Comparative transcriptome analysis of leaves from two grapevine species reveals an early gene expression profile associated with Elsinoe ampelina resistance

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Abstract

Background: Anthracnose (Elsinoe ampelina) causes extensive damage to grapevine (Vitis vinifera) production worldwide, but the defense mechanisms exhibited by grape are not well understood. Results: In present study, the transcriptome differences of two grape species that exhibit either strong resistance (HR) or sensitivity (HS) to E . ampelina were determined at different time points up to 72 hours post infection (hpi) using RNA-seq profiling. Approximately 172 million high quality reads were obtained from a total of 40 samples. As a result, 3414 differentially expressed genes (DEGs) were identified, with 2,246 in the HR grape V . q uinquangularis Shang-24 accession and 2,019 in the HS grape V . vinifera Red Globe. More up-regulated than down-regulated genes were identified both in the HR and HS samples at each time point except 48 hours post infection. Conclusions: Gene ontology (GO) function and pathway enrichment analysis suggested that the grape transcriptional response to E . ampelina infection involves genes encoding protein kinases, transcription factors, metabolite synthesis, and phytohormone signaling. Although most of the GO functional categories and enriched pathways in response to E . ampelina infection were the same in the two species, the response was apparent much earlier in the HR grape (6 hours post infection and 24 hours post infection) than in the HS grape (48 hours post infection and 72 hours post infection), which may be associated with the contrasting resistance phenotypes. This study provides new insights into the grape defense system involved in responses to E . ampelina infection, and has identified several candidate genes that may be exploited in future biotechnological approaches to increase disease resistance in grapevine.

Background

As one of the most widely cultivated fruit crops, Grapevine (Vitis vinifera L.) has considerable economic, ecological and social value. According to the OIV (International Organization of Vine and Wine) 2018 report, the area used for grapevine production in China has increased 177% to 830,000 hectares since 2000, second only to the 974,000 hectares used for its cultivation in Spain (http://www.oiv.int). However, bacterial, fungal and viral pathogen diseases, such as powdery mildew (Erysiphe necator), downy mildew (Plasmopara viticola), Botrytis cinerea, and anthracnose (Elsinoe ampelina) are major limiting factors in grapevine production and quality.

E. ampelina is a biotrophic fungus originating in European grapes that can cause devastating disease in most of the world’s grape producing areas, but especially in areas with high rainfall [1, 2]. The pathogen attacks all young parts of the grapevine, such as leaves, tendrils, fruits and petioles. As the disease progresses, the symptoms usually appear as numerous circular spots, which enlarge and become sunken, resulting in lesions with round edges [1]. High temperatures and humidity are known to contribute to occurrence of the disease and associated fungal growth [2]. The breeding of grape cultivars that are resistant to anthracnose is an important objective, and previous studies have shown that while European V. vinifera cultivars are highly susceptible, American grapes and Vitis hybrids exhibit varying levels of resistance [3]. Chinese wild Vitis species possess various levels of E. ampelina tolerance and inheritance of this trait is high, making these species a valuable resource for studying E. ampelina tolerance [4].

Among wild Vitis species, V. quinquangularis has been shown to possess potentially durable, non-race-specific E. ampelina resistance, and disease-resistance genes have successfully been transferred from Chinese wild Vitis species to V. vinifera [5, 6]. Thus, understanding the genetic and molecular basis of disease resistance and identifying the associated genes responsible in resistant germplasm has the potential to enhance molecular breeding and to develop new grape varieties with greater E. ampelina resistance.

Plants have evolved elaborate defense systems to fight pathogen attacks and these often involve a signal transduction cascade triggered by infection [7]. The first layer in activating a plant response is to recognize the pathogen-induced damage. General elicitors (or pathogen-associated molecular patterns, PAMPs) and specific elicitors encoded by pathogen Avr genes are recognized by a wide repertoire of receptors [8]. Defense responses include strengthening cell walls [9], the synthesis of pathogenesis-related (PR) proteins and antimicrobial compounds, such as phytoalexins [10], and the hypersensitive response (HR), where cells undergo programmed cell death in the infected region to block pathogen proliferation [11]. The plant defense system is regulated by a suite of phytohormones, including jasmonic acid (JA), salicylic acid (SA), ethylene (ETH), abscisic acid (ABA), brassinosteroids (BR), and gibberellin [12–14].

To generate a platform for molecular assisted selection of disease resistance, we previously constructed a suppression subtractive hybridization cDNA library that was constructed from E. ampelina-infected leaves and identified 20 E. ampelina-defense-related genes [15]. Related studies included an analysis of gene expression in Chinese wild V. quinquangularis, which identified nine differentially expressed cDNA fragments that may be functionally associated with E. ampelina resistance [16], and the identification of a randomly amplified polymorphic DNA (RAPD) marker (OPV02-600) linked to an E. ampelina resistance gene in Chinese wild V. quinquangularis [17]. Research efforts focused on gene expression profiling of V. flexuosa have identified R-related genes, such as VfRLK, VfRPS5-like, and VfCXE, which may contribute to E. ampelina resistance through SA and HR signaling pathways. Expression levels of these genes show a strong correlation with the accumulation of lignin, phytoalexin levels and anthocyanin biosynthesis during E. ampelina infection [18]. Other genes that have been linked to E. ampelina resistance encode chitinase, stilbene synthase, chalcone synthase, polygalacturonase-inhibitor protein, lipid transfer-protein, adenosine triphosphate synthase, glutamine synthetase, and ribulose 1–5 bisphosphate-carboxylase [19–21].

The completion of the grape genome sequence has facilitated study of the molecular mechanism of grape disease resistance at the genome level [22]. Genome-wide analysis of gene expression using RNA-sequencing (RNA-seq) has been widely implemented in grape, including studies of biotic stress responses. In addition, mechanisms of downy mildew resistance in V. amurensis were investigated using Solexa sequencing and a total of 15,249 differentially expressed genes (DEGs) were associated with infection responses [23]. Another study of three Chinese wild Vitis species infected by powdery mildew identified more than 1,600 distinct transcripts that were absent, or highly divergent, from sequences in the grape reference PN40024 genome. Annotations of these genes suggested based on homology to

functionally characterized genes indicated that they were involved in defense responses and secondary metabolism [24]. Although RNA-Seq has been used to analyze the expression profiles of stress response genes in grape, there are few such studies related to E. ampelina infection. Transcriptome analysis of V. flexuosa infected with E. ampelina highlighted the diversity of genes and pathways regulated by E. ampelina infection [25], while another study reported significant changes in the expression of transcripts associated with SA- and JA-mediated pathways and flavonoid biosynthesis in grapevine following E. ampelina infection [18].

In this current study, we performed comparative transcriptome profiling of two grapevine species exhibiting substantially different degrees E. ampelina susceptibility were compared: the largely resistant (termed HR) V. quinquangularis Shang-24 and the highly susceptible (termed HS) V. vinifera Red Globe. DEGs were identified, highlighting those that likely play roles in the host immune response. The outcome of the study supports the initial premise that RNA-Seq technology has great potentiality to mine the target genes.

Results

Transcriptome profiling of grape leaves infected by E. ampelina at different time points

To elucidate how the grape transcriptome responses to the E. ampelina infection, we performed RNA-Seq analysis using leaves from the HR and HS grapevines infected by E. ampelina at 6, 12, 24, 48 and 72 hpi. In total, we generated 20 transcriptome libraries including the controls and the treatments. After filtering out ~ 5% low quality reads, we gained ~ 181 million clean raw reads (Table 1), resulting in a total of ~ 3.2–13.8 million reads per library (Table 1). An average of 82% of the clean reads were mapped to the reference V. vinifera 12 × PN40024 genome [26]. The percentage of mapped reads was lower for the HR libraries than for the HS libraries (Table 1). To further examine the robustness of the RNA-Seq dataset, the correlation coefficients of the transcriptome profiles among the 40 samples were calculated and determined to be 0.95 between two biological replicates (Additional file 1: Table S1).

Table 1 Number of clean reads and mapping ratio following Anthracnose - infected in grape

Sample	Condition	Time points	Raw reads	Clean reads		Mapped reads	
				Number	Percentage	Number	Percentage
HR Shang-24	CK	6hpi	14,140,510	11,860,015	83.87	9,314,097	78.53
		12hpi	13,439,081	12,872,840	95.79	10,437,556	81.08
		24hpi	7,673,331	7,635,641	99.51	5,956,482	78.01
		48hpi	11,451,907	11,378,160	99.36	9,038,595	79.44
		72hpi	7,415,596	7,380,947	99.53	5,683,711	77.01
	T	6hpi	16,079,101	13,845,510	86.11	10,810,352	78.08
		12hpi	11,131,879	10,599,666	95.22	8,193,318	77.30
		24hpi	9,351,927	9,308,207	99.53	6,704,884	72.03
		48hpi	9,774,637	9,708,791	99.33	7,767,761	80.01
		72hpi	12,785,825	12,724,984	99.52	9,904,203	77.83
HS Red Globe	CK	6hpi	4,476,951	4,449,520	99.39	3,884,779	87.31
		12hpi	4,689,692	4,657,025	99.30	4,107,847	88.21
		24hpi	3,240,809	3,220,684	99.38	2,815,530	87.42
		48hpi	10,264,316	9,754,753	95.04	8,519,628	87.34
		72hpi	8,490,490	8067883	95.02	7,152,137	88.65
	T	6hpi	5,877,452	5,844,292	99.43	5,214,179	89.22
		12hpi	4,943,739	4,910,176	99.32	4,276,914	87.10
		24hpi	4,762,133	4,729,857	99.32	4,116,020	87.02
		48hpi	11,469,648	10,841,953	94.53	9,707,162	89.53
		72hpi	9,548,548	9,069,120	94.98	8,017,126	88.40

Global transcriptome changes in grape during E. ampelina infection

A total of 3414 DEGs were found using DESeq analysis from the HR and HS grapes in at least one time point, among which 2,246 were detected in the HR grape samples and 2,019 DEGs in the HS grape samples (Fig. 1a, Additional file 2: Table S2). To identify E. ampelina-responsed genes, we compared the numbers of DEGs to their control at the same time point. Figure 1 showed that the numbers of up-regulated genes were consistently higher than down-regulate in either HR or HS cultivars, except for the HR48 (HR at 48 hpi) sample.

Venn diagram analysis of the DEGs among the five HR time points revealed limited overlap (24 DEGs) across all five time points, but a much greater overlap (427 sequences) between the HR12 and HR24 time points (Fig. 2a). Similarly, we identified 32 genes in common between all five HS time points and 132 between the HS12 and HS24 samples (Fig. 2b). The greatest number of DEGs were distinctive for specific datasets (i.e. 722 in HR12, 1,328 in HR24, 711 in HS6, and 945 in HS72 compared to the controls) with relatively little overlap (8 DEGs; Fig. 2c) across all the samples. These results suggest that gene expression in HR and HS is different with specific genes up- or down-regulated depending on the infection period. We next analyzed the expression patterns of these common DEGs using Genesis software [27],

and found that the 24 common DEGs identified from the HR grape, were all up-regulated throughout the duration of the experiment, except for a NAC transcription factor, which was down-regulated at the late stage of infection (Fig. 2a). Similarly, the 32 common DEGs identified from the HS samples, the majority of which were transcription factors, were also induced at all time points, except one glycine rich protein transcript, which was down-regulated at 48 h (Fig. 2b). Our data indicated that there was little difference in the identity and number of common DEGs between HR and HS grapes. Furthermore, the eight common DEGs identified from both HR and HS grapes showed significant up-regulation during all the time points in both species (Fig. 2c), suggesting a potential role in grape defense against E. ampelina, although the biological functions of these genes are still unknown.

Genesis K-means analysis of the 3414 DEGs revealed four major expression pattern groups (Fig. 3a). Expression profiles of most genes in group I (390 DEGs) were relatively stable during all the five time points in the HS grape, but up- and/or down-regulated significantly in the HR grape from 6 to 24 hpi. Based on this expression variety in the HR grape, we divided group I DEGs into three sub- clusters. Majority of the 188 DEGs in cluster 1 showed stable expression 6–12 hpi, then were highly induced at 12–24 hpi. Cluster 2 genes (165) showed rapid greater expression at 6–12 hpi, while many genes in cluster 3 (37 DEGs) were continuously induced from 6 to 48 hpi in the HR grape. Group II expression (263 DEGs) profiles varied dramatically in both the HR and HS grapes during the whole infection time. These genes could be classified into three sub-clusters. Cluster 4 DEGs (117) showed similar expression profiles in both the two grape species at whole time, except between 6–12 hpi, where they were induced in the HR grape but suppressed in the HS grape. However, the expression profiles of genes in cluster 5 (79 genes) were largely opposite between the two grape species during the whole time. Cluster 6 genes (165) were substantially up-regulated during the initial stage of infection, but down-regulated at 48 hpi in both species. Group III expression (548 DEGs) profiles showed the opposite expression patterns from group I genes. Most were relatively stable in the HR grape after infection but were up- or down-regulated in HS grape. The expression profiles of genes in cluster 7 (62) and cluster 8 (395) varied similarly in the HS grape during the E. ampelina infection, being up-regulated at 6–12 hpi and 24–72 hpi, but down-regulated at 12–24 hpi, although the changes in expression were more rapid for cluster 7 than for cluster 8 genes. Cluster 9 genes (91) were up-regulated to a minor degree at 6–12 hpi, then remained stable at 12–48 hpi followed by significant up-regulation at 48–72 hpi. Group IV genes (2,213) did not show expression differences between the HR and the HS grapes. Within the three group IV sub-clusters, the patterns of those in cluster 10 genes (847) were very similar between the two species, while most genes in cluster 11 (865) only showed similar expression patterns in both species after 12 hpi. Expression of most cluster 12 DEGs (501) were induced at earlier stages (6–24 hpi) in the HR grape then suppressed during the later stages of infection (48–72 hpi). In contrast, these genes showed suppressed expression at 6–48 hpi followed by induction at 48–72 hpi in the HS grape.

To more clearly identify DEGs linked to E. ampelina infection, the heatmap of the 851 common DEGs between two grapes after E. ampelina infection (Fig. 1a) was generated from the RPKM data (Fig. 3b). Base on the heatmap, the 851 common DEGs were divided into six classes (Fig. 3b; Additional file 3: Table S3). Most genes in Class I, V and VI, involved in stress response and metabolic processes such as

phenylalanine ammonia-lyase, receptor-like protein kinase, chalcone synthase, heat shock proteins and cytochrome P450 superfamily proteins, were highly abundant and induced in the HR grape at 6, 12 and 24 hpi. Notably, the expression of these DEGs was induced in the HS grape much later at 72 hpi. However, the expression pattern of Class II genes was largely opposite between two grape species at 6 and 12 hpi. Class III (i.e. 60S ribosomal protein, histone) genes first exhibited down-regulation in both the HR and HS grapes and were significantly down-regulated at 24 hpi. Class IV genes (e.g. ETH responsive transcription factor 1, NAC transcription factor and WRKY transcription factor) were highly induced at 6 hpi in the HS grape and at 24 hpi in the HR grape. Overall, most DEG expression profiles present in both grape species were consistently up- or down-regulation, but the response time was much faster in the HR grape (6 hpi and 24 hpi) than in the HS grape (48 hpi and 72 hpi).

Functional classification and pathway enrichment analysis

The 3,414 DEGs related to E. ampelina infection of grape were arranged into 98 functional categories based on the GO (Gene Ontology) analysis (Additional file 4: Table S4). They comprised three major enrichment categories: ‘molecular functions’ (25), ‘biological processes’ (47) and ‘cellular components’ (26). In the ‘molecular function’ category, genes associated with ‘binding’ (12.7%), ‘protein binding’ (12.4%), ‘nucleotide binding’ (9%), ‘DNA binding’ (5.3%), ‘nucleic acid binding’ (0.9%), ‘RNA binding’ (0.9%), ‘carbohydrate binding’ (0.9%), ‘lipid binding’ (0.6%), ‘chromatin binding’ (0.2%), ‘receptor binding’; (0.1%), and ‘oxygen binding’ (0.04%) were classified as binding, which was the most abundant category (Fig. 4a). Other abundant groups were ‘catalytic activity’ (11.1%), ‘transferase activity’ (7.3%), ‘hydrolase activity’ (6.9%), ‘kinase activity’ (4.4%), ‘receptor activity’ (3.2%), ‘transporter activity’ (2.4%), and two transcription-related groups that include ‘transcription factor activity’ (2.2%) and ‘transcription regulator activity’ (1.84%). In the ‘biological processes’ categories, ‘response to stress’ (8.3%), ‘response to abiotic stimulus’ (4.0%), ‘response to endogenous stimulus’ (3.5%), ‘response to biotic stimulus’ (2.6%), and ‘response to external stimulus’ (1.4%) were grouped in the plant host defense category, which involves plant responses to various abiotic and pathogen/insect stresses. Gene functions in ‘metabolic process’ (6.9%), ‘lipid metabolic process’ (2.1%), ‘protein metabolic process’ (2.0%), ‘carbohydrate metabolic process’ (1.9%) and ‘DNA metabolic process’ (1.2%) represented the second most abundant functional gene group (Additional file 4: Table S4). In addition to these two major categories, additional categories identified included ‘biosynthetic process’ (5.2%), ‘transport’ (3.5%), ‘transcription’ (3.4%), ‘signaling transduction’ (3.3%), and two cell structure groups that included ‘cellular process’ (8.4%) and ‘cellular component organization’ (3.1%). In the ‘cellular component’ category, the abundant groups included ‘membrane’, ‘nucleus’, ‘plasma membrane’, and ‘cytoplasm’ (Fig. 4b; Additional file 4: Table S4). We also found that all the 47 GO biological process terms were present in the two different grapes and that the first five most abundant GO terms were the same, including ‘response to stress’, ‘cellular process’, ‘metabolic process’, ‘biosynthetic process’, and ‘response to abiotic stimulus’ (Fig. 4c; Additional file 5: Table S5). However, genes related to ‘metabolic process’, ‘transcription’, and ‘cellular amino acid and derivative metabolic process’ were more abundant in the HS grape than in the HR grape, where ‘cellular process’, ‘cell cycle’, ‘DNA metabolic process’, and ‘cell death’ were more enriched. These results indicate that many biological

processes changed in both the resistant and susceptible grapes after E. ampelina infection, most of which are the same, including some defense-related processes.

For further functional categorization, pathway enrichment analyses were performed using Plant MetGenMAP. As a result, all the significant DEGs from both the HR and HS samples were grouped into 22 biochemical pathways ($P < 0.05$; Fig. 5a). DEGs HR grape were assigned to 10 significantly changed pathways, and 21 in the HS grape (Fig. 5a). Some of them were involved in plant responses to pathogen infection, and have also been reported by other studies. For example, variations in expression of genes involved in plant hormone and flavonoid biosynthesis following Erysiphe necator-infection in V. pseudoreticulata have been previously reported [28]. Major pathways related to plant responses following E. ampelina infection in HR grape included: flavonoid biosynthesis (53), salicylate biosynthesis (21), phenylpropanoid biosynthesis (21), suberin biosynthesis (23), triacylglycerol degradation (20) and jasmonic acid biosynthesis (9) (Fig. 5b). Pathways unique to the E. ampelina-infected HS grape included methanol oxidation to formaldehyde (8), oxidative ethanol degradation III (8), removal of superoxide radicals (9), cyanide degradation (9) and monoterpene biosynthesis (10). Pathways common to the two genotypes included flavonoid biosynthesis, asparagine biosynthesis, salicylate biosynthesis, phenylpropanoid biosynthesis, suberin biosynthesis, and triacylglycerol degradation (Fig. 5a). Most DEG expression patterns from these metabolic pathways were significantly up-regulated (Fig. 5b). For example, the flavonoid biosynthesis (P1) pathway and the relevant DEG transcripts were enriched at all time points in HR grape after E. ampelina infection. The DEGs involved in P1 were also up-regulated in HS grape, but not until 48 hpi. Only a few DEGs involved in the asparagine biosynthesis pathway were down-regulated in both genotypes at 24 hpi and 72 hpi. Interestingly, jasmonic acid biosynthesis was only enriched in HR grape. We also noticed that there was a higher number of enriched pathways in the HR grape than in the HS grape during the initial stage after infection: eight pathways significantly changing at 6 h in HR and only three in HS grape. Moreover, 71 DEGs from significantly changed pathways were observed in HR grape at 24 hpi, which was also the highest number of all-time points. In the HS grape, the number of enriched pathways gradually increased from three at 6 hpi to 13 at 72 hpi, and the number of DEGs changed from nine at 6 hpi to 113 at 72 hpi. Pathway enrichment analysis revealed that transcripts involved in flavonoid biosynthesis and phenylpropanoid biosynthesis play very important roles in the grape responses to E. ampelina infection. The resistant and susceptible grape varieties responded differently as the significantly changed pathways were observed earlier in the HR grape (6 and 24 hpi) than in the HS grape (48 and 72 hpi).

Genes potentially involved in grape resistance to E. ampelina infection

GO functional and pathway enrichment analyses revealed that there were a lot of defense-related genes undergoing transcriptional changes in both the HR and HS grapes after E. ampelina infection. Most of these genes belong to Class I, II, IV, V and VI described in Fig. 3b (Additional file 3: Table S3), including protein kinases, transcription factors, metabolite synthases, and hormone and pathogen-related genes (Fig. 6; Additional file 6: Table S6). Below, the gene families of interest for potential engineering of pathogen resistance are further described.

Protein kinases and protein

In plant pathogen-induced immunity, the roles of kinases have been well defined. In particular, it has been shown that receptor-like kinases (RLKs) trigger initial immune responses.[29].Interestingly, there were many up-regulated RLKs in both the HR and HS grapes after searching our DEGs data. In general, most of them were significantly induced in the HR grape during earlier stages, and only some were up-regulated in HS grape at later stages. Similarly, several calmodulin-binding proteins and pathogenesis related (PR) proteins including beta-1, 3-glucanase, chitinase, thaumatin-like protein and disease resistance protein were also up-regulated in HR grape during the earlier stages (Fig. 6).

Transcription factors

Transcription factors are important regulators of both biotic and abiotic stress responses as part of the immune system [30].

Transcription factors (TFs) are an integral part of the immune system that are vital in regulating plant defense responses. Many TFs were found in our analysis, which include members of the WRKY, MYB, ERF1, NAC domain, zinc finger and bHLH families (Fig. 6). Most of them play critical roles in plant defense against biotic stress in previous reports [7, 31–34], and many were induced by E. ampelina infection in the HR grape at the earlier stages (Fig. 6). In contrast, a number of the WRKY TFs were up-regulated earlier in HS grape than in HR grape and about one third of them only showed differential expression in the HS grape (Fig. 6). The zinc finger-containing TFs were suppressed at earlier time points after infection in the HR grape but up-regulated in the HS grape, and among the 37 MYBs identified, five were down-regulated at 24 hpi but only in the HR grape (Fig. 6). Besides, we also found lots of heat-shock TFs (HSF) described as crucial regulators in phytohormone signaling. Figure 6 showed the detailed expression patterns of these TFs.

Metabolism and oxidative stress

Among the E. ampelina infection-related genes, there were several genes participating in plant metabolism (Fig. 5). Examples of these genes were involved in phenylpropanoid pathway, such as flavonoid biosynthesis and phenylpropanoid biosynthesis (Fig. 5a). Most of them were induced in the HR grape at 6 hpi and/or 24 hpi but not up-regulated until 48 hpi and/or 72 hpi in the HS grape, suggesting that metabolite synthesis-related genes may also take part in the grape defense against E. ampelina (Fig. 5b).

Reactive oxygen species (ROS) are important in plant defense against biotic stress because they can protect host cellular components from oxidative damaged caused by free radicals released during the oxidative burst [35].As one of the major ROS scavengers, glutathione is known to play a crucial role in plant response to pathogen infection [36]. We found an enzyme gene involved in glutathione synthesis (glutathione S-transferase; GSTU) was highly induced at earlier time points (6–24 hpi) in the HR grape and later (48–72 hpi) in the HS grape (Fig. 6). Other ROS-related genes including cinnamyl alcohol dehydrogenase and peroxidase, showed similar expression patterns (Fig. 6), while one peroxidase was highly induced during all the time after infection in both the HR and HS grapes (Fig. 2c). Finally, four

catalases were identified as being up-regulated only in the HS grape at 12 and 24 hpi (Fig. 6). Together, these data reveal that the expression of genes involved in phenylpropanoid and flavonoid pathways is extremely dynamic in grape after E. ampelina infection.

Phytohormone signaling related genes

Expression of multiple phytohormone signaling related genes were also affected by E. ampelina infection (Fig. 6). For example, an ETH signal transduction-related gene (ERF1) was up-regulated in both genotypes but with the delay in the HR grape. Eight TIFY proteins, three of which were jasmonate ZIM-domain (JAZ) genes, which function as repressors of JA signaling were induced in the HR grape at 24 hpi and in the HS grape at 72 hpi. In addition, jasmonate signal-related genes, lipoxygenases (LOX3), allene oxide cyclase (AOC4) and allene oxide synthase (AOS), showed similar expression patterns (Fig. 6). Disease susceptibility 1 (EDS1) and pathogen-related protein 1 (PR1) are genes associated with SA mediated defense responses, and were up-regulated in both grape varieties. In addition, three genes related to the ABA signaling pathway were induced earlier in the HS grape (Fig. 6). Our results also highlighted the involvement of GA in grape defense responses to E. ampelina as beta-glucosidase (BGL) expression varied substantially during E. ampelina infection (Fig. 6).

Discussion

Generation of a comprehensive transcriptome profile of E. ampelina infected grape leaves

While V. labrusca, Vitis sp. (hybrids) and Chinese wild Vitis species, especially V. quinquangularis, are more anthracnose tolerant than V. vinifera cultivars [4], much remains to be learnt about the molecular mechanisms of the grape response to infection. Genes involved in protein binding and oxidoreductase activity were the most abundant functional gene group in V. flexuosa after E. ampelina infection. However, a single grape genotype was used and only one time point analyzed[25]. In the current study, 2,246 and 2,019 DEGs from resistant and susceptible grape varieties, respectively, were analyzed at five different time points after E. ampelina infection.

The different HR and HS genetic backgrounds provided an opportunity to identify genes involved in grape defense responses. Differential expression analyses revealed that the number of DEGs gradually increased during the early stage of E. ampelina infection in the HR grape, reached a maximum at 24 h and then decreasing, compared to the control, while more DEGs were present in the HS than in the HR variety at 6 h and 72 h. More up- than down-regulated genes were identified during the whole time except at 48 h. Our results are in accordance with previous reports that infections by other pathogens, such as powdery and downy mildews, drive transcriptional induction more than suppression [37, 38]. GO functional and pathway enrichment analyses indicated that responses to stress, metabolic processes, transcription factor and transmembrane transport related genes were associated with the DEGs. A comparison of our analysis with previous transcriptome studies of pathogen infection in grape, suggests that the observed resistance in the HR grape was induced and not based on differences in basal gene expression between resistant and susceptible species [28, 38]. We found many that cytochrome P450

(CYP450) genes were expressed in both genotypes, indicating that CYP450 superfamily proteins may regulate defenses against E. ampelina infection (Fig. 6). CYPP450 proteins have been suggested to mediate the synthesis and metabolism of many primary and secondary metabolites related to defense against a range of pathogenic microbes and insects [39, 40], and the CYP736B gene has been shown to be involved in the grape defense response to Xylellafastidiosa [41].

Pathways involved in grape E. ampelina resistance are similar to those used in responses to other stresses

GO functional analysis of the DEGs gave an overview of the grape response to E. ampelina, and ROS-related genes were prominent in both HR and HS samples. Moreover, data from the pathway enrichment analysis showed that ROS and oxidative-related pathways were significantlyup-regulated (Fig. 5). The expression of many ROS scavenging related genes, taking peroxidase genes for example, was up-regulated in both HR and HS grapes during infection, suggesting that E. ampelina infection may increase the accumulation of ROS. In many plants studied to date, Nucleotide Binding Site/Leucine-Rich Repeat (NBS-LRR) proteins and RLKs have been shown to act as resistance (R-) genes to recognize the pathogen-derived elicitors [42, 43]. NBS-LRRs function in defense responses, while RLKs have been implicated in a wide range of signaling pathways as transmembrane proteins with putative extracellular domains and intracellular protein kinase domains [42]. Recently, a study of grapevine defense responses to E. ampelina identified six genes encoding RLKs that were highly induced [44].We found both NBS-LRRs and RLKs to be abundant in both HR and HS grapes, consistent with a role in resistance to E. ampelina infection.

Transcription factors regulate the expression of many abiotic and biotic stress-responsive genes [45]. Many WRKY, MYB, NAC, bHLH, and zinc-finger proteins were identified in this study and their expression patterns are shown in Fig. 6. In contrast to previous studies of downy mildew responses, which revealed many WRKY transcripts to be strongly induced by infection, and much more so in the resistant V. riparia grape than in the susceptible V. vinifera grape [38], our results indicated that most of the WRKY factors were up-regulated in both genotypes, but at earlier time points in the HS than in HR leaves. We speculate that the different types of damage inflected by oomycete- and fungal- pathogens may explain the discrepancy in these transcriptional responses. WRKY factors are regulated by interaction with MAP kinase in other species [46, 47]; here we found that both WRKYs and MPK1 were induced in grape by E. ampelina infection, which is consistent with previous studies [28, 38] in accordance.

Calcium is a secondary messenger that participates in responses to abiotic and biotic stresses [48, 49], which is consistent with our observation that calmodulin-binding proteins, calcium-transporting ATPase and calmodulins (CaMs), were up-regulated in both HR and HS grapes, with a rapid early induction. Our previous study identifying differentially expressed grape genes associated with E. ampelina resistance through suppressive subtraction hybridization (SSH) revealed that CaM expression was strongly induced at 6 hpi in resistant material, while its transcripts remained at similar levels to the control throughout the entire time course in susceptible material [15].

Phytohormone signaling pathways are involved in E. ampelina resistance

E. ampelina infection also induced the expression of genes associated with phytohormone signaling pathways [15], which play roles in the adaptation of plants to biotic and abiotic stresses [50]. JA, SA, and ETH are involved in stress signaling upon pathogen infection, while ABA regulates responses to abiotic stresses, such as drought, low temperatures, and osmotic stress[12, 13]. Auxin and GA are generally thought of as negative regulators of plant innate immunity [50]. In this current study, JA-related genes (JAZ, LOX, AOC and AOS) were up-regulated at early time points in the HR grape and at late time points in the HS grape. We previously reported that JAZ7is a candidate gene for conferring for resistance to powdery mildew infection in grape [51], which is consistent with studies showing that many herbivory- and pathogen-induced responses in grapevines are mediated by the JA pathway [28, 52]. In contrast, SA signaling is typically involved in plant resistance to biotrophic pathogens[53], with the positive SA regulator EDS1 conferring basal defense against various pathogens by interacting with a subset of plant resistance (R) genes [54]. Three EDS1-like genes with distinct expression patterns were identified in V. flexuosa inoculated with E. ampelina, and two of them(VfEDL2 and VfEDL3)are known to be induced by infection [55]. In our study, up-regulation of EDS1 at the early time point in the HR grape is indicative of an SA pathway response in the resistant grape. Our results also indicated the involvement of ERFs (ETH pathway), which were more abundantly expressed during the initial E. ampelina infection stage in the HS grape than in the HR grape. This agrees with the results of previous studies indicating that ERFs are involved in grape responses to Botrytis cinerea infection [56]. Taken together, our results highlighted the involvement of ABA, auxin and GA in grape defenses against E. ampelina and the complexity of the phytohormone signaling pathways.

Stress responses include secondary flavonoid metabolites

Many secondary metabolites, such as flavonoids, phenylpropanoids and terpenoids, play roles in plant defense against herbivores, pests and pathogens [40, 57]. In the current study, GO functional and pathway enrichment analyses confirmed the contribution of secondary metabolites, via flavonoid and phenylpropanoid biosynthesis, to the defense of grape against E. ampelina. Notably, the flavonoid metabolite-related chalcone synthase gene, CHS, and the phenylpropanoid metabolite-related phenylalanine ammonia-lyase gene (PAL) were up-regulated at the earlier stages (6 and 24 hpi) in the HR grape and during the later stages (48 and 72 hpi) in the HS grape. Previous reports have demonstrated a rapid accumulation of flavonoid phytoalexins, lignin, and phenolic compounds in grape infected with powdery mildew [58]. Moreover, stilbene synthase (STS) expression was induced in the anthracnose-tolerant ‘Noble’ cultivar, but remained stable in susceptible cultivars after E. ampelina infection [19].Compared to previous reports, in which flavonoid and phenylpropanoid biosynthesis pathways were represented by DEGs [28, 38], our study provide more detailed information pertaining to the timing of phenylpropanoid biosynthesis induction in resistant and susceptible grapes .

Unique transcriptional changes in different grape species in response to E. ampelina infection

Transcript profiling of grape infected with other diseases has revealed differences in transcriptional changes between compatible and/or incompatible interactions [37, 38]. As expected, our data showed

that the transcriptomes of both HR and HS grapes respond to E. ampelina infection and that there are overlaps in these responses. Although both species responded to infection with broad changes in gene expression, the response was much faster in the HR grape, with a peak of gene induction at 24 hpi. Many genes encoding RLKs and leucine-rich repeat receptor-like proteins were specifically induced in HR grape at early stages of infection, particularly at 24 hpi (Fig. 6). These genes function to mediate pathogen recognition and trigger defense responses in many species [59]. In addition, most of the signal transduction, primary and secondary metabolism, and defense-related transcripts showed similar expression patterns (Fig. 3b; Fig. 6). A previous comparative transcriptome analysis of cold stress resistant and susceptible Para rubber tree (Hevea brasiliensis) genotypes revealed a more rapid and intensive response in the resistant rubber tree [60], and similar results have been reported for grape resistance to downy mildew infection [38]. Interestingly, only transcription factors, such as WRKY and ERF genes, were induced earlier in the HS grape (Fig. 6). Taken together the data show that grape resistance to E. ampelina is not a single genetic trait, but the results of a complex network, including diverse physiological and biochemical processes. Pathogen resistance involves not only the recognition and activation of resistance genes, but also appropriate timing of their expression and that of the associated signal transduction pathways. Most of the GO functional categories and enriched pathways found in response to E. ampelina infection were the same in the two grape genotypes; however, the responses occurred much earlier in the HR grape (6 and 24 hpi) than in the HS grape (48 and 72 hpi), which may account for the differences in resistance between the two species.

Conclusions

In summary, genes encoding protein kinases, transcription factors, metabolite synthesis, and phytohormone signaling were involved in response to Elsinoe ampelina. Moreover, all DEGs containing functional variants were identified, so as relate biological processes and pathways. This paper provides evidence for further functional research and adds our knowledge for Elsinoe ampelina resistance in grape.

Materials And Methods

Plant materials

Grapevines used in this study were Chinese wild V. quinquangularis accession ‘Shang-24’, and V. vinifera cv ‘Red Globe’. ‘Shang-24’ is highly resistant to E. ampelina while ‘Red Globe’ is susceptible [4, 15]. Both ‘Shang-24’ and ‘Red Globe’ were maintained in the grape germplasm resource orchard at the Northwest A&F University, located in Yangling, China (34° 20’ N, 108°24’ E). All the plant materials used and collected in this work comply with China’s guidelines and legislation.

Pathogen inoculations

E. ampelina used in this study was isolated from diseased grapevine plants in Yangling, China. For E. ampelina inoculation, young V. quinquangularis‘Shang-24’ and V. vinifera cv ‘Red Globe’ leaves were

inoculated as previously described [15]. Leaves sprayed with sterile water were used as controls. To collect samples that represented the E. ampelina infection process, leaves were harvested at 6 h, 12 h, 24 h, 48 h and 72 hours post inoculation (hpi) based on the proposed E. ampelina infection cycle and our previous studies [1, 2, 15]. Leaves were excised from the third to the fifth internode beneath the apex, at a time where the shoots were 25–35 cm in length. At each time point, eight leaves from eight separate plants were combined to form one sample, then immediately frozen in liquid nitrogen and stored at -80 °C until use. The experiment was repeated to generate two biological replicates.

RNA isolation and sequencing

Total RNA was extracted as previously described from leaves of E. ampelina-inoculated and mock-inoculated leaves [61]. The integrity of the total RNA was assessed by electrophoresis on 1.0% agarose gels and quantified with a NanoDrop spectrophotometer (ND-7000, NanoDrop, USA). Strand-specific RNA-Seq libraries were constructed as described in ref. 62 and two biological replicates were sequenced on the Illumina HiSeq 2000 platform (at the Genomics Resources Core Facility at Weill Cornell Medical College, NY, USA) using the single-end mode [62].

Bioinformatics analysis

The raw reads were selectively filtered using Trimmomatic to remove adaptor and low-quality sequences [63], and after discarding reads shorter than 40 bp, the resulting reads were aligned to the ribosomal RNA database [64] using Bowtie [65] to remove ribosomal sequences. The resulting high-quality cleaned reads (final reads) were mapped to the V. vinifera 12 × PN40024 genome using Tophat [26, 66]. The counts of mapped reads from each sample were derived and normalized to reads per kilobase of exon model per million mapped reads (RPKM). DEGs between E. ampelina-inoculated and mock-inoculated samples at each time point were identified using DESeq 1.8.3 software [67] and the raw count data. Raw P values were adjusted for multiple testing using a false discovery rate (FDR) [67, 68] and genes with an FDR < 0.05 and fold-changes > 2.0 were considered to be differentially expressed. Gene ontology (GO) term and pathway enrichment analysis were conducted using Plant MetGenMAP [69]. The DEGs were clustered based on their expression patterns by the Genesis K-means method [27].

List Of Abbreviations

hours post infection, hpi; differentially expressed genes, DEGs; Gene ontology, GO; pathogen-associated molecular patterns, PAMPs; jasmonic acid, JA; salicylic acid, SA; ethylene, ETH; abscisic acid, ABA; brassinosteroids, BR; randomly amplified polymorphic DNA, RAPD; receptor-like kinases, RLKs; pathogenesis related, PR; heat-shock TFs, HSF; Reactive oxygen species, ROS; Reactive oxygen species, ROS; jasmonate ZIM-domain, JAZ; allene oxide cyclase, AOC4; allene oxide synthase, AOS; beta-glucosidase, BGL; Nucleotide Binding Site/Leucine-Rich Repeat, NBS-LRR; calmodulins, CaMs; suppressive subtraction hybridization, SSH; Transcription factors, TFs

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

X.W. and M.G. designed the research; R.W. and C.J. provided technical assistance; M.G. and C.C. analyzed the data; Z.F. provided the experimental materials; M.G. and X.W. wrote the article with contributions of all the authors. All authors read and approved the final manuscript.

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Supplementary Files Legend

Additional file 1: Table S1. Correlation coefficients of transcriptome profiles among RNA-Seq samples from different grapes.

Additional file 2: Table S2. List of all DEGs detected by RNA-Seq analysis after *E. ampelina* infection in the HR and HS grapes compared with control.

Additional file 3: Table S3. Expression pattern of DEGs common to both HR and HS grapes following hierarchical clustering.

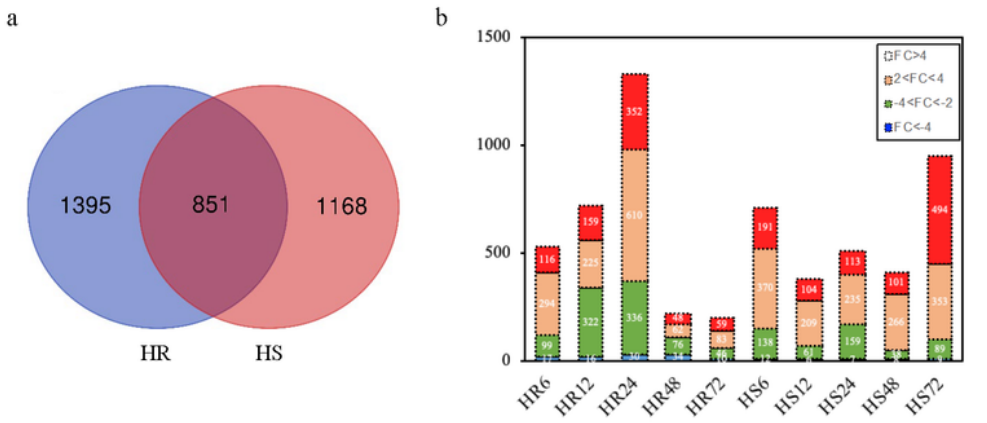
Additional file 4: Table S4. Gene ontology (Go) analysis of all DEGs at FDR < 0.05.

Additional file 5: Table S5. Functional categorization of DEGs after *E. ampelina* infection in the HR and HS grapes based on biological process of Gene Ontology (GO).

Additional file 6: Table S6. Detailed list of resistance-related genes in the HR and HS grapes that are induced or suppressed in response to *E. ampelina* infection.

Figures

Figure 1



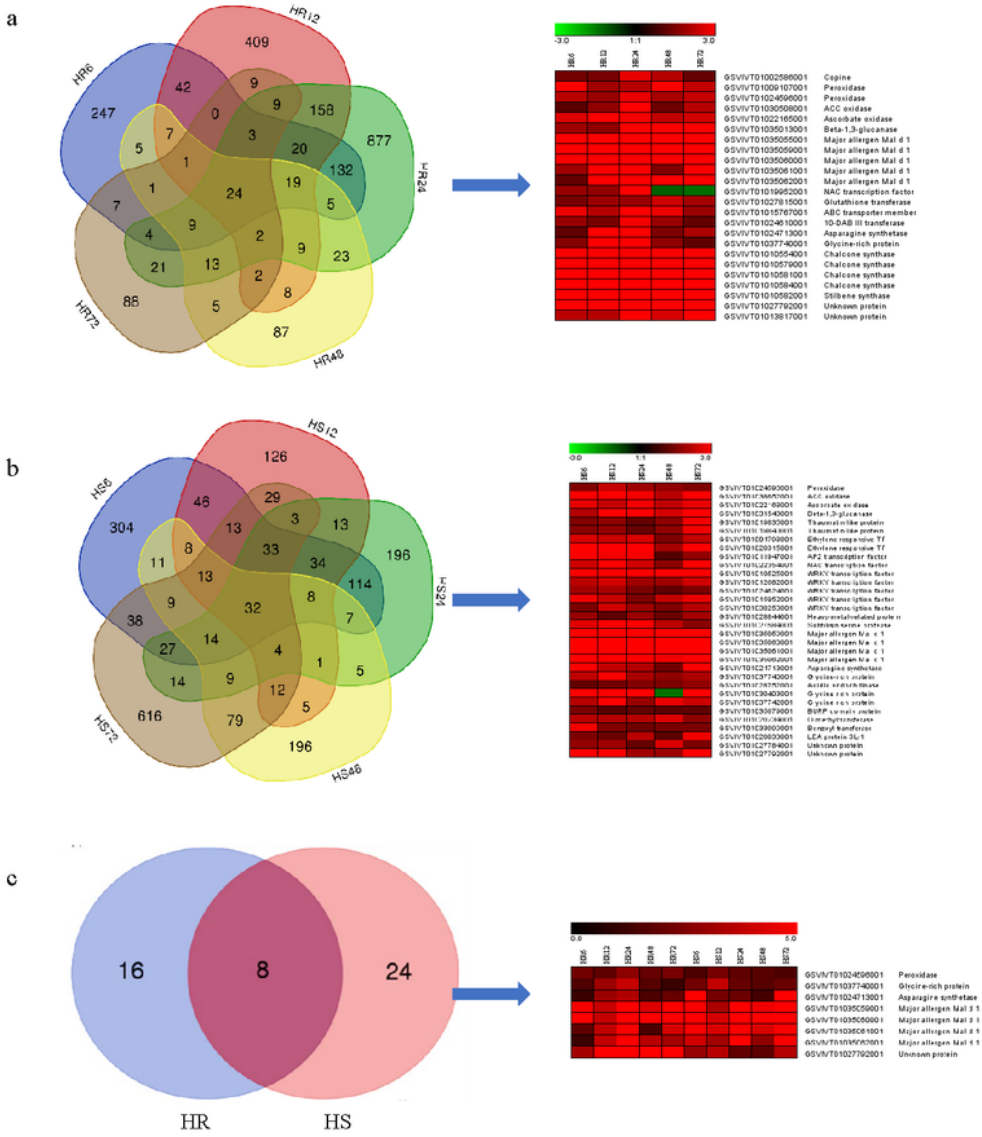
1

Figure 1

Number of differentially expressed genes (DEGs) in the leaves of HR and HS grapes after Elsinoe ampelina infection at different time points (P value ≤ 0.05 and fold change (FC) ≥ 2). (a) Venn diagram showing the number of common DEGs following E. ampelina infection in the HR and HS grapes. (b) Number of DEGs in the HR and HS grapes after E. ampelina infection. Expression FC (fold change) calculated as log2 of the ratio of gene expression when compared to the respective control samples. The

values indicated in the boxes represent the number of DEGs at different time points in the HR and HS grapes.

Figure 2



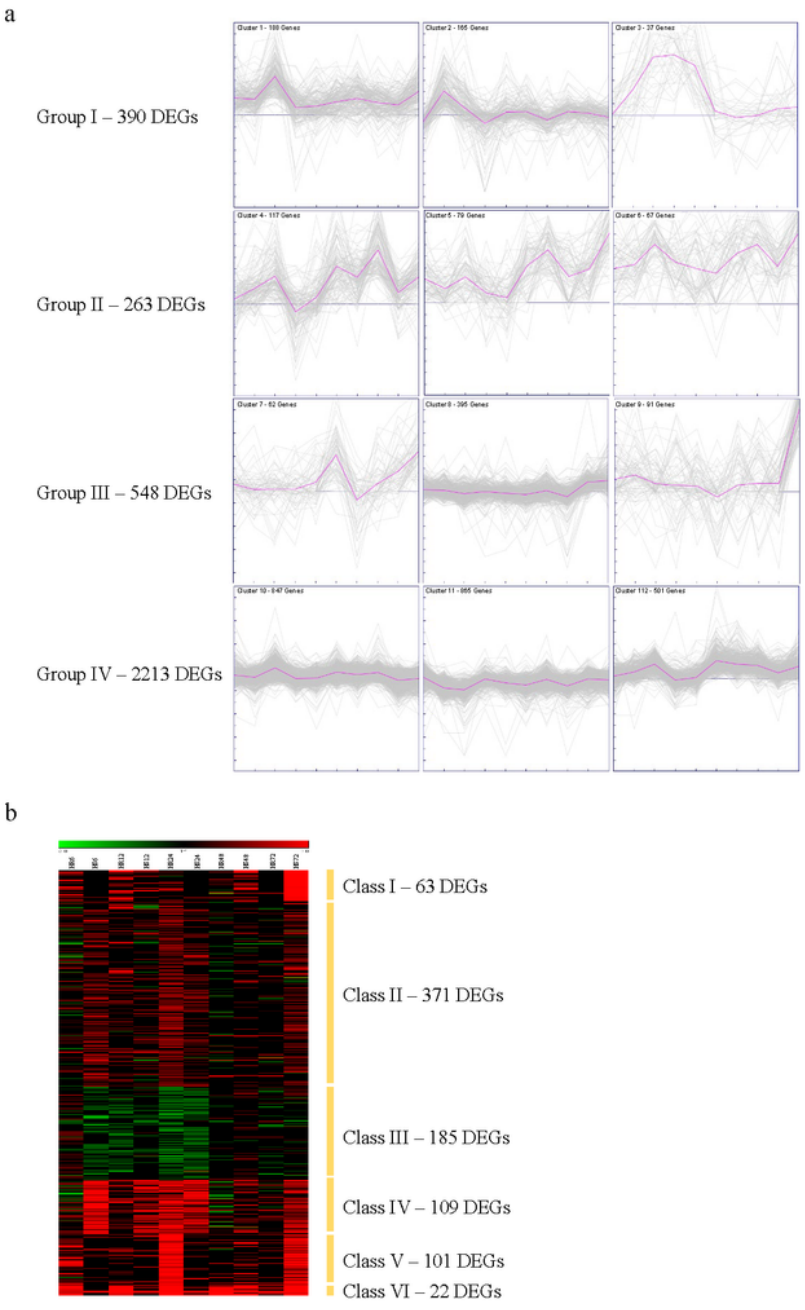
2

Figure 2

Change in expression and comparative analysis of differentially expressed genes (DEGs) between the HR and HS grapes following Elsinoe ampelina infection at different time points. Venn diagram and heatmap showing common or uniquely regulated genes at different time points in the E. ampelina infected HR

grape (a) or HS grape (b) leaves. (c) Venn diagram and heatmap showing the number and the expression profiles of the common DEGs following E. ampelina infection in the HR and HS grapes. Red and green colors indicate up- and down-regulated genes.

Figure 3



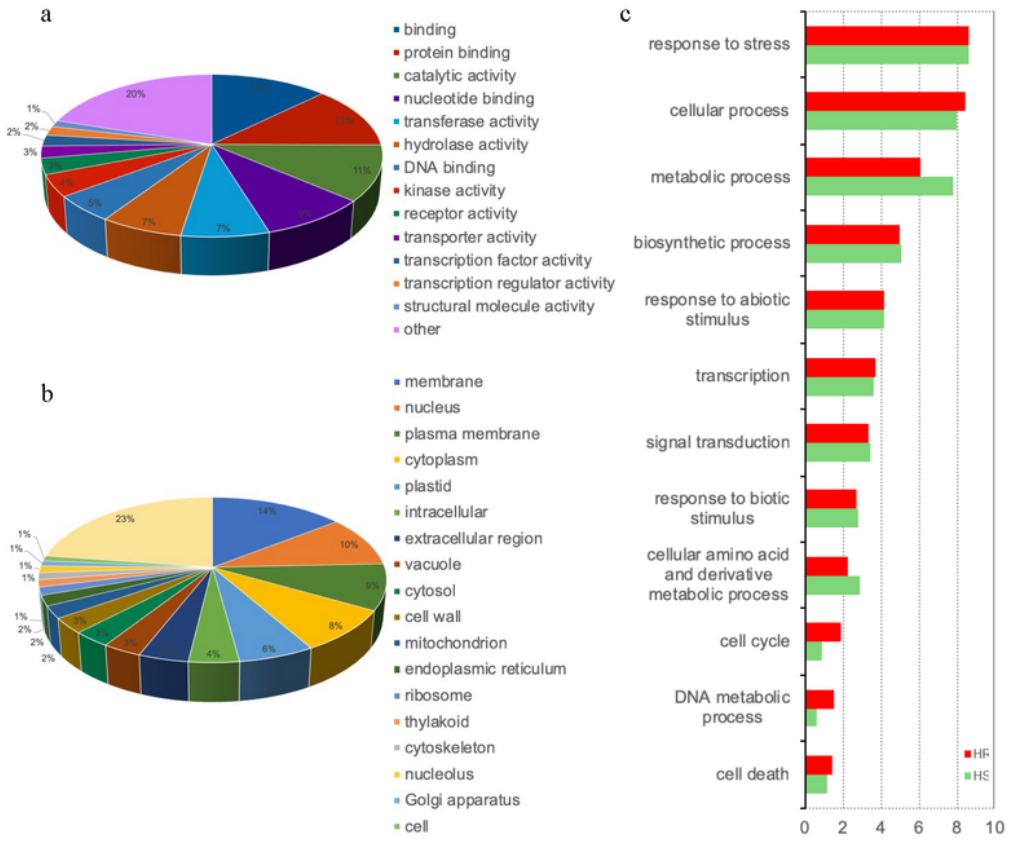
3

Figure 3

Cluster analysis of differentially expressed genes (DEGs) between the HR and HS grapes at five time points, based on the K-means method or hierarchical clustering. (a) Cluster analysis of 3,414 DEGs

between the HR and HS grapes. The numbers following ‘HR” refer to the hours after Elsinoe ampelina infection. In all panels, grey lines indicate the expression levels of individual genes and magenta lines indicate a ‘consensus’ of all the DEGs within a specific subcluster. (b) Heatmap of 851 DEGs found in both the HR and HS grapes. Red and green colors indicate up- and down-regulated genes, respectively. The heatmap was generated from the RPKM (Reads Per Kilobase Million) data.

Figure 4

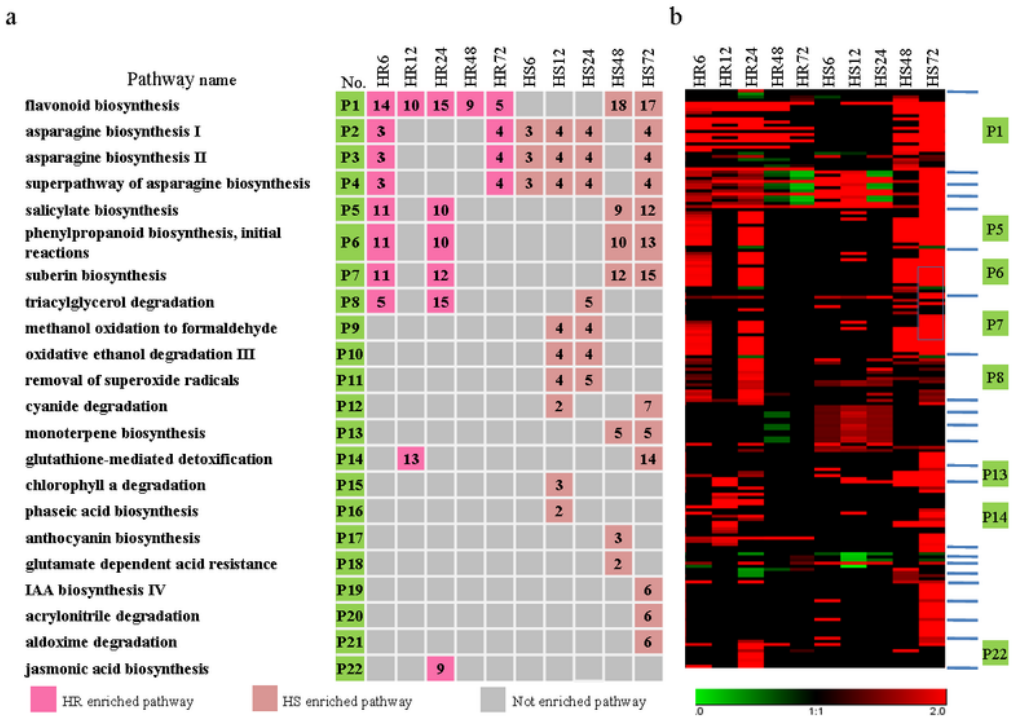


4

Figure 4

Analysis of GO (gene ontology) functional classifications. Functional categorization of all differentially expressed genes following Elsinoe ampelina infection of the HR and HS grapes based on (a) Molecular function (b) Cellular component or (c) Biological process.

Figure 5



5

Figure 5

Pathway enrichment analysis of the HR and HS grape leavesat different time points after Elsinoe ampelina infection. (a) Enriched pathways at different time points after E. ampelina infection in the HR

and HS grapes. (b) Expression profiles of genes involved in different pathways. P1-P22, number of different pathways.

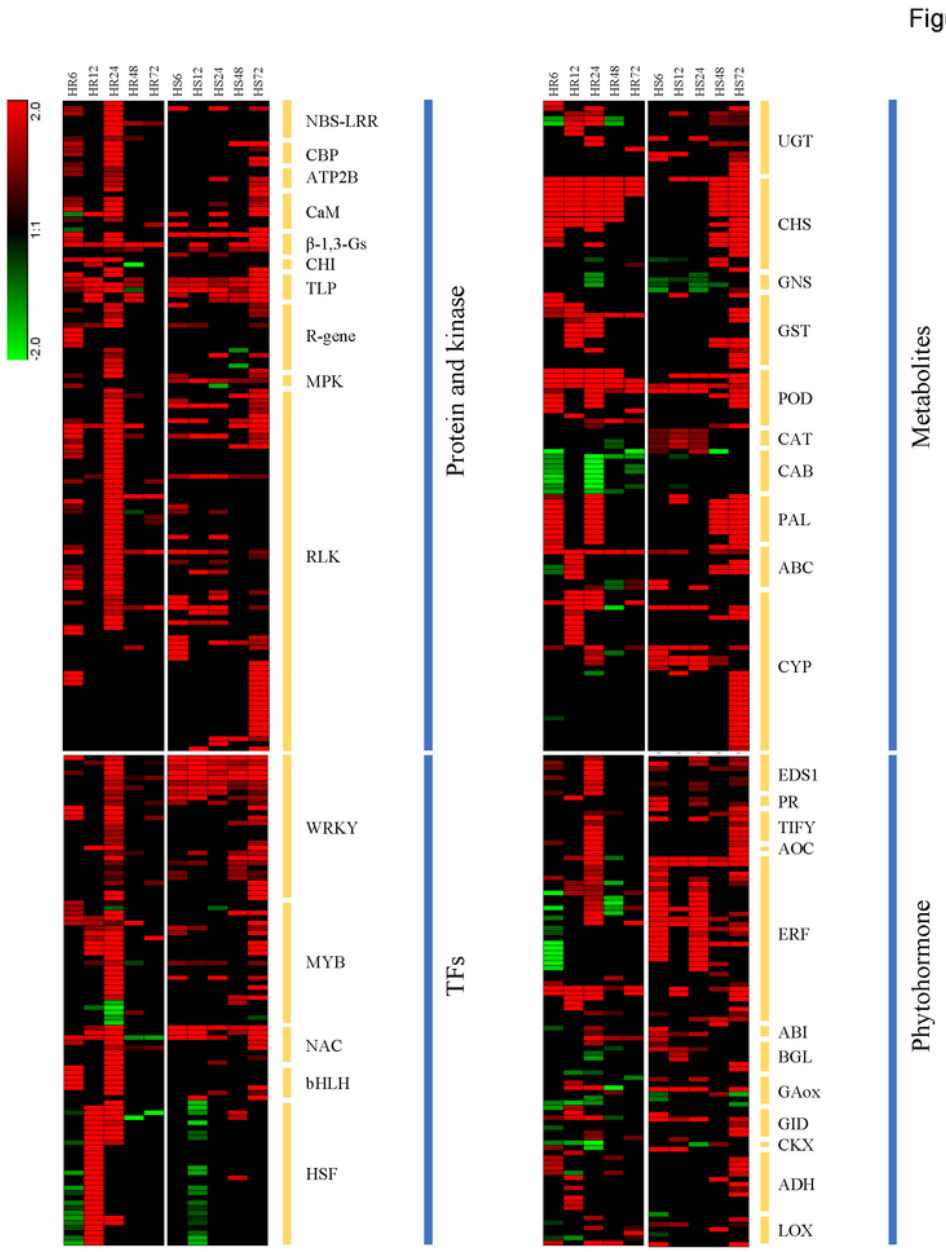


Figure 6

Heatmap of clustered GO (gene ontology) terms for genes potentially linked to resistance that are differentially expressed in HR and HS grape leaves infected with Elsinoe ampelina infected.

Supplementary Files

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

- Additionalfile1SupplementaryTable1.xlsx
- Additionalfile2SupplementaryTable2.xlsx
- Additionalfile3SupplementaryTable3.xlsx
- Additionalfile6SupplementaryTable6.xlsx
- Additionalfile5SupplementaryTable5.xlsx
- Additionalfile4SupplementaryTable4.xlsx