



# Deciphering the Molecular Signatures Associated With Resistance to *Botrytis cinerea* in Strawberry Flower by Comparative and Dynamic Transcriptome Analysis

Guilin Xiao, Qinghua Zhang, Xiangguo Zeng, Xiyang Chen, Sijia Liu and Yongchao Han\*

## OPEN ACCESS

### Edited by:

Raj Majumdar,  
United States Department of  
Agriculture, United States

### Reviewed by:

Hyong Woo Choi,  
Andong National University,  
South Korea  
Paul Galewski,  
United States Department of  
Agriculture (USDA), United States

### \*Correspondence:

Yongchao Han  
hyc660@126.com

### Specialty section:

This article was submitted to  
Plant Pathogen Interactions,  
a section of the journal  
Frontiers in Plant Science

Received: 03 March 2022

Accepted: 19 April 2022

Published: 27 May 2022

### Citation:

Xiao G, Zhang Q, Zeng X, Chen X,  
Liu S and Han Y (2022) Deciphering  
the Molecular Signatures Associated  
With Resistance to *Botrytis cinerea* in  
Strawberry Flower by Comparative  
and Dynamic Transcriptome Analysis.  
*Front. Plant Sci.* 13:888939.  
doi: 10.3389/fpls.2022.888939

Hubei Key Laboratory of Vegetable Germplasm Enhancement and Genetic Improvement, Institute of Industrial Crops, Hubei Academy of Agricultural Sciences, Wuhan, China

Gray mold caused by *Botrytis cinerea*, which is considered to be the second most destructive necrotrophic fungus, leads to major economic losses in strawberry (*Fragaria × ananassa*) production. *B. cinerea* preferentially infects strawberry flowers and fruits, leading to flower blight and fruit rot. Compared with those of the fruit, the mechanisms of flower defense against *B. cinerea* remain largely unexplored. Therefore, in this study, we aimed to unveil the resistance mechanisms of strawberry flower through dynamic and comparative transcriptome analysis with resistant and susceptible strawberry cultivars. Our experimental data suggest that resistance to *B. cinerea* in the strawberry flower is probably regulated at the transcriptome level during the early stages of infection and strawberry flower has highly complex and dynamic regulatory networks controlling a multi-layered defense response to *B. cinerea*. First of all, the higher expression of disease-resistance genes but lower expression of cell wall degrading enzymes and peroxidases leads to higher resistance to *B. cinerea* in the resistant cultivar. Interestingly, CPKs, RBOHDs, CNGCs, and CMLs comprised a calcium signaling pathway especially play a crucial role in enhancing resistance by increasing their expression. Besides, six types of phytohormones forming a complex regulatory network mediated flower resistance, especially JA and auxin. Finally, the genes involved in the phenylpropanoid and amino acids biosynthesis pathways were gene sets specially expressed or different expression genes, both of them contribute to the flower resistance to *B. cinerea*. These data provide the foundation for a better understanding of strawberry gray mold, along with detailed genetic information and resistant materials to enable genetic improvement of strawberry plant resistance to gray mold.

**Keywords:** strawberry (*fragaria × ananassa*), flower, *Botrytis cinerea*, RNA sequencing, resistance-related genes

## INTRODUCTION

*Botrytis cinerea* is a necrotrophic fungal pathogen that causes gray mold disease in more than 1,400 plant species, including almost all vegetable and fruit crops (Elad et al., 2016). This polyphagous pathogen is regarded as the second most destructive phytopathogen globally, as it is found worldwide and damages fruit, flower, and leaf both before and after harvest (Dean et al., 2012). Data suggest that gray mold disease causes \$10 billion to \$100 billion in losses per year (Weiberg et al., 2013).

Plants have evolved a multi-layered defense system against *B. cinerea*. First, the plant cell wall acts as a physical barrier to *B. cinerea* infection (Underwood, 2012; Blanco-Ulate et al., 2014). Next, once the pathogen penetrates cell walls, plants detect this attack and trigger signaling pathways that induce defense responses at the plant cell walls (Hematy et al., 2009). Plant pattern recognition receptors (PRRs) such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs) recognize pathogen-associated molecular patterns (PAMPs) and host damage-associated molecular patterns (DAMPs), activating PAMP-triggered immunity, key transcriptional regulators in the defense against *B. cinerea* (Mengiste, 2012; Lai and Mengiste, 2013). The signals from these RLKs and RLPs are then transduced to mitogen activated protein kinase (MAPK)-dependent and/or -independent cascades and activate *WRKY33*, resulting in the up-regulation of genes involved in camalexin biosynthesis (Birkenbihl et al., 2012; Zhou et al., 2020). Plant hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) also contribute to the plant resistance to *B. cinerea* (AbuQamar et al., 2017). However, to date, little information has been obtained about the effects of *B. cinerea* infection on these processes in strawberry (*Fragaria × ananassa*) (Underwood, 2012; González et al., 2013; Li et al., 2013; Petrasch et al., 2019; Lu et al., 2020), a popular small fruit crop with short production cycles, extremely high nutrition, and good flavor.

*Botrytis cinerea* preferentially infects strawberry flowers and fruits, leading to flower blight and fruit rot, which are the two most important causes of yield and economic losses. Studies have demonstrated that *B. cinerea* inoculum primarily enters the strawberry flower organs; the infected petals, stamens, and calyxes and then facilitate primary infection in fruits (Petrasch et al., 2019). The previous studies have focused primarily on the interaction of on *B. cinerea* with strawberry fruit (Liang et al., 2018; Xiong et al., 2018; Haile et al., 2019) while paying little attention to its interaction with the flower.

Moreover, the researches on resistance mechanisms have been hampered by the lack of fully resistant strawberry resources (Bestfleisch et al., 2015). As documented by Bristow et al. (1986), the levels of resistance to *B. cinerea* varies between strawberry cultivars as detailed as follows: Fungal growth in stamens appears to be strongly inhibited—so that the fungus never reaches the receptacle—in some cultivars, but not in others.

In our previous work, we established a method to evaluate *B. cinerea* resistance in strawberry flower and found different levels of resistance in different cultivars (data not shown). Nonetheless, the molecular signatures have not been deciphered until now. The recent research has demonstrated that next-generation RNA

sequencing (RNA-seq) can serve as a powerful tool to elucidate the transcriptional reprogramming induced by biotic and abiotic stresses (Kou et al., 2018; Guo et al., 2019). The RNA-seq has been used to study the interactions between *B. cinerea* and plant hosts including the model plants *Arabidopsis* (Windram et al., 2012) and tomato (Blanco-Ulate et al., 2013; Smith et al., 2014) and the non-model plants lettuce (De Cremer et al., 2013), cucumber (Kong et al., 2015), grape (Haile et al., 2017), and kiwi (Zambounis et al., 2020). However, this approach has so far provided little information about *B. cinerea* infection of strawberry (Xiong et al., 2018; Haile et al., 2019), and particularly strawberry flower.

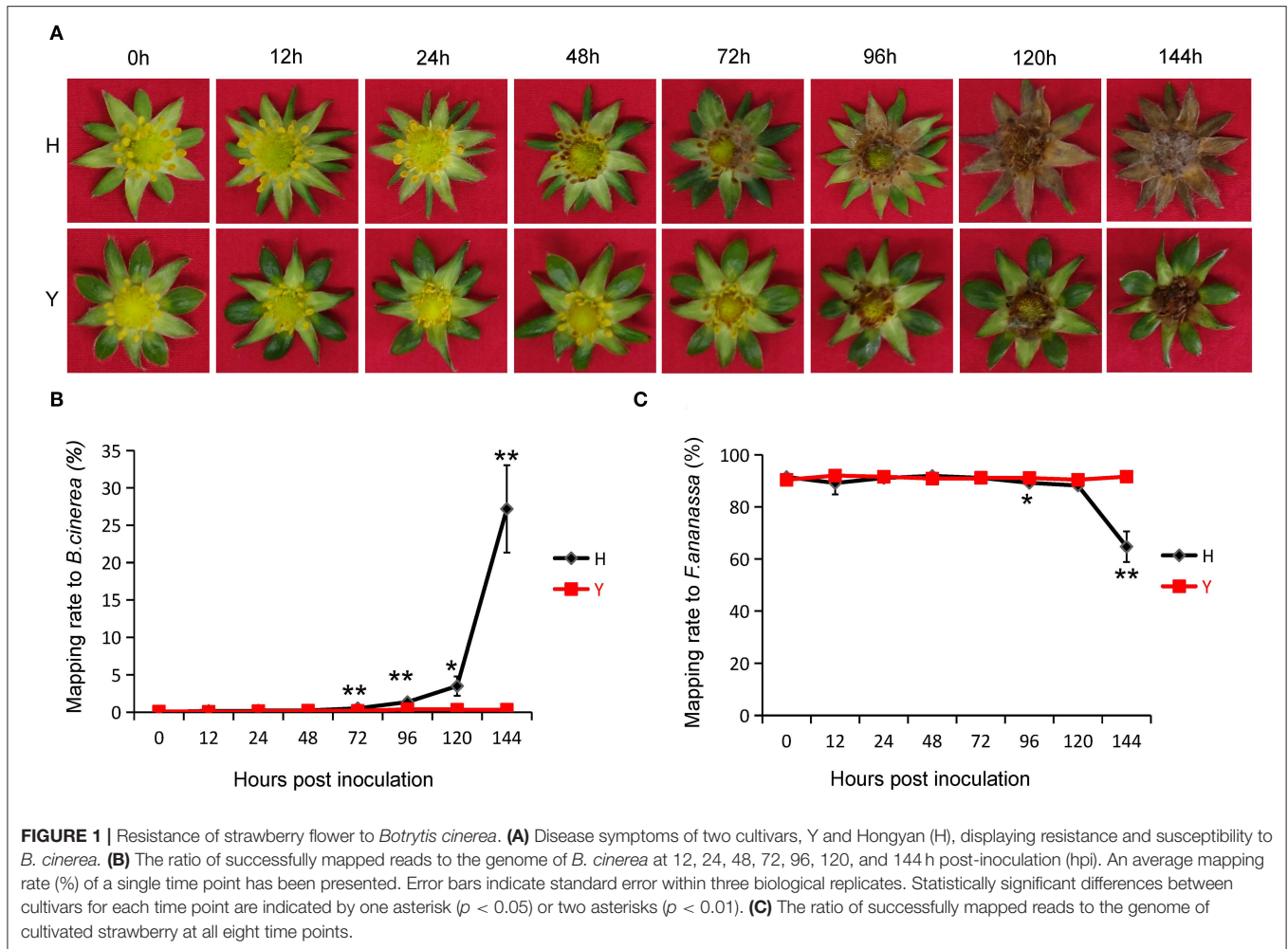
In our previous study, we identified two cultivars with different levels of resistance to *B. cinerea*: the resistant Yanli (Y) and the susceptible H. In the present study, we conducted a time-series and comparative transcriptome analysis of these two strawberry cultivars designed to uncover the molecular mechanisms of strawberry resistance to *B. cinerea*. By analyzing the transcriptomes of these cultivars at eight time points, we obtained a global view of gene expression changes involved in *B. cinerea* resistance in the strawberry flower. Our findings enhance the understanding of molecular interactions between *B. cinerea* and strawberry flower and provide gene resources for improving strawberry resistance.

## RESULTS

### Flowers of Two Strawberry Cultivars Showed Different Degrees of *B. cinerea* Resistance

Although strawberry petals could be colonized by conidia of *B. cinerea*, the quick abscission of petals after blossoming reduced the probability of infection by this means. Therefore, we first removed petals from the *in vitro* flowers of two strawberry cultivars, Y and H, and then inoculated these apetalous flowers with *B. cinerea* to examine their resistance. The two cultivars responded differently: cultivar H was susceptible to *B. cinerea*, showing mild symptoms at 24 h post inoculation (hpi) and typical gray mold symptoms at 48 hpi, whereas cultivar Y was resistant, with no detectable symptoms at 48 hpi, mild symptoms at 72 h, and typical gray mold symptoms only after 96 hpi (**Figure 1A**). The fact that the obvious differences in symptoms were observed from 48 hpi indicated that resistance to *B. cinerea* in the strawberry flower is probably regulated at the transcriptome level during the early stages of infection.

To investigate the transcriptome dynamics of strawberry flowers after *B. cinerea* inoculation, we performed RNA-seq with total RNA isolated from the flowers of the two cultivars at eight widely spaced time points, 0 (before inoculation), 12, 24, 48, 72, 96, 120, and 144 hpi with three independent biological replicates at each time point. A total of 397.55 GB clean data were obtained from all 48 samples. The clean reads were mapped to the *B. cinerea* and *Fragaria × ananassa* genome (Camarosa, FAN\_r1.1) with HISAT2 (Kim et al., 2015) and the mapping rates were calculated, respectively. As expected, the mapping rate of clean reads aligned to *B. cinerea* genome increased and the mapping



rate to the strawberry genome decreased with the extension of inoculation time (Figures 1B,C). There was no significant difference between Y and H until 72 hpi. At 144 hpi, the mapping ratio to the *B. cinerea* genome had increased to  $27.19 \pm 5.84\%$  in H and  $0.34 \pm 0.99\%$  in Y and the mapping ratio to the strawberry genome had decreased to  $64.73 \pm 5.90\%$  in H and  $91.63 \pm 0.71\%$  in Y.

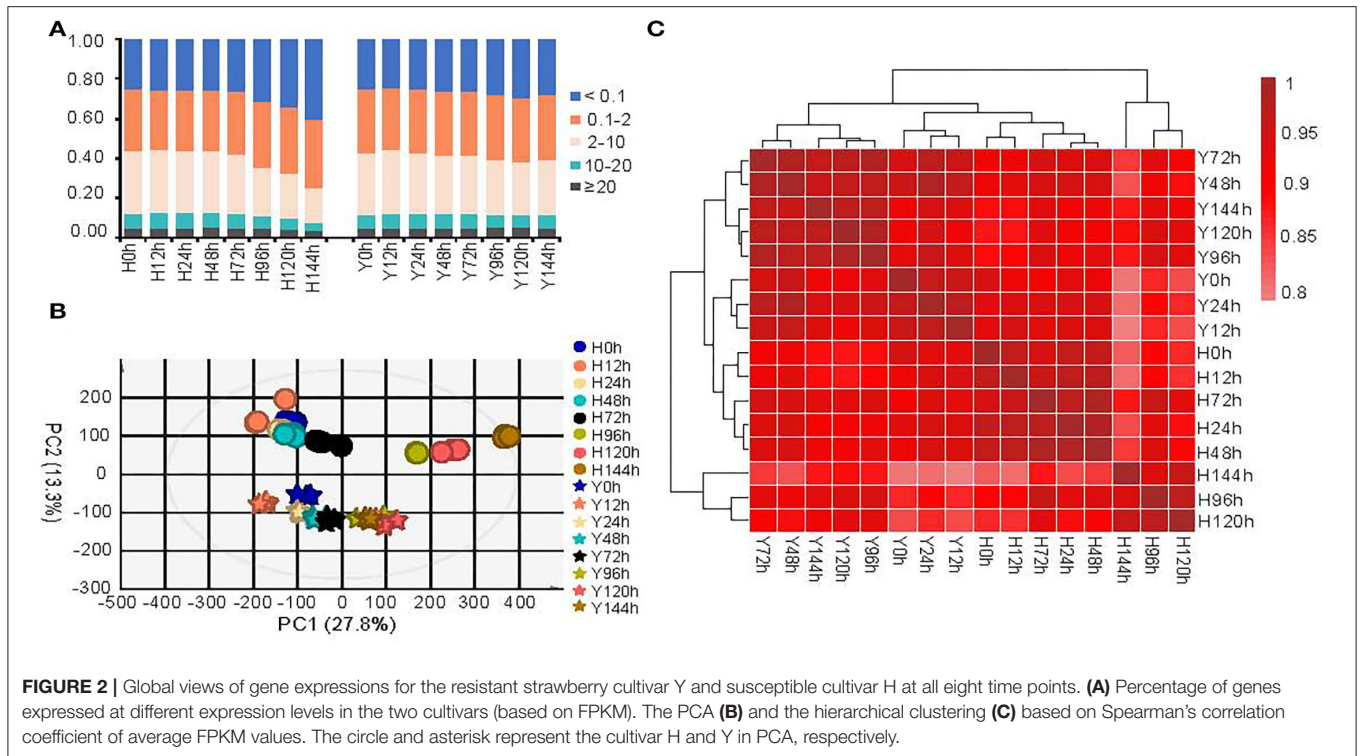
### Global Transcriptomic Changes of Strawberry Flower After Infection by *B. cinerea*

We assembled the mapped reads and quantified them in fragments per kilobase of transcript length per million mapped reads (FPKM) using StringTie (Pertea et al., 2015). A total of 90,767 genes were identified including 87,797 known genes and 2,970 previously identified genes. Among them, ~86% genes expressed (FPKM > 0.1) in at least one of the 16 samples.

The percentage of genes expressed ranged from 75.0% (at 0 hpi) to 59.5% (at 144 hpi) in H and 75.3% (at 12 hpi) to 70.5% (at 120 hpi) in Y (Figure 2A). About 3.8–5.1% of genes showed very high (FPKM  $\geq 20$ ) expression both before and after infection

by *B. cinerea* in both cultivars. The number of genes exhibiting high ( $10 \leq \text{FPKM} < 20$ ), moderate ( $2 \leq \text{FPKM} < 10$ ), and low ( $0.1 \leq \text{FPKM} < 2$ ) expression were similar in all samples, except the susceptible cultivar H showed lower overall expression after 72 hpi, which was in accordance with the mapping rates of clean reads (Figure 2A). Overall, the total proportion of genes showed very high, high, and moderate expression was greatest at 12 hpi, followed by 24 hpi, revealing transcriptome-induced resistance to *B. cinerea* at the early infection stages.

To inspect the global difference between H and Y before and after *B. cinerea* infection, we performed principal component analysis (PCA) and hierarchical clustering based on Spearman's correlation coefficient of the average FPKM values for all genes (Figures 2B,C). We observed substantial differences between the two cultivars, with the H and Y samples being divided into two groups in the PCA (Figure 2B). However, there was also a highly significant correlation between H and Y, as evidenced by correlation coefficients >0.78 (Figure 2C). Interestingly, the results of PCA as well as hierarchical clustering showed that the transcriptomes of H at 96, 120, and 144 hpi differed substantially from those at the other time points, in accordance with the mapping rate for *B. cinerea*. Furthermore, cultivar H showed



severe symptoms after 96 hpi. Consequently, we conducted a further differential gene expression analysis at 0–72 hpi. Replicate 3 of H12 was removed because it was separated from the other two replicates in PCA and had relatively low correlation with the other samples in H (**Supplementary Table 1**).

Twelve genes were selected for validation of their expression by qRT-PCR (**Supplementary Figure 1**). The results of Pearson correlation analysis demonstrate a significant correlation ( $r = 0.468$ ,  $p < 0.05$ ) between qRT-PCR and RNA-seq. The transcript levels of *EDS1*, *CML41*, *PYL4*, *PAL1*, *ARF5*, *GH3.5*, *BGLU12*, *4CL1*, *SGT1*, and *PP2CA* were high in the resistant cultivar Y, whereas the transcript levels of *WRKY33* and *RPM1* were high in the susceptible cultivar H.

## Resistant Cultivar Y Exhibited More Sophisticated Expression Patterns

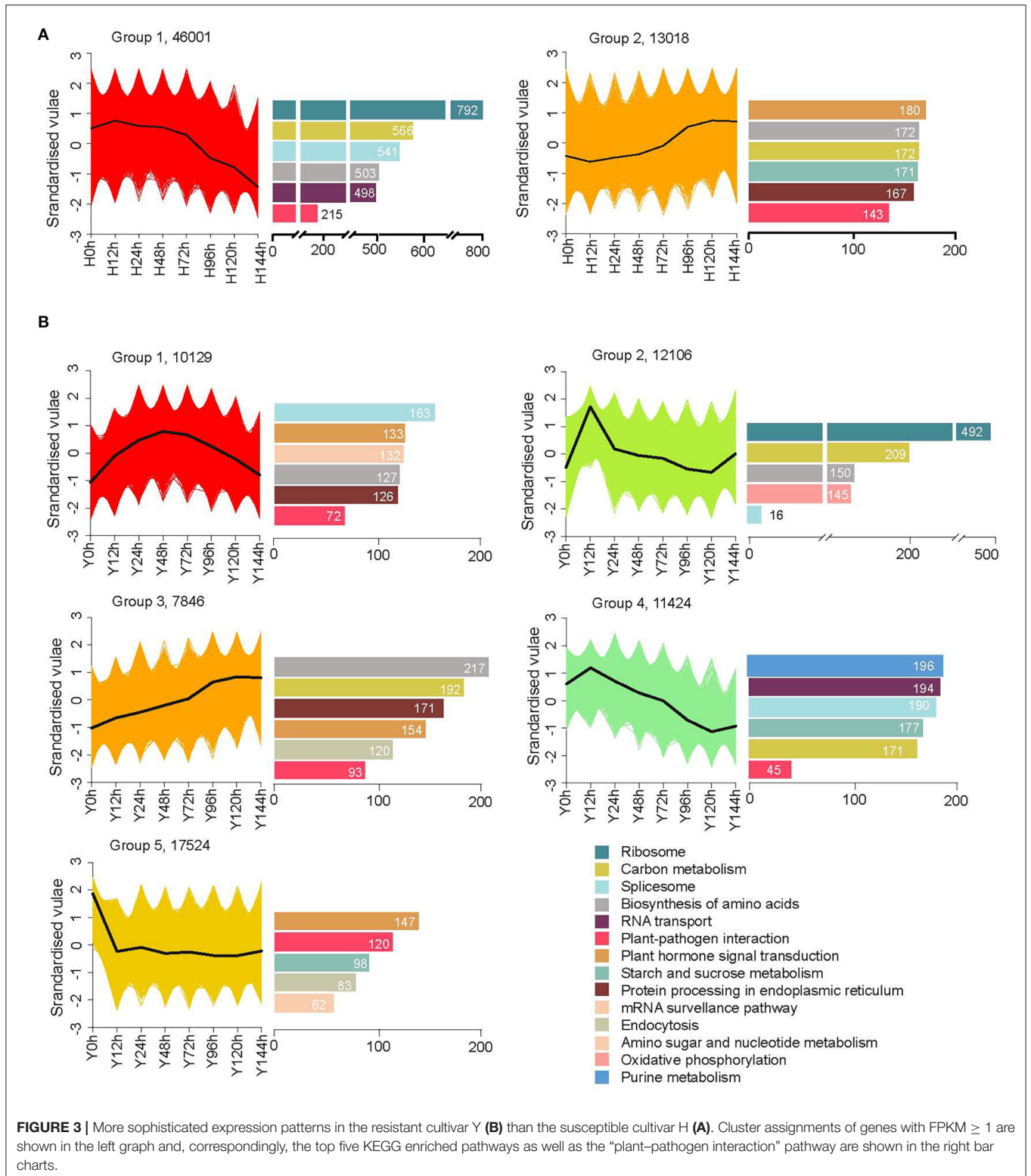
To identify expression clusters for each cultivar, we applied *K*-means clustering with the time series gene expression datasets. The cluster assignments of genes with  $\text{FPKM} \geq 1$  and the top five Kyoto Encyclopedia of Gene and Genomes (KEGG)-enriched pathways as well as the “plant–pathogen interaction” pathway are displayed in **Figure 3**. A total of 59,122 genes were clustered into five groups in the resistant cultivar Y, but only two groups in susceptible cultivar H, indicating that Y has a more complex response than H at the transcriptome level. As the time since inoculation increased, the expression levels in H decreased for 77.9% (46,001) and increased for 22.1% (13,018) of the genes. In Y, one-third of the genes (17,524) belonged to group 5, which

was characterized by up-regulation at 12 hpi followed by down-regulation until 120 hpi, and the other genes were almost equally divided between groups 1 and 4.

Approximately 60% of the genes in the “plant–pathogen interaction” pathway were clustered in the down-regulated group 1, and the other ~40% were clustered in the up-regulated group 2 in H. Again, Y exhibited a more range of diverse expression changes than H. Over one-third of “plant–pathogen interaction” genes were clustered in group 3, where genes were drastically down-regulated at 12 hpi and then showed slight fluctuations in expression, with a maximum at 24 hpi. One-quarter and one-fifth of genes were in groups 2 and 1, respectively, and smaller numbers of genes were assigned to groups 5 and 4. The diverse expression clusters of Y are likely to account for resistance to *B. cinerea* for *in vitro* strawberry flower.

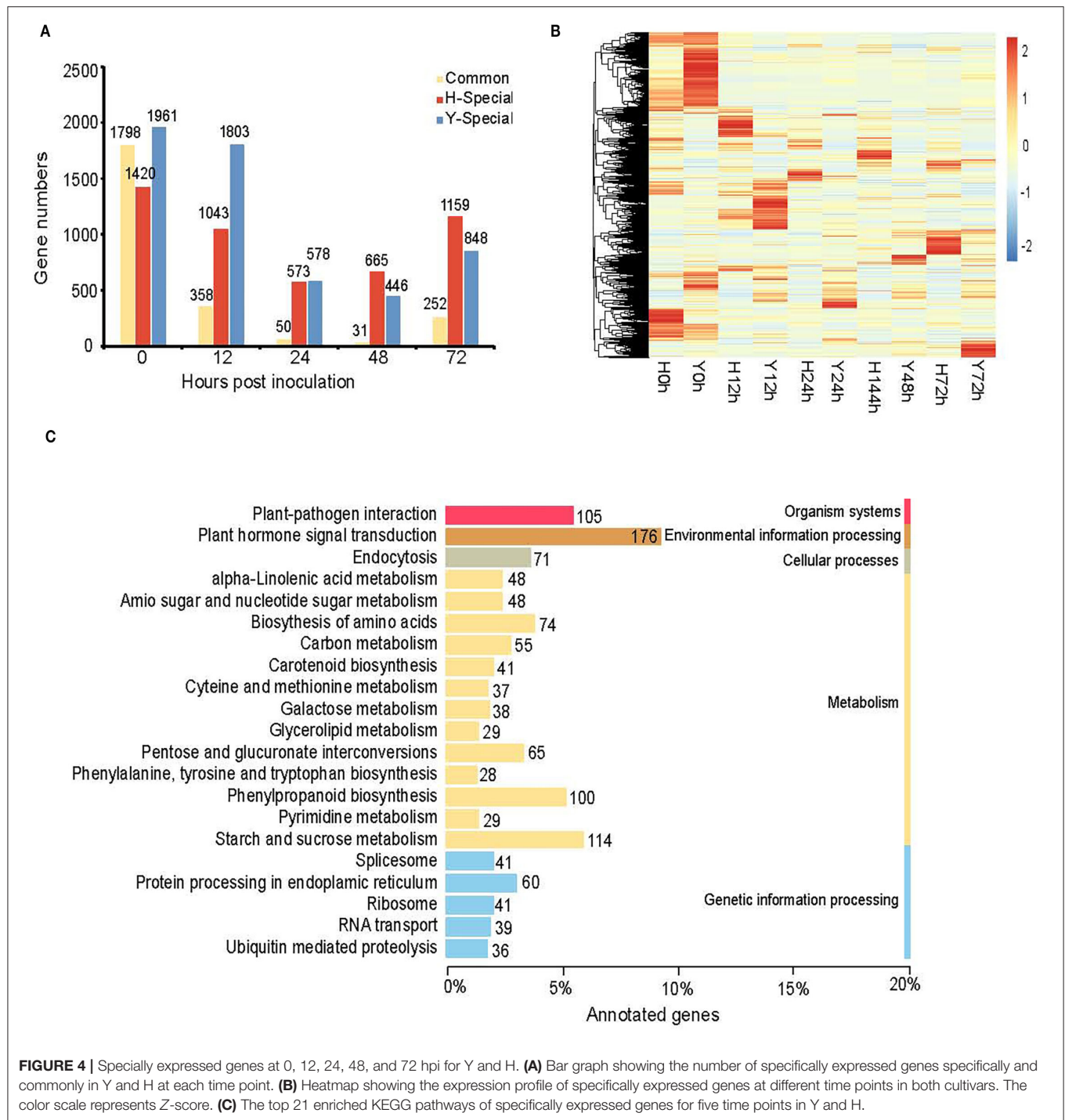
## Five KEGG Pathways Enriched of Gene Sets Specially Expressed (SEGs) as Well as Different Expression Genes (DEGs)

We identified gene sets specially accumulated at 0–72 hpi by applying a stage-specificity (SS) scoring algorithm to uncover the transcriptional differences that characterized each time point for resistant cultivar Y and susceptible cultivar H and potentially mitigated *B. cinerea* infection. Based on the criterion  $\text{SS} \geq 0.5$ , we identified 11,950 SEGs for two cultivars at five time points. Among them, 8,125 and 7,349 were included for Y and H, respectively (**Figure 4**). The trend of changes in SEG numbers in Y was coincident with that in H as described in the following: First, both decreased and then increased.



However, the turning point occurred later in the resistant cultivar Y (48 hpi) than in the susceptible cultivar H (24 hpi). In Y, 628 (7.7%) and 477 (5.9%) genes showed high transcript

accumulation at 24 and 48 hpi, respectively. In H, 8.5% (623) genes were specifically expressed at 24 hpi and 9.5% (696) at 48 hpi.



A large difference between Y and H was observed at 12 hpi, and the number of SEGs for Y was more than that of H from 0 hpi to 24 hpi, whereas the number of SEGs for H was more than Y at 48 and 72 hpi, indicating that the induced resistance for strawberry flower was acquired at the early infection stages (Figure 4A). A visual display of the expression profiles for all these genes in both cultivars is shown in Figure 4B. Furthermore, we

analyzed the KEGG (<http://www.genome.jp/kegg/>) pathway of SEGs at five time points and identified the enrichment pathways (Figure 4C). The pathway “plant hormone and signal transduction” was the most enriched, followed by “starch and sucrose metabolism,” “plant–pathogen interaction,” “phenylpropanoid biosynthesis,” and “biosynthesis of amino acids.” The numbers of SEGs in these five pathways were 176, 114, 105, 100, and 74, respectively.

We further identified DEGs between the resistant cultivar Y and the susceptible cultivar H for each time point from 0 to 72 hpi (Figure 5). A total of 12,922 genes were differentially expressed between Y and H at one or more of the five time points.

The number of genes up-regulated in Y was greater than the number down-regulated in Y at all five time points, indicating that the resistance is likely acquired through the activation of a larger number of defense-related genes (Figure 4A). For four of the five time points (0, 24, 48, and 72 hpi), the number of DEGs varied from 6,089 to 6,781, whereas it was only 4,983 at 12 hpi. A Venn diagram of common and specific DEGs shows 337 common DEGs for the four inoculation time points but not for the “no inoculation” time point (0 hpi) (Figure 4B). The top seven enriched KEGG pathways of all DEGs at the five time points were “starch and sucrose metabolism,” “plant hormone and signal transduction,” “phenylpropanoid biosynthesis,” “biosynthesis of amino acids,” “carbon metabolism,” “ribosome,” and “plant–pathogen interaction.” The number of DEGs in each pathway was 162, 137, 129, 125, 124, 114, and 105, respectively (Figure 4C). Among them, “plant hormone and signal transduction,” “starch and sucrose metabolism,” “plant–pathogen interaction,” “phenylpropanoid biosynthesis,” and “biosynthesis of amino acids” were enriched for both SEGs and DEGs, indicating that these pathways may be involved in resistance to *B. cinerea* for strawberry flower. There were 798 genes included in these five pathways (Supplementary Table 2).

## Transcriptional Regulatory Network Controlling Strawberry Flower Resistance to *B. cinerea*

To elucidate the gene co-expression network that arises in response to the infection of *B. cinerea* in strawberry flower, a weighted gene co-expression network analysis (WGCNA) was utilized within DEGs and SEGs (Figure 6). A total of 22,496 genes were included in the WGCNA. Finally, 3,748 genes were grouped into 10 modules that were defined using color codes, along with a gray module representing the remaining uncorrelated genes (Figure 6A). The most genes (1,202) were contained in the yellow module, followed by the skyblue (910) and orange (356) modules. A few genes were contained in the darkgray (98) and royalblue (131) modules. Among the 10 modules, 6 were significantly ( $p < 0.05$ ) correlated with *B. cinerea* resistance (Figure 6B). The royalblue module was the most significant one, followed by the black and yellow modules. The other three closely related modules were the lightyellow, darkgreen, and darkturquoise modules. Consequently, 152 genes in the associated modules in WGCNA overlapped with genes in the “plant–pathogen interaction,” “plant hormone and signal transduction” starch and sucrose metabolism,” “phenylpropanoid biosynthesis,” and “biosynthesis of amino acids” pathways (Supplementary Table 2). A clear regulation of these pathways and candidate genes were displayed in Figure 7.

In the plant resistance to the necrotrophic pathogen *B. cinerea*, the transcription factor *WRKY33* and *MYC2* were well-studied genes. Here, we identified eight *WRKY* transcription

factors including *WRKY33* and three *MYC2* genes. There were another three well-known disease-resistance proteins including the R protein for resistance to *Pseudomonas syringae* pv. *maculicola* 1 (*RPM1*) and the heat shock protein 90 (*HSP90*) conferring biotic stress tolerance. All these disease-resistance genes improve *B. cinerea* resistance in strawberry flower by increasing the expression: highest in the resistant cultivar Y before inoculation except *WRKY34* and one gene annotated as *RPM1*. Additionally, 14 cell wall-related genes including 7 pectinesterases (PMEs/PPMEs), 4 polygalacturonases (PGs), and 3 UDP-glucuronate 4-epimerases (GAEs) were highly expressed in the susceptible cultivar H before inoculation which may lead to the sensitivity. Different from these genes mentioned here, five out of the six peroxidases (PODs) were induced by *B. cinerea*. The expression of them increased more rapidly in the susceptible cultivar H than the resistant cultivar Y leading to the higher expression in H than Y at 24 and 48 hpi. Our results suggest calcium signaling plays crucial role in strawberry flower resistance to *B. cinerea*. A total of 30 genes including 5 calcium-dependent protein kinases (CPKs), 2 respiratory burst oxidase homolog proteins D (RBOHDs), 3 cyclic nucleotide-gated ion channels (CNGCs), and 20 calcium-binding proteins (CMLs) had the same expression pattern as the disease-resistance genes except one *CNGC*.

Furthermore, our results also reveal that plant defense to *B. cinerea* is mediated by hormonal regulatory networks. First, an SA marker gene pathogenesis-related genes 1 (*PR1*) was specially accumulated in the susceptible cultivar H before inoculation. Also, *EDS1* was another gene in the SA signaling, one of them showed the same expression module as *PR1* whereas two of them highly expressed in the resistant cultivar Y before inoculation. Except for this gene mentioned here, *MYC2*, there were 12 TIFY family genes as well as one JA-amido synthetase (*JAR1*) belonging to the JA biosynthesis pathway genes. Three of them were differentially expressed between two cultivars. Besides, a total of 12 genes in the auxin pathway belonged to the 3 gene families as follows: Auxin-responsive proteins (SAURs and IAAs), auxin response factors (ARFs), and indole-3-acetic acid-amido synthetases (GH3s). More than half of the auxin-signaling genes displayed the same expression module as disease-resistance genes. Furthermore, the expression of four ethylene-responsive transcription factors (ERFs) increased with time after inoculation and higher in H than Y. The last but not the least, two receptors, each in the ABA and GA signaling pathway, namely, ABA receptor (*PYL*) and gibberellin receptor (*GID1C*) had different expression levels between cultivars. The *PYL* had the highest expression in the resistant cultivar in Y at 48 hpi while the *GID1C* had the highest expression in H before inoculation.

Finally, genes in the phenylpropanoid biosynthesis and amino acids biosynthesis pathways were involved in the resistance to *B. cinerea* in strawberry flower. There were six amino acid-biosynthesis genes, including three chorismate mutases (CMs), one aspartokinase (*AK2*), one amino acid acetyltransferase (*NAGS2*) and one S-adenosylmethionine synthase 2 (*MSAMS2*). All these amino acid-biosynthesis genes showed the expression trend consistent with disease-resistance genes. Genes in the phenylpropanoid biosynthesis

pathway mainly comprised of lignin biosynthesis genes, such as  $\beta$ -glucosidases (BGs), phenylalanine ammonia-lyases (PALs), caffeic acid 3-O-methyltransferases (COMTs), lignin-forming anionic peroxidases (LigPODs), and cinnamyl alcohol dehydrogenase (*CAD*). Four and three of them reached the highest expression before inoculation in the resistant cultivar Y and the susceptible H, respectively. The other six genes increased their expression after inoculation and showed the highest expression at 48 hpi.

## DISCUSSION

### In Response to Infection, the Resistant Strawberry Cultivar First Up-Regulated Disease-Resistance Genes and Down-Regulated Cell Wall-Degrading Enzymes and Peroxidases

Three genes that are known to be involved in the interaction between *B. cinerea* and host plants, *EDS1* (Bhandari et al., 2019; Baggs et al., 2020; Lapin et al., 2020), *WRKY33* (Birkenbihl et al., 2012; Zhou et al., 2020) and *MYC2* (Lorenzo et al., 2004) were identified by WGCNA analysis with the dynamic and comparative transcriptome data. These genes were highly expressed in the resistant cultivar Y before inoculation in addition to *RPM1* and *HSP90*, disease-resistance proteins for other pathogens (Huang et al., 2014; El Kasmi et al., 2017; Ul Haq et al., 2019). Therefore, it seems likely that most disease-resistance genes in strawberry flower were prepared to resist invading pathogens before inoculation. In addition, we found several genes involved in hormone and  $Ca^{2+}$  signaling showed similar expression patterns. We speculate that this phenomenon may exist specially at the flower stage accompanied by the transition from vegetative to reproductive development. However, more experiments are needed to confirm this possibility.

The plant cell wall serves as the first line of defense against pathogen penetration. Our results indicate that the susceptible cultivar H increases the expression of cell wall-degrading enzymes including PGs, PMEs/PPMEs, and GAEs, leading to the flower sensitivity. The previous studies have documented that these enzymes mediate immunity by degrading cell walls or affecting cell wall integrity and structure (Bethke et al., 2014, 2016; Lionetti et al., 2017). The pectinesterase *AtPME17* and three pectinesterase inhibitors have been proven to be involved in Arabidopsis resistance to *B. cinerea* (Lionetti et al., 2017; Del Corpo et al., 2020). Same as the cell wall-degrading enzymes, PODs showed higher expression in the susceptible cultivar H than Y, indicating that PODs negatively regulate *B. cinerea* resistance. This is consistent with a previous study that illustrated that PODs play a crucial role in the generation of  $H_2O_2$  or other ROS in the immune response (Daudi et al., 2012).

In conclusion, disease-resistance genes were up-regulated in the resistant cultivar Y but cell wall-degrading enzymes and peroxidases were down-regulated, which is supported by previous studies. Unfortunately, few PRRs important for

PTI were found though serine/threonine-protein kinases were included in **Supplementary Table 2**.

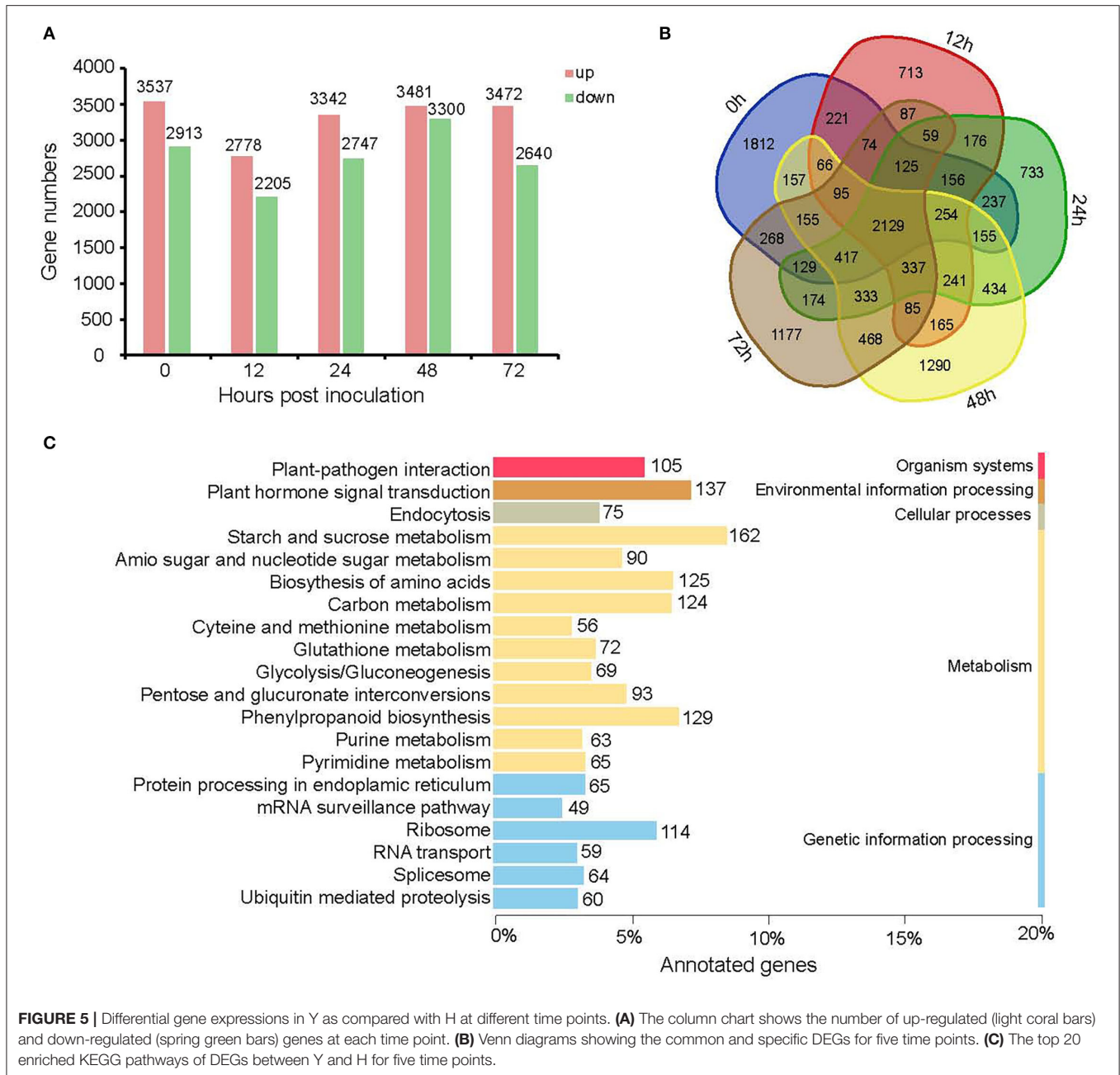
### The Calcium Signaling Pathway Plays a Crucial Role in the Resistance to *B. cinerea* in Strawberry Flower

Although  $Ca^{2+}$  is well known to serve as a second messenger in cell signaling, how  $Ca^{2+}$  encodes complex information with high specificity in various signaling processes, such as development and biotic interactions, remains an area of intense study. The most striking result to emerge from our study was that 30 genes belonging to the calcium signaling pathway accounted for about 20% of all the 152 genes selected by WGCNA. Although little attention has been focused on the calcium signaling pathway genes in the interaction between strawberry and *B. cinerea*, our knowledge on the  $Ca^{2+}$  dependent-immunity mechanisms advanced recently (Liang et al., 2018; Xiong et al., 2018; Haile et al., 2019). Considering the model plant Arabidopsis as an example, active BOTRYTIS-INDUCED KINASE1 (BIK1) and CPKs phosphorylate  $Ca^{2+}$ -bound RBOHD to boost ROS production. The CaM-gated CNGC2–CNGC4 complex is then activated, leading to the sustained cytosolic  $Ca^{2+}$  elevation and the relay of  $Ca^{2+}$ -dependent immunity (Tian et al., 2020). Interestingly, 30 calcium signaling pathway genes found in this study belonged to the CPKs, RBOHDs, CNGCs, and CMLs. Moreover, these genes displayed the same expression module as disease-resistance genes: highly expressed in the resistant cultivar Y. The CMLs had the largest number (20) of genes. The expression of *CML41* in the resistant cultivar Y was 51.2 and 27.9, as shown by the FPKM value in H at 0 hpi and was 2.7 times higher than that in H at 24 hpi, which was validated by qRT-PCR. Consistent with our results, recent research has shown that two CALMODULIN-LIKE genes (*CML46* and *CML47*) and *cbp60a* (CALMODULIN-BINDING PROTEIN60) contribute to two different modes of negative regulation of immunity (Lu et al., 2018). Same as CMLs, RBOHDs, and CPKs are  $Ca^{2+}$  sensors decoding the spatial and temporal calcium concentration changes in the cytoplasm to convey  $Ca^{2+}$  signals into specific cellular responses (Lu et al., 2018; Bredow and Monaghan, 2019; Lee et al., 2020). The CNGCs mediate calcium entry and provide a critical link between the PAMP–PRR complex and calcium-dependent immunity programs in the PTI signaling pathway (Tian et al., 2019). According to these data, we infer that CPKs, RBOHDs, CNGCs, and CMLs comprise a calcium signaling pathway that plays a crucial role in enhancing resistance to *B. cinerea* by their increased expression.

### Resistance to *B. cinerea* Is Mediated by Hormonal Regulatory Networks

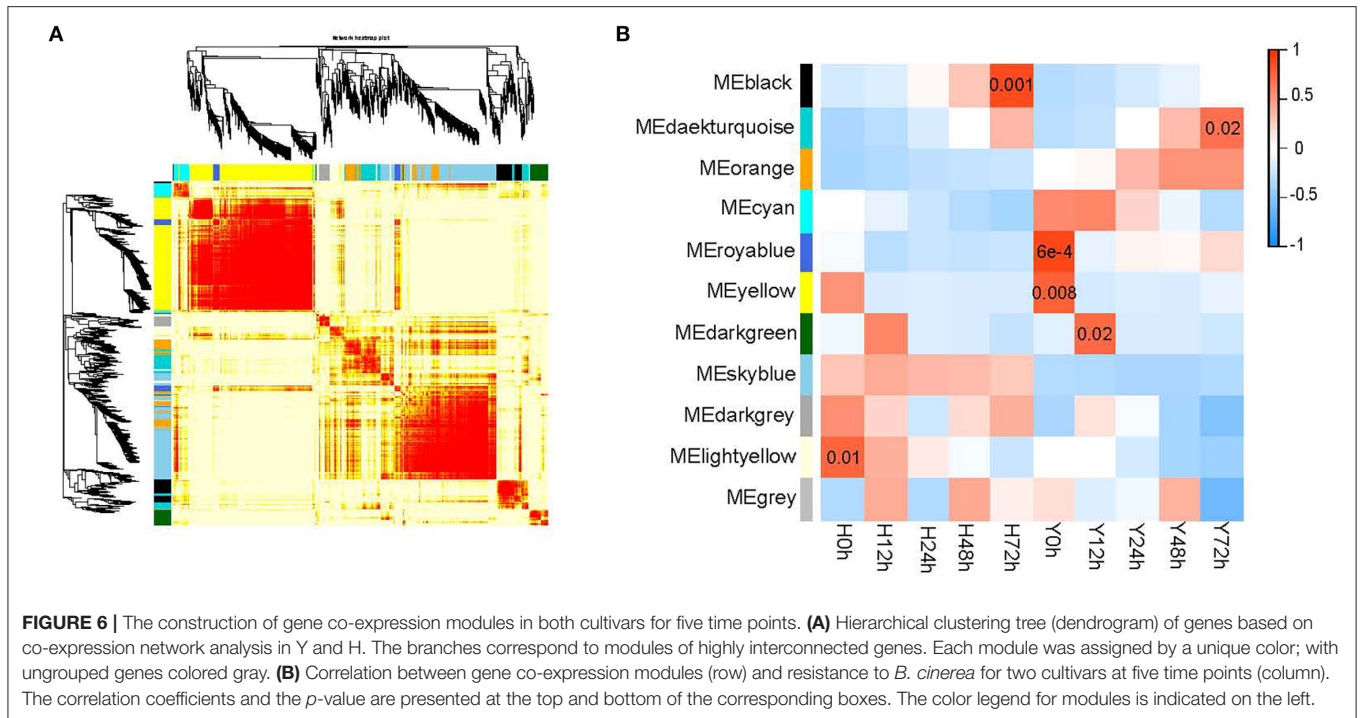
The most enriched pathway we identified in this study is the “plant hormone and signal transduction” pathway, which involves six types of phytohormones, namely, SA, JA, auxin, ET, ABA, and gibberellic acid (GA). These results corroborate with previous findings (AbuQamar et al., 2017), suggesting an essential role of phytohormones in the plant response to *B. cinerea*. Also, SA is well known to regulate plant defense response





to pathogens including *B. cinerea*. This research identified the following two well-known genes in the SA signaling pathway: *EDS1* and *PRI* (Breen et al., 2017). A marker gene in the SA pathway, *PRI*, was highly expressed in the susceptible cultivar H, indicating negative regulation of SA in strawberry resistance. This finding was confirmed by earlier work (Ferrari et al., 2003; Breen et al., 2017). The genes in the JA and auxin pathways were the most and second most enriched with 12 and 13 genes, respectively, implying primary roles in the resistance. Twelve TIFY genes in the JA pathway that have been proven to be involved in the resistance to bacterial blight in rice (Yamada et al., 2012) and powdery mildew in *Vitis vinifera*

(Yu et al., 2019) displayed a similar expression module as *MYC2*, a transcription factor in this pathway. The majority of auxin-related genes were differentially expressed between two cultivars. These results provide further support for our hypothesis that auxin contributes to defense response against necrotrophic pathogens (Nafisi et al., 2015). IAA and GH3 have been reported to regulate plant disease-resistance in cassava (Fan et al., 2020) and Arabidopsis (Zhang et al., 2007), respectively. By contrast, four ERFs were induced by inoculation in this study. The previous research demonstrated that ERF1 confers resistance to several necrotrophic fungi in Arabidopsis (Berrocal-Lobo et al., 2002), and that ERF099 is an important regulator involved in *B.*



*cinerea* resistance in rose petal (Li et al., 2020). Finally, *PYL4* and *GID1C*, the ABA and GA receptor genes, have been demonstrated to mediate Arabidopsis immune responses toward necrotrophic and biotrophic pathogens (Garcia-Andrade et al., 2020) and rice resistance to blast fungus (Tanaka et al., 2006), respectively. These results suggest that six types of phytohormones form a complex regulatory network that mediates flower resistance to *B. cinerea*, especially JA and auxin.

## The Phenylpropanoid and Amino Acid Biosynthesis Pathways Are Involved in the Resistance to *B. cinerea*

We also found that genes in the phenylpropanoid biosynthesis pathway were enriched. Similarly, Dong and Lin (2021) documented that phenylpropanoid metabolism contributes to plant development and the interplay between plants and the environment, including biotic and abiotic stresses. We found 15 genes, including 7 BGs, 3 PALs, 2 COMTs, 2 LigPODs, and a *CAD1*, belonging to the phenylpropanoid biosynthesis pathway. The BGs serve as detonators of plant chemical defense against pathogens and herbivores (Morant et al., 2008). The FaBG3, which encodes  $\beta$ -glucosidase in *F. ananassa*, has been shown to regulate fruit ripening, dehydration stress, and *B. cinerea* fungal infection in strawberries via modulation of ABA homeostasis and transcriptional regulation of ripening-related genes (Li et al., 2013). The previous reports have indicated that lignin is deposited at the infection site to inhibit the penetration and growth of pathogens (Mutuku et al., 2019; Lee et al., 2020; Xiao et al., 2021), and relevant genes, such as PALs, *CAD*, and COMTs, have been confirmed, through functional genomics

experiments, to be associated with disease-resistance (Tonnessen et al., 2015; Li et al., 2019; Hoch et al., 2021).

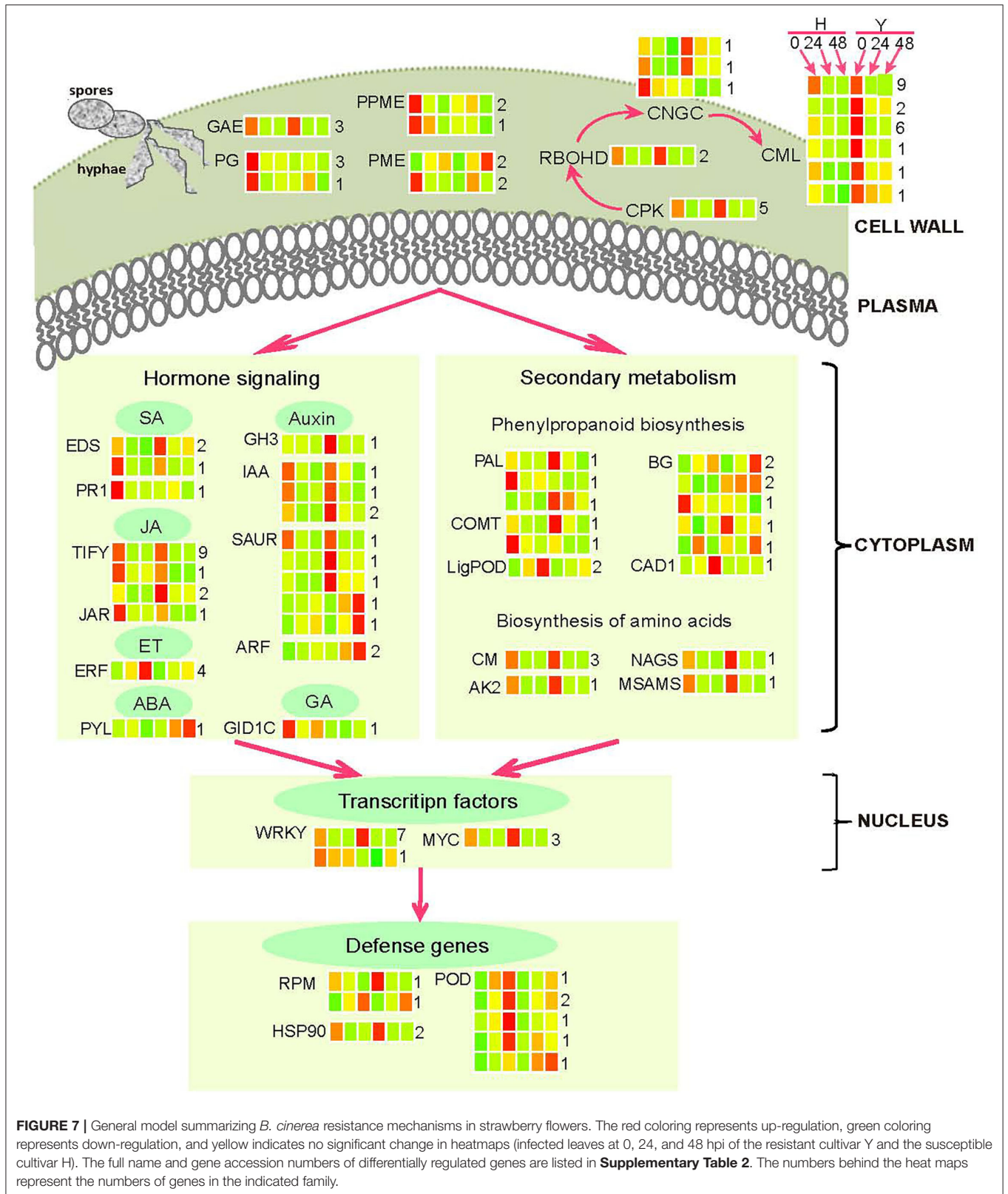
The amino acid metabolic pathways have been considered to be integral to the plant immune system (Zeier, 2013). We found that the CMs were DEGs and AK2 was a SEG. Overexpression of CM lead to increased resistance to *Xanthomonas oryzae* in rice (Jan et al., 2020) and *B. graminis* f. sp. *Hordei* in barley (Hu et al., 2009). Additionally, the loss-of-inhibition allele of AK2 exhibited strong resistance to *Hyaloperonospora arabidopsidis* (*Hpa*) (Stuttman et al., 2011). Combining our findings with the previous reports, we conclude that the phenylpropanoid and amino acid biosynthesis pathways are involved in the resistance to *B. cinerea*.

## MATERIALS AND METHODS

### Plant Materials and Pathogen Inoculation

Two strawberry cultivars, Y and H, with contrasting resistance to *B. cinerea* were acquired. Strawberry plants were grown in the field during the winter season (September–April) in two consecutive years. The field management was conducted according to local practices to ensure normal crop growth. *In vitro* inoculation of *B. cinerea* was performed when plants reached peak flowering.

*Botrytis cinerea* strain BC-1 was isolated in March 2017 and maintained at 4°C in darkness. At 2 or 3 weeks before inoculation, BC-1 was subcultured on potato dextrose agar (PDA) in the dark at 20 ± 1°C under an alternating 1-h darkness/12-h light photoperiod to produce conidia. Spore inoculums were prepared by harvesting conidia in water and filtered through double gauze to remove hyphae.



**FIGURE 7 |** General model summarizing *B. cinerea* resistance mechanisms in strawberry flowers. The red coloring represents up-regulation, green coloring represents down-regulation, and yellow indicates no significant change in heatmaps (infected leaves at 0, 24, and 48 hpi of the resistant cultivar Y and the susceptible cultivar H). The full name and gene accession numbers of differentially regulated genes are listed in **Supplementary Table 2**. The numbers behind the heat maps represent the numbers of genes in the indicated family.

Then, the concentration of the pathogen suspension was adjusted to  $10^6$  spores  $ml^{-1}$  through counting on a hemacytometer.

The stamens of flowers with petals removed were spray inoculated so that the suspensions were evenly spaced over the stamens. To investigate the transcriptome dynamics, time-series

inoculations were employed. The whole flowers without petals were harvested before inoculation (0 hpi) as well after inoculation by *B. cinerea* at 12, 24, 48, 72, 96, 120, and 144 hpi, immediately frozen in liquid nitrogen, and then later subjected to RNA extraction and sequencing. There were three biological replicates for each time point for both cultivars.

## The RNA Sequencing, Read Mapping, and Differential Gene Expression Analysis

Total RNA was extracted from each sample (~100 mg) using RNAPrep Pure Plant Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The library products were prepared for sequence analysis *via* NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). An Illumina NovaSeq 6000 instrument was used for sequencing, and paired-end reads were generated. These raw reads were processed by removing reads containing the adapter sequences, reads with more than 5% poly-N, and low-quality reads (reads with more than 50% of low-quality bases of quality value  $\leq 5$ ) to obtain clean reads. At the same time, Q20, Q30, GC content, and sequence duplication level of the clean data were calculated. A total of 397.55 GB high-quality clean reads (average 5.95 GB reads from each sample) were generated for from 48 samples and all the downstream analyses were based on these clean data.

Filtered reads were mapped to the octoploid strawberry genome *Fragaria* × *ananassa* available at <https://datadryad.org/resource/10.5061/dryad.b2c58pc> (Edger et al., 2019) and *B. cinerea* B05.10 from NCBI by HISAT2 (Kim et al., 2015) and then assembled by StringTie (Pertea et al., 2015). The gene expression level was calculated as FPKM by StringTie. The genes exhibiting a difference in expression level of at least 2-folds, with corrected *p*-value after adjusting with false discovery rate  $\leq 0.01$ , were considered to be DEGs by DESeq2. The genes specifically expressed (SEGs) at any time point in both cultivars were identified *via* the SS scoring algorithm of Zhan et al. (2015), which compares the expression of a gene at a given time point with its maximum expression level at other time points. The genes with SS score  $\geq 0.5$  were regarded as specifically expressed at one time point. All raw reads were deposited to the National Center for Biotechnology Information Sequence Reads Archive (SRA) with accession number PRJNA761556. **Supplementary Table 4** presented the sample name and their corresponding accession number.

## Gene Annotation and Functional Enrichment Analysis

All assembled genes were annotated based on the following database: Nr (<http://ftp.ncbi.nih.gov/blast/db/>, NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (<http://pfam.xfam.org/>, Protein family), KOG/COG (<http://www.ncbi.nlm.nih.gov/KOG/>, <http://www.ncbi.nlm.nih.gov/COG/>, Clusters of Orthologous Groups of proteins), Swiss-Prot (<http://www.uniprot.org/>, A manually annotated and reviewed protein sequence database), KO (<http://www.genome.jp/kegg/>, KEGG Ortholog database), and GO (<http://www.geneontology.org/>, Gene Ontology). On the basis of annotation, we conducted gene ontology (GO) enrichment

and KEGG pathway enrichment analysis for both DEGs and SEGs. The GO enrichment analysis of the differentially expressed genes (DEGs) was implemented by the Goseq R packages based on Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. The KEGG pathway enrichment analysis of DEGs was implemented with KOBAS software (Mao et al., 2005).

## Co-expression Regulatory Network Construction by WGCNA

The coexpression regulatory network was constructed by WGCNA (Langfelder and Horvath, 2008) for both DEGs and SEGs based on their correlation patterns. A total of 22,496 genes were used for analysis. The soft threshold was set to 30 to make the network fit to a scale-free topology. The GO and KEGG pathway enrichment analyses were performed for each module as introduced here. In addition, we detected the association of each module with the phenotype of resistance to *B. cinerea* (0 and 1 were designated as resistant and susceptible phenotypes, respectively, for Y and H). A positive correlation indicated that genes in a module have higher expression in the resistant cultivar than the susceptible one.

## Absolute Quantitative qRT-PCR Analysis

Absolute qRT-PCR experiments were applied for validating the results of RNA-seq. The total RNA for each sample were extracted as described here. The gene-specific primers designed using Primer Express (v3.0) software are listed in **Supplementary Table 3**. There were three biological replicates for each time point and cultivar, with three technical replicates for each biological replicate. The transcript level of each gene was normalized by comparison with the internal control gene, housekeeping gene gene11892 (Gao et al., 2020), and fold change was calculated according to the  $2^{-\Delta\Delta CT}$  method.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA761556.

## AUTHOR CONTRIBUTIONS

YH conceived and supervised the project. GX performed the inoculation experiment, qRT-PCR, analyzed the transcriptomic data, and wrote the draft manuscript. QZ initialed the inoculation experiment and revised the manuscript. XZ, XC, and SL managed plant propagation and field operation for plant growth as well as the inoculation experiment. All authors have read and approved the submitted version.

## FUNDING

This work was supported by Grants from the National Natural Science Foundation of China (31701882), National

Key Research and Development Program of China (2018YFD1000300), Hubei Province Key R&D Program (2021BBA099), and Agricultural Science and Technology Innovation Center Program of Hubei Province (2019-620-00 0-001-08).

## REFERENCES

- AbuQamar, S., Moustafa, K., and Tran, L. S. (2017). Mechanisms and strategies of plant defense against *Botrytis cinerea*. *Crit. Rev. Biotechnol.* 37, 262–274. doi: 10.1080/07388551.2016.1271767
- Baggs, E. L., Monroe, J. G., Thanki, A. S., O'Grady, R., Schudoma, C., Haerty, W., et al. (2020). Convergent loss of an EDS1/PAD4 signaling pathway in several plant lineages reveals coevolved components of plant immunity and drought response. *Plant Cell* 32, 2158–2177. doi: 10.1105/tpc.19.00903
- Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of Ethylene-Response-Factor 1 in Arabidopsis confers resistance to several necrotrophic fungi. *Plant J.* 29, 23–32. doi: 10.1046/j.1365-313x.2002.01191.x
- Bestfleisch, M., Luderer-Pflimpfl, M., Höfer, M., Schulte, E., Wünsche, J. N., Hanke, M. V., et al. (2015). Evaluation of strawberry (*Fragaria L.*) genetic resources for resistance to *Botrytis cinerea*. *Plant Pathol.* 64, 396–405. doi: 10.1111/ppa.12278
- Bethke, G., Grundman, R. E., Sreekanta, S., Truman, W., Katagiri, F., and Glazebrook, J. (2014). Arabidopsis pectin methylesterases contribute to immunity against *Pseudomonas syringae*. *Plant Physiol.* 164, 1093–1107. doi: 10.1104/pp.113.227637
- Bethke, G., Thao, A., Xiong, G., Li, B., Soltis, N. E., Hatsugai, N., et al. (2016). Pectin biosynthesis is critical for cell wall integrity and immunity in *Arabidopsis thaliana*. *Plant Cell* 28, 537–556. doi: 10.1105/tpc.15.00404
- Bhandari, D. D., Lapin, D., Kracher, B., von Born, P., Bautor, J., Niefind, K., et al. (2019). An EDS1 heterodimer signalling surface enforces timely reprogramming of immunity genes in Arabidopsis. *Nat. Commun.* 10:772. doi: 10.1038/s41467-019-08783-0
- Birkenbihl, R. P., Diezel, C., and Somssich, I. E. (2012). Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol.* 159, 266–285. doi: 10.1104/pp.111.192641
- Blanco-Ulate, B., Morales-Cruz, A., Amrine, K. C., Labavitch, J. M., Powell, A. L., and Cantu, D. (2014). Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts. *Front. Plant Sci.* 5:435. doi: 10.3389/fpls.2014.00435
- Blanco-Ulate, B., Vincenti, E., Powell, A. L., and Cantu, D. (2013). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Front. Plant Sci.* 4:142. doi: 10.3389/fpls.2013.00142
- Bredow, M., and Monaghan, J. (2019). Regulation of plant immune signaling by calcium-dependent protein kinases. *Mol. Plant Microbe Interact.* 32, 6–19. doi: 10.1094/MPMI-09-18-0267-FI
- Breen, S., Williams, S. J., Outram, M., Kobe, B., and Solomon, P. S. (2017). Emerging insights into the functions of pathogenesis-related protein 1. *Trends Plant Sci.* 22, 871–879. doi: 10.1016/j.tplants.2017.06.013
- Bristow, P. R., McNicol, R. J., and Williamson, B. (1986). Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould development. *Ann. Appl. Biol.* 109, 545–554. doi: 10.1111/j.1744-7348.1986.tb03211.x
- Daudi, A., Cheng, Z., O'Brien, J. A., Mammarella, N., Khan, S., Ausubel, F. M., et al. (2012). The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. *Plant Cell* 24, 275–287. doi: 10.1105/tpc.111.093039
- De Cremer, K., Mathys, J., Vos, C., Froenicke, L., Michelmore, R. W., Cammue, B. P., et al. (2013). RNAseq-based transcriptome analysis of *Lactuca sativa* infected by the fungal necrotroph *Botrytis cinerea*. *Plant Cell Environ.* 36, 1992–2007. doi: 10.1111/pce.12106
- Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13, 414–430. doi: 10.1111/j.1364-3703.2011.00783.x
- Del Corpo, D., Fullone, M. R., Miele, R., Lafond, M., Pontiggia, D., Grisel, S., et al. (2020). AtPME17 is a functional Arabidopsis thaliana pectin methylesterase regulated by its PRO region that triggers PME activity in the resistance to *Botrytis cinerea*. *Mol Plant Pathol.* 21, 1620–1633. doi: 10.1111/mpp.13002
- Dong, N. Q., and Lin, H. X. (2021). Contribution of phenylpropanoid metabolism to plant development and plant-environment interactions. *J. Integr. Plant Biol.* 63, 180–209. doi: 10.1111/jipb.13054
- Edger, P. P., Poorten, T. J., VanBuren, R., Hardigan, M. A., Colle, M., McKain, M. R., et al. (2019). Origin and evolution of the octoploid strawberry genome. *Nat. Genet.* 51, 541–547. doi: 10.1038/s41588-019-0356-4
- El Kasmi, F., Chung, E. H., Anderson, R. G., Li, J., Wan, L., Eitas, T. K., et al. (2017). Signaling from the plasma-membrane localized plant immune receptor RPM1 requires self-association of the full-length protein. *Proc. Natl. Acad. Sci. U.S.A.* 114, E7385–E7394. doi: 10.1073/pnas.1708288114
- Elad, Y., Vivier, M., and Fillinger, S. (2016). “Botrytis, the good, the bad and the ugly,” in *Botrytis – the Fungus, the Pathogen and its Management in Agricultural Systems*, eds S. Fillinger and Y. Elad (Cham: Springer) Chapter 1, 1–15. doi: 10.1007/978-3-319-23371-0\_1
- Fan, S., Chang, Y., Liu, G., Shang, S., Tian, L., and Shi, H. (2020). Molecular functional analysis of auxin/indole-3-acetic acid proteins (Aux/IAAs) in plant disease resistance in cassava. *Physiol. Plant* 168, 88–97. doi: 10.1111/ppl.12970
- Ferrari, S., Plotnikova, J. M., De Lorenzo, G., and Ausubel, F. M. (2003). Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J.* 35, 193–205. doi: 10.1046/j.1365-313X.2003.01794.x
- Gao, Q., Luo, H., Li, Y., Liu, Z., and Kang, C. (2020). Genetic modulation of RAP alters fruit coloration in both wild and cultivated strawberry. *Plant Biotechnol. J.* 18, 1550–1561. doi: 10.1111/pbi.13317
- Garcia-Andrade, J., Gonzalez, B., Gonzalez-Guzman, M., Rodriguez, P. L., and Vera, P. (2020). The role of aba in plant immunity is mediated through the pyr1 receptor. *Int. J. Mol. Sci.* 21:5852. doi: 10.3390/ijms21165852
- González, G., Fuentes, L., Moya-León, M. A., Sandoval, C., and Herrera, R. (2013). Characterization of two PR genes from *Fragaria chiloensis* in response to *Botrytis cinerea* infection: a comparison with *Fragaria x ananassa*. *Physiol. Mol. Plant Pathol.* 82, 73–80. doi: 10.1016/j.pmp.2013.02.001
- Guo, J., Qi, J., He, K., Wu, J., Bai, S., Zhang, T., et al. (2019). The Asian corn borer *Ostrinia furnacalis* feeding increases the direct and indirect defence of mid-whorl stage commercial maize in the field. *Plant Biotechnol. J.* 17, 88–102. doi: 10.1111/pbi.12949
- Haile, Z. M., Nagpala-De Guzman, E. G., Moretto, M., Sonogo, P., Engelen, K., Zoli, L., et al. (2019). Transcriptome profiles of strawberry (*Fragaria vesca*) fruit interacting with *Botrytis cinerea* at different ripening stages. *Front. Plant Sci.* 10:1131. doi: 10.3389/fpls.2019.01131
- Haile, Z. M., Pilati, S., Sonogo, P., Malacarne, G., Vrhovsek, U., Engelen, K., et al. (2017). Molecular analysis of the early interaction between the grapevine flower and *Botrytis cinerea* reveals that prompt activation of specific host pathways leads to fungus quiescence. *Plant Cell Environ.* 40, 1409–1428. doi: 10.1111/pce.12937
- Hematy, K., Cherk, C., and Somerville, S. (2009). Host-pathogen warfare at the plant cell wall. *Curr. Opin Plant Biol.* 12, 406–413. doi: 10.1016/j.pbi.2009.06.007
- Hoch, K., Koopmann, B., and von Tiedemann, A. (2021). Lignin composition and timing of cell wall lignification are involved in *Brassica napus* resistance to stem rot caused by *Sclerotinia sclerotiorum*. *Phytopathology* 111, 1438–1448. doi: 10.1094/PHYTO-09-20-0425-R
- Hu, P., Meng, Y., and Wise, R. P. (2009). Functional contribution of chorismate synthase, anthranilate synthase, and chorismate mutase to penetration

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.888939/full#supplementary-material>

- resistance in barley-powdery mildew interactions. *Mol. Plant Microbe Interact.* 22, 311–320. doi: 10.1094/MPMI-22-3-0311
- Huang, S., Monaghan, J., Zhong, X., Lin, L., Sun, T., Dong, O. X., et al. (2014). HSP90s are required for NLR immune receptor accumulation in Arabidopsis. *Plant J* 79, 427–439. doi: 10.1111/tbj.12573
- Jan, R., Khan, M. A., Asaf, S., Lee, I. J., Bae, J. S., and Kim, K. M. (2020). Overexpression of OsCM alleviates BLB stress via phytohormonal accumulation and transcriptional modulation of defense-related genes in *Oryza sativa*. *Sci. Rep.* 10:19520. doi: 10.1038/s41598-020-76675-1
- Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317
- Kong, W., Chen, N., Liu, T., Zhu, J., Wang, J., He, X., et al. (2015). Large-scale transcriptome analysis of cucumber and *botrytis cinerea* during infection. *PLoS ONE* 10:e0142221. doi: 10.1371/journal.pone.0142221
- Kou, S., Chen, L., Tu, W., Scossa, F., Wang, Y., Liu, J., et al. (2018). The arginine decarboxylase gene ADC1, associated to the putrescine pathway, plays an important role in potato cold-acclimated freezing tolerance as revealed by transcriptome and metabolome analyses. *Plant J.* 96, 1283–1298. doi: 10.1111/tbj.14126
- Lai, Z., and Mengiste, T. (2013). Genetic and cellular mechanisms regulating plant responses to necrotrophic pathogens. *Curr. Opin. Plant Biol.* 16, 505–512. doi: 10.1016/j.pbi.2013.06.014
- Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics* 9:559. doi: 10.1186/1471-2105-9-559
- Lapin, D., Bhandari, D. D., and Parker, J. E. (2020). Origins and immunity networking functions of EDS1 family proteins. *Annu. Rev. Phytopathol.* 58, 253–276. doi: 10.1146/annurev-phyto-010820-012840
- Lee, D., Lal, N. K., Lin, Z. D., Ma, S., Liu, J., Castro, B., et al. (2020). Regulation of reactive oxygen species during plant immunity through phosphorylation and ubiquitination of RBOHD. *Nat. Commun.* 11:1838. doi: 10.1038/s41467-020-15601-5
- Li, D., Liu, X., Shu, L., Zhang, H., Zhang, S., Song, Y., et al. (2020). Global analysis of the AP2/ERF gene family in rose (*Rosa chinensis*) genome unveils the role of RcERF099 in *Botrytis* resistance. *BMC Plant Biol.* 20:533. doi: 10.1186/s12870-020-02740-6
- Li, Q., Ji, K., Sun, Y., Luo, H., Wang, H., and Leng, P. (2013). The role of FaBG3 in fruit ripening and *B. cinerea* fungal infection of strawberry. *Plant J.* 76, 24–35. doi: 10.1111/tbj.12272
- Li, T., Wang, Q., Feng, R., Li, L., Ding, L., Fan, G., et al. (2019). Negative regulators of plant immunity derived from cinnamyl alcohol dehydrogenases are targeted by multiple Phytophthora Avr3a-like effectors. *N. Phytol.* doi: 10.1111/nph.16139. [Epub ahead of print].
- Liang, Y., Guan, Y., Wang, S., Li, Y., Zhang, Z., and Li, H. (2018). Identification and characterization of known and novel microRNAs in strawberry fruits induced by *Botrytis cinerea*. *Sci. Rep.* 8:10921. doi: 10.1038/s41598-018-29289-7
- Lionetti, V., Fabri, E., De Caroli, M., Hansen, A. R., Willats, W. G., Piro, G., et al. (2017). Three pectin methyltransferase inhibitors protect cell wall integrity for arabidopsis immunity to *Botrytis*. *Plant Physiol.* 173, 1844–1863. doi: 10.1104/pp.16.01185
- Lorenzo, O., Chico, J. M., Sanchez-Serrano, J. J., and Solano, R. (2004). Jasmonate-insensitive1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* 16, 1938–1950. doi: 10.1105/tpc.022319
- Lu, B., Wang, Y., Zhang, G., Feng, Y., Yan, Z., Wu, J., et al. (2020). Genome-wide identification and expression analysis of the strawberry FvbZIP gene family and the role of key gene FvbZIP46 in fruit resistance to gray mold. *Plants* 9:1199. doi: 10.3390/plants9091199
- Lu, Y., Truman, W., Liu, X., Bethke, G., Zhou, M., Myers, C. L., et al. (2018). Different modes of negative regulation of plant immunity by calmodulin-related genes. *Plant Physiol.* 176, 3046–3061. doi: 10.1104/pp.17.01209
- Mao, X., Cai, T., Olyarchuk, J. G., and Wei, L. (2005). Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics* 21, 3787–3793. doi: 10.1093/bioinformatics/bti430
- Mengiste, T. (2012). Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* 50, 267–294. doi: 10.1146/annurev-phyto-081211-172955
- Morant, A. V., Jorgensen, K., Jorgensen, C., Paquette, S. M., Sanchez-Perez, R., Moller, B. L., et al. (2008). Beta-glucosidases as detonators of plant chemical defense. *Phytochemistry* 69, 1795–1813. doi: 10.1016/j.phytochem.2008.03.006
- Mutuku, J. M., Cui, S., Hori, C., Takeda, Y., Tobimatsu, Y., Nakabayashi, R., et al. (2019). The structural integrity of lignin is crucial for resistance against *Striga hermonthica* parasitism in rice. *Plant Physiol.* 179, 1796–1809. doi: 10.1104/pp.18.01133
- Nafisi, M., Fimognari, L., and Sakuragi, Y. (2015). Interplays between the cell wall and phytohormones in interaction between plants and necrotrophic pathogens. *Phytochemistry* 112, 63–71. doi: 10.1016/j.phytochem.2014.11.008
- Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T. C., Mendell, J. T., and Salzberg, S. L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 33, 290–295. doi: 10.1038/nbt.3122
- Petrasch, S., Knapp, S. J., van Kan, J. A. L., and Blanco-Ulate, B. (2019). Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen *Botrytis cinerea*. *Mol. Plant Pathol.* 20, 877–892. doi: 10.1111/mpp.12794
- Smith, J. E., Mengesha, B., Tang, H., Mengiste, T., and Bluhm, B. H. (2014). Resistance to *Botrytis cinerea* in *Solanum lycopersicoides* involves widespread transcriptional reprogramming. *BMC Genomics* 15:334. doi: 10.1186/1471-2164-15-334
- Stuttman, J., Hubberten, H. M., Rietz, S., Kaur, J., Muskett, P., Guerois, R., et al. (2011). Perturbation of *Arabidopsis* amino acid metabolism causes incompatibility with the adapted biotrophic pathogen *Hyaloperonospora arabidopsidis*. *Plant Cell* 23, 2788–2803. doi: 10.1105/tpc.111.087684
- Tanaka, N., Matsuoka, M., Kitano, H., Asano, T., Kaku, H., and Komatsu, S. (2006). gid1, a gibberellin-insensitive dwarf mutant, shows altered regulation of probenazole-inducible protein (PBZ1) in response to cold stress and pathogen attack. *Plant Cell Environ.* 29, 619–631. doi: 10.1111/j.1365-3040.2005.01441.x
- Tian, W., Hou, C., Ren, Z., Wang, C., Zhao, F., Dahlbeck, D., et al. (2019). A calmodulin-gated calcium channel links pathogen patterns to plant immunity. *Nature* 572, 131–135. doi: 10.1038/s41586-019-1413-y
- Tian, W., Wang, C., Gao, Q., Li, L., and Luan, S. (2020). Calcium spikes, waves and oscillations in plant development and biotic interactions. *Nat Plants* 6, 750–759. doi: 10.1038/s41477-020-0667-6
- Tonnessen, B. W., Manosalva, P., Lang, J. M., Baraoidan, M., Bordeos, A., Mauleon, R., et al. (2015). Rice phenylalanine ammonia-lyase gene OsPAL4 is associated with broad spectrum disease resistance. *Plant Mol. Biol.* 87, 273–286. doi: 10.1007/s11103-014-0275-9
- Ul-Haq, S., Khan, A., Ali, M., Khattak, A. M., Gai, W. X., Zhang, H. X., et al. (2019). Heat shock proteins: dynamic biomolecules to counter plant biotic and abiotic stresses. *Int. J. Mol. Sci.* 20:5321. doi: 10.3390/ijms20215321
- Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085
- Weiberg, A., Wang, M., Lin, F. M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–123. doi: 10.1126/science.1239705
- Windram, O., Madhou, P., McHattie, S., Hill, C., Hickman, R., Cooke, E., et al. (2012). Arabidopsis defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell* 24, 3530–3557. doi: 10.1105/tpc.112.102046
- Xiao, S., Hu, Q., Shen, J., Liu, S., Yang, Z., Chen, K., et al. (2021). GhMYB4 downregulates lignin biosynthesis and enhances cotton resistance to *Verticillium dahliae*. *Plant Cell Rep.* 40, 735–751. doi: 10.1007/s00299-021-02672-x
- Xiong, J. S., Zhu, H. Y., Bai, Y. B., Liu, H., and Cheng, Z. M. (2018). RNA sequencing-based transcriptome analysis of mature strawberry fruit infected by necrotrophic fungal pathogen *Botrytis cinerea*. *Physiol. Mol. Plant P* 104, 77–85. doi: 10.1016/j.pmpp.2018.08.005
- Yamada, S., Kano, A., Tamaoki, D., Miyamoto, A., Shishido, H., Miyoshi, S., et al. (2012). Involvement of OsJAZ8 in jasmonate-induced resistance to bacterial blight in rice. *Plant Cell Physiol.* 53, 2060–2072. doi: 10.1093/pcp/pcs145
- Young, M. D., Wakefield, M. J., Smyth, G. K., and Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* 11:R14. doi: 10.1186/gb-2010-11-2-r14

- Yu, Y., Wan, Y., Jiao, Z., Bian, L., Yu, K., Zhang, G., et al. (2019). Functional characterization of resistance to powdery mildew of vvtify9 from *Vitis vinifera*. *Int. J. Mol. Sci.* 20:4286. doi: 10.3390/ijms20174286
- Zambounis, A., Ganopoulos, I., Valasiadis, D., Karapetsi, L., and Madesis, P. (2020). RNA sequencing-based transcriptome analysis of kiwifruit infected by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* 111:101514. doi: 10.1016/j.pmpp.2020.101514
- Zeier, J. (2013). New insights into the regulation of plant immunity by amino acid metabolic pathways. *Plant Cell Environ.* 36, 2085–2103. doi: 10.1111/pce.12122
- Zhan, J., Thakare, D., Ma, C., Lloyd, A., Nixon, N. M., Arakaki, A. M., et al. (2015). RNA sequencing of laser-capture microdissected compartments of the maize kernel identifies regulatory modules associated with endosperm cell differentiation. *Plant Cell* 27, 513–531. doi: 10.1105/tpc.114.135657
- Zhang, Z., Li, Q., Li, Z., Staswick, P. E., Wang, M., Zhu, Y., et al. (2007). Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis-Pseudomonas syringae* interaction. *Plant Physiol.* 145, 450–464. doi: 10.1104/pp.107.106021
- Zhou, J., Wang, X., He, Y., Sang, T., Wang, P., Dai, S., et al. (2020). Differential phosphorylation of the transcription factor WRKY33 by the protein kinases CPK5/CPK6 and MPK3/MPK6 cooperatively regulates camalexin biosynthesis in *Arabidopsis*. *Plant Cell* 32, 2621–2638. doi: 10.1105/tpc.19.00971
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.
- Copyright © 2022 Xiao, Zhang, Zeng, Chen, Liu and Han. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.