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Ectopic and transient expression of *VvDIR4* gene in *Arabidopsis* and grapes enhances resistance to anthracnose via affecting hormone signaling pathways and lignin production

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Abstract

Background DIR (Dirigent) proteins play important roles in the biosynthesis of lignin and lignans and are involved in various processes such as plant growth, development, and stress responses. However, there is less information about VvDIR proteins in grapevine (*Vitis vinifera* L).

Results In this study, we used bioinformatics methods to identify members of the DIR gene family in grapevine and identified 18 *VvDIR* genes in grapevine. These genes were classified into 5 subfamilies based on phylogenetic analysis. In promoter analysis, various plant hormones, stress, and light-responsive *cis*-elements were detected. Expression profiling of all genes following *Colletotrichum gloeosporioides* infection and phytohormones (salicylic acid (SA) and jasmonic acid (JA)) application suggested significant upregulation of 17 and 6 *VvDIR* genes, respectively. Further, we overexpressed the *VvDIR4* gene in *Arabidopsis thaliana* and grapes for functional analysis. Ectopic expression of *VvDIR4* in *A. thaliana* and transient expression in grapes increased resistance against *C. gloeosporioides* and *C. higginsianum*, respectively. Phenotypic observations showed small disease lesions in transgenic plants. Further, the expression patterns of genes having presumed roles in SA and JA signaling pathways were also influenced. Lignin contents were measured before and after *C. higginsianum* infection; the transgenic *A. thaliana* lines showed higher lignin content than wild-type, and a significant increase was observed after *C. higginsianum* infection.

Conclusions Based on the findings, we surmise that *VvDIR4* is involved in hormonal and lignin synthesis pathways which regulate resistance against anthracnose. Our study provides novel insights into the function of *VvDIR* genes and new candidate genes for grapevine disease resistance breeding programs.

Keywords *Vitis vinifera* L., Dirigent gene, *Colletotrichum gloeosporioides*, Disease resistance, *VvDIR4*

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Background

During the process of plant evolution, plants have developed complex and effective defense mechanisms to mitigate environmental impacts. These mechanisms include oxidative cleavage, lignin synthesis, lignin deposition, and release of acids as signaling pathways [1–3]. Lignin is an important component of plant cell walls and is a randomly polymerized macromolecule consisting of highly substituted phenylpropane units. Lignin is one of the most abundant organic polymeric materials on land and primarily constitutes the secondary cell wall. It enhances cell wall hardness through cross-linking with cellulose and hemicellulose and improves the ability to resist adverse biotic and abiotic stresses [4]. Dirigent (DIR) proteins were initially discovered in *Forsythia* and *Pinus* and were found to have roles in lignin synthesis [5]. Subsequent studies revealed the widespread distribution of DIR proteins in vascular plants, including ferns, gymnosperms, and angiosperms [6]. Research has also found that DIR proteins participate in responses to diseases, pests, and certain abiotic stresses [7]. Most DIR genes do not contain introns [8]. Ralph et al. [9] and Sahani et al. [10] conducted phylogenetic analysis of 150 DIR proteins from different species and classified the DIR gene family into six subfamilies: DIR-a, DIR-b/d, DIR-c, DIR-e, DIR-f, and DIR-g, with DIR-c being specific to monocotyledonous plants. Genes from the DIR-a subfamily were primarily involved in lignin-related reactions, while limited information is available about the specific functions of genes within other subfamilies.

The main function of DIR genes is to regulate plant stress responses by participating in the control of lignin and suberin biosynthesis pathways [11]. DIR genes exhibit inducible responses to pathogen attacks, for example *PsDIR1* gene in peas being was induced in response to infections by *Cladosporium fulvum* and *Alternaria solani* [12]. The DIR gene in *Brassica rapa* enhanced resistance to *Verticillium dahliae* and *Alternaria brassicicola* [13]. *BrDIR2* gene in oilseed rape was induced after infection with *Fusarium oxysporum f.sp. conglutinans* and in cabbage, four homologous genes to *BrDIR* were found to respond to the same fungus infection [14, 15]. The *HfrDIR* genes in wheat responded to induced infection by *Hessian fly* [16]. In strawberry, 33 *FvDIR* genes were identified and *FvDIR13* showed response to *Colletotrichum gloeosporioides* infection [17]. Eleven *SlDIR* genes in *Solanum lycopersicum* enhanced resistance against *Pst* DC3000 infection [18]. The *CsDIR16* gene in *Cucumis sativus* showed upregulated expression under cucumber mosaic virus stress [19]. In *Vigna angularis*, the expression of *VaDIR14*, *VaDIR16*, and *VaDIR33* was significantly induced following fungus infection [20]. Moreover, certain DIR genes may exhibit responsiveness to the induction of disease-related hormones. For example, the expression of the *SHDIR11* and *SHDIR16* genes in *Saccharum officinarum* was induced by SA and MeJA [21]. *FvDIR1* in *Fragaria vesca* was induced by MeJA, while *FvDIR7* and *FvDIR24* were significantly induced by SA [17]. In *Populus*, *PeDIR19* showed significant upregulation following exogenous hormones (MeJA and ABA) application [22]. Overexpression of *GmDIR22* in *Glycine max* plants enhanced resistance to *Phytophthora sojae* via increasing total lignin accumulation [23]. Overexpression of *TaDIR13* in *Nicotiana tabacum* increased lignin accumulation and enhanced resistance to *Pseudomonas syringae* [24]. Overexpression of *GhDIR1* gene in cotton increased lignin contents, enhanced lignification of the epidermis and vascular bundles, and improved tolerance to *Verticillium wilt* [25]. Six DIR genes in *Pinus species* are strongly induced after mechanical damage and bark beetle attack [9]. These all findings suggest the critical roles of DIR genes in plant stress resistance.

Grapes (*Vitis vinifera* L.) are among the most economically valuable cultivated fruit trees, having a rich cultivation history and diverse range of uses. Most grape production worldwide involves European grapes (*V. vinifera* ssp.), which are renowned for their high quality and yield, resulting in significant economic benefits. However, European grapes are more susceptible to fungal diseases, especially anthracnose, when compared to *V. labrusca* L. Anthracnose stands out as a major grape disease, posing a serious threat to the viticulture industry [26]. Anthracnose, also known as late rot, is primarily caused by *C. gloeosporioides* and is widespread globally. *C. gloeosporioides* mostly affects mature fruits, causing the growth of black and brown anthracnose spots on the infected fruits and leaves. It also leads to surface ulceration of the fruits, juice extravasation, and mold contamination, all of which severely impact grape yield and quality. The role of DIR genes in disease resistance has been well-studied in many crops [11, 17, 27, 28]. However, there is limited information available on grape DIR genes. The critical roles of DIR genes in disease resistance and the importance of grapes justify the need for DIR genes identification and functional characterization in grapevine.

This study utilized bioinformatics methods to identify the *VvDIR* gene family members in grapes, and analyzed their encoded protein's physicochemical properties, gene structure, promoter elements, evolutionary relationships, and subcellular localization predictions. The expression analysis of *VvDIR* genes was analyzed following inoculation with *C. gloeosporioides* and hormone (SA and JA) treatments. Further, we investigated the functionality of the *VvDIR4* gene in disease resistance mechanisms by overexpressing it in *A. thaliana* and grape leaf tissues through stable and transient transformation. This research contributes to the further study of the DIR gene

Table 1 Basic details and physicochemical properties of VvDIR proteins

Name	Accession No.	Chr.	Location	CDS/bp	ORF/aa	Mw/kDa	pI	Instability index	Predicted location
VvDIR 1	GSVIVG01019450001	2	775165.779587	720	239	27.21	9.69	25.06	chlo
VvDIR 2	GSVIVG01029030001	5	10938188.10941512	768	255	26.14	6.19	21.8	chlo
VvDIR 3	GSVIVG01025047001	6	5021172.5031333	3283	1093	121.24	8.28	37.99	plas
VvDIR 4	GSVIVG01025394001	6	1125322.1126160	582	193	21.2	6.73	25.82	chlo
VvDIR 5	GSVIVG01025397001	6	1094444.1095244	618	205	23.19	9.47	33.91	plas
VvDIR 6	GSVIVG01025391001	6	1143640.1150253	702	233	26.09	8.93	44.29	plas
VvDIR 7	GSVIVG01025393001	6	1126772.1130774	369	131	14.31	8.73	34.86	extr
VvDIR 8	GSVIVG01033445001	8	20638631.20639387	369	122	13.53	9.1	35.36	chlo
VvDIR 9	GSVIVG01033447001	8	20633800.20634450	651	216	24.45	8.89	28.98	plas
VvDIR 10	GSVIVG01033451001	8	20602689.20603258	570	189	20.72	9.19	29.98	chlo
VvDIR 11	GSVIVG01033453001	8	20562778.20572390	438	145	16.56	10.89	27.79	chlo
VvDIR 12	GSVIVG01030446001	12	5615239.5615622	384	127	14.28	9	32.47	plas
VvDIR 13	GSVIVG01030448001	12	5638289.5638931	534	177	19.57	8.69	29.46	plas
VvDIR 14	GSVIVG01030449001	12	5644843.5645490	540	179	19.74	7.85	30	plas
VvDIR 15	GSVIVG01000846001	13_random	2867042.2867807	549	182	19.96	6.65	29.32	extr
VvDIR 16	GSVIVG01009625001	18	10015414.10021454	1968	655	73.73	9.47	42.93	chlo
VvDIR 17	GSVIVG01005967001	Un	23020082.23026773	3846	1281	104.94	5.7	60.84	cyto
VvDIR 18	GSVIVG01005968001	Un	23028234.23029456	972	323	36.9	4.57	43.56	chlo

Nucl: nucleus, Extr: extracellular, Chlo: chloroplast, Mito: mitochondria, plas: plasmid

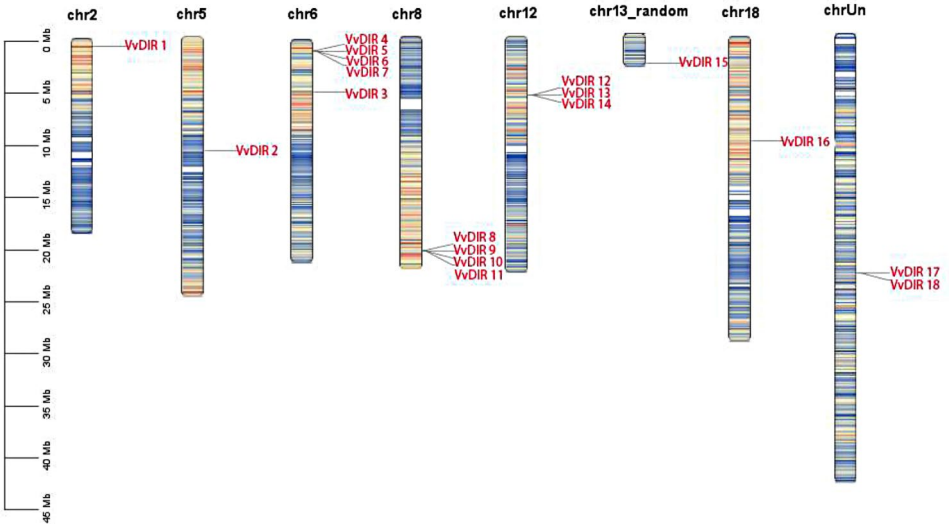

Fig. 1 Chromosomal distribution of the *VvDIR* genes. The size of the chromosome is shown using a vertical scale (Mb)

family in grapes, laying the foundation for understanding its role in anthracnose disease resistance.

Results

Identification and physiochemical properties of VvDIR proteins

18 DIR genes were identified in the grape genome and were unevenly distributed on 8 chromosomes. The genes were named according to their chromosomal positions (VvDIR1 to VvDIR18). The physicochemical properties and basic information including the deduced protein length, Mw, pI, and instability index of all the *VvDIR* proteins are mentioned in Table 1. The VvDIR proteins have coding sequence lengths ranging from 369 bp (VvDIR11) to 3846 bp (VvDIR17). The pI values range from 4.57 (VvDIR18) to 10.89 (VvDIR11), while the MW ranges from 14.28 kDa (VvDIR12) to 121.32 kDa (VvDIR3). The instability index varied from 21.8 (VvDIR2) to 60.84 (VvDIR17). Most (15 out of 18) of the VvDIR proteins were predicted to be present in chloroplasts and plasmids.

Chromosomal distribution and evolutionary history of *VvDIR* genes

Eighteen *VvDIR* genes were mapped unequally on eight grape chromosomes (Fig. 1) and Chromosome 6 contained the highest number of *VvDIR* members (5 genes), followed by chromosome 8 (4 genes) and chromosome

(3 genes). The *VvDIR* genes were clustered at the top of chromosome 6 and the lower half of chromosome 8, suggesting that these chromosomes underwent secondary duplication events (Fig. 1). In plants, tandem duplication and segmental duplication are the two most common causes of gene family expansion [29]. Only one event of segmental duplication (*VvDIR5*/*VvDIR10*) was identified (Fig. 2).

Phylogenetic relationships and classification of VvDIR proteins

Based on phylogenetic trees constructed among the DIR proteins of grapes, rice, and *A. thaliana*, the DIR proteins were divided into five subfamilies (DIR-a, b/d, e, f, and g) (Fig. 3). The VvDIR proteins were unevenly distributed in the subfamilies (DIR-a, b/d, e, f, and g) and the DIR-a subfamily contained the highest *VvDIR* genes (*VvDIR2*, *4*, *6*, *9*, *13*, and *14*). The DIR-e subfamily contained five *VvDIR* (*VvDIR1*, *3*, *7*, *8*, and *15*) genes followed by DIR-g subfamily which has four *VvDIR* genes (*VvDIR5*, *11*, *17*, and *18*). The DIR-b/d, DIR-f, DIR-c subfamilies contained 2, 1, and 0 members, respectively. The alignment of clustering patterns of phylogenetic trees with previous classifications indicates the accuracy of the results [9, 30, 31].

Gene structure architecture of *VvDIR* members

The conserved motifs, domains, and evolutionary relationships of the 18 *VvDIR* members were analyzed to get deep insights (Fig. 4). The MEME suite was utilized to detect motifs in VvDIR proteins (Fig. 4B). Details of each subject sequence are listed in Table S2. These conserved motifs annotated by the SMART program. It was found that motif 3 was present in 15 members, followed by motif 4 which was present in 10 members. Motifs 5, 6, and 7 were present in 4, 3, and 4 *VvDIR* genes, respectively. The lowest number of motifs (2) was found in *VvDIR17*. The motifs of the same group showed more similarities with each other, indicating that they may have similar functions. For example, motif 9 is only found in members of subfamily DIR-e and subfamily DIR-f, while motif 6 is only found in members of subfamily DIR-b/d and subfamily DIR-a. The presence of different motifs in different DIR genes suggests that they may have diverse biochemical and biological roles.

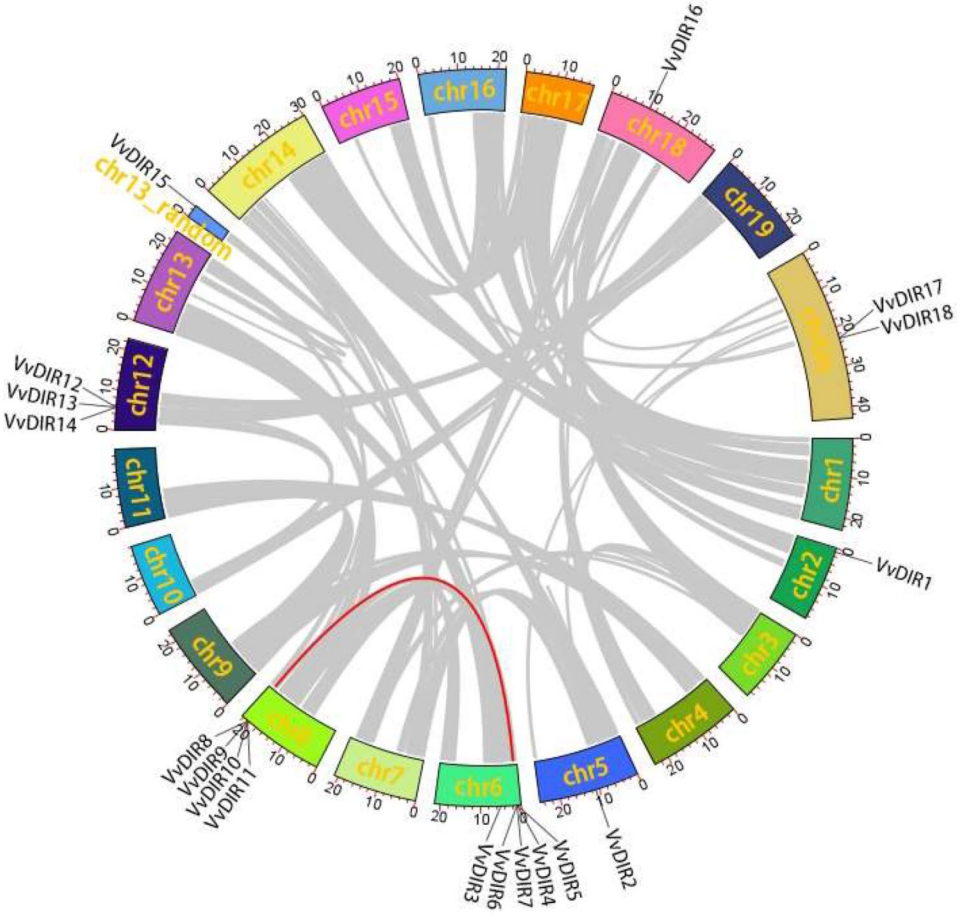

Fig. 2 Collinearity analysis of *VvDIR* genes. A red line connects the syntenic relationships of *VvDIR* genes

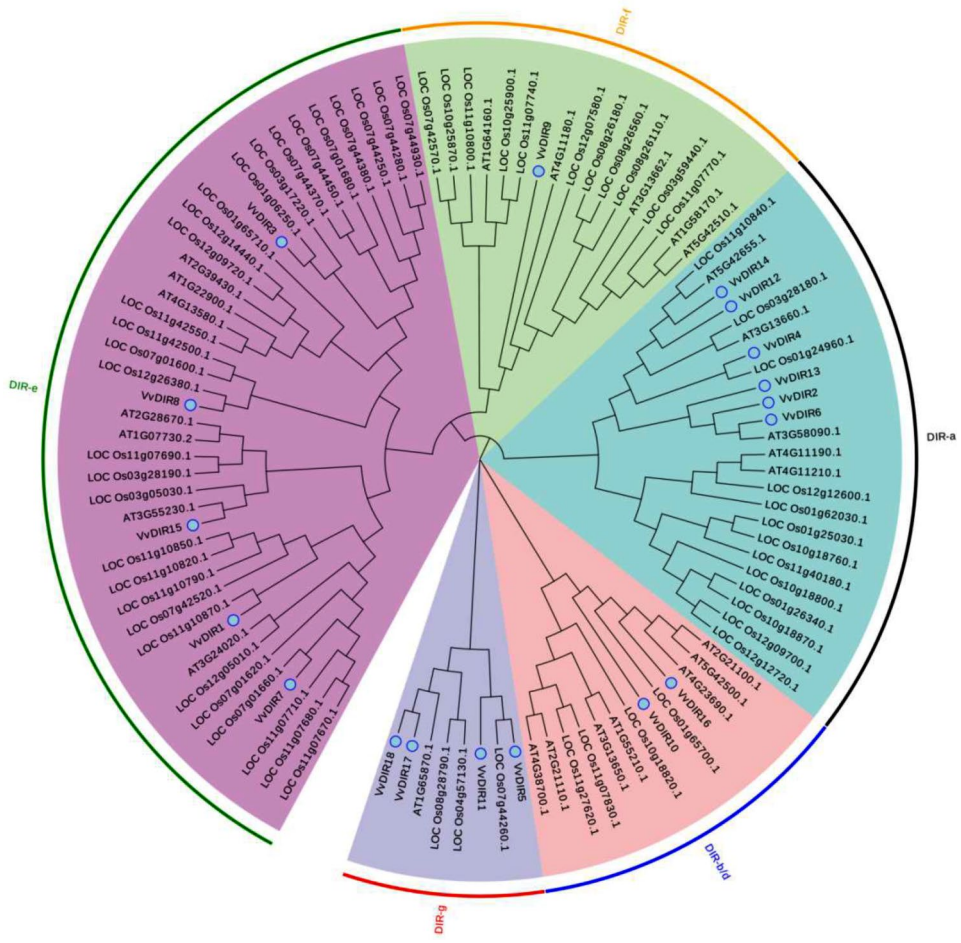

Fig. 3 Phylogenetic analysis of DIR genes in *A. thaliana*, *O. sativa*, and *V. vinifera*. The full-length amino acid sequences of DIR genes from *A. thaliana* (At), *O. sativa* (Os), and *V. vinifera* (Vv, blue) were aligned by Clustal W. MEGA 7.0 was used to create the phylogenetic tree, using the maximum-likelihood method with 1000 bootstrap repetitions. Different subfamilies are marked with different colors

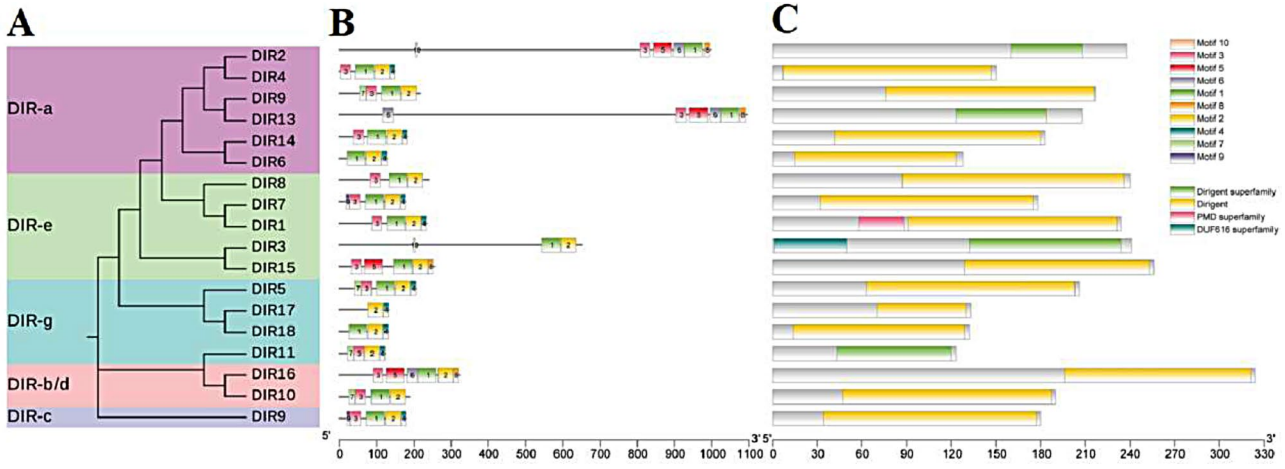

Fig. 4 Gene structure, conserved motifs, and phylogenetic relationship of *VvDIR* genes. (**A**) Phylogenetic tree of *VvDIR* genes. The phylogenetic tree was generated using the maximum-likelihood method with 1000 bootstrap replicates utilizing the full-length protein sequences of *VvDIR* genes. The *VvDIR* genes can be divided into five subgroups, which are denoted with different colors, respectively. (**B**) Motif distribution patterns. (**C**) Domains of the *VvDIR* genes

Analysis of *cis-acting* elements

The promoter region of the *VvDIR* genes contains various regulatory elements, including TGA-element, AuxRE, AuxRR-core, TATC-box, GARE-motif, P-box, ABRE, CGTCA-motif, TGACG-motif, SARE, and TCA-element, which are associated with responses to auxin (IAA), gibberellin (GA), abscisic acid (ABA), methyl jasmonate (MeJA), and salicylic acid (SA) (Fig. 5). Specifically, 15 CGTCA motifs were identified in the promoter regions of 10 members, while 12 members contained 29 TCA-elements. These findings suggest the critical roles of *VvDIR* genes play in plant stress responses by enhancing the synthesis of lignin and lignans. Moreover, anaerobic-induced elements were present in most of the members, followed by MeJA-responsiveness.

Expression analysis of *VvDIR* genes following *C. gloeosporioides* and signaling molecules application

Grape leaves following *C. gloeosporioides* infection showed different expression patterns of *VvDIR* genes (Fig. 6). All genes except *VvDIR17* showed upregulation upon inoculation with *C. gloeosporioides*. Most of the genes displayed the same pattern of upregulation and showed peak expression at 48 h post inoculation (hpi). However, there were differences as well, for example, *VvDIR 5*, *8*, *11*, *12*, *14* initially showed upregulation followed by subsequent downregulation, and *VvDIR11*, *12*, *14* displayed the highest expression at 6 hpi. The *VvDIR4* showed the highest increase in expression levels at 48 hpi and *VvDIR 5*, *8* showed the peak expression at 12 hpi.

Further, we also analyzed the response of six DIR genes (*VvDIR2*, *4*, *6*, *7*, *11*, *and 13*) to the application of two hormones (SA and MeJA), as shown in Fig. 7. *VvDIR2* showed the highest response to SA at 6 hpi. *VvDIR6* was upregulated at 3 hpi in response to MeJA, while *VvDIR4* and *VvDIR11* also displayed a significant response to MeJA and had the highest response at 3 hpi (80.54) and 24 hpi (71.09), respectively. On the other hand, *VvDIR7* and *VvDIR13* had a significant response to SA, reaching a peak at 3 hpi (6151.26 and 9.50, respectively), but only showed a slight response to MeJA stress. The observed changes in the expression levels of *VvDIR4* to C. *gloeosporioides* infection and hormone application suggest its important roles in the disease resistance mechanism.

Ectopic expression of *VvDIR4* in*A. thaliana* enhances the resistance to *C. higginsianum*

Expression profiling revealed that the *VvDIR4* gene showed significant change in expression in strawberry following *C. gloeosporioides* infection, and the expression was upregulated with external SA and JA treatment. Therefore, the *VvDIR4* gene was selected for further investigation and ectopically expressed in *A. thaliana*. Among the 27 transgenic T2 lines, three independent transgenic lines (T3-10, T3-14, and T3-25) having the highest expression levels of *VvDIR4* were selected for subsequent experiments (Fig. 8C). The leaves of the selected transgenic and wild-type lines were inoculated with *C. higginsianum*. For phenotypic observations, leaf samples were taken after 3 days (Fig. 8A). Both transgenic and wild-type plants responded to *C. higginsianum*, displaying black lesions around the inoculation sites. However, the disease symptoms in wild-type plants were more severe than those in transgenic plants, with the largest lesion diameter observed in the wild-type plants and the smallest in T3-10 (Fig. 8A and B).

To further investigate the molecular mechanism of *VvDIR4* transgenic plants following *C. higginsianum*

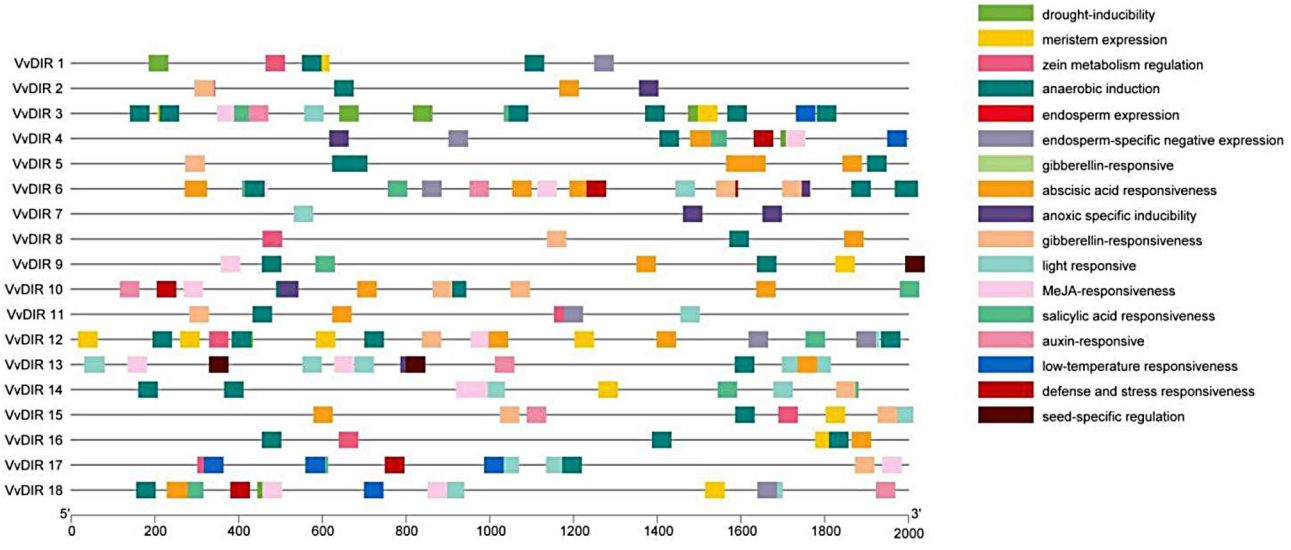

Fig. 5 Promoter analysis of grape DIR genes

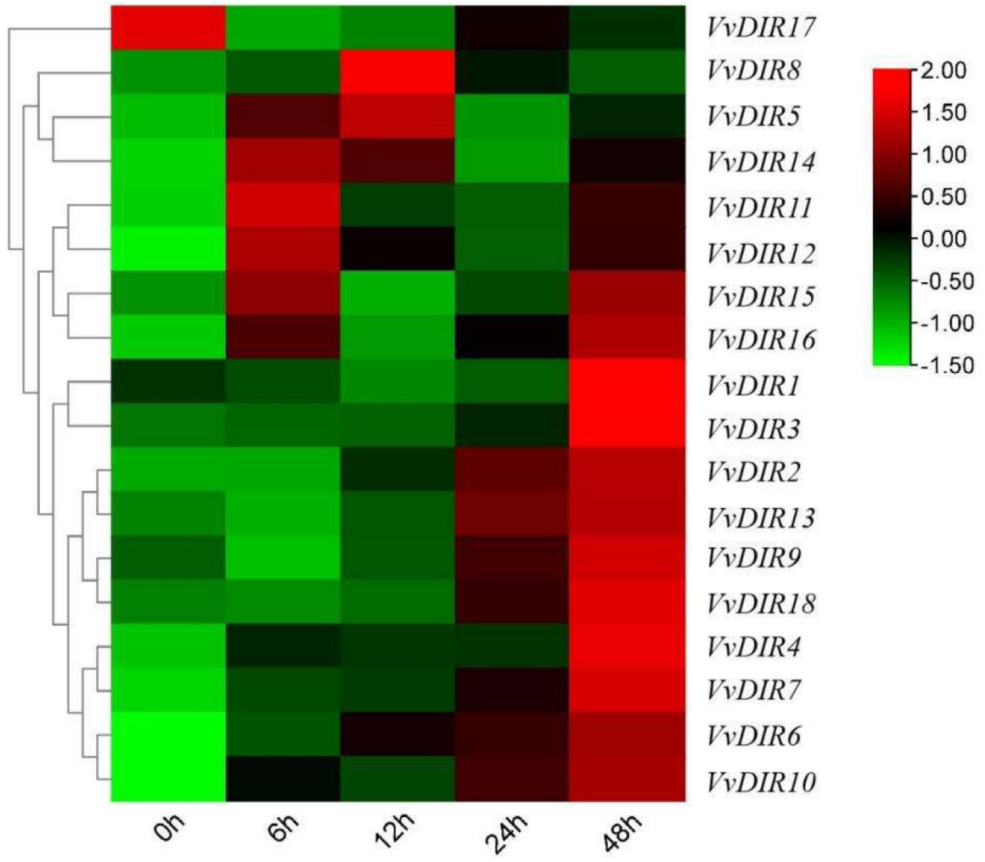

Fig. 6 *VvDIR* genes expression profiles in response to *C. gloeosporioides* inoculation. qRT-PCR was used for expression analysis and a heatmap was generated using TBtools. The red and green color scale indicate high and low expression levels, respectively

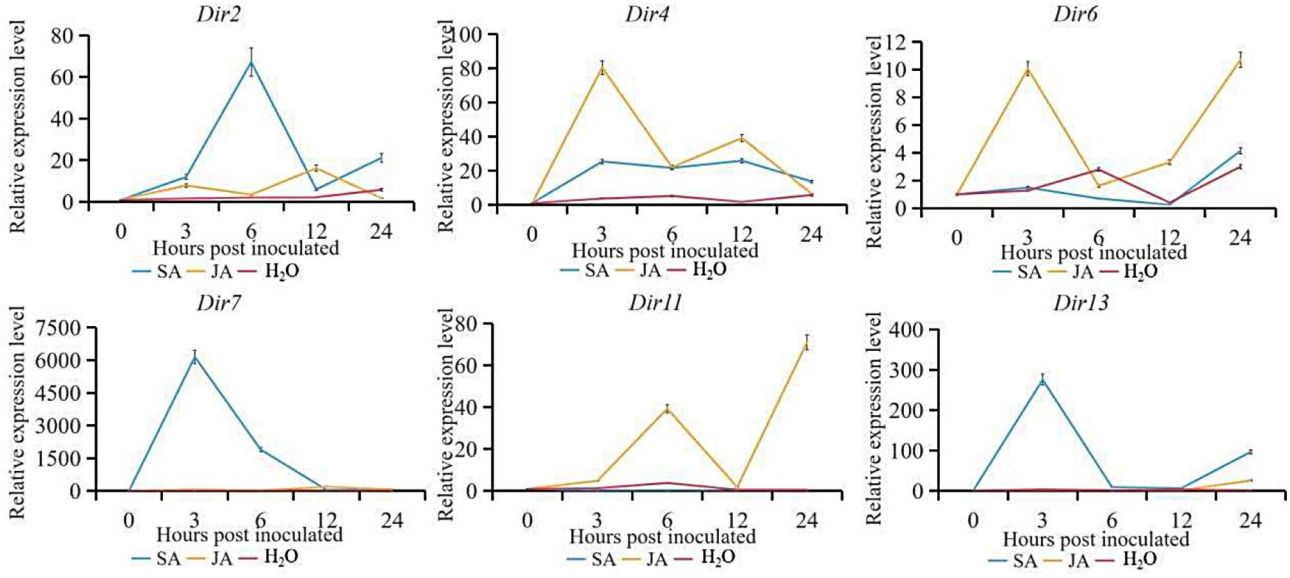

Fig. 7 *VvDIR* genes expression profiles in response to SA and MeJA treatment by qRT-PCR. Error bars represent the standard deviation (SD) of three biological replicates

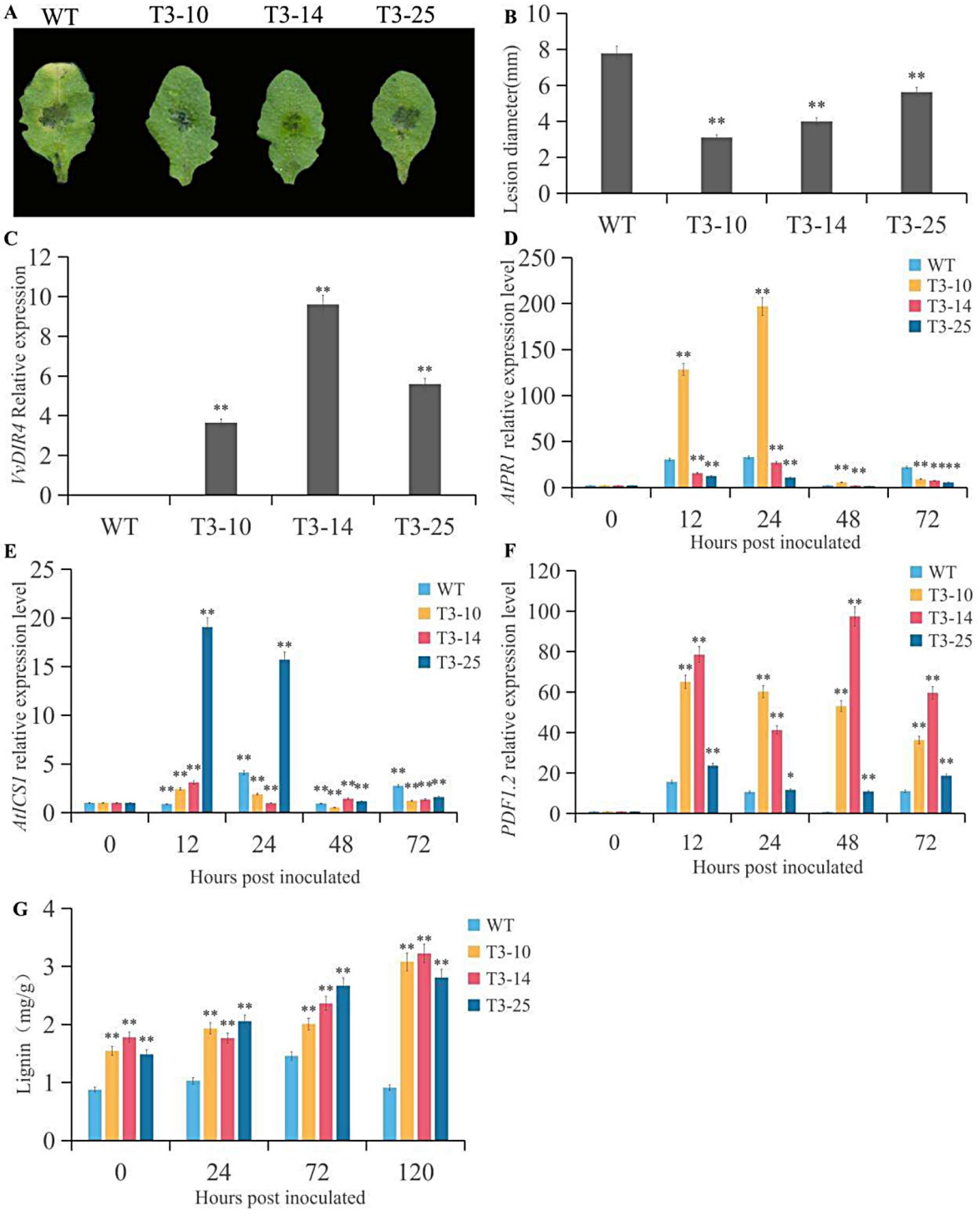

Fig. 8 Ectopic expression of *VvDIR4* gene in *A. thaliana* and expression levels at 0, 12, 24, 48, and 72 hpi. Data are the mean values and SDs of three replications and asterisks denote a statistically significant difference (* $p < 0.05$, ** $p < 0.01$, Student's *t*-test) between wild-type and transgenic lines. (**A**) Symptoms of disease in WT and transgenic *A. thaliana* leaves after 72 hpi. (**B**) The average lesion diameter on leaves after three days of inoculation. (**C**) The transcript level of *VvDIR4* in leaves of WT and transgenic lines. (**D**) Expression analysis of *AtPR1* gene. (**E**) Expression analysis of *AtICS1* gene. (**F**) Expression analysis of *AtPDF1.2* gene. (**G**) Lignin content in WT and transgenic *A. thaliana* (whole plants) after *C. higginsianum* inoculation

inoculation, we measured the transcription levels of important genes having presumed roles in the SA (*AtPR1* and *AtICS1*) and JA (*AtPDF1.2*) signaling pathways (Fig. 8D-F). In the T3-10 line, the *AtPR1* gene was significantly upregulated at 12 hpi and reached its highest expression at 48 hpi (Fig. 8E). At 12 hpi, the expression levels of the *AtICS1* gene in all three transgenic lines reached their maximum, significantly higher than that in the wild-type line (Fig. 8D). The expression levels of the *AtPDF1.2* gene in all transgenic lines were higher than those in the wild-type line at all time points, with the highest expression levels occurring at 48 hpi in T3-14 and at 12 hpi in the other two lines (Fig. 8F).

Overexpression of *VvDIR4* increases acid-soluble lignin content in *A. thaliana*

Members of the DIR-a subfamily are involved in lignin synthesis [17, 32]. To verify whether the *VvDIR4* gene is also involved in lignin synthesis, we measured the lignin content in wild-type and transgenic *A. thaliana* plants after *C. gloeosporioides* inoculation. The lignin content in transgenic *A. thaliana* started to increase at 24 hpi and reached its highest level at 120 hpi (Fig. 9G). In contrast,

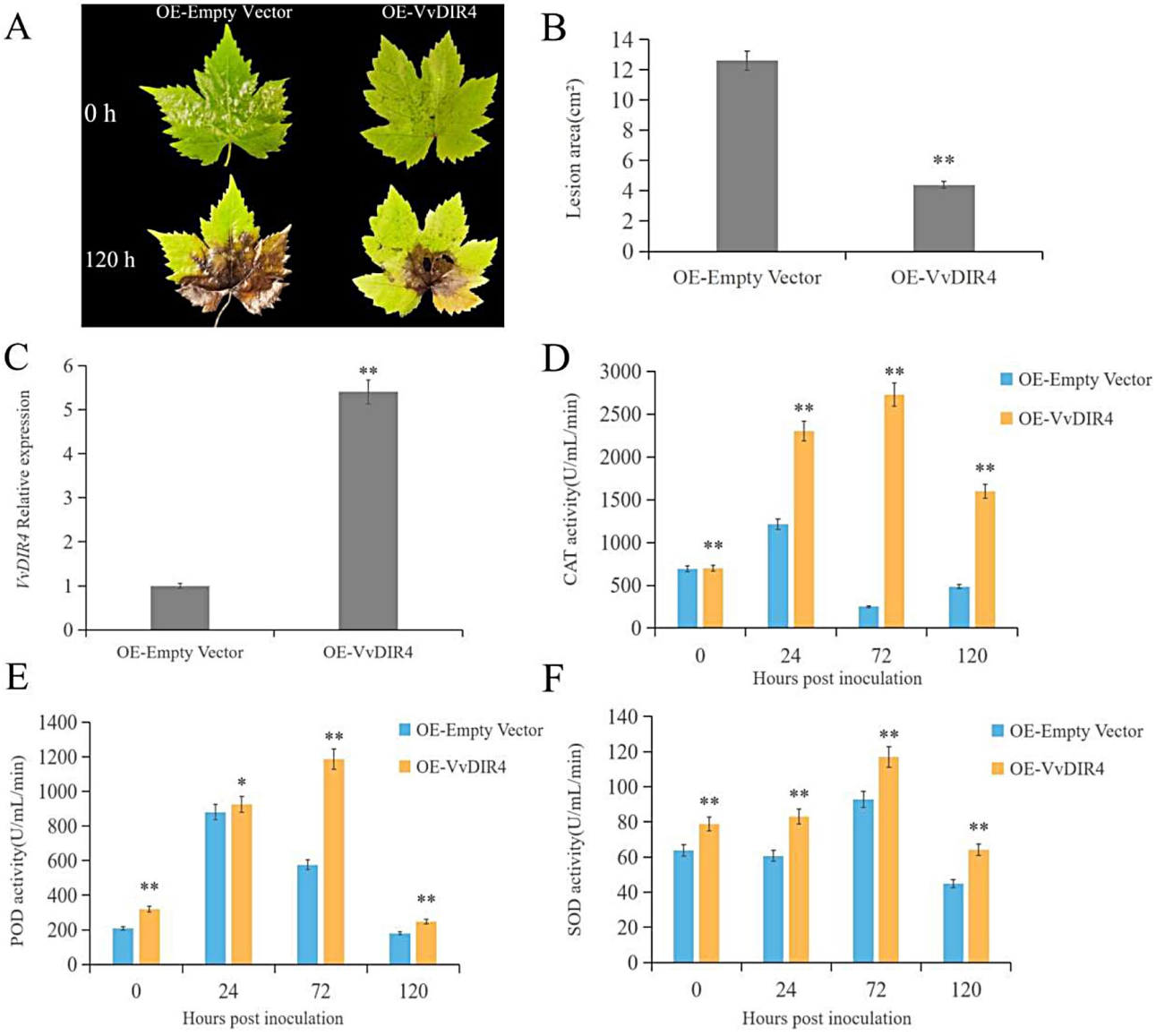

Fig. 9 The *VvDIR4* gene was transiently overexpressed in grapes cultivars Thompson seedless. These leaves were inoculated with a PDA disk containing *C. gloeosporioides* mycelium and harvested at 0, 24, 72, and 120 h. Data are the mean values and SDs of three replications. Asterisks denote a statistically significant difference (* $p < 0.05$, ** $p < 0.01$, Student's *t* test) between the empty vector control and transgenic lines. (**A**) Disease symptoms of the control and transiently transformed leaves at 120 hpi. (**B**) Average lesion area on the leaves at 120 hpi. (**C**) Expression of *VvDIR4* in the control and transiently transformed grape leaves. (**D**-**F**) CAT activity (**D**), POD activity (**E**), and SOD activity (**F**) after inoculation with *C. gloeosporioides*

the lignin content in wild-type plants did not show significant changes and remained lower than that in transgenic plants at all time points (Fig. 9G), indicating *VvDIR4* gene may enhance disease resistance in *A. thaliana* by regulating lignin synthesis.

The overexpression of *VvDIR4* gene has been shown to enhance the resistance of grapevine against *C. gloeosporioides*

For further functional verification of the *VvDIR4* gene, a pCAMBIA2300-*VvDIR*4-GFP overexpression vector was constructed and transiently transformed into the *C. gloeosporioides* susceptible grape cultivar 'Thompson Seedless' leaves using the *Agrobacterium*-mediated transient transformation method. Transient overexpression of the empty vector pCAMBIA2300-GFP in leaves served as the control. After 24 h of transient transformation, the leaves were inoculated with agar plates containing *C. gloeosporioides* mycelium. The expression level of *VvDIR4* in transiently transformed grape leaves was significantly higher than that in the control (Fig. 9C). Phenotypic observations revealed that overexpression of *VvDIR4* enhanced grape resistance against *C. gloeosporioides*, as the damage severity of the control leaves was significantly greater than that of the transiently overexpressed *VvDIR*4 leaves (Fig. 9A and B).

Mittler et al. [33] reported that plants can restrict or even kill pathogens by inducing the production of reactive oxygen species (ROS) and have ROS scavenging systems that maintain a dynamic balance with ROS production. Furthermore, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are important antioxidant enzymes in plants [34]. Therefore, the activities of CAT, SOD, and POD in grape leaves after *C. gloeosporioides* infection were also estimated. CAT, SOD, and POD activities in the leaves overexpressing *VvDIR4* were higher than those in the control group during the infection process and reached their peaks at 72 h (Fig. 9D-F). These results suggest the involvement of *VvDIR4* in the *C. gloeosporioides* infection resistance mechanism.

Discussion

The DIR genes have key roles in the biosynthesis of plant lignin and phenylpropanoids and serve as key regulatory factors that can be activated in response to biotic and abiotic stresses [9, 11, 35]. The activation makes them significantly involved in stress resistance, particularly in the defense against pathogens. The involvement of DIR genes in the biotic stress resistance mechanism has been studied in various plant species, including rice [31], soybean [36], and pepper [37]. However, there is less information available about their roles in the grapevine. Here, 18 *VvDIR* family genes were identified in the grapevine genome using bioinformatics methods and were analyzed in terms of their physicochemical properties, gene structures, phylogenetic relationships, motif distribution, expansion patterns, and expression profiling. Moreover, the role of the *VvDIR4* gene in anthracnose resistance was validated by overexpressing *A. thaliana* and grapevine. Our results provide new candidate genes for disease resistance breeding and basic information about the roles of *VvDIR* genes in anthracnose resistance.

All genes were unevenly distributed on eight chromosomes including three pairs of tandemly duplicated genes and one pair of segmentally duplicated genes (Figs. 1 and 2). These results suggest that both tandem and segmental duplication have played a role in the expansion of DIR gene family in grapes. There are 25, 45, 34, 22, and 18 DIR genes in *A. thaliana*, *Vigna radiata*, *Medicago sativa*, *Brassica napus*, and *V. vinifera* respectively [9, 14, 36, 38]. These diverse numbers of DIR genes in different plants suggest the DIR family changed extensively during genome evolution. There are 6 subfamilies in most of the plants, but grapes contained only five of six DIR subfamilies, and perhaps DIR-c was lost during grape evolution (Fig. 3).

Motif distribution patterns can provide some clues about gene functions. Most members of the same subfamily have almost similar motif distribution patterns with few exceptions suggesting close evolutionary relationships among genes of the same family [39]. Promoter analysis suggested that most of the genes are responsive to phytohormones (GA, SA, and MeJA) suggesting their involvement in hormone signaling pathways. These results are consistent with the findings of Cheng et al. [39]. We conducted a study on the changes in gene expression levels in in detached grape leaves following *C. gloeosporioides* inoculation, nearly all genes exhibited significant alterations in expression. 12 genes showed significant upregulation at 48 hpi, suggesting their key roles in anthracnose disease resistance mechanism (Fig. 6).

Plant hormone signal transduction pathways play an important role in systemic acquired resistance (SAR) in plants [40]. SA and MeJA are endogenous plant hormones and have important roles in plant immune responses. SA is primarily involved in the basic defense against biotrophic pathogens, whereas MeJA controls the response to necrotrophic pathogens [41, 42]. In our study, following SA and MeJA treatments, certain *VvDIR* genes showed differential expression patterns. Specifically, *VvDIR2*, *4*, *7*, and *13* genes showed upregulation against SA application, and *VvDIR4* and *6* displayed upregulation to MeJA treatment. Interestingly *VvDIR4* showed a positive response to both hormones, suggesting a role in hormonal signaling pathways of JA and MeJA (Fig. 7). These results further validate our predication made on *cis*-elements analysis that DIR genes have a role in hormone signaling pathways, especially SA and MeJA.

In conclusion, *VvDIR* genes may play important roles in biotic and abiotic stress resistance in grapes.

To get more insights, *VvDIR4* was ectopically and transiently expressed in *A. thaliana* and grapevine leaves, respectively. The transgenic plants of *A. thaliana* exhibited smaller lesions and higher disease resistance compared to wild-type plants (Fig. 8A). These results further support the role of *VvDIR4* in disease resistance. Moreover, to further justify the role of *VvDIR4* the expression patterns of genes having presumed roles in hormone signaling pathways were studied. Following *C. higginsianum* infection *AtPR1* and A*tICS1* showed significantly differential expression patterns in transgenic *A. thaliana* plants as compared to wild type (Fig. 8D, E). These findings are in line with previous results that *AtPR1* and *AtICS1* are involved in the SA pathway in *A. thaliana* [43, 44]. Moreover, *AtPDF1.*2 also showed higher expression in transgenic plants, suggesting the role of *VvDIR4* in JA signaling pathways. The high expression of *PDF1.2* indicates activation of the JA hormone pathway [44]. These are also consistent with the findings of Shi et al. [17].

Transient overexpression of the *VvDIR4* gene in susceptible grapevine cultivar 'Thompson Seedless' improved its resistance against *C. gloeosporioides*. After 120 h of inoculation with *C. gloeosporioides*, the transiently overexpressed leaves showed significantly smaller lesion areas compared to the control (empty vector) (Fig. 9A-B). ROS network also plays an important role in plant defense signaling [33]. Upon pathogen infection, ROS is rapidly generated to prevent pathogen entry or induce resistance genes inhibiting pathogen growth [45]. However, high levels of ROS can be toxic and negatively impact subcellular components and metabolism in plants [46]. To cope with this, plants have ROS scavenging systems that maintain a dynamic balance with ROS production. SOD, POD, and CAT are important antioxidant enzymes in plants [34]. In our study, after *C. gloeosporioides* infection, the transient overexpression of *VvDIR4* in leaves increased CAT, SOD, and POD activities compared to the control (Fig. 9D-F). These findings suggest that *VvDIR4* may enhance plant resistance to *C. gloeosporioides* by increasing the activity of antioxidant enzymes to suppress the production of ROS.

Conclusions

DIR proteins, particularly those in the DIR-A subfamily, play important roles in lignin biosynthesis. During pathogen infections, they contribute to enhanced plant resistance by promoting lignin accumulation [11, 47]. *VvDIR4*, belonging to the DIR-A subfamily, was investigated for its involvement in lignin synthesis. We measured lignin content in both wild-type and transgenic *A. thaliana* plants using the method described by Syros et al. [48], both before and after inoculation with *C. higginsianum*. The transgenic lines exhibited higher lignin content than the wild type, with a significant upregulation observed after *C. higginsianum* infection. These results endorse that *VvDIR4* may play critical roles in grape lignin formation, thereby enhancing resistance to *C. gloeosporioides* infection through increased lignin biosynthesis. Our study offers fundamental insights into *VvDIR* genes, with a detailed functional analysis of *VvDIR4*. We surmise that *VvDIR4* contributes to anthracnose resistance in grapes by influencing lignin synthesis and influencing JA and SA signaling pathways. These findings are valuable for grape disease resistance breeding programs.

Methods

Plant materials and seedlings treatment

The grape varieties (*V. vinifera* L.), "Red Globe" and "Thompson Seedless," planted at the research experimental base of Fujian Agriculture and Forestry University were used. *A. thaliana* plants were grown for 4 weeks in a growth chamber at 21 °C, 70% relative humidity, and a 12-hour light/12-hour dark cycle. The pathogens were sprayed on *A. thaliana* and grape plants, respectively. The concentration of inoculated pathogens (*C. gloeosporioides* and *C. higginsianum*) was 1×106 conidia/mL. Grape plants were kept in a growth chamber at 28 °C and 85% humidity after inoculation, while *A. thaliana* was placed in a growth chamber with a 16-hour light (23 °C)/8-hour dark (21 °C) cycle. To evaluate the biological activity of *VvDIR4* against anthracnose, 28 *A. thaliana* transgenic lines and Col-0 wild-type plants were infected with *C. higginsianum*, following the recommendations of Casado-Diaz et al. [49] and Wen et al. [50]. Samples were collected at 0, 12, 24, 48, and 72 h post-inoculation (hpi) from transgenic and wild-type *A. thaliana* plants. Grape leaves were sprayed with a 5 mM salicylic acid (SA) or 50 mM methyl jasmonate (MeJA) solution, while control plants were sprayed with distilled water. Each grape leaf was sprayed with 1.5 mL of each exogenous hormone using a hand sprayer. Leaf samples were collected at 0, 3, 6, 12, and 24 h after spraying. To extract RNA, all collected samples were immediately frozen in liquid nitrogen and stored at -80 °C. Each experiment was conducted with three biological and technical replicates.

Identification of DIR genes in grapevine

The genome, proteome, and annotation information for *A. thaliana*, *Oryza sativa*, and *V. vinifera* L. were obtained from the TAIR (https://www.arabidopsis.org), PlantTFDB (http://planttfdb.gao-lab.org), and Phytozome (https://phytozome-next.jgi.doe.gov). The Hidden Markov Model (HMM) profile for the Dirigent domain (PFAM 03018) was obtained from the Pfam database (http://pfam.xfam.org/) and used to search grape protein databases [51]. The local Blast analysis was performed

using TBtools [52]. Sequences with an E-value less than $1e^{-5}$ were selected as candidate sequences and redundant sequences were removed. The presence of a complete DIR domain in all putative DIR genes was checked using the SMART (http://smart.embl.de/) and CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) websites. Based on this analysis 18 *VvDIR* genes were selected for further study [53, 54]. The ProtParam tool provided by the ExPASy Server (https://web.expasy.org/protparam/) was utilized to calculate the physicochemical properties of *VvDIR* genes, including protein molecular weight (Mw), amino acid counts, and isoelectric point (pI) values. The subcellular location of proteins was predicted using the Plant-mPLoc Server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) [55].

Phylogenetic studies and multiple sequence alignment

One phylogenetic tree was generated among DIR protein sequences of *A. thaliana*, *O. sativa*, and *V vinifera* L. and another phylogenetic tree was generated among VvDIR proteins. The trees were generated with MEGA 7 (http://www.megasoftware.net.) using the neighbor-joining (NJ) and maximum likelihood (ML) methods, respectively. Bootstrap values were set at 1000 replications to evaluate the statistical reliability of the phylogenetic trees [56]. The resulting trees were visualized using Evolgenius online software (https://evolgenius.info//evolview-v2/#login).

Distribution of the *VvDIR* genes on chromosomes and duplication events

The chromosomal locations of *VvDIR* gene family members were identified according to physical location information from the Phytozome (https://phytozome-next.jgi.doe.gov). The chromosomal distribution map of the *VvDIR* gene family members were performed using TBtools [52]. Tandemly duplicated *VvDIR* genes were analyzed using MCScanX software [57], Duplication and collinearity analysis of *VvDIR* genes according to Basic Circos function module in TBtools.

Conserved motif distribution and gene structure analysis

For *cis*-regulatory elements analysis, the 2 kb upstream sequence of each *VvDIR* gene was extracted using the "GXF Sequences Extract" tool in TBtools software [52] and the obtained sequences were analyzed using the online PlantCare database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). 'TBtools *Basic BIO sequence View*' tool was utilized for visualization purposes.

RNA extraction and PCR analysis

RNA was extracted from "Red Globe " grape leaves using the RNApre Pure Plant Total RNA Extraction Kit (Tiangen, Beijing, China) following company guidelines. cDNA was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Dalian, China). For qPCR analysis cDNA was diluted 6 times. Gene-specific primers were designed using Primer Premier 5.0 software (Table S1). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Premix Ex Taq (2×) kit and the Applied Biosystems StepOne Plus instrument. The reaction mixture (20 μL) contained 10μL of SYBR Pre-mix Ex Taq (2×), 0.4 μL of each primer (10 μmol/L), 2 μL cDNA template, and 7.2 μL ddH_2O. The PCR was carried out under the following conditions: predenaturation at 95 °C for 2 min; denaturation at 95 °C for 5 s, annealing at 60 °C for 34 S, for 40 cycles; melt curve analysis: 95 °C for 15 S, 60 °C for 20 S, 95 °C for 15 S. Each reaction was performed three times and grape *Actin* (Accession no. XM_002282480) was used as an internal reference. The gene expression profiles were visualized using TBtools software and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [58].

Plasmid construction and transformation

The *VvDIR4* ORF sequence (582 bp) was amplified using LA Taq (TaKaRa Bio, Dalian, China) and then cloned into pMD20-T (TaKaRa Bio, Dalian, China). The resulting sequence was verified by sequencing (FuZhou ShangYa BioInc., Fuzhou, China) and submitted to GenBank (Acc. NO. OR903152). To generate the transgenic plants, the sequence was cloned into the binary vector pCAMBIA1300-HA (CAMBIA company) using specific primers *VvDIR4*-clone-F and *VvDIR4*-clone-R containing *BamH I* and *Kpn I* restriction sites (Table S1). The recombinant plasmid was transformed into *Agrobacterium tumefaciens* GV3101 using a freeze-thaw technique [59] and then transferred into wild *A. thaliana* using the floral dip method [60]. The transgenic plants were screened on a solid MS medium containing 50 mg/L hygromycin [61].

Measurement of the acid-soluble lignin content after *C. higginsianum* inoculation

Samples of leaves and stems were collected from both wild-type and transgenic plants of *A. thaliana* to analyze the lignin content using the acetyl bromide method [62]. After inoculating *A. thaliana* with *C. higginsianum*, samples were collected at three different time points: 0, 12, and 24 hpi, for a total of 128 plants. The extraction of lignin was performed following the instructions provided in the lignin extraction kit from Herui Bioscience (Herui Bio, Fujian, China), and the amount of lignin was measured and calculated accordingly.

Transient expression of *VvDIR4* in grapes

The *VvDIR4* gene was cloned into the pCAMBIA2300-HA vector (CAMBIA company) using specific primers

VvDIR4-GFP-F and *VvDIR4-GFP-R* containing *Kpn I* and *Xba I* sites (Table S1). The recombinant plasmid was transformed into *A. tumefaciens* GV3101 using the freeze-thaw technique. The transformed *Agrobacterium* was then transiently transformed into "Thompson Seedless" grape leaves using a vacuum infiltration method [63]. The transformed "Thompson Seedless" grape leaves were incubated in the dark for 2 days and used for pathogen infection experiments. Leaf samples were collected at 0, 24, 72, and 120 h for antioxidant enzyme activity detection. Herui Bio Kit (Fujian, China) was used according to the manufacturer's instructions for enzyme activity detection. Each experiment used 10 transiently transformed leaf samples as biological replicates, and each experiment was conducted thrice.

Statistical analysis

The averages and standard deviations of at least three independent replicates were used in all the experiments. Statistical analysis was performed using SPSS version 21.0. Student's t-test was used to detect the differences between the mean of the expression level, and the overall statistical significance of the data was determined at $^{*}p<0.05$ and $^{**}p<0.01$ levels.

Abbreviations

aa	Amino acid
Chr	Chromosome numbers
qPCR	Quantitative real-time polymerase chain reaction
HMM	Hidden Markov Models
Hpi	Hours post inoculation
hpt	Hours post treatment
ICS1	Isochorismate synthase
ICS1	Isochorismate synthase 1
LOX3	Lipoxygenase 3
MEME	Multiple Em for Motif Elicitation
MeJA	Methyl jasmonate
SA	Salicylic acid
MW	Molecular weight
ORF	Open reading frame
PDF1.2	Plant defensing 1.2
pI	Isoelectric point
PR1	Pathogenesis-related gene 1

Supplementary Information

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Supplementary Material 1

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Author contributions

Z.Q.M and W.Z.F planned, carried out the experiments, analyzed the data, interpreted the results and wrote the manuscript, performed the experiments. L.N and B.A were involved in revision of the manuscript. D.X.C and L.J.H were involved in the laboratory experiments. C.Q.X contributed to acquisition and analysis. L.Y and W.Z.F projected administration, supervision, visualization, and writing review & editing. All authors have read and approved the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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