogens, signal transduction, and induction of genes that leads to production of molecules with antimicrobial activity. During PM and DM infection, under favorable environmental conditions, the conidiospores of *Erysiphe necator* (PM fungus) and *Plasmopara viticola* (DM fungus) germinate on the grapevine leaf surface, leading to initial interaction of

Pathogen-associated molecular patterns (PAMPs) and grapevine defense molecules (PRRs), thus providing first layer of defense i.e Pathogen Triggered Immunity (PTI) [2] [3] However, the activated defense is not sufficiently robust to prevent the fungal proliferation in plant. Additionally, to enhance its invasion in plant, fungus delivers certain effector proteins inside the cell via different mechanisms such as Type 3 effector proteins, haustoria etc. During that time, another defense mechanism known as Effector Triggered Immunity (ETI) is activated that triggers the expression of resistance (R) proteins often resulting in apoptosis of the infected cell, thereby blocking the further proliferation of the pathogen [4] [5].

Various signal transduction pathways that are activated during ETI lead to re-programming of the host plant transcriptome and activation of defensive genes. It is believed that the resistance of plant against biotrophic pathogens is mediated through Salicylic acid (SA) signaling in which SA acts as secondary messenger [6]. As *Erysiphe necator* and *Plasmopara viticola* are both biotrophic in nature, therefore the defence mechanism activated in plant in response to mildew infection is signaled through SA. There are cascade of events that occur during this signaling and various genes get activated that acts upstream and downstream of SA. It has been reported previously that the R-gene mediated resistance response is activated via 2 key 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 demonstrated that any perturbation of the interaction between EDS1, NDR1 and R proteins can affect the activation of signaling pathway thereby suggesting the importance of these 2 central regulators of plant defense response [8] [9] [10] [11]. Some 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*), which get functionally activated upstream of SA to promote SA accumulation.

Additionally, SA signaling requires one major protein downstream of SA i.e *Nonexpressor of PR Gene1 (NPR1)* gene. This gene is a key regulator of SA mediated signal transduction which got activated after accumulation of SA [12]. Before activation, *NPR1* is present in cytoplasm in oligomeric form, after accumulation of SA, *NPR1* reduces to monomeric units that get translocated into the nucleus where it interacts with various TGA and WRKY transcription factors (TFs) and ultimately leads to expression of *Pathogenesis Related (PR)* genes, the final players of plant defense response [13] [6] [14].

Altogether, accumulation of SA and expression of *PR* genes leads to development of Systemic Acquired Resistance (SAR) i.e a broad spectrum resistance in plants [15].

Previously, we characterized 63 PM-responsive *NBS-LRR* genes from different grapevine cultivars by analyzing transcriptome of these cultivars inoculated with *E. necator*. In this study, we investigated the interaction between various defense responsive genes activated in response to PM and DM infection and proposed a pathway for the same. Additionally, we characterized the identified genes on the basis of chromosomal locations, gene and protein structure analysis, physicochemical properties and functional

domain annotation using BLAST2GO. Moreover, we also depicted numerous *cis*-acting regulatory elements (CARE) for the identified genes in response to powdery and downy mildew stress.

Material And Methods

Identification of PM and DM responsive defensive genes in V. vinifera

Protein coding sequences of various gene families i.e EDS1, NDR1, NPR1, RAR1, TFs, PR reported in grapes were extracted from National Center for Biotechnology Information (NCBI) genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/Vitis_vinifera/protein/) and grape genome browser 12X i.e genoscope (<http://www.cns.fr/externe/GenomeBrowser/Vitis/>) and generated a fasta file. To identify PM and DM responsive defensive genes, we downloaded the RNA-seq data of Thompson Seedless and Pinot Noir varieties of *V. vinifera* with project numbers of SRP116308 and PRJEB24540 with the use of NCBI Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra>). The transcriptomic data of Thompson Seedless was responsive to PM and was available at 1 time point i.e 36 hours post inoculation (hpi) (Northwest A&F University). Similarly, the RNA seq data of Pinot Noir is DM-responsive and is available at 3 time points i.e 1dpi, 2dpi and 3dpi [16]. Previously, we reported the occurrence of 386 *NBS-LRR* genes in *V. vinifera* [17]. We used these gene sequences to identify PM and DM-responsive *NBS-LRR* gene sequences. The abundance of all fungal responsive defensive genes were calculated by RSEM (RNA-Seq by Expectation-Maximization) software of the Trinity-V2.4 package as fragments per kilobase of transcript per million fragments mapped (FPKM) [18]. Subsequently, EdgeR was used to measure the differential gene expression by applying the cut-off value of four folds of change in expression level and a P value of 0.001. The differential gene expressions of all classes of genes identified in our study were represented as heat maps and Hierarchical Clustering Explorer 3.5 was used to generate heat maps (<http://www.cs.umd.edu/hcil/hce/>) [19]. Further, on the basis of heat maps generated, the fold change and number of up and down regulated defensive genes were determined in response to powdery and downy mildew infection in *V. vinifera*.

Co-expression analysis

Next, we conducted co-expression (CE) analysis of differentially expressed defensive genes to determine the sequence of events that occurs during a signaling cascade in response to PM and DM-infection. The CoExpress 1.5.2 software tool was used with default parameters (<http://Bioinformatics.lu/CoExpress>). The data 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 done using the software cytoscape 3.7.1 (<https://cytoscape.org/>).

Plant material and treatment

As SA is playing a pivotal role in defense against fungal infections, we studied its effect on Thompson Seedless variety of *V. vinifera*. The 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 SA treatment was given at the final concentration of 100 mg/litre at 5 different time intervals i.e 1hpi, 2hpi, 1dpi, 2dpi and 3dpi and subsequently the control and treated leaves were collected, snap frozen and stored in -80°C till further use. Additionally, we collected the healthy and fungal infected leaf samples of Thompson Seedless variety of *V. vinifera* from National Research Centre for Grapes (NRCG), Pune.

RNA isolation, cDNA synthesis and quantitative real time PCR analysis

On the basis of genes identified in co-expression analysis, we performed quantitative real time PCR (qPCR) of PM and DM-responsive differentially expressed defensive genes to measure the relative expression. Leaf samples were used to isolate the total RNA by following Ghawana et al. 2011 protocol and subsequently, the cDNA was prepared by using Superscript III first strand cDNA synthesis kit (Invitrogen USA) [20]. 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/>). We performed qPCR experiment 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, 57°C for 20 sec and 72°C for 20 sec. To analyze the results of qPCR, we used REST 2009 software (Qiagen) and $2(-\Delta\Delta\text{CT})$ method to obtain relative gene expression ratios of target genes with respect to control genes (<http://www.REST.de.com>) [21] [22].

Gene and protein structure analysis

We determined the gene structure by identifying the exons and introns present in the gene sequences. The coding sequence of each gene was aligned to its genomic sequence using an online tool 'Splign' (<http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>). Further verification of the aligned result was done by using Ensembl Plants portal (http://plants.ensembl.org/Vitis_vinifera/Gene). The visualization of position of exons and introns was done by using GSDS2.0 server (<http://gsds.cbi.pku.edu.cn/>) [23]. Additionally, we plotted the intron phases for our gene sequences with the same tool. Next, the conserved protein motifs were discovered by using Multiple Expectation Maximization for Motif Elicitation (MEME) Suite (<http://meme-suite.org/tools/meme>) with default parameters. [24].

Chromosomal mapping of PM and DM-responsive defensive genes

To determine the positions of PM and DM-responsive defensive gene sequences chromosome-wide, chromosomal mapping was done. Primarily, the gene sequences were searched against non-redundant (nr) database by performing BLASTN and short-listed the hits depicting maximum identity and subsequently located the gene on respective chromosome. Additionally, the confirmation of chromosomal position of genes was done through Grape Genome Browser i.e. Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) and Ensembl Plants portal (https://plants.ensembl.org/Vitis_vinifera/Location/Genome).

Functional characterization

For the functional annotation of PM and DM-responsive defensive genes, BLAST2GO (<https://www.blast2go.com/blast2go-pro>) tool was used [25]. Basically, 3 steps are involved in BLAST2GO annotation: BLAST for finding homologous sequences, Mapping to assign GO terms for each hit obtained, Annotation to assign function to query sequences on the basis of their cellular localizations, molecular functions and biological processes.

Promoter analysis

The *cis*-acting regulatory elements of PM and DM-responsive defensive genes were predicted using PlantCare database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [26]. We extracted a 2-kb sequence upstream of translation start site after aligning the genomic sequences with coding sequences and analyze it eventually for the presence of different regulatory elements.

Physicochemical characteristics of PM and DM-responsive defensive genes

The physicochemical characteristics of PM and DM-responsive defensive genes were depicted using ProtParam bioinformatics tool (<https://web.expasy.org/protparam/>), which provides the information regarding the number of amino acids, theoretical pI (isoelectric point), molecular weight and instability index of protein [27].

Results And Discussion

*Identification of fungal responsive defensive genes in *V. vinifera**

To better understand the molecular mechanism of powdery and downy mildew resistance in grapevine, we performed genome-wide identification of PM and DM-responsive defensive genes in grapes in response to salicylic acid mediated signal transduction pathway, the ultimate goal of which is to provide ETI in plants. By using Genoscope and NCBI, we retrieved 9, 10, 20, 4, 3, 177 (47, 59, 71) and 44 EDS, NDR1, NPR, RAR1, PAD, TFs (TGA, WRKY, NAC) and PR protein coding sequences of *V. vinifera* and developed a fasta file. Additionally, we also used 386 *NBS-LRR* gene sequences identified in our previous study. Next, to identify fungal responsive defensive genes, normalized expression analysis of the above mentioned gene classes was conducted. To accomplish this task, we extracted the transcriptomic data of one PM-susceptible (Thompson seedless) and one DM-susceptible (Pinot Noir) *V. vinifera* varieties from NCBI and conducted the normalized expression analysis (FPKM) analysis. Consequently, we identified varied number of differentially expressed PM-responsive and DM-responsive defensive genes in *V. vinifera*. As, NBS-LRR proteins are the primary proteins recognized by avirulent proteins of the pathogen, hence, we identified 2 highly expressing PM-responsive and 28 DM-responsive *NBS-LRR* genes that were differentially regulated in the present study (Fig. 1). Our results showed that most of the fungal responsive *NBS-LRR* genes were up-regulated during later stages of fungal infection as compared to initial phase. Various fungal responsive *NBS-LRR* genes have also been identified previously in many

plants such as apple, Arabidopsis, wheat, grapes, Cucumber etc [28] [29] [30] [31] [32], [33] [34]. 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 that is responsive to PM in Kishmish vatkana' and 'Dzhandzhal kara' grapevine varieties [32]. Likewise, in *V. amurensis* and *V. riparia* grapevine varieties, *NBS-LRR* gene cluster was identified in response to DM infection.

Later on, after identification of *NBS-LRR* genes, our research is being directed towards identification of molecules that leads to activation of NBS-LRR proteins. In search of that, two key genes i.e *EDS1* and *NDR1* were identified that were showing differential expression pattern in response to PM and DM-infection. We identified 4 *EDS1* and 7 *NDR1* differentially expressed genes in response to PM infection; 2 *EDS1* and 5 *NDR1* in case of DM-infection (Fig. 1). Our results also showed that all *EDS1* genes got up-regulated during PM-infection as compared to control condition and out of 7, 5 *NDR1* genes got up-regulated during PM-infection. Similarly, during DM-infection, all genes got up-regulated during later stages (3 dpi) of infection.

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 [35]. So, to study the interaction of EDS1 and PAD4, we identified differentially expressed *PAD4* genes in grape genome. As a result, we got 2 *PAD4* genes through FPKM analysis in PM infected samples out of which one gene is upregulated but surprisingly, we did not get any *PAD4* gene expressing in DM-infected sample (Fig. 1).

There is one another protein that is found in our study acting upstream of SA i.e RAR1, a co-chaperone of HSP90. We found 1 PM-responsive and 1 DM-responsive differentially expressed transcript encoding *RAR1* in case of both PM and DM infection and both the transcripts were showing up-regulation during fungal stresses (Fig. 1). Till now, all these genes lead to accumulation of SA in plant cell that is essential for local and SAR in plant.

Further, downstream of SA, another important protein activated is NPR1. This gene is considered as master regulator of SA-mediated immune response. There are reports on *NPR1* gene that tells us that over-expression of this gene in Arabidopsis, wheat, tomato and apple confers broad spectrum resistance against fungal and bacterial pathogens [36] [37] [38] [39]. In our analysis, we found 4 PM-responsive and 4 DM-responsive *NPR* (comprising of *NPR1* and *NPR2*) genes that got differentially expressed during fungal infection and all *NPR* genes were seen to be upregulated gradually from initial to later stages of both powdery and downy mildew infection (Fig. 1).

NPR1 and *NPR2* further interacts with different classes of transcription factors. Through differential expression analysis, we identified 20 PM-responsive and 39 DM-responsive TFs that belongs to 3 different classes i.e bZIP, WRKY and NAC. The interaction of NPR proteins with several TFs finally activates various PR proteins that provides SAR to plant.

The description of plant PR proteins was first made in tobacco leaves infected by tobacco mosaic virus [40]. Since then, these proteins have been identified in many monocot and dicot plants. Usually, the accumulation of PR proteins is induced in plants during conditions of pathogenic infection, thereby playing an eminent role in improving the defensive ability of plant. [41]. In our study, we found 7 PM-responsive and 19 DM-responsive differentially expressed PR proteins that belongs to 5 different classes of PR proteins i.e PR1 (Antifungal), PR2 (β -1,3-Glucanase), PR5 (Thaumatin-like), PR10.1 (Ribonuclease-like) and PR10.7 (Ribonuclease-like) (Fig. 1). The role of all the PR proteins identified in our study has been previously identified in various grape varieties as documented in literature. Previously, PR1 proteins exhibiting anti-fungal property has been identified and cloned in *V. vinifera* [42]. However, till date, its role in signaling pathway was not determined in grapes. Our study suggested that PR1 is activated during SA mediated signaling cascade in response to 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 directly affects growth of powdery mildew infection at the pathogen infection site [43]. Similarly, PR-5 proteins enhance fungal membrane permeability and causing osmotic rupture of fungal plasma membrane providing immunity against fungal attack [44]. PR-10 proteins acts directly against pathogens or can contributes to plant defense in programmed cell death during HR [45]. Previously, *PR-10* gene was isolated from *V. pseudoreticulata* that is showing resistance to *P. viticola* infection [46]. All these studies of PR proteins supported our results of PR proteins activated in response to SA in grapes.

Overall in our study, we identified 47 PM-responsive and 98 DM-responsive differentially expressed genes in *V. vinifera* accessions used in our study (Fig. 1). Overall, our results suggested that most of the genes got upregulated during PM and DM infection conditions as compared to control ones.

Co-expression analysis

Next, we conducted co-expression (CE) analysis of differentially expressed PM and DM-responsive defensive genes using the software tool CoExpress 1.5.2. Subsequently, we screened some highly co-expressing genes in *V. vinifera* that might be regulating the powdery and downy mildew infection through SA signaling cascade. The visualization of the co-expressed genes was done by using the software Cytoscape 3.7.1 with default parameters (Fig. 2). Amongst all, we got 6 PM-responsive and 7 DM-responsive *NBS-LRR* genes that were found to be showing strong co-interaction with *EDS1* and *NDR1* genes residing downstream *NBS-LRR* genes. It has been reported previously that *EDS1* interacts with TIR domain containing R genes and *NDR1* associates with CC containing R genes [7] [47] [48] [49]. Interestingly, we also got the similar results of *EDS1* and *NDR1* interactions with different subsets of *NBS-LRR* genes through co-expression analysis. We found that in case of PM-responsive genes, out of 6 *NBS-LRR* genes, 4 genes consisting of CC domain are interacting with *NDR1* protein whereas 2 genes containing TIR domain are interacting with *EDS1* proteins (Fig. 2A). Similarly, we got 7 *NBS-LRR* genes in response to DM infection, amongst which 4 genes having CC domains were interacting with *NDR1* and 3 genes consisted of TIR domain are associated with *EDS1* (Fig. 2B).

Next, as it was described above that EDS1 associates with PAD4, we got one PM-responsive *PAD4* gene i.e XP_010652916.1 that is strongly interacting with *EDS1* gene (NP_001267967.1) (Fig. 2A).

We also find a strong interaction of *EDS1* and *NDR1* genes with *RAR1* gene. This protein stabilizes the NBS-LRR proteins, thus playing an immense role in ETI [50] [51] [52] [53] [54] [55]. All these proteins interacting till now leads to accumulation of SA in cell which 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 it interacts with various transcription factors that eventually leads to activation of various PR proteins [56]. Through co-expression analysis, we identified 2 NPR1 proteins and 1 NPR2 protein interacting with SA in response to PM-infection and 1 NPR2 protein in response to DM-infection (Fig. 2). Both NPR1 and NPR2 were reported previously to participate in SA signal transduction in plants like Arabidopsis, rice, apple, poplar etc [57] [58] [59] [60] [61] [39].

The co-expression results depicted that NPR1 and NPR2 proteins interacts with TGA, WRKY and NAC transcription factors in case of both powdery and downy mildew infection. 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. 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 [62]. Similarly WRKY and NAC transcription factors were also found to interact with *NPR1* gene in previous studies [63] [64]. The interaction of NPR proteins with several TFs finally activates various PR proteins that provides SAR to plant. We got 6 PM-responsive and 4 DM-responsive highly co-expressing PR proteins which belongs to 5 different PR protein families i.e PR1, PR2, PR5, PR10.1 and PR10.7 (Fig. 2). Altogether, we screened 24 PM-responsive and 22 DM-responsive differentially expressed genes in *V. vinifera* accessions that were found to be highly interacting amongst each other in response to SA mediated signal transduction during powdery and downy mildew stress (Fig. 2).

qPCR expression analysis

On the basis of FPKM and co-expression analysis, we selected some highly interacting defensive genes that are activated during a signaling cascade in response to fungal infection for validation by qPCR. We chose 14 PM-responsive and 13 DM-responsive genes for real time expression analysis under various stress conditions (Fig. 3). The primer sequences, amplicon length and annealing temperature of PM and DM-responsive defensive genes is listed in table S3 and table S4.

There are reports that tell us about the importance of SA during pathogenic attack. 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 [65] [66] [67]. 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 [68] [69] [70]. It has been reported previously that exogenous application of SA or SA analogs induces SAR and restores resistance in numerous mutants compromised in signaling steps upstream of SA production [49] [71] [72] [73]. We also applied SA

exogenously on leaves of Thompson seedless variety at 5 different time intervals i.e 1hpi, 2hpi, 1dpi, 2dpi and 3dpi to study the change in expression patterns of various defense related genes.

Consequently, we found that most of the genes got upregulated during 2dpi PM infection, 2 hpi and 3 dpi SA treatment (Fig. 3A). Similarly, for DM, genes were mostly upregulated during late fungal infection, 1hpi and 3 dpi SA treatment (Fig. 3B). The possible assumption would be that after fungal infection, SA levels rise in plant leading to defense reaction. So, when we gave SA treatment to our samples, initially genes got upregulated in 1hr or 2 hr; and later on to provide SAR to plant, the expression level peaks up at 3dpi in both fungal infections.

Additionally, our results of powdery and downy mildew infection through quantitative real time PCR are in accordance with results obtained through FPKM analysis. Most of the genes got up-regulated during PM and DM infection as compared to control condition in both FPKM and real time analysis.

Chromosomal distribution of PM and DM-responsive genes

After identification of PM and DM-responsive defensive genes obtained through FPKM and co-expression analysis studies, we finally got 24 PM-responsive and 22 DM-responsive defensive genes in the grape genome. We characterized the identified genes on the basis of chromosomal distribution which showed that PM and DM-responsive defensive genes are distributed widely in the grape genome. Amongst all, PM-responsive *NBS-LRR* genes were located on different 5 chromosomes i.e. 5, 11, 14, 15 and 19 (Fig. 4A). Similarly, PR proteins were distributed on chromosome 4, 5, 7, 8 and 18. *EDS1* is present on chromosome 17; *NDR1* on chromosome 7; *PAD4* on chromosome 16 and *NPR1* and *NPR2* were located on chromosomes 11, 7 and 10 (Fig. 4A). Likewise, DM-responsive defensive genes were also unevenly distributed on the grape genome. Amongst all, DM-responsive *NBS-LRR* genes were located on chromosomes 5, 13, 14, 15 and 19 (Fig. 4B). Likewise, PR proteins were located on chromosomes 4, 7, 8 and 18; the chromosomes shared between PM and DM-responsive PR proteins are same (Fig. 4B). *EDS1* genes were present on chromosome 17; *NDR1* on chromosomes 9 and 10; *RAR1* on chromosome 16 and *NPR2* on chromosome 10 (Fig. 4B). Altogether, out of 24 PM-responsive defensive genes, maximum number (5) of genes were present on chromosome 5, followed by 4 genes on chr7, 3 and 2 genes each on chromosome numbers 11 and 18 (Fig. 4A). Similarly, for DM-responsive defensive genes, 3 genes were present on chromosome 5; 2 genes each on chromosomes 7, 10, 17 and 18 followed by a single gene number on various other chromosomes (Fig. 4B). Additionally, we identified 1 gene of DM-responsive pathway that was not placed on any scaffold.

Physicochemical characteristics of PM and DM-responsive defensive genes

The average length, molecular weight, physical index (pI) and instability index of PM-responsive defensive proteins was 467 amino acids, 526.69 kDa, 6.6 and 46.57 respectively (Table S1). We found the maximum and minimum polypeptide length to be 1413 and 112 amino acids. Likewise, the maximum and minimum molecular weight depicted was 159.516 kDa and 17.221 kDa. The maximum pI determined was 9.45 whereas minimum was 4.84 respectively. To find out whether the protein is stable or not, we

deduced the instability index. The maximum and minimum instability index found was 91.98 and 24.90 and out of 24, 6 such proteins were identified that were found to be stable and 18 proteins were unstable (Table S1).

Similarly, The average length, molecular weight, physical index (pI) and instability index of DM-responsive defensive proteins was 576 amino acids, 650.81 kDa, 7.49 and 46.15 respectively (Table S2). Through our analysis, we got the maximum and minimum polypeptide length to be 2130 and 136 amino acids. Similarly, the maximum and minimum molecular weight depicted was 242.899 kDa and 15.758 kDa. The maximum pI determined was 10.19 whereas minimum was 5.06 respectively. Our results suggested that it could be easier to clone fungal-responsive defensive genes due to small size of these proteins and subsequently various transgenic studies could be done.

The values of 57.17 and 31.49 were found to be the maximum and minimum instability index. Our results suggested that out of 22, 4 such proteins were identified that were found to be stable and 18 proteins were unstable (Table S2). Most of the genes that were found stable are ones belonging to NDR1 and PR protein family. The PR protein families that were found to be stable includes PR1, PR2, PR10.1 and PR10.7.

Gene structure and motif analysis

The structural analysis of PM and DM-responsive defensive genes was depicted in terms of introns and exons that were found to be widely diverged. The three processes are known to be responsible for diversified structures of exons and introns: insertion/ deletion, exonization/pseudo-exonization and exon/intron gain/loss [74] [75] [76].

For PM-responsive defensive genes, the intron numbers were 1 to 7 with the maximum intron number of 7 in 1 gene; in contrast, the minimum intron number was one in 3 genes. The most frequent intron number in maximum sequences is 2 and 3. Moreover, 5 such genes were identified that lacked introns (Fig. 5A). Similarly, for DM-responsive defensive genes, the intron numbers were 1 to 7 with the maximum intron number of 7 in 1 gene; in contrast, the lowest intron number was one in 2 genes. The most frequent intron number in maximum sequences was 2. We also identified 9 intron-less genes (Fig. 5B).

Next, we depicted various intron phases based on the location of intron with respect to reading frame of gene. As a result, 3 types of intron phases were found in our sequences. Mostly, the introns were in phase 0, followed by introns in phase 1 and phase 2. For PM-responsive defensive genes, approximately, 54% of the introns were present in 0 phase, 36% in 1st phase and 10% in 2nd phase, respectively (Fig. 5A). For DM-responsive defensive genes, approximately, 58% of the introns were present in 0 phase, 32% in 1st phase and 10% in 2nd phase, respectively (Fig. 5B).

Next, the candidate protein sequences were examined for the presence of various motifs by using 'MEME' server and Pfam database. The results were further verified through InterProScan for the presence of desired motif. As a result, we identified 18 different conserved motifs dispersed throughout the protein

sequences in PM and DM-responsive defensive genes (Fig. 5). The various conserved domains identified amongst different classes of defensive proteins includes NB-ARC and LRR8 and Rx-N in NBS-LRR proteins; Lipase 3 and EDS EP domains in EDS and PAD4 proteins; LEA2 domain in NDR1 proteins; BTB, DUF3420, Ank2, BetV1 and NPR1 interacting domains in NPR1 and NPR2 proteins; CHORD domain in RAR1 protein; bZIP1, bZIPC, NAM and WRKY in TFs; Thaumatin, Glycohydro 17, Betv1 and CAP in different classes of PR proteins. The block diagrams of the conserved motifs are shown in Fig. 5. The presence of these motifs is necessary for defensive proteins to perform various important activities.

Functional prediction of defensive genes

To understand the diverse functions of proteins at molecular level, we performed Blast2GO that depicted the biological process, molecular function and cellular component, the 3 major characteristics of genes. Various GO terms were assigned to our gene sequences to determine the possible functions of PM and DM-responsive defensive genes. The results of GO terms allocations showed that maximum number of sequences were involved in biological processes; followed by sequences in cellular component and molecular function category in both PM and DM-responsive genes.

For PM-responsive genes, in biological process category, we obtained 16% sequences that were involved in response to stimulus (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 [Supp. Fig. 1A(a)]. Likewise, the GO term annotations in cellular component category suggested that most of our gene sequences were performing their respective functions in cell (23%), cell part (23%), organelle (20%), membrane (7%), extracellular region (7%), membrane part (6%) etc. [Supp. 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 results of molecular process category showed that maximum number of sequences (52%) were involved in binding activity (GO:0005488), followed 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) [Supp. Fig. 1A(c)].

Similarly, for DM-responsive genes, in biological process annotations, the percentage of gene sequences involved in various processes were 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 [Supp. Fig. 1B(a)]. Likewise, 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 [Supp. 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 [Supp. Fig. 1B(c)].

Altogether, our results depicted that the PM and DM-responsive defensive genes identified in our study might be playing an eminent role in biological, cellular and molecular processes.

Prediction of cis-acting regulatory elements

In response to biotic and abiotic stresses, a complex regulatory mechanism is activated in plants, therefore the characterization of PM and DM stress-responsive regulatory elements will provide an important information regarding upstream regulation of PM and DM-responsive defensive proteins. We chose an upstream sequence of upto 2 kb sequence from the transcription start site for identifying *cis* elements that are associated with stress induced gene expression in promoter regions of defensive genes. As a result of our analysis, we found 2 primary *cis*-acting elements i.e TATA and CAAT boxes in promoter and enhancer regions of all gene sequences. Next, we focused on the identification of PM and DM stress specific promoter elements. Consequently, 5 types of fungal stress-responsive regulatory elements namely salicylic acid responsive element (TCA element), defense and stress responsive element (TC-rich repeats), wound and pathogen responsive element (WUN-motif and W-box), stress responsive element (GT1 Box), As-1 and TGA were identified in PM and DM-responsive promoter sequences; the transcription factors binds to these cis-regulatory elements and activates the stress tolerant pathway. Amongst all, we got salicylic acid responsive element in 15 each PM-responsive and DM-responsive promoter sequences, wound and pathogen responsive element in 17 PM-responsive and 16 DM-responsive sequences, defense and stress responsive element in 12 PM-responsive and 10 DM-responsive sequences, As-1 in 10 each in PM-responsive and DM-responsive sequences and TGA motif in 6 PM-responsive and 9 DM-responsive sequences (Fig. 6). Previously, the role of various TFs have been identified in response to PM and DM-stress; NAC TFs bind to GT1 box and bZIP TFs associates with TGA elements; WRKY binds to W-box in response to fungal infection [77] [78] [79]. In our analysis, we observed the presence of these elements in defensive proteins which strongly supported that these genes got up-regulated in situation of PM and DM stress as a defense mechanism. Altogether, we could say that there were various types and number of regulatory elements present in promoter regions of defensive genes involved in signaling cascade, indicating that these genes must be playing an eminent role in regulatory mechanisms in response powdery and downy mildew stress condition.

Conclusion

In our study, grapevine genome was explored to identify PM and DM-responsive defensive genes that could be used to improve disease resistance. Consequently, 47 PM-responsive and 98 DM-responsive differentially expressed genes were identified in 2 different *V. vinifera* accessions amongst which 24 PM-responsive and 22 DM-responsive defensive genes are found to be highly interacting with each other. Also, we explored the various characteristic features of the identified PM and DM-responsive defensive genes such as gene organization, motif analysis, gene regulation, chromosomal localization and functional analysis. Altogether, we can summarize that the pathogen responsive defensive genes identified in our present study could be beneficial in enhancing powdery and downy mildew resistance in grapevine.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE: Not Applicable

CONSENT FOR PUBLICATION: Not Applicable

AVAILABILITY OF DATA AND MATERIAL: Not applicable

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AUTHOR CONTRIBUTIONS: KS; conceived the idea, designed the experiments, analyzed the results and finalized the manuscript. N Goyal and GB, collected and analyzed the data, compiled the results. AKU provided the plant material analyzed the results. N Goyal and N Garewal performed wetlab experiments and wrote the manuscript.

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Figures

(A)



(B)

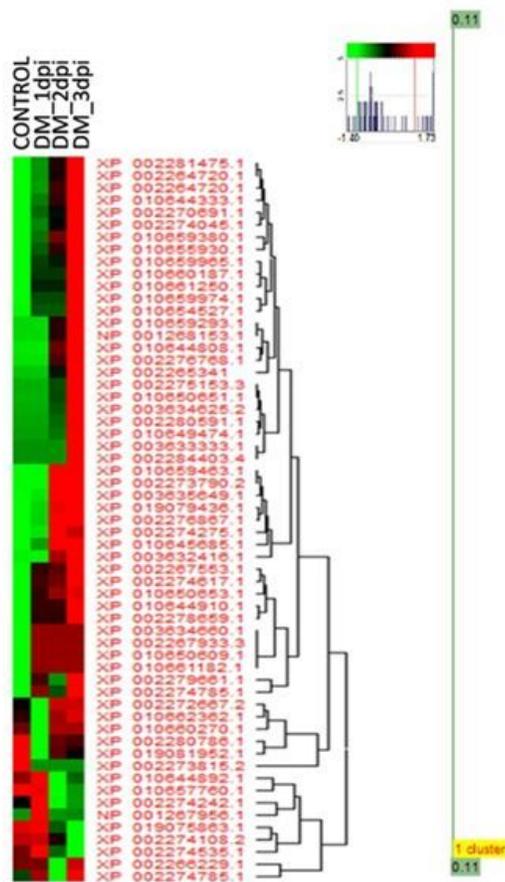
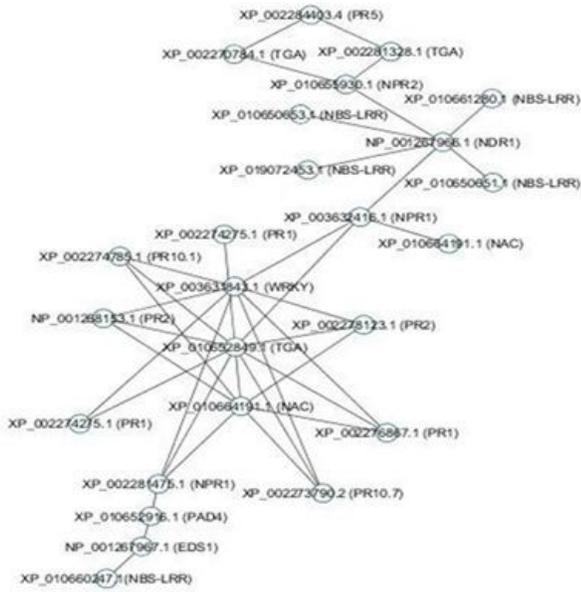


Figure 1

Heat maps representing expression levels of various defensive genes in *Vitis vinifera*. (a) PM-responsive defensive genes at time points of 0 hr and 36 hpi. (b) DM-responsive defensive genes in at time points of 0hr, 1dpi, 2dpi and 3dpi. By increasing the brightness of green and red colours, the declined and elevated expression levels were indicated.

(A)



(B)

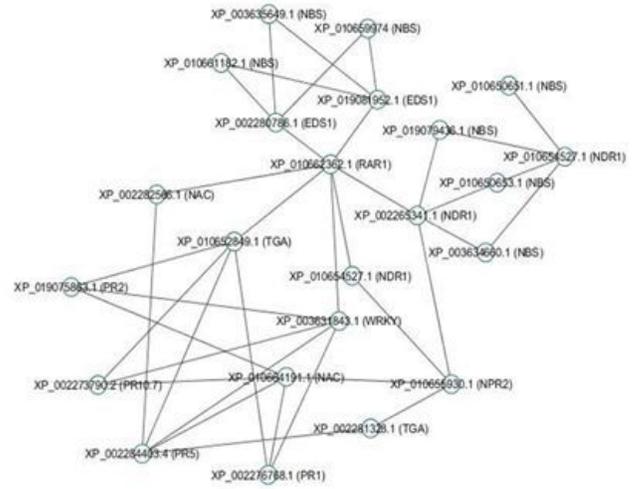


Figure 2

Co-expression analysis of defensive 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 defensive genes visualized by cytoscape 3.7.1. (b.) Interaction amongst DM-responsive defensive genes was shown.

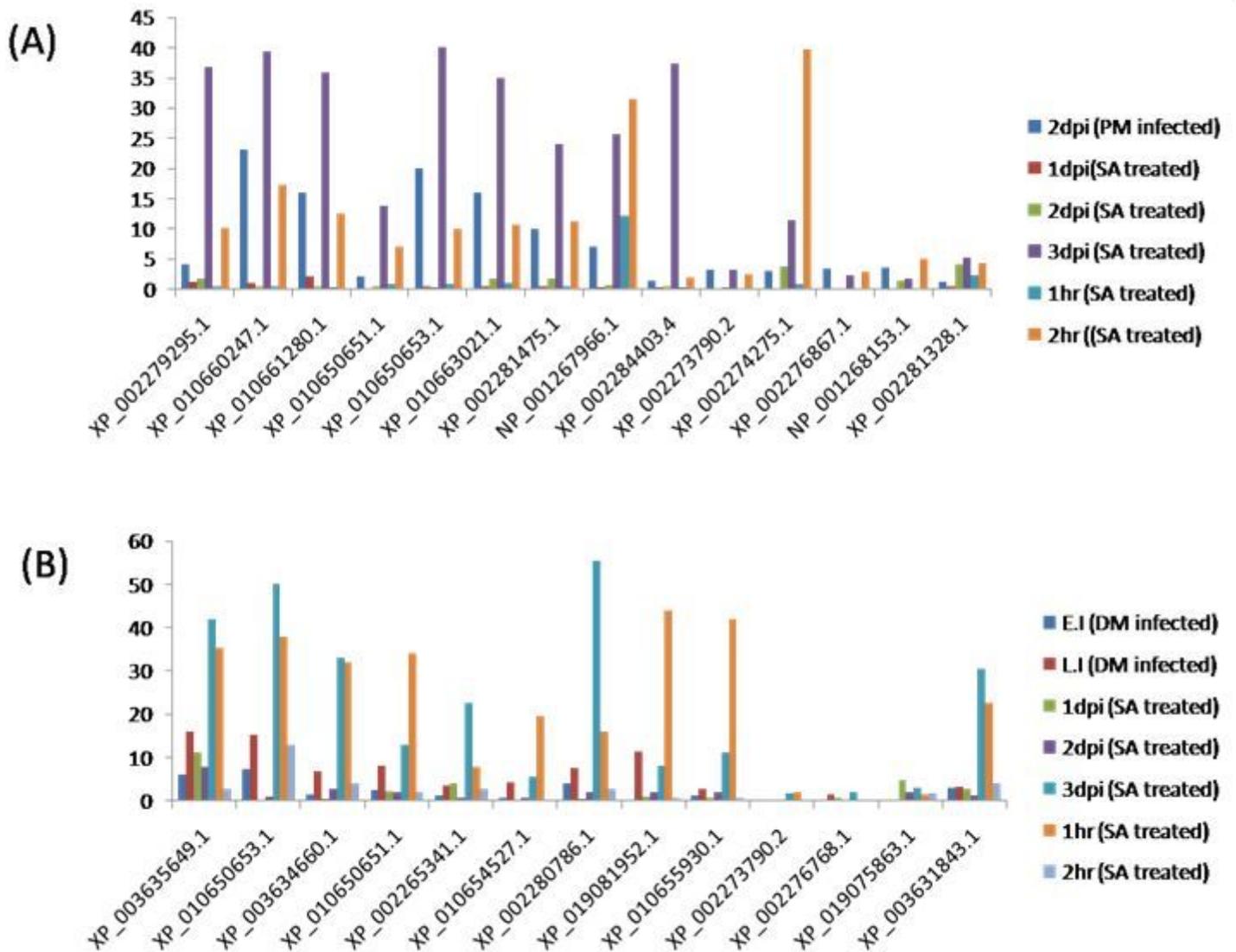


Figure 3

Relative expression levels of defensive 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 defensive genes during PM infection and salicylic acid (SA) treatment. (b.) Real time graph of DM-responsive defensive genes during DM infection and salicylic acid (SA) treatment.

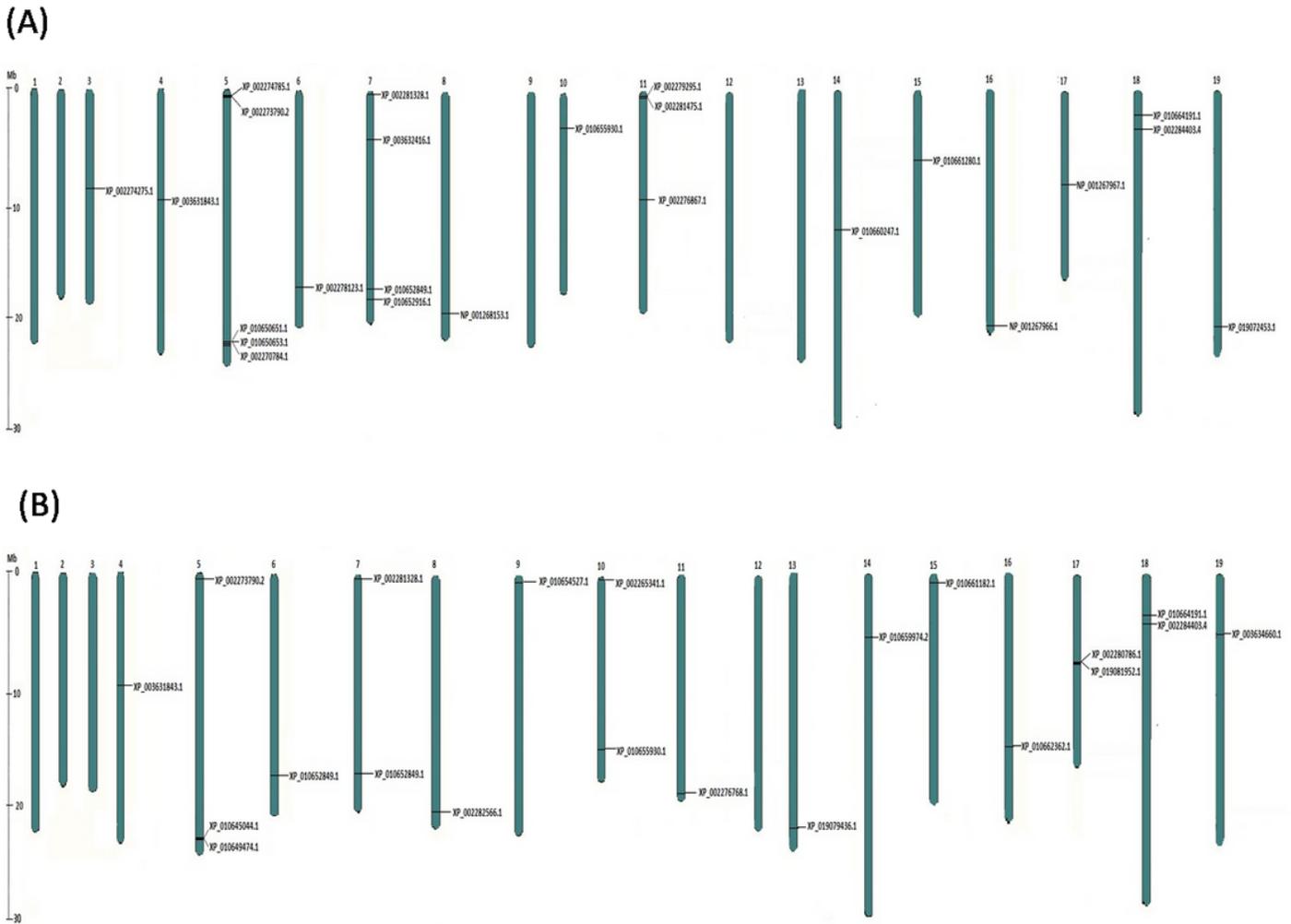


Figure 4

Chromosomal distribution of fungal-responsive defensive genes on 19 chromosomes of *V. vinifera*. (a.) Chromosomal locations of PM-responsive defensive genes. (b.) Chromosomal mapping of DM-responsive defensive genes. The chromosomal position of defensive genes was shown by black lines; chromosome number are exhibited on the top of chromosome and the size of chromosome is shown by vertical line.

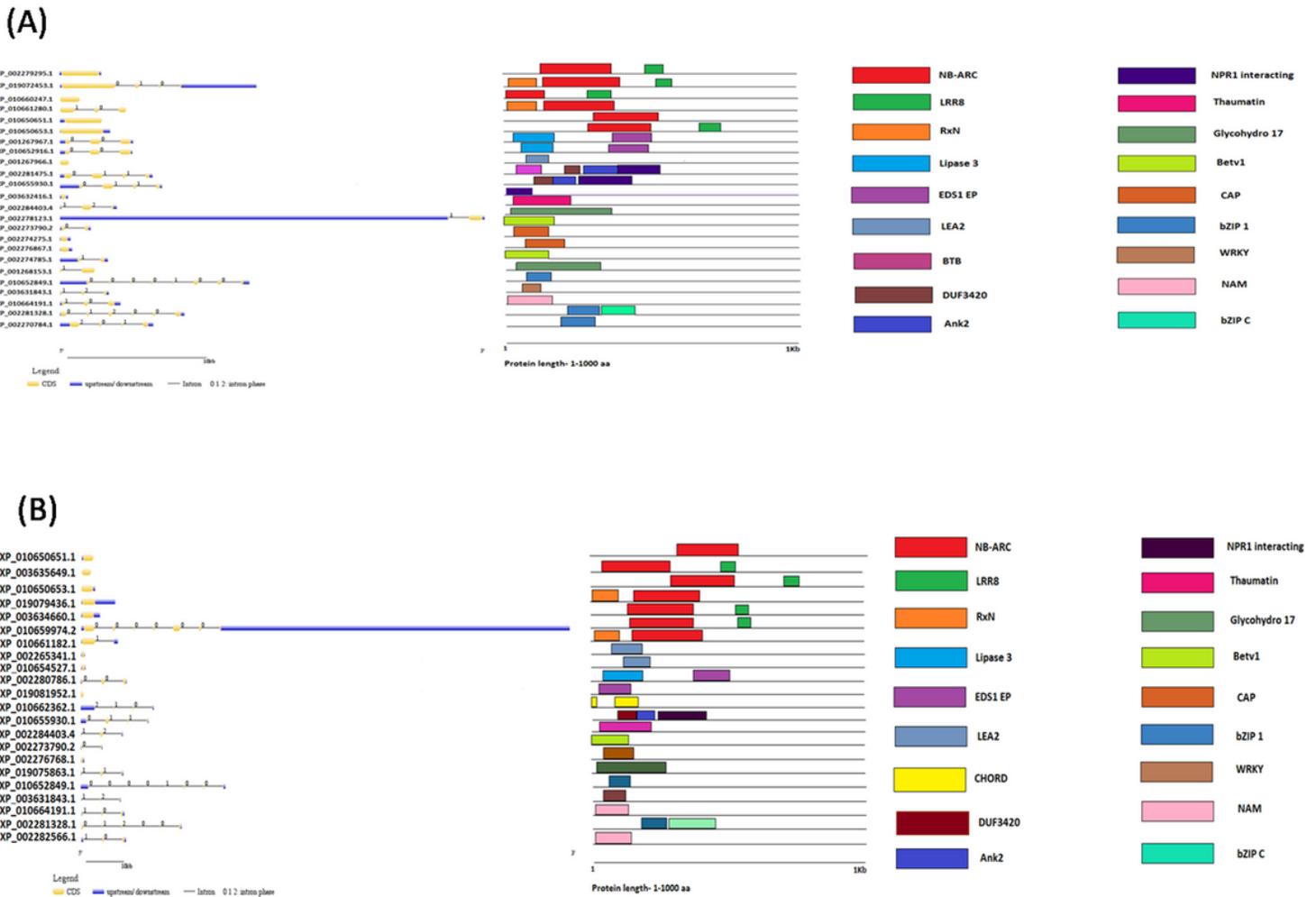


Figure 5

Gene structure and motif analysis of fungal-responsive defensive genes. The organization of introns and exons is represented by GSDS 2.0 server and motif analysis was done by using Pfam database and MEME motif based sequence analysis tool (a.) Gene structure and conserved domains of PM-responsive defensive genes. (b.) Gene structure and conserved domains of DM-responsive defensive genes. Exons are indicated by yellow boxes that are separated by thin lines of introns and UTRs are represented by blue boxes. Conserved motifs for both PM and DM-responsive genes was shown with different colored boxes.

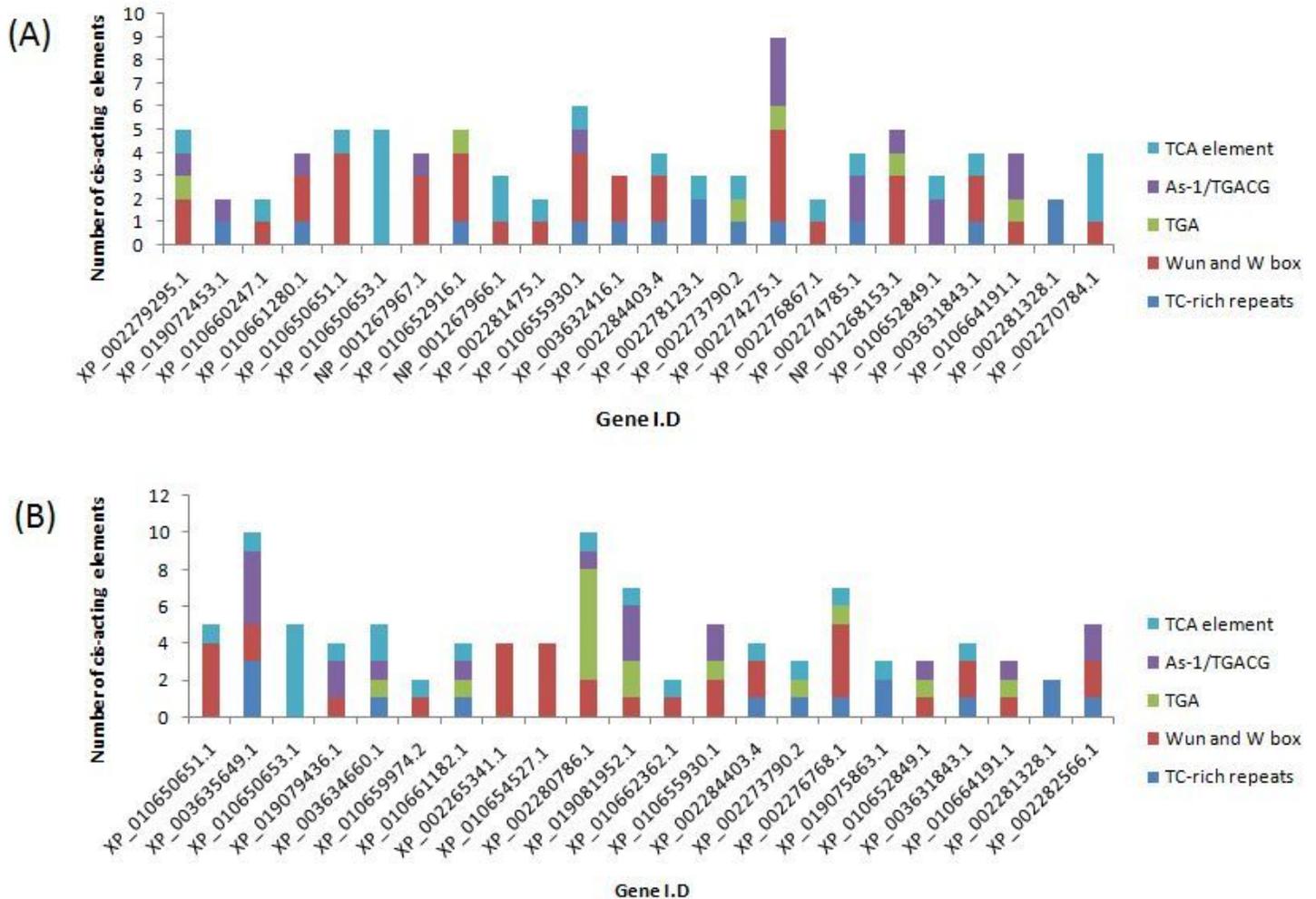


Figure 6

Promoter analysis of fungal stress responsive defensive genes. (a.) PM-stress responsive (b.) DM-stress responsive cis elements were shown in different colors. Each color represents different cis-element.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplFigS1.jpg](#)
- [SupplementaryTableS1.docx](#)
- [SupplementaryTableS3.docx](#)
- [SupplementaryTableS2.docx](#)
- [SupplementaryTableS4.docx](#)