The Impact of *Elsinoë ampelina* Infection on Key Metabolic Properties in *Vitis vinifera* ‘Red Globe’ Berries via Multiomics Approaches

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Accepted 16 September 2021.

Grape anthracnose caused by *Elsinoë ampelina* (Shear) is one of the most serious fungal diseases that lead to the quality reduction and yield losses of grape (*Vitis vinifera* ‘Red Globe’) berries. In the present study, metabolome and transcriptome analyses were conducted using grape berries in the field after infection with *E. ampelina* at 7, 10, and 13 days to identify the metabolic properties of berries. In total, 132 metabolites with significant differences and 6,877 differentially expressed genes were detected and shared by three comparisons. The analyses demonstrated that phenylpropanoid, flavonoid, stilbenoid, and nucleotide metabolisms were enriched in *E. ampelina*-infected grape berries but not amino acid metabolism. Phenolamide, terpene, and polyphenole contents also accumulated during *E. ampelina* infection. The results provided evidence of the enhancement of secondary metabolites such as resveratrol, α -viniferin, ϵ -viniferin, and lignins involved in plant defense. The results showed the plant defense-associated metabolic reprogramming caused by *E. ampelina* infection in grape berry and provided a global metabolic mechanism under *E. ampelina* stimulation.

Keywords: amino acid, fungus–plant interactions, genomics, grape anthracnose, infection, metabolomics, microscopy and imaging, phenylpropanoid, plant antifungal responses, proteomics, transcriptomics

Grapevines are one of the most important fruit crops worldwide, with a global cultivated area of approximately 7.4 million ha (OIV International Organization of Vine and Wine 2019).

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Funding: This work was supported by the National Natural Science Foundation of China grant number 32072534 and the Shanxi National Science Foundation grant number 2020JM-155.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary tables and supplementary figures are published online.

The author(s) declare no conflict of interest.

Grape berries are an important source of nutraceutical properties and bioactive compounds that have health benefits for humans (Bucić-Kojić et al. 2013). In 2019, the grape cultivation area in Shaanxi Province was 47,000 ha, ranking second in China, and the annual output value is over 5 billion CNY (National Bureau of Statistics of China 2019). *V. vinifera* ‘Red Globe’ has become the largest fresh grape cultivar in this region. However, grapevines are prone to several fungal diseases that cause quality damage and yield losses. Grape anthracnose results from *Elsinoë ampelina* infections of young tissues and is one of the important plant diseases worldwide, especially in high-humidity areas (Thind 2015). Most *Vitis vinifera* table and wine grape cultivars are highly susceptible to anthracnose, including Red Globe, Thompson Seedless, Pinot Noir, and Cabernet Sauvignon (Han et al. 2021; Kono et al. 2013; Li et al. 2021).

E. ampelina is a hemibiotrophic fungal pathogen and only infects *Vitis* spp. and hybrids. Other *Elsinoë* spp. cause disease on fruit perennials such as cane spot on raspberry caused by *E. veneta*, citrus scab by *E. fawcettii* and *E. australis*, and pear and apple spots caused by *E. pyri* (Li et al. 2018). Anthracnose symptoms initially appear on berries as small, circular reddish spots that enlarge and become sunken lesions with a gray-white center, which look like a bird’s eye (Santos et al. 2020). With high disease severity, grape berry infection by *E. ampelina* leads to delayed berry ripening, berry drop, and low quality for fresh fruit and wine production (Carisse and Morissette-Tomas 2013). Grape anthracnose causes 10 to 15% yield losses but severe infection can cause up to 100% yield losses on highly susceptible cultivars in a year of high disease prevalence (Magarey et al. 1993).

In addition to yield losses, *E. ampelina* induces physicochemical changes in grape leaves, including lower sugar, phenolic, carotenoid, chlorophyll, and ascorbic acid concentrations. In addition, there are higher phospholipid and malondialdehyde levels compared with uninfected leaves (Daulta and Chauhan 1981; Kansal and Lal 1979; Murria et al. 2018). Histopathological evidence showed that phenolic compounds accumulate in infected leaf areas (Braga et al. 2019). Gene expression involved in metabolic and biosynthetic processes are induced in infected grape leaves in response to *E. ampelina* infection (Ahn et al. 2014). Studies thus far have primarily focused on the effect of *E. ampelina* on leaves (Gao et al. 2012; Li et al. 2021) but comprehensive investigation of transcriptomic and metabolomic changes in berries has not been as well defined.

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Fruit susceptibility to infection is critically important to understand for the development of control strategies. Transcriptome and metabolome approaches have revealed some mechanisms of susceptible grapevines toward fungal infection (Agudelo-Romero et al. 2015; Blanco-Ulate et al. 2015). Many studies have reported the responsive mechanisms in grapevines triggered by *Botrytis cinerea* (Hong et al. 2012), *Plasmopara viticola* (Nascimento et al. 2019), and *Erysiphe necator* (Fung et al. 2008) through transcriptomic and metabolomic approaches but no reports have addressed the global transcriptomic and metabolic changes in fruits infected with *Elsinoë ampelina*.

The aim of this study was to investigate and understand the comprehensive influence of *E. ampelina* on grape berries under field conditions. Our data represent the first report of the transcriptome and metabolome analyses in grape berry response to *E. ampelina*. In this work, we provide novel insights into the responses and metabolic changes of berries during the interaction of the grape–*E. ampelina* pathosystem.

RESULTS

Phenotypic characterization of grape berries infected by *E. ampelina*.

Small brown spots appeared on the surface of *E. ampelina*-infected *V. vinifera* ‘Red Globe’ berries at 7 days postinoculation (dpi), whereas no symptoms were observed on the grape berries without *E. ampelina* infection. The spots enlarged rapidly from the sites of infection with dark-brown edges (Fig. 1A). Acervuli were observed on the lesions of the berries at 10 dpi by aniline blue and fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA-FITC) staining (Fig. 1B and C). The conidia of *E. ampelina* began to germinate and produce germ tubes at 1 dpi on the surface of grape berries (Fig. 2A and B). The germinating spores formed hyphae, and the proliferative hyphae formed hyphal cushions, which are an important foundation for the formation of acervuli (Fig. 2C).

Metabolic and transcriptional profiling analyses of infected and mock-treated grape berry samples.

We performed metabolome and transcriptome analyses on the berries of *V. vinifera* ‘Red Globe’ at 7, 10, and 13 dpi with or without *E. ampelina* to study the grape–*E. ampelina* interaction. In terms of metabolites, cluster heatmap and principal component analysis (PCA) showed that the biological replicates clustered together, with similar metabolic profiles for each time point. The replicates between *E. ampelina*-infected and noninfected berries displayed different separation (Supplementary Figs. S1 and S2). In total, 725 metabolites were identified using a liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based, widely targeted metabolomics method (Supplementary Table S1), and 365 metabolites showed significant difference (Fig. 3A). Among these metabolites, 139, 184, and 201 metabolites were increased in concentration at 7, 10, and 13 dpi, respectively, compared with noninfected control (Supplementary Fig. S3). In total, 132 metabolites, including flavonoids, amino acids, phenylpropanoids, nucleotide, derivatives, and lipids (Fig. 3B; Supplementary Table S2), were significantly different and shared by the three time points. Several processes involved in the biosyntheses of phenylpropanoid, flavonoid, flavone, flavonol, and amino acids were triggered by *E. ampelina* (Fig. 3C).

The 18 samples of grape berries (3 control and 3 inoculated each at 7, 10, and 13 dpi with three biological replicates) generated a range of 7.08 to 9.20 Gb of clean bases per sample by RNA sequencing (RNA-seq). Approximately 90% of the cleaned reads were mapped to the reference genome of

V. vinifera (Supplementary Table S3). Correlations were observed between the biological replicates and PCA analysis showed obvious separation between infected grape berries and controls (Supplementary Fig. S4). In total, 11,696, 12,002, and 9,713 differentially expressed genes (DEGs) were obtained at 7, 10, and 13 dpi, respectively, compared with the controls, and 6,877 DEGs (26.12% of total detected transcripts) were shared by three comparisons (Supplementary Fig. S5A). The DEGs were grouped into four clusters according to their patterns of expression. The expression of DEGs in clusters 1 and 2 increased whereas the expression in cluster 4 decreased during *E. ampelina* infection at 7, 10, and 13 dpi. We detected 6,381, 6,406, and 5,436 upregulated DEGs and 5,315, 5,596 and 4,277 downregulated DEGs at 7, 10, and 13 dpi, respectively (Supplementary Fig. S5B and C). As with metabolomic pathways, the enriched DEGs were involved in phenolic acid biosynthesis, flavonoid biosynthesis, pyruvate metabolism, and amino acid biosynthesis (Supplementary Fig. S6).

E. ampelina induced changes in phenolic acid, flavonoid, and stilbenoid metabolisms.

The activation of phenylpropanoid metabolism leads to flavonoid, lignin, and stilbenoid biosyntheses. The levels of 17 phenolic acids, 11 flavones, nine flavonols, four stilbenoids, three flavonoids, two flavanones, two proanthocyanidins, and one isoflavone were significantly different in *E. ampelina*-infected grape berries compared with healthy berries (Table 1). Except for the decrease in triclin 5-O-rutinoside and robinin, the other 47 metabolites significantly increased after inoculation with *E. ampelina*. *p*-Coumaraldehyde and coniferin were the two most abundant phenylpropanoids. Isorhamnetin, dihydroquercetin, and chrysoeriol were the three most abundant flavonoids. Resveratrol, piceatannol, ϵ -viniferin, and α -viniferin were the stilbenoids with relatively high contents (Fig. 4).

Approximately 95.74% (90 of 94) of the transcripts of phenylpropanoid, flavonoid, and stilbenoid biosynthesis genes were considerably upregulated in *E. ampelina*-infected grape berries (Supplementary Table S4). Four DEGs were selected for the validation of expression by quantitative real-time (qRT)-PCR, and the expression of *Vv4CL*, *VvCHS*, *VvFLS*, and *VvF3H* was upregulated in *E. ampelina*-infected grape berries (Supplementary Fig. S7). Several MYB transcription factors are known as regulators of phenylpropanoid pathways in grape berries. In our results, the activators *VvMYB5a*, *VvMYB5b*, *VvMYBP1*, *VvMYB14*, and *VvMYB15* were induced whereas the suppressor genes of the phenylpropanoid pathways *VvMYBC2-L1* and *VvMYB4a* were downregulated in *E. ampelina*-infected grape berries (Supplementary Table S4). The results suggested that the metabolites and synthesis-related genes involved in phenylpropanoid metabolism were triggered by *E. ampelina* in grape berries.

Changes in amino acid metabolism during *E. ampelina* infection.

In our results, 20 amino acids and derivatives were detected with significant difference (Supplementary Table S2). Ten amino acids—namely, D-(+)-phenylalanine, L-phenylalanine, L-proline, proline, L-threonine, L-(+)-arginine, L-(–)-tyrosine, L-methionine, L-tryptophan, and especially histidine—were significantly decreased during *E. ampelina* infection (Fig. 5B). Twenty-four genes were known in the amino acid metabolism (Fig. 5A). Twenty-five DEGs involved in amino acid metabolism were detected in response to *E. ampelina* infection (Fig. 5C). Among these genes, four DEGs involved in the biosyntheses of arginine, aspartic acid, tryptophan, and serine were downregulated, whereas the other genes were upregulated.

Fig. 1. Disease symptoms and acervuli observation in *Elsinoë ampelina*-infected *Vitis vinifera* 'Red Globe' berries at various days postinoculation. **A**, Symptom development of grape anthracnose in grape berries at 7, 10, and 13 days postinoculation (dpi). *-Ea* indicates noninfected grape berries and *+Ea* represents infected grape berries. Acervuli observation of *E. ampelina* on *V. vinifera* 'Red Globe' berries stained with **B**, aniline blue and **C**, fluorescein isothiocyanate-labeled wheat germ agglutinin at 7, 10, and 13 dpi. Scale bars: A = 1 cm, B = 10 μ m, and C = 50 μ m.

Alterations in nucleotide and derivate metabolisms during *E. ampelina* infection.

We detected 14 nucleotides and derivatives in three comparisons with significant difference (Supplementary Table S2). Three metabolites (uridine diphosphate glucose, uridine, and

pseudouridine) were accumulated in response to *E. ampelina* infection, and six metabolites associated with purine metabolism were significantly higher in *E. ampelina*-infected berries than healthy berries (Supplementary Fig. S8A and B). In terms of biosynthesis genes, only four genes were identified in nucleotide

Fig. 2. Conidial development and infection of *Elsinoë ampelina* on *Vitis vinifera* 'Red Globe' berries at various time points. Conidial germination of *E. ampelina* was observed by **A**, scanning electron microscopy and **B**, fluorescein isothiocyanate-labeled wheat germ agglutinin staining. **C**, Infection was observed by aniline blue staining. Abbreviations: A = acervuli, H = hyphae, and HC = hyphal cushion. Scale bars: A, 0 and 72 h postinoculation (hpi) = 5 μ m and 24 and 48 hpi = 5 μ m; B = 10 μ m; C, 1 and 3 days postinoculation (dpi) = 20 μ m and 5, 7, 10, and 13 dpi = 10 μ m.

and derivate metabolisms. The expression of genes that encode uridine nucleosidase, uridine kinase, and xanthine dehydrogenase/oxidase were activated, whereas 5'-nucleotidase was suppressed (Supplementary Fig. S8C).

Defense responses of grape berries to *E. ampelina* infection.

In this study, the contents of three phenolamides (putrescine, N-acetylputrescine and agmatine) and two terpenes (nimbin and phytol) were significantly higher in *E. ampelina*-infected berries than in healthy berries (Fig. 6). Most of the DEGs related to

reactive oxygen species (ROS) and antioxidant activity, including glutathione peroxidase, peroxidase, catalase, and superoxide dismutase, were upregulated; however, the DEGs that encode toll interleukin 1 (TIR) and nucleotide-binding adaptor shared by APAF-1, resistance proteins, and CED-4 (NB-ARC) domain proteins were downregulated in *E. ampelina*-infected berries at 7, 10, and 13 dpi (Supplementary Fig. S9).

The contents of four polyphenols (epicatechin gallate, procatechuic acid, catechin gallate, and gallic acid) and two components of lignin (pinoresinol and coniferin) significantly accumulated more in *E. ampelina*-infected berries than

Fig. 3. Metabolic profiling of grape berries after infection with *Elsinoë ampelina*. **A**, Venn diagram of different metabolites ($P < 0.05$; $|\log_2[\text{fold change}]| \geq 1$) in *E. ampelina*-infected berries and noninfected berries (control) at 7, 10, and 13 days postinoculation (dpi). **B**, Composition of 132 different metabolites shared by three comparisons between *E. ampelina*-infected berries and noninfected berries at 7, 10, and 13 dpi. **C**, Heatmap of the different metabolites involved in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in *E. ampelina*-infected berries compared with noninfected berries at 7, 10, and 13 dpi. The heatmap scale is provided on the upper right. Numbers indicate metabolites involved in different KEGG pathways.

in healthy berries (Figs. 4 and 6), and lignin accumulation was observed using phloroglucinol-HCl staining (Supplementary Fig. S10A). The upregulation of eight DEGs that encode lac-cases with potential functions in lignin biosynthesis provided further evidence of the activation of lignin metabolism in response to *E. ampelina* infection (Supplementary Fig. S10B).

DISCUSSION

E. ampelina mainly infects young grape tissues such as leaves, petioles, stems, tendrils, and berries (Santos et al. 2017). Among these, berries are an important harvest object. As important as berries are, it is currently not fully understood how susceptible berries alter their metabolic processes in defense against

infection by *E. ampelina*. Therefore, the current study provided transcriptomic and metabolomic profiles of berries to investigate molecular mechanism of berries after being attacked by *E. ampelina*. In this study, the first symptom was observed from 7 dpi and acervuli formation was found at 10 dpi (Fig. 1). However, on leaves, first symptoms and acervuli were observed at 3 and 4 dpi (Li et al. 2019), indicating that symptom development was delayed in grape berries compared with leaves. The difference may due to the thicker waxes and more “hard” wax components (oleanolic acid) on the berries than on leaves (Possingham et al. 1967). Previous research has revealed highly significant correlations between cuticles and epicuticular waxes and the resistance of grape to *B. cinerea* (Herzog et al. 2015). Skin features of grape tissues seem to influence the development

Table 1. Changes induced by *Elsinoë ampelina* in 49 metabolites involved in phenolic acid, flavonoid, and stilbenoid metabolisms

Compounds	Ions (Da) ^a		Rt (min) ^b	MW (Da) ^c	Log ₂ fold change ^d		
	Q1	Q3			7 dpi	10 dpi	13 dpi
Feruloyl syringic acid	375.20	137.10	3.43	374.20	1.82	2.31	3.01
Hydroxy-methoxycinnamate	195.10	145.30	3.19	194.10	1.65	2.52	2.46
Caffeate	179.00	135.00	3.16	180.04	1.18	1.22	1.54
Ferulic acid	193.10	134.00	4.03	194.06	2.47	2.59	3.21
3-(4-Hydroxyphenyl)propionic acid	165.10	119.00	3.84	166.06	12.84	4.34	12.86
3-Hydroxy-4-methoxycinnamic acid	193.10	134.00	4.03	194.06	2.40	2.59	3.15
p-Coumaric acid	163.00	119.00	3.83	164.05	3.17	3.70	4.01
Coniferyl alcohol	179.10	146.00	3.86	180.08	1.61	2.46	3.51
Pinorensinol	357.10	151.00	5.36	358.14	2.29	2.86	3.07
Coniferin	341.00	179.00	2.72	342.13	4.92	6.88	7.22
Esculin (6,7-Dihydroxycoumarin-6-glucoside)	339.10	177.00	2.71	340.08	1.96	2.66	3.06
Sinapinaldehyde	207.10	192.00	4.43	208.00	2.33	3.95	4.52
p-Coumaraldehyde	147.10	129.00	4.25	148.00	3.74	12.95	6.63
Methyl p-coumarate	179.10	147.10	5.48	178.18	1.21	1.04	1.25
(E)-p-coumaric acid	165.00	146.80	3.86	164.05	2.66	3.35	3.48
4-Hydroxy-3-methoxycinnamaldehyde	177.10	162.00	4.65	178.06	1.29	2.78	2.70
Acetosyringone	195.10	137.00	4.27	196.07	11.24	12.63	12.91
Selgin 5-O-hexoside	479.10	317.10	3.54	478.10	1.99	3.73	3.61
C-hexosyl-chrysin O-feruloylhexoside	755.20	177.10	4.58	754.20	2.50	2.73	3.96
Syringetin 5-O-hexoside	509.20	347.10	4.01	508.20	2.39	2.41	3.42
Tricin	331.10	315.00	5.69	330.10	1.43	1.56	3.36
Syringetin 7-O-hexoside	509.30	347.20	4.10	508.30	2.57	2.87	3.21
Chrysoeriol 7-O-hexoside	463.20	301.10	4.25	462.20	2.82	2.98	3.71
Hesperetin C-hexosyl-O-hexosyl-O-hexoside	789.10	626.80	2.63	788.10	2.08	2.23	2.77
Tricin 5-O-rutinoside	637.10	329.20	3.98	638.10	-1.36	-1.29	-5.81
Amentoflavone	537.10	375.00	5.81	538.09	10.37	11.90	11.97
Apigenin 5-O-glucoside	433.10	271.00	3.75	432.11	18.35	19.93	20.21
Chrysoeriol	299.10	284.00	5.71	300.06	4.80	5.32	5.48
Myricetin	317.00	151.00	4.43	318.04	1.22	1.45	1.59
Dihydroquercetin (Taxifolin)	303.10	125.00	4.09	304.06	5.57	4.05	5.53
Isorhamnetin 3-O-neohesperidoside	623.20	314.00	3.75	624.17	2.11	5.38	4.15
Isorhamnetin O-hexoside	479.20	317.20	4.11	478.20	2.02	2.66	3.88
Isorhamnetin 5-O-hexoside	479.20	317.20	4.00	478.20	2.05	2.75	3.82
Isorhamnetin	315.10	151.00	5.81	316.06	8.81	3.87	9.23
Aromadecin (Dihydrokaempferol)	287.10	259.00	4.58	288.06	3.54	3.31	3.71
Rhamnetin (7-O-methoxyl quercetin)	317.00	243.00	6.19	316.06	11.27	11.70	12.94
Kaempferol-3-O-robinoside-7-O-rhamnoside (Robinin)	741.20	433.00	3.31	740.22	-2.12	-2.26	-3.28
Deoxyrhapontin	405.10	243.10	5.09	404.15	2.25	2.14	2.14
Isorhamnetin 3-O-glucoside	477.10	314.00	4.19	478.11	2.52	3.26	4.21
5,7-Dihydroxychromone	177.00	88.90	4.47	178.03	13.80	14.89	15.33
Eriodictol	287.10	135.00	5.03	288.06	2.43	3.10	4.83
Naringenin chalcone	271.10	151.00	5.45	272.07	2.00	1.03	2.15
Procyanidin A1	575.00	285.00	3.69	576.13	2.60	2.75	2.77
Procyanidin B3	577.10	407.10	2.87	578.14	1.29	1.28	1.62
Formononetin 7-O-glucoside (Ononin)	429.10	267.00	4.54	430.13	2.02	1.54	1.44
Resveratrol	227.10	185.00	4.74	228.08	5.87	6.74	6.41
Piceatannol	243.10	159.10	4.26	244.07	7.53	6.71	7.34
ε-Viniferin	455.10	215.20	5.41	454.14	5.45	6.12	7.43
α-Viniferin	679.20	453.40	5.67	678.19	6.13	6.67	7.96

^a Q1 = precursor ions and Q3 = product ions.

^b Retention time.

^c Molecular weight.

^d Fold change of relative content of listed metabolites in infected berries compared with healthy berries at 7, 10, and 13 days postinoculation (dpi) with significant difference ($P < 0.05$).

of grape anthracnose. The symptoms of grape anthracnose on berries were estimated with percentage of lesion over the whole cluster areas from 0 to 7 grades (Wang et al. 1998). In this study, the three time points (7, 10, and 13 dpi) represented the low grade, middle grade, and high grade, respectively, on diseased berries. The samples of these three time points were used to analyze the metabolic and transcriptional changes during the disease process. However, time points of sampling should be conducted before visible symptoms in order to study early signal transduction or recognition response on *E. ampelina*-infected berries.

Mitra 2007). Secondary metabolites are also synthesized as a part of the plant defense strategy in combating pathogens. In this study, metabolic variations in phenolic acid, flavonoid, and stilbenoid pathways were induced by *E. ampelina* infection. Integrated metabolite profiling and transcriptome analysis showed that 49 metabolites and 90 transcripts associated with those metabolic pathways were activated by *E. ampelina* infection. The results were consistent with previous reports in grape berries (Agudelo-Romero et al. 2015; Blanco-Ulate et al. 2015) and leaves (Li et al. 2021) in response to fungal pathogens. Several MYB transcription factors are known as regulators of phenylpropanoid pathways in grape berries, including *VvMYB5a* (Deluc et al. 2006), *VvMYB5b* (Deluc et al. 2008), *VvMYBPA1* (Bogs et al. 2007), *VvMYB14*, and *VvMYB15* (Höhl et al. 2013) as activators and *VvMYBC2-L1* and *VvMYB4a* as suppressors (Cavallini et al. 2015; Huang et al. 2014). In our results, *VvMYB5a*, *VvMYB5b*, *VvMYBPA1*, *VvMYB14*, and *VvMYB15*

were induced as expected whereas *VvMYBC2-L1* and *VvMYB4a* were downregulated in *E. ampelina*-infected grape berries. The expression patterns of these genes were consistent with the previous results of grape response to *B. cinerea* (Blanco-Ulate et al. 2015). In addition, phenolamides and terpenoids, which are important secondary metabolites that are related to plant defense (Celedon et al. 2017; Dong et al. 2015), also were accumulated in *E. ampelina*-infected berries. Finally, morphological evidence did not show symptoms and infection hyphae in resistant Chinese wild *V. quinquangularis* accession Shang-24 (Han et al. 2021), whereas typical disease symptoms and infection hyphae were found in this study (Fig. 1). Taken together, activation of defense responses in susceptible berries are inducing tolerance rather than resistance to *E. ampelina*.

In addition to prominent functions in protein biosynthesis, amino acids play pivotal roles in plant abiotic and biotic stress responses (Hildebrandt et al. 2015). For example, proline

Fig. 5. Changes in amino acid metabolism in grape berries during *Elsinoë ampelina* infection. **A**, Pathway for amino acid metabolism based on Kyoto Encyclopedia of Genes and Genomes pathways. Amino acids in blue are those detected in this study, whereas amino acids in black were not found in this study. **B**, Heatmap of amino acids during *E. ampelina* infection. Displayed amino acids are significantly differentially regulated at every time point when compared with healthy berries ($P < 0.05$); dpi = days postinoculation. **C**, Heatmap of differentially expressed genes involved in amino acid metabolism during *E. ampelina* infection. Displayed genes are significantly differentially regulated at every time point when compared with healthy berries ($P < 0.05$). P5C = pyrroline-5-carboxylate, Glu = glutamine, Phe = phenylalanine, Pro = proline, Thr = threonine, His = histidine, Arg = arginine, Tyr = tyrosine, Met = methionine, Trp = tryptophan, Asp = aspartic acid, Gln = glutamine, Cys = cysteine, and Ser = serine. Numbers near each arrow refer to enzyme names as follows: 1, proline dehydrogenase; 2, pyrroline-5-carboxylate dehydrogenase; 3, ornithine aminotransferase; 4, arginase; 5, carbamoyl-phosphate synthase; 6, asparaginase; 7, aspartate aminotransferase; 8, argenase dehydrogenase; 9, chorismate mutase; 10, argenase dehydrogenase; 11, anthranilate synthase α ; 12, anthranilate phosphoribosyltransferase; 13, indole-3-glycerol phosphate synthase; 14, tryptophan synthase; 15, aspartate-semialdehyde dehydrogenase; 16, aspartokinase/homoserine dehydrogenase; 17, cystathionine γ -synthase; 18, S-adenosylmethionine synthase; 19, S-adenosylmethionine-dependent methyltransferase; 20, adenosylhomocysteinase; 21, cysteine desulfurase; 22, serine hydroxymethyltransferase; 23, threonine aldolase; and 24, threonine dehydratase.

accumulation is related to plant susceptibility to pathogen infection (Haudecoeur et al. 2009). Arginine is a precursor for the synthesis of nitric oxide (NO) and polyamines (Winter et al. 2015). NO is an activator of local and systemic immune responses (Jedelská et al. 2021) and polyamines are scavengers of ROS (Shi et al. 2013). Exogenous arginine treatment also induced tomato resistance to *B. cinerea* (Zheng et al. 2011). In our results, the contents of 10 amino acids (such as proline and arginine) were significantly reduced in *E. ampelina*-infected grape berries compared with healthy grape berries. These results were consistent with previous reports that the levels of free amino acids are lower in *E. ampelina*-infected grape leaves than in healthy grape leaves (Murria et al. 2018). Upregulated genes were found in amino acid biosynthesis but the content of several amino acids was reduced. These results may be explained by the fact that amino acids are needed for the production of many plant defense compounds and metabolites (Zhou et al. 2015), and the enzymatic activity involved with amino acid synthesis may be affected by toxins or effectors of pathogens (van Andel 1966). Furthermore, plant amino acids have been shown to be consumed by *B. cinerea* during infection

(Dulermo et al. 2009). Liu et al. (2010) reported that lower contents of glutamine, alanine, and proline enhanced resistance to diverse pathogens. Therefore, the lower contents of amino acids may limit the amino acids consumed by pathogens as a defense strategy. However, some amino acids accumulated in host plants after inoculation with different pathogens compared with non-inoculated plants (Ward et al. 2010). It seems that accumulated or reduced amino acids depended upon the type of pathogen used and both were involved in plant defense (Rojas et al. 2014).

In this study, 14 metabolites associated with nucleic acid metabolism were significantly accumulated in *E. ampelina*-infected berries. It is also known that nucleoside analogs exhibit antifungal activities (Srivastava et al. 2007). For example, some uridine derivatives significantly inhibited the growth of bacterial and fungal strains (Kawsar et al. 2018). It is likely that nucleosides and nucleotides are involved in plant defense. In addition to participating in nucleic acid synthesis, purine and pyrimidine are energy sources and the precursors for the synthesis of primary and secondary products (Stasolla et al. 2003). The activation of nucleotide metabolism is needed for the host to establish

Fig. 6. Polyphenol, phenolamides, and terpene contents in *Elsinoë ampelina*-infected (+Ea) and noninfected (–Ea) *Vitis vinifera* ‘Red Globe’ berries at 7, 10, and 13 days postinoculation (dpi). Data represent the mean values $\pm$ standard error of three independent measurements. An asterisk (*) indicates significant difference ($P < 0.05$) by one-way analysis of variance between *E. ampelina*-infected and noninfected berries.

a defense system (Zrenner et al. 2006) such as providing upstream substrates for the synthesis of downstream defense metabolites. Another possible speculation is that changes in the host's nucleotide metabolism are believed to be induced by the fungus for nutrient acquisition or to modify systems for its own benefit. More comprehensive studies are needed to prove the role of nucleotide and nucleoside analogs in plant defense.

The defense response causes photosynthesis and respiration reduction, energy deprivation, growth inhibition, and yield loss (Deng et al. 2017). In response to fungal infection, the large shift of gene transcription is also an energy-consuming process (Bolton 2009). For example, in total, 6,877 DEGs were found in this study and 6,847 DEGs were detected in *V. vinifera* berries in response of *B. cinerea* (Blanco-Ulate et al. 2015). In particular, susceptible grape plants have more responsive transcripts associated with defense response than resistant grape plants (Fung et al. 2008). In addition, the energy exhaustion by host defense against *E. ampelina* may delay berry development ripening, which are the common impacts of grape anthracnose on grape berries (Carisse and Morissette-Tomas 2013; Magarey et al. 1993). Therefore, the defense cannot effectively prevent *E. ampelina* infection and symptom development in susceptible *V. vinifera* 'Red Globe' despite the fact that the reprogramming of metabolites and transcripts is involved in defense activation in *E. ampelina*-infected grape berries.

In conclusion, this work provides the first combined metabolomic and transcriptomic analysis of the impacts of *E. ampelina* on grape berries. The reprogramming of primary metabolism in *E. ampelina*-infected grape berries seems to provide substrates for the synthesis of secondary metabolites such as phenolic acids, flavonoids, and stilbenoids, which play important roles in plant defense. A number of metabolites and genes were induced in grape berries in response to *E. ampelina*. Although abundant secondary metabolites are beneficial to human health (Bucić-Kojić et al. 2013) and needed as a defense response against pathogens, this defense leads to the decline of growth and the loss of yield (Zhang et al. 2020). In addition, unclear gaps remained in our understanding of the reprogramming of grape during *E. ampelina* infection. Nucleotides, for instance, are important primary metabolites but little is known about how the changes are regulated by the host infected with *E. ampelina* or other pathogens for its own benefit. Overall, the information provided within this study will be useful to improve the physiological and molecular relevance of studies in the future.

MATERIALS AND METHODS

Fungal infection and sample collection.

Field experiments were conducted using 6-year-old *V. vinifera* 'Red Globe' plants in the germplasm nursery of the Northwest A&F University, Shaanxi, China (latitude = 34.16°, longitude = 108.07°). The plants were clonally propagated and spaced 2 to 4 m apart in the field. *E. ampelina* strain YL-1 was isolated from diseased Red Globe grapevines and maintained in potato dextrose agar (PDA) at 25°C as previously described (Li et al. 2019). Conidia were harvested from 25-day-old colonies, which were grown in PDA bottles at 21°C for 24 h in the dark (Li et al. 2018). Conidial concentration was adjusted to 2×10^6 conidia/ml in sterile water with a hemocytometer. Inoculation was conducted on berries by spraying the conidial suspension using a small sprayer at the development stage of EL33 (Coombe 1995). Every cluster was inoculated with 5 ml of conidial suspension. Control clusters were sprayed with sterile water. The clusters were then enclosed with individual plastic bags for 1 week (Agudelo-Romero et al. 2015). Samples were collected at 7, 10, and 13 dpi. For each time point of inoculated and control plants, five clusters were collected and the experiment was repeated

three times. All inoculated and control clusters were harvested, immediately frozen in liquid nitrogen, and kept at -80°C for metabolomic and transcriptomic analysis. For histochemical observation, all fresh samples were fixed in FAA solution (90 ml of 70% ethanol, 5 ml of formalin, and 5 ml of acetic acid) or 2.5% (vol/vol) glutaraldehyde in phosphate buffer (pH 7.2, 100 mM).

Histochemical observation.

WGA-FITC (Sigma-Aldrich) staining and scanning electron microscopy (SEM) were used to investigate the conidial development of *E. ampelina* on the surface of berries. Five pieces from five inoculated berries were observed at 0, 24, 48, and 72 h postinoculation (hpi). Each assay was performed three times. For WGA-FITC staining, small pieces (0.3 by 0.3 cm) were cut from the grape berries, fixed in 50% ethanol for 2 h, then cleared in 20% KOH for 2 days (Kobae et al. 2016). The specimens were soaked in phosphate buffer solution (PBS, pH 7.5) for 30 min, then stained with WGA-FITC at 20 µg/ml (g/vol) overnight at room temperature in the dark. The pieces were washed three times with PBS, then observed with an Olympus BX-53 microscope (Olympus) under blue light excitation. For SEM observation, small pieces were fixed in 2.5% (vol/vol) glutaraldehyde in phosphate buffer (pH 7.2, 100 mM) for 24 h at room temperature, dehydrated using a graded ethanol from 30 to 90% (vol/vol) for 15 min, dehydrated three times using absolute ethanol, dried with a CO₂ critical point dryer (EM CPD300; Leica), then observed by SEM (SU8010; Hitachi) as previously described (Li et al. 2019).

Aniline blue and WGA-FITC staining were used for the acervuli observation of *E. ampelina* on infected berries. Briefly, small pieces were dehydrated in an ethanol series, embedded in paraffin, and sectioned using a Leica EM UC7 microtome. The 5-µm sections were separately stained with 150 mM K₂HPO₄ (pH 9.5) containing 0.1% (wt/vol) aniline blue for 30 min and WGA-FITC at 20 µg/ml (g/vol) using the previously described method (Li et al. 2019). The samples were observed with a BX-53 microscope (Olympus) at 7, 10, and 13 dpi for WGA-FITC staining and 1, 3, 5, 7, 10, and 13 dpi for aniline blue staining. Five pieces from five inoculated berries were observed at each time points. Each assay was performed three times.

Phloroglucinol-HCl was used to label lignin for lignin staining. The sections were obtained as mentioned above, stained for 30 min with phloroglucinol reagent (10 g of phloroglucinol and 95 ml of absolute ethanol), washed with HCl, and examined with a light microscope (Olympus BX-53) as previously described (Cadot et al. 2006). Five pieces from five inoculated berries were observed at 7, 10, and 13 dpi. Each assay was performed three times.

RNA extraction, Illumina sequencing, and transcriptome data analysis.

Six samples of inoculated and control grape clusters (7, 10, and 13 dpi) were used for transcriptome analysis to investigate *E. ampelina* gene expression in grape berries. Each sample was composed of 20 berries. Total RNA was extracted from three biological replicates of each sample using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA degradation and contamination were assessed by 1% agarose gel electrophoresis. Purity and concentration were determined using NanoDrop (Thermo Scientific) and Qubit 2.0 fluorometers (Life Technologies, Carlsbad, CA, U.S.A.), respectively. cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Transcriptome sequences were obtained using an Illumina HiSeq2500 at the Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) from 18 RNA samples.

High-quality reads were aligned against the *E. ampelina* YL-1 genome and *V. vinifera* reference genome (12 × PN40024). Gene expression levels were calculated using the Cufflinks program based on the expected number of fragments per kilobase of transcript sequence per millions of base pairs sequenced (Trapnell et al. 2012). Transcripts with a corrected $P < 0.05$ and $|\log_2[\text{fold change}]| \geq 1$ were considered significant DEGs relative to the controls. The DEGs were then subjected to Gene Ontology enrichment with the threshold of $P < 0.05$. Venn diagram and hierarchical clustering were used to illustrate the differential gene expression patterns between different groups. Heatmaps were generated using the TBtools software (Chen et al. 2018).

qRT-PCR analysis.

qRT-PCR was performed to validate the RNA-seq transcriptome data, with four genes in the phenylalanine pathway as the target genes. Total RNA was extracted from three biological replicates of grape berries under control and inoculation condition using Trizol reagent (Invitrogen). cDNA was synthesized using PrimerScript Reverse Transcriptase (TaKaRa) according to the manufacturer's protocol. qRT-PCR was performed with SYBR qPCR Master Mix (Vazyme). The *elongation factor 1- α* gene was used to normalize gene expression (Yan et al. 2017). The primers used are listed in Supplementary Table S5. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Metabolomic analysis of infected and mock-treated grape berry samples.

E. ampelina-inoculated and control grape berry samples were subjected to metabolomics analysis with three biological replicates at 7, 10, and 13 dpi. Each sample was composed of 20 berries. For each sample, approximately 100 mg of frozen grape berry was crushed using a mixer mill (MM 400; Retsch) with a zirconia bead for 1.5 min at 30 Hz and extracted overnight at 4°C with 1.0 ml of 70% aqueous methanol. Samples were then centrifuged at $10,000 \times g$ for 10 min as previously described (Chen et al. 2013). The supernatants were absorbed (CNWBOND carbon-graphitized carbon black solid-phase extraction cartridge, 250 mg, 3 ml; Anpel, Shanghai, China) and filtrated (SCAA-104, 0.22- μm pore size; Anpel) before LC–electrospray ionization (ESI)–MS/MS analysis.

The sample extracts (2 μl in volume) were analyzed using an LC-ESI-MS/MS system (high-performance liquid chromatography [HPLC], Shim-pack ultrafast LC Shimadzu CBM30A system; MS, Applied Biosystems 6500 Q TRAP) on a Waters Acquity UPLC HSS T3 C18 HPLC column (1.8 μm , 2.1 by 100 mm) with mobile phases consisting of water (0.04% acetic acid, solvent A) and acetonitrile (0.04% acetic acid, solvent B). The gradient program was as follows: 95:5% (A:B) (vol/vol) at 0 min, 5:95% (A:B) (vol/vol) at 11.0 min, 5:95% (A:B) (vol/vol) at 12.0 min, 95:5% (A:B) (vol/vol) at 12.1 min, and 95:5% (A:B) (vol/vol) at 15.0 min. The flow rate was 0.40 ml/min, and the column temperature was 40°C. The effluent was alternatively connected to a triple-quadrupole linear ion trap (Q TRAP) MS (API 6500 Q TRAP LC/MS/MS system) equipped with a TurboIonSpray ESI interface operating in positive ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source was turbo spray; ion spray voltage was 5500 V; source temperature was 500°C; ion source gases were gases I and gas II; and curtain gas was set at 55, 60, and 25.0 psi (Wang et al. 2018).

Metabolite quantification and annotation were performed by MetWare Biotechnology Co., Ltd. (Wuhan, China). For quantitative analyses, the characteristic ions of each metabolite were screened through the mass spectrometer signals obtained by

screening the characteristic ions of each metabolite, filtering the peak type, and removing the redundant signals using the multiple reaction monitoring mode (Supplementary Table S1). The integration and correction of chromatographic peaks were performed using MultiQuant version 3.0.2 (AB SCIEX, Concord, Ontario, Canada). Metabolites in infected grape berry with corrected $|\log_2[\text{fold change}]| \geq 1$ and $P < 0.05$ were considered significantly changed relative to the mock-treated grape berry (Yang et al. 2020). The relative contents of metabolites were represented as chromatographic peak area integrals. All of the data were transformed to $\log_2$ fold change for further analysis.

Statistical analysis.

Hierarchical cluster analysis and PCA of metabolites were conducted using the R package as previously described (Wang et al. 2018). Correlation analysis of qRT-PCR and RNA-seq data were performed using GraphPad Prism 7.00 (GraphPad Software Inc.). All data were analyzed using SPSS 17.0 software, and data were expressed as the mean $\pm$ standard deviation of triplicates. The differences between the means were analyzed using one-way analysis of variance, and a P value < 0.05 was considered to be significant.

Data availability.

Raw RNA-seq data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under the accession number PRJNA678563.

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