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Novel Fungal Pathogenicity and Leaf Defense Strategies Are Revealed by Simultaneous Transcriptome Analysis of *Colletotrichum fructicola* and Strawberry Infected by This Fungus

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Colletotrichum fructicola, which is part of the *C. gloeosporioides* species complex, can cause anthracnose diseases in strawberries worldwide. However, the molecular interactions between *C. fructicola* and strawberry are largely unknown. A deep RNA-sequencing approach was applied to gain insights into the pathogenicity mechanisms of *C. fructicola* and the defense response of strawberry plants at different stages of infection. The transcriptome data showed stage-specific transcription accompanied by a step-by-step strawberry defense response and the evasion of this defense system by fungus. Fungal genes involved in plant cell wall degradation, secondary metabolism, and detoxification were up-regulated at different stage of infection. Most importantly, *C. fructicola* infection was accompanied by a large number of highly expressed effectors. Four new identified effectors function in the suppression of Bax-mediated programmed cell death. Strawberry utilizes pathogen-associated molecular patterns (PAMP)-triggered immunity and effector-triggered immunity to prevent *C. fructicola* invasion, followed by the initiation of downstream innate immunity. The up-regulation of genes related to salicylic acid provided evidence that salicylic acid signaling may serve as the core defense signaling mechanism, while jasmonic acid and ethylene pathways were largely inhibited by *C. fructicola*. The necrotrophic stage displayed a significant up-regulation of genes involved in reactive oxygen species activation. Collectively, the transcriptomic data of both *C. fructicola* and strawberry shows that even though plants build a multilayered defense against infection, *C. fructicola* employs a series of escape or antagonizing mechanisms to successfully infect host cells.

Keywords: ***Colletotrichum fructicola*, strawberry, effector, pathogenicity, RNA-seq, resistance**

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INTRODUCTION

Anthracnose caused by *Colletotrichum* spp. is a devastating disease of cultivated strawberry (*Fragaria* × ananassa Duch) (Henz et al., 1992; Xiao et al., 2004). The *C. gloeosporioides* species complex is the most prevalent agent of strawberry anthracnose in China (Xie et al., 2010). *C. gloeosporioides* usually produce a reddish-brown necrosis of crown tissue following infection. Under greenhouse conditions or in summer nurseries, *C. gloeosporioides* may also produce necrosis on stolons, lesions on fruit, or black leaf spots (MacKenzie et al., 2006). *C. fructicola*, a gloeosporioides aggregate formerly known as *C. gloeosporioides*, is responsible for strawberry anthracnose in Korea and Japan (Nam et al., 2013; Gan et al., 2017). *C. fructicola* is also an important causal agent of strawberry anthracnose in Hubei, China. In inoculated strawberry plants, *C. fructicola* showed strong pathogenicity to both the leaves and petioles of strawberries, with mortality occurring in 77.8% of plants 30 days after inoculation (Han et al., 2016). Many *Colletotrichum* spp. utilize a hemibiotrophic infection strategy (O'Connell et al., 2004). Firstly, the pathogen uses a melanized appressorium to penetrate host tissue (Kubo and Takano, 2013). Then, the pathogen produces biotrophic primary hyphae. Within a few days, narrower, secondary hyphae form and proliferate through necrotrophic growth (Perfect et al., 1999; O'Connell et al., 2004).

The interplay between pathogen virulence and host resistance during the co-evolution of fungal plant pathogens and their hosts has been complicated (Koeck et al., 2011). Successful pathogens can dampen basal defenses by producing effectors that inhibit pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). However, in turn plants can detect such effectors and mount a second layer of defense called effector-triggered immunity (ETI) (Katagiri and Tsuda, 2010). These inducible defenses are also associated with wide-ranging transcriptional and hormonal reprogramming in plants (Pieterse et al., 2012). Phytohormones are a central regulator of immunity. Salicylic acid (SA) is the core plant defense hormone against biotrophic and hemi-biotrophic pathogens; meanwhile, jasmonic acid (JA) is important for resistance to necrotrophic pathogens (Glazebrook, 2005). In the context of basal resistance, cross talk between the SA signaling pathway and other hormone signaling pathways, such as JA, auxin, or abscisic acid (ABA), are often mutually antagonistic (Pieterse et al., 2012).

The interactions between strawberries and *Colletotrichum* spp. have recently been reported (Anwar and Ding, 2004; Li et al., 2013). Anwar and Ding (2004) cloned the strawberry class II chitinase genes, *FaChi2-1* and *FaChi2-2*, and found that both were up-regulated at the mRNA level after *C. fragariae* or *C. acutatum* infection. Using a homologous cloning approach, several nucleotide–binding site–leucine–rich repeat (NBS-LRR) resistance genes were found to be involved in the ecotype-specific responses to *C. gloeosporioides* in strawberries (Li et al., 2013). These preliminary studies provide some information on *Colletotrichum*–strawberry interactions. However, strawberries' resistance to *Colletotrichum* has been reported to be mostly polygenic and quantitatively inherited (Zebrowska et al., 2006; Amil-Ruiz et al., 2011). Therefore, a comprehensive approach is required to fully understand the interaction between the pathogen and the strawberry.

RNA-seq has been used to study *Colletotrichum*–host interactions. O'Connell et al. (2012) investigated *C. higginsianum* and *C. graminicola* at three stages of development in *Arabidopsis thaliana* and maize, respectively. This study revealed that the pathogenicity–related genes of the fungi are successively transcribed and linked to pathogenic transitions (O'Connell et al., 2012). A recent study investigated the expression profiles of mango fruit during *C. gloeosporioides* infection and showed that most of the defense-related genes, such as those encoding ethylene response factors (ERFs), NBS-LRRs, and pathogenesis–related proteins (PRs), were up-regulated after *C. gloeosporioides* infection (Hong et al., 2016). However, these transcriptome studies were focused on either the pathogen or the host; few studies have simultaneously analyzed the response of both the pathogen and the host using RNA-seq. Alkan et al. performed a simultaneous transcriptomic analysis of *C. gloeosporioides* and the tomato plant and demonstrated stage-specific transcription and concurrent changes in fruit response (Alkan et al., 2015). Nevertheless, to date, no comprehensive stage-specific transcriptomic study has been performed to evaluate the interactions between *C. fructicola* and strawberry.

We performed dual RNA-seq profiling to simultaneously determine the transcriptomes of strawberry and *C. fructicola* throughout the infection process. These data provide novel insights into the infection process of *C. fructicola* and the regulation of strawberry defense genes during infection.

RESULTS AND DISCUSSION

Proceeding of *C. fructicola* Infection on Strawberry Leaves

The conidia of *C. fructicola* were oval with a smooth surface at 0 h. By 12 h post inoculation (hpi), conidia geminated and generated a germ tube from both tips. By 24 hpi, mature, melanized appressoria could be found. By 72 hpi, the hyphae had a morphology reminiscent of secondary hyphae in other *Colletotrichum* spp. was observed. By 96 hpi, secondary hyphae were predominant and the leaves developed visible anthracnose symptoms (**Figure 1**).

Interactive Transcriptomic Analyses

For simultaneous analysis of host plant strawberry and its pathogen *C. fructicola*, RNA was isolated from mycelium grown *in vitro* (pathogen control), mock inoculation (strawberry control) or inoculated strawberry leaves at three different time points (24, 72, and 96 hpi). Three biological replicates were pooled and sequenced. We obtained 5.37–14.01 Gb high quality reads from each treatment and aligned them against the reference genomes. The high quality reads were separately aligned to the *C. fructicola* Nara gc5 genome (Gan et al., 2013) and the diploid strawberry progenitor *Fragaria vesca* accession Hawaii 4 genome (Shulaev et al., 2011). For mycelium samples (control of pathogen), 83.77% of reads could be mapped to the *C. fructicola* genome; meanwhile, for samples from infected leaves, the percentage of reads mapped ranged from 0.03% (at 24 hpi) to

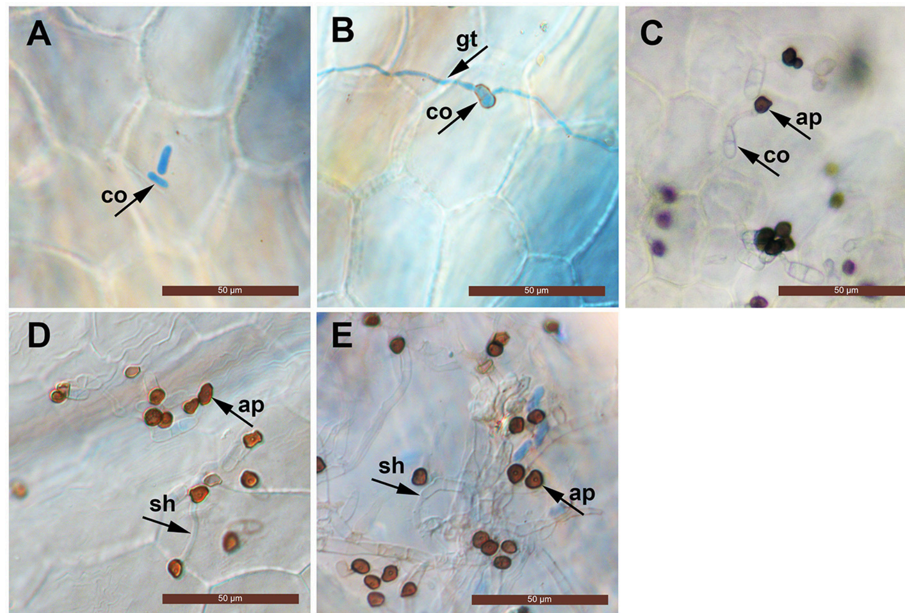

FIGURE 1 | Light micrographs showing infection structures of *C. fructicola* on the leaves of susceptible strawberry cultivar "JiuXiang." co, conidia; ap, appressoria; gt germ tube. sh, secondary hyphae. Bars = 50 μm. **(A)** Ungerminated conidia at 0 hpi. **(B)** Germination of conidia with germ-tube from one tip at 12 hpi. **(C)** Formation of mature, melanized appressoria 24 hpi. **(D)** Formation of a hyphae which had a morphology that is reminiscent of secondary hyphae (sh) at 72 hpi. **(E)** Development of secondary hyphae (sh) at 96 hpi.

0.15% (at 96 hpi) (**Table 1**). For mock inoculated strawberry leaves, 62.62% of reads could be mapped to the *F. vesca* genome; meanwhile, the percentage of reads mapped from samples of infected leaves were61.29% (24 hpi), 60.04% (72 hpi) and 60.12% (96 hpi).

When comparing inoculated strawberry leaves with mycelium grown *in vitro* (pathogen control), there were 7811 differentially expressed genes (DEGs) in *C. fructicola* (2-fold change) (Table S2) (**Figure 2A**). Venn diagrams of *C. fructicola* up-regulated genes at each fungal colonization stage compared with the mycelium samples revealed an overlap, but also showed distinct stage-specific expression (**Figure 2B**). In total, 725, 408, and 630 genes were exclusively expressed at 24, 72, and 96 hpi, respectively. We further examined the functional roles of all the DEGs based on 45 categories belonging to three groups using gene ontology (GO) enrichment analysis (**Figure 2C**). The "catalytic activity," binding, "transporter activity," and "transcription regulator activity" categories were the most enriched GO terms in the "molecular function" group. The "metabolic process," "cellular process," and "biological regulation" categories were the most enriched GO terms in the "biological process" group. The "cell," "organelle," and "cell part" categories were the most enriched GO terms in the "cell component" group (**Figure 2C**). To validate gene expression data obtained through differential expression analysis, eight candidate effector genes were tested using qRT-PCR. *Actin* and *α-tubulin* were used as reference genes. All eight of the selected genes showed trends similar to those found in the RNA-seq data. The qPCR results were highly consistent with RNA-Seq results, with pearson correlation coefficients ranging from 0.83 to 0.85. These results suggest that the transcriptome analysis was reliable (**Figures 2D,E**).

When comparing inoculated strawberry leaves vs. mock inoculation, 2273 DEGs were found in the strawberry (2-fold change) (**Figure 3A**, Table S14). Venn diagrams of strawberry genes up-regulated in response to each fungal colonization stage were compared with the non-inoculated mock treatment (**Figure 3B**): 245 genes were up-regulated at 24 hpi. By comparison, 707 and 890 genes were up-regulated at 72 and 96 hpi, respectively; 587 genes were up-regulated at both 72 and 96 hpi, indicating an overlap between those two time points. The DEGs annotated to the GO database were distributed among 47 functional types, including "growth," "cell," "response to stimulus," "metabolic process," and "signaling" (**Figure 3C**) (Ke et al., 2014). To validate the gene expression data obtained through differential expression analysis, eight plant hormones and cell apoptosis–related genes were tested for expression profiling using qPCR. *GADPH* were used as the reference gene. The trends in expression levels of the eight selected genes were similar to those found in the RNA-seq data. The qPCR results were highly consistent with RNA-Seq results, with Pearson correlation coefficients ranging from 0.86 to 0.92. The results suggest that the transcriptome analysis was reliable (**Figures 3D,E**).

C. fructicola Transcriptomics

Adhesion to the Host Surfaceand Remodeling of Cell Walls

Fungal hydrophobins are small, secreted, hydrophobic proteins that may be involved in the adhesion of spores to the leaf

TABLE 1 | RNAseq read counts and percentage mapping statistics to *C. fructicola* and strawberry genome.

	Samples	Clean reads	Genome	Total mapped reads	Total unmapped reads	Unique_match
Control	JiuXiang (host)	98573510 (100%)	*Fragaria_vesa*	61726447 (62.62%)	36847063 (37.38%)	54204716 (54.99%)
	CGMCC3.17371 (pathogen)	42264966 (100%)	Naragc5	35405362 (83.77%)	68596041 (16.23%)	21244745 (50.27%)
Infected mixed transcriptome	24 hpi	88846458 (100%)	*Fragariavesa*	544520336 (61.29%)	34394425 (38.71%)	47606785 (53.58%)
			Naragc5	28221 (0.039%)	88818237 (99.97%)	21726 (0.02%)
	72 hpi	953905321 (100%)	*Fragariavesa*	57270501 (60.04%)	38120031 (39.96%)	48761603 (51.12%)
			Naragc5	53153 (0.06%)	95337379 (99.94%)	45235 (0.05%)
	96 hpi	109226054 (100%)	*Fragariavesa*	65830542 (60.12%)	39104592 (39.93%)	49520288 (50.56%)
			Naragc5	114160 (0.15%)	97823884 (99.88%)	107802 (0.11%)

surface (Tucker and Talbot, 2001). In *Magnaporthegrisea*, the class I hydrophobin MPG1 and the class II hydrophobin MHP1 are both required for full pathogenicity (Talbot et al., 1993, 1996; Kim et al., 2005). In our data, a gene encoding MPG1 (CGGC5_8072) was expressed at high levels throughout the entire course of infection (Table S3). Another secreted fungal protein that binds to hydrophobic surfaces is encoded by HsbA in *Aspergillus oryzae*. This secreted protein binds to artificial polybutylene succinate-co-adipate (PBSA) hydrophobic surfaces and has been shown to recruit a polyesterase, which degrades PBSA and enables the fungus to use it as a carbon source (Ohtaki et al., 2006). Based on the occurrence of the HsbAPfam motif (PF12296), nine homologs of HsbA were discovered in the transcriptome of *C. fructicola*. Analysisof the transcriptomic data (Table S3) suggests that eight HsbA-encoding genes are up-regulated at 24 hpi and that the ninth gene is up-regulated only at a later stage (72 hpi). These data suggest that HsbA-like protein-mediated adhesion likely plays a role during *C. fructicola* appressorium development.

In the first step of invasion, pathogens need plant cell wall–degrading enzymes (PCWDEs) to degrade the plant cell walls and facilitate penetration and migration; meanwhile, remodeling of the pathogen's cell wall is necessary for evasion of PTI (Bellincampi et al., 2014; Oliveira-Garcia and Valent, 2015). The most prevalent PCWDEs during the early stages of *C. fructicola* infection (24 hpi) are pectin-degrading enzymes, such as carbohydrate esterase (CE) family 1, glycoside hydrolase (GH) family GH3, GH31, and several pectate lyases (PL1, PL3) (Table S4). Pectin is the main component of plant cell walls. The depolymerization of pectin makes the plant cell wall more vulnerable and accessible to other PCWDEs (Malinovsky et al., 2014). An increase in the number and diversity of the GH families that facilitate plant cell wall breakdown was observed at 96 hpi, including GH28 (polygalacturonase), GH31 (α-glucosidase), GH47 (α-mannosidase), and GH105 (rhamnogalacturonylhydrolase), as well as pathogen wall modification, such as GH-3 (β-1,3-glucan) and GH-18 (chitinase) (Figure S1, Table S4).

The transcription of an abundant class of genes encoding enzyme inhibitors was increased, including glucanase inhibitor proteins that bind to and inhibit host endo-β-1,3-glucanases of glycosyl hydrolase (GH) family 16; this was especially prevalent during 72 hpi. These proteins may impede the degradation of the fungal cell wall and prevent it from being recognized by the plant (Esquerré-Tugayé et al., 2000). Notably, three genes containing the carbohydrate-binding module 50 (CBM50), which binds to chitin (LysM domain-containing proteins), were up-regulated at 72 and 96 hpi (Table S4). The binding of chitin by pathogen proteins could help it to avoid recognition by host chitin receptors and suppress chitin-induced host immunity (de Jonge and Thomma, 2009).

Primary Metabolism: Fatty Acid Oxidation and the Glyoxylate Cycle Were Activated During the Early Stage of *C. fructicola* Infection

During the infection process, pathogenic fungi usually encounter nutrient deprivation in the host before gaining access to sufficient nutrients to successfully colonize living tissue (Lee et al., 2009). We observed significant up-regulation of the key genes involved in two primary metabolic pathways (fatty acid oxidation and the glyoxylate cycle).

During the first step of infection, fungi produce extracellular degrading enzymes. We observed significant up-regulation of cutinase–coding genes during *C. fructicola* infection (Table S4). This process provides the materials needed for fungal fatty acid oxidation. Thereafter, fatty acid synthesis and subsequent transport were bothactivated at 24 and 72 hpi, respectively, and were characterized by the up-regulation of the genes encoding Acyl-CoA dehydrogenase and Enoyl-CoA hydratase. Acyl-CoA is the basic currency of carbon metabolism within the cell (Strijbis and Distel, 2010). In our study, two genes encoding key enzymes of the glyoxylate cycle (citrate synthase and isocitratelyase) were highly induced at 24 hpi. This indicates that the use of Acyl-CoA, which is derived from fatty acid β-oxidation, may provide carbon skeletons for anabolic processes via the glyoxylate cycle early in infection (**Figure 4**, Table S5).

The expression patterns demonstrate that *in vitro*, where abundant nutrition sources are available in the culture medium, *C. fructicola* tends to rely on glycolysis and the tricarboxylic acid cycle for energy production. By contrast, during the early stages of infection where there are limited sources of nutrition, *C. fructicola* tends to rely on the mobilization of stored lipids through fatty acid oxidation to form Acyl-CoA, which is further assimilated via the glyoxylate cycle to supply energyto cells (Table S6).

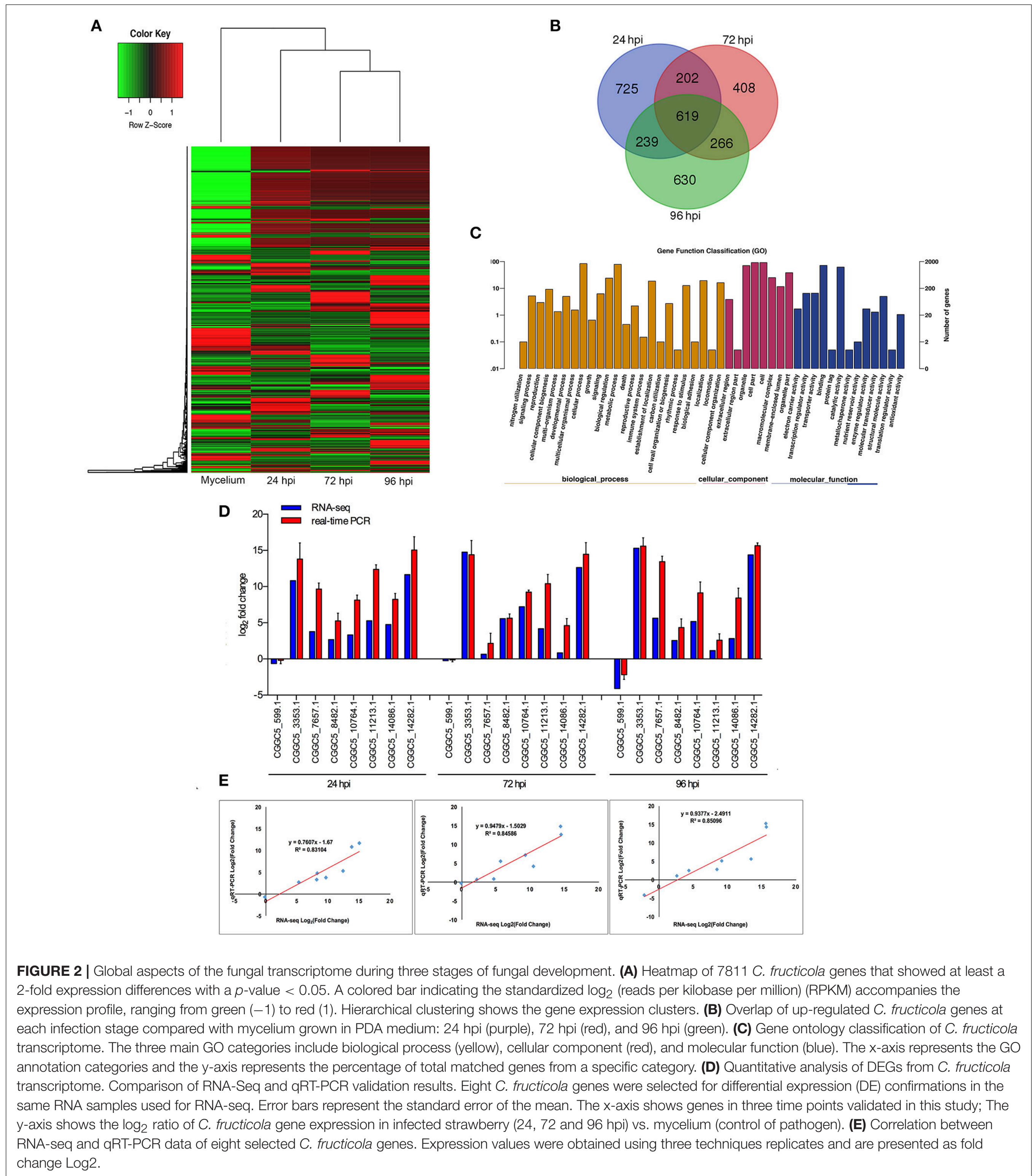

FIGURE 2 | Global aspects of the fungal transcriptome during three stages of fungal development. **(A)** Heatmap of 7811 *C. fructicola* genes that showed at least a 2-fold expression differences with a *p*-value < 0.05. A colored bar indicating the standardized $\log_2$ (reads per kilobase per million) (RPKM) accompanies the expression profile, ranging from green (−1) to red (1). Hierarchical clustering shows the gene expression clusters. **(B)** Overlap of up-regulated *C. fructicola* genes at each infection stage compared with mycelium grown in PDA medium: 24 hpi (purple), 72 hpi (red), and 96 hpi (green). **(C)** Gene ontology classification of *C. fructicola* transcriptome. The three main GO categories include biological process (yellow), cellular component (red), and molecular function (blue). The x-axis represents the GO annotation categories and the y-axis represents the percentage of total matched genes from a specific category. **(D)** Quantitative analysis of DEGs from *C. fructicola* transcriptome. Comparison of RNA-Seq and qRT-PCR validation results. Eight *C. fructicola* genes were selected for differential expression (DE) confirmations in the same RNA samples used for RNA-seq. Error bars represent the standard error of the mean. The x-axis shows genes in three time points validated in this study; The y-axis shows the $\log_2$ ratio of *C. fructicola* gene expression in infected strawberry (24, 72 and 96 hpi) vs. mycelium (control of pathogen). **(E)** Correlation between RNA-seq and qRT-PCR data of eight selected *C. fructicola* genes. Expression values were obtained using three techniques replicates and are presented as fold change Log2.

Secondary Metabolism: Melanin Biosynthesis Is a Pivotal Process During Appressorium Development

Secondary metabolites produced by *Colletotrichum* are known to contribute to pathogenicity (Gan et al., 2013). There were 11 secondary metabolite backbone genes up-regulated in our transcriptomic data (Table S7). Three polyketide synthases (PKSs)-encoding genes (CGGC5_9803, CGGC5_12144, and CGGC5_14485) were significantly up-regulated at 24 hpi.

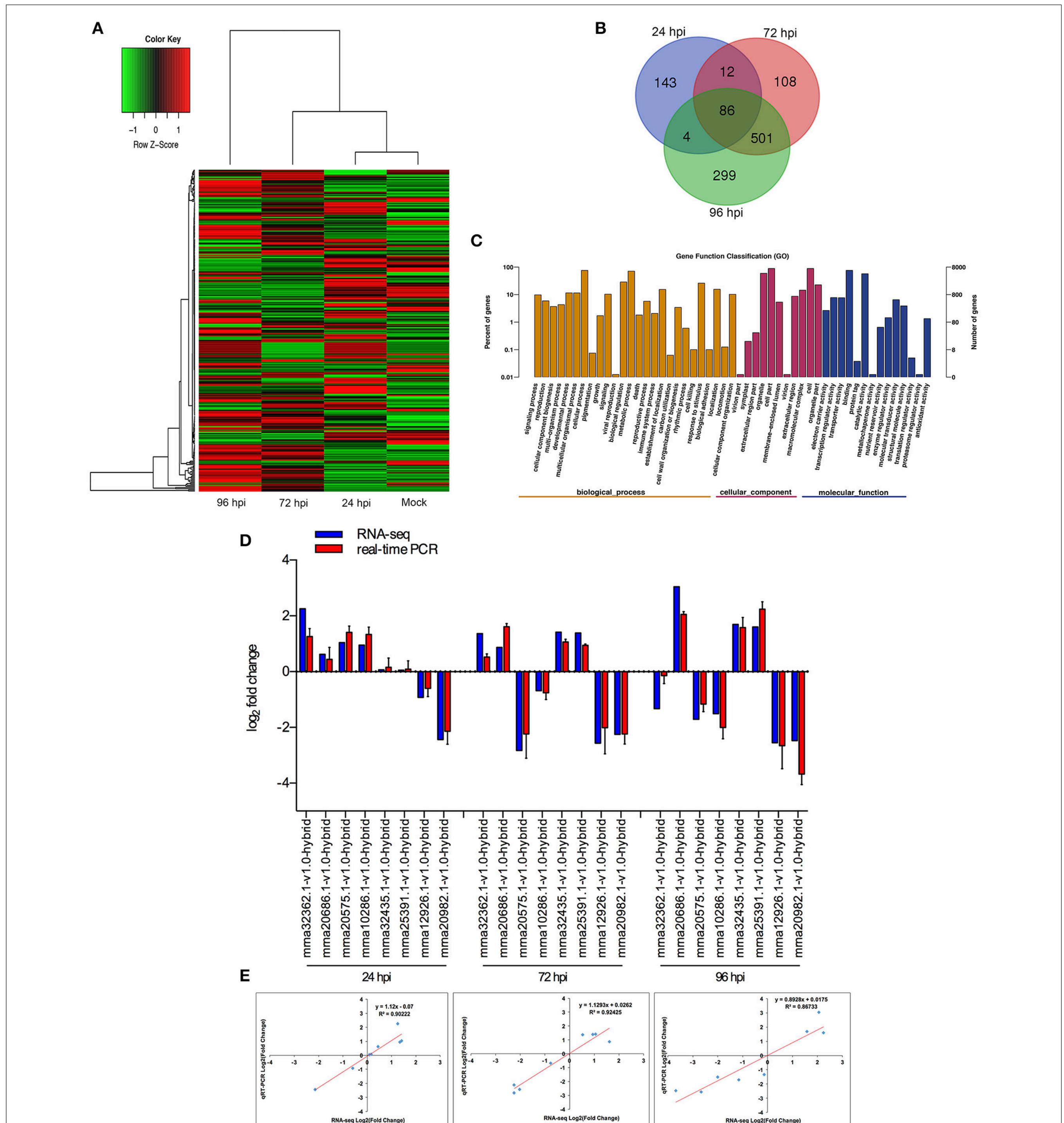

FIGURE 3 | Global aspects of the strawberry transcriptome during three stages of fungal development. **(A)** Heatmap of 2273 strawberry genes that showed at least 2-fold expression difference with *p*-value < 0.05. A colored bar indicating the standardized $\log_2$ (reads per kilobase per million) (RPKM) accompanies the expression profile, ranging from green (−1) to red (1). Hierarchical clustering shows gene expression clusters. **(B)** Overlap of up-regulated genes in response to each stage compared with mock treatment with water containing Tween−20: 24 hpi (purple), 72 hpi (red), and 96 hpi (green). **(C)** Gene ontology classification of strawberry transcriptome. The three main GO categories include biological process (yellow), cellular component (red), and molecular function (blue). The x-axis represents the GO annotation categories and the y-axis represents the percentage of total matched genes from a specific category. **(D)** Quantitative analysis of DEGs from strawberry transcriptome. Comparison of RNA-Seq and qRT-PCR validation results. Eight strawberry genes were selected for DE confirmations in the same RNA samples used for RNA-seq. Error bars represent the standard error of the mean. *x*-axis shows genes in three time points validated in this study; *y*-axis shows the $\log_2$ ratio of strawberry gene expression in infected strawberry (24, 72, and 96 hpi) vs. mock inoculated strawberry (control of strawberry). **(E)** Correlation between RNA-seq and qRT-PCR data to eight selected strawberry genes. Expression values were obtained using three techniques replicates and are presented as fold change Log2.

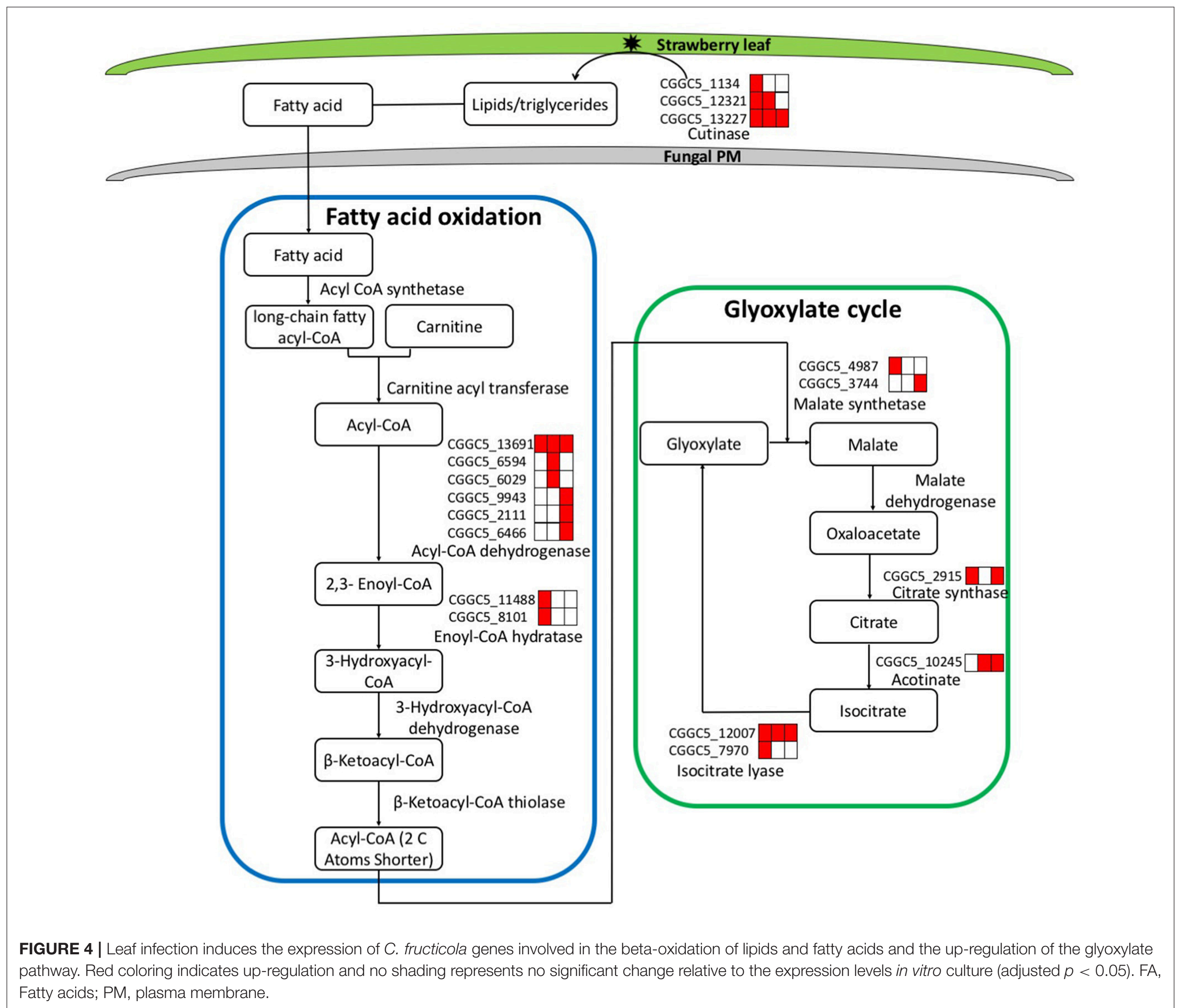

FIGURE 4 | Leaf infection induces the expression of *C. fructicola* genes involved in the beta-oxidation of lipids and fatty acids and the up-regulation of the glyoxylate pathway. Red coloring indicates up-regulation and no shading represents no significant change relative to the expression levels *in vitro* culture (adjusted $p < 0.05$). FA, Fatty acids; PM, plasma membrane.

CGGC5_14485 was homologous with ALB1, which encodes a PKS that catalyze the first step in the melanin biosynthesis of *M. grisea* (Chumley, 1990). The other five secondary metabolite backbone-forming genes were highly up-regulated at 96 hpi; this coincides with the necrotrophic stage of *C. fructicola* (Table S7).

Secondary metabolites associated with sporulation can be placed into three broad categories, including pigment. Melanin, a pigment located in the cell wall of appressoria, can provide mechanical strength that aids in tissue penetration (Eisenman and Casadevall, 2012). Many fungi synthesize melanin via the dihydroxynaphthalene (DHN) pathway. DHN is produced from 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) via a sequence of reduction and dehydration reactions with the intermediates scytalone,1,3,8-trihydroxynaphthalene and vermelone (Eisenman and Casadevall, 2012). The expression profiles of *C. fructicola* genes encoding enzymes of the DHN melanin biosynthesis pathway were all up-regulated at 24 hpi (Table S8). Melanin biosynthesis is a pivotal process during appressorium development and seems to play a role in *C. fructicola* pathogenesis.

Transporters and Detoxification of Compounds Protect *C. fructicola* From Host Defense

Plant pathogens must protect themselves, especially during infection when they are likely to encounter host defense mechanisms (Göhre and Robatzek, 2008). One way fungi accomplish this task while overcoming intercellular toxin accumulation is via efflux pumps, in particular ATP-binding cassette (ABC) transporters and transporters of the major facilitator superfamily (MFS) (Coleman and Mylonakis, 2009). In our data, several ABC-transporter-encoding genes were up-regulated at all three time points post infection (Table S9). The ABC transporters in other fungi have shown to play an

important role in fungal resistance. For example, BcatrB from *Botrytis cinerea* (homolog of CGGC5_15191) and NhABC1 from *Fusariumsolani f. sp. pisi* (homolog of CGGC5_9023) were shown to be responsible for the efflux of plant-derived defense compounds (Stefanato et al., 2009; Coleman et al., 2011). A search for genes with the Pfam membrane transporter motifs PF00083 (a signature of saccharide) and PF07690 (MFS signature) identified 83 up-regulated genes (Table S10). Of these, 56 were up-regulated at 24 hpi. Genes encoding sugar and MFS transporters were also highly up-regulated; (Figure S2A). In *Cercospora nicotianae*, a MFS transporter gene *CTB4* (homologs of CGGC5_9801), encodes a cercosporin efflux pump that contributes to self-protection from cercosporin, subsequently facilitating the virulence of these pathogens on their host plants (Choquer et al., 2007). The significant up-regulated ABC and MFS transporter genes at 24 hpi may suggest that these transporters play a critical role in protection against plant defense compounds during the early stage of infection (Del Sorbo et al., 2000).

Cytochrome P450s (CYP450) are involved in detoxification and contribute to host interactions (Nelson, 1999). We observed 36 genes encoding CYP450 (PF00067) that were simultaneously up-regulated at 72 hpi. A recent publication showed that P450s were specifically involved in virulence as well as both asexual and sexual development. Most importantly, P450s seem to play redundant roles in the degradation of xenobiotics in *Fusarium graminearum*; *CYP51* genes in *F. graminearum* (homologs of CGGC5_10696, CGGC5_2390) mediate differential sensitivity to sterol demethylation inhibitors (Shin et al., 2017). CYP450 contributes to the production of mycotoxins and the detoxification of host metabolites in other fungi (Crešnar and Petric, 2011). The up-regulation of several P450 genes in our data may suggest a special role for theCYP450-mediated detoxification produced by the plant at this stage (Table S11, Figure S2B).

Defining *C. fructicola* Candidate Effectors

Fungal effectors are secreted molecules that modulate the interaction between the fungus and its host (Lo Presti et al., 2015). There are 2042 genes (13.2%) that encode secreted proteins in the genome of Nara gc5 (Gan et al., 2013). The current transcriptomic data show that some homologs of known effectors from other phytopathogens were differentially expressed after *C. fructicola* infection. For example, the gene encoding CGGC5_2199 (homolog of Cmu1) was up-regulated at 24 hpi. The effector Cmu1 counteracts salicylic acid (SA)-dependent immunity in the host, functioning as a chorismate mutase to reduce the levels of the SA precursor chorismate (Djamei et al., 2011). The genes encoding CGGC5_10914 (a homolog of Ecp6) were up-regulated at 96 hpi; this is consistent with the previously reported expression patterns of this gene. Previous reports showed that the expression of Ecp6 gene was gradually up-regulated and maximal at 13 dpi during the interaction of *Cladosporium fulvum* with tomato (Bolton et al., 2008). Ecp6 has identified to be a novel virulence factor through sequestering chitin oligosaccharides to prevent elicitation of plant immunity (de Jonge et al., 2010). The genes encoding for CGGC5_10914 (a homolog of CHEC30) were up-regulated at 72 and 96 hpi. *C. higginsianum* effector CHEC30 was appeared to be preferentially expressed at biotrophic stage (Kleemann et al., 2012). The expression patterns of other homologs of known effectors are listed in Table S12.

In addition to these known effectors, candidate effectors were identified in the transcriptome of *C. fructicola*via a pipeline (**Figure 5A**) that shares common features with those described for filamentous plant pathogens (Petersen et al., 2011; Guyon et al., 2014). We first refined the prediction by combining the predictions from SignalP4.1 and TMHMM to identify 729 proteins that had secretion signals but did not have transmembrane helices (Petersen et al., 2011). Next, GPIsom removed 87 proteins harboring glycophosphatidyl inositol anchor motifs, which likely represent surface proteins rather than secreted effectors (Fankhauser and Mäser, 2005). This resulted in 642 predicted secreted proteins (SPs). A Circos plot was generated to visualize the peptide sequence similarities between these SPs and their homologs. These homologs show a 90% peptide sequence identity, which indicates that these SPs have striking similarities tothose of other *Colletotrichum* species (**Figure 5B**). In total, 294 of the 642 SPs were small (<300 aminoacid) secreted proteins (SSPs). We observed 85 SSPs that were highly expressed during infection. These 85 SSPs were evaluated with the EffectorP prediction tool, which is usable for both apoplastic and cytoplasmic effectors (Sperschneider et al., 2016). 52 SSPs were ultimately identified as the most likely set of *C. fructicola* effectors (**Figure 5C**). Post-translational modification of effectors influences their function. Glycosylation likely impacts effector function by modifying protein stability, as well as conformation or resistance to host proteases (Doehlemann et al., 2014). Here, 90.6% of candidate effectors carried at least one predicted N/O-glycosylation site (**Figure 5C**, Table S13).

To identify the function of the candidate effectors, four highly expressed SSPs were chosen for a transient assay in *Nicotiana benthamiana*. The signal peptides of these candidate effectors were first removed to enable the genes to be expressed stably in plant cells. The four candidate effectors that partially or fully suppressed Bax-mediated programmed cell death in tobacco leaves (**Figure 5D**).

Strawberry Transcriptomics

Cell Wall Fortification

Improving cell wall lignin content is one of the common plant defense mechanisms. In our study, inoculation induced the expression of strawberry 4-coumarate-CoA ligase and disease resistance-responsive (dirigent-like) proteins. These proteins are involved in lignin biosynthesis (Lee et al., 1995; Davin et al., 1997). Several dirigent-like protein-encoding genes were up-regulated during the three stages of infection (Table S15).

A number of cell wall synthesis genes were also down-regulated during the course of *C. fructicola* infection. For example, six of the 10 cellulose-synthase-like genes, including CSLC5 and CSLD3 homologs (Dai et al., 2011; Almeida et al., 2015), were down-regulated at 72 and 96hpi (Table S15). Similar changes (i.e., replacement of cell wall components) have

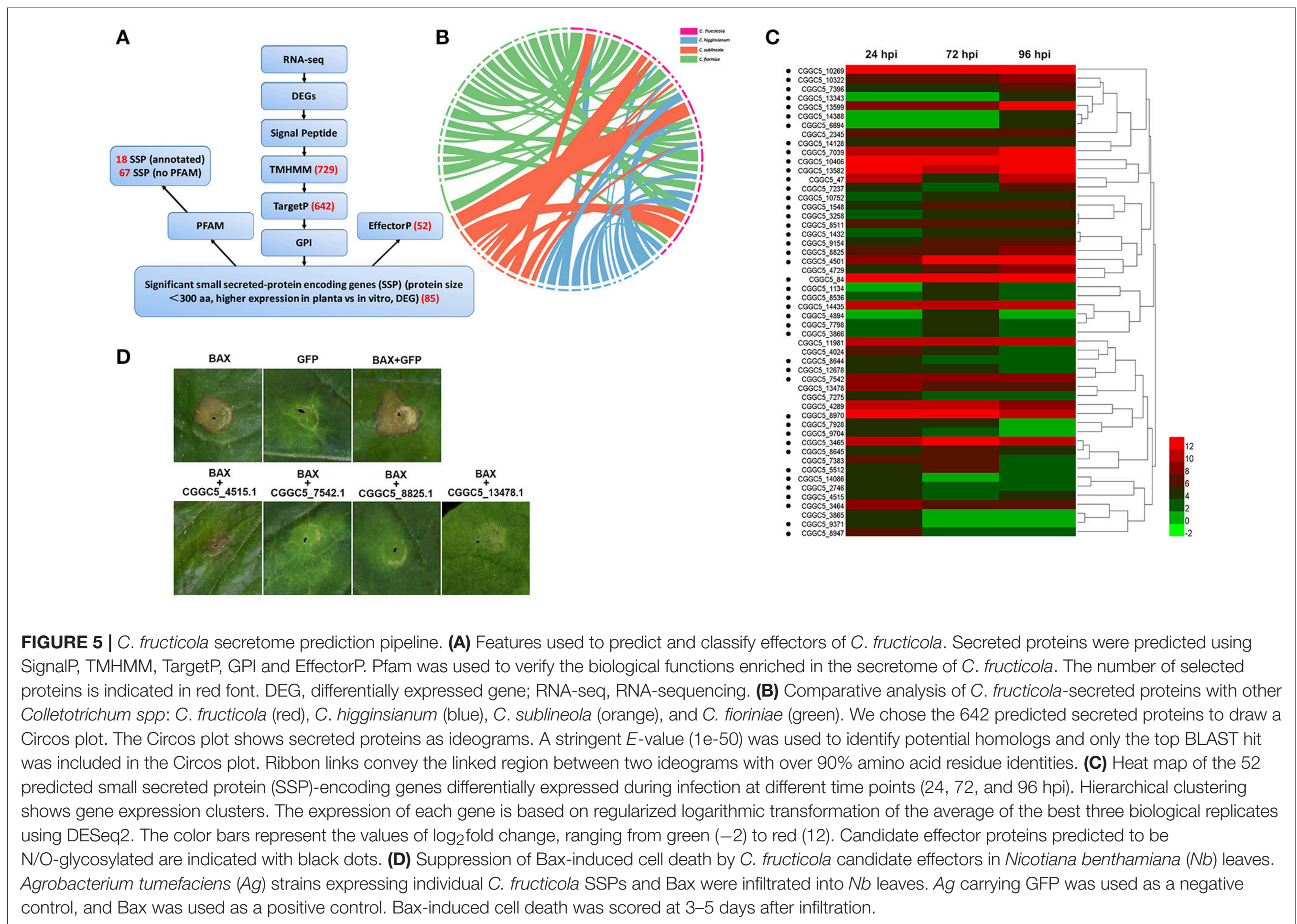

FIGURE 5 | *C. fructicola* secretome prediction pipeline. **(A)** Features used to predict and classify effectors of *C. fructicola*. Secreted proteins were predicted using SignalP, TMHMM, TargetP, GPI and EffectorP. Pfam was used to verify the biological functions enriched in the secretome of *C. fructicola*. The number of selected proteins is indicated in red font. DEG, differentially expressed gene; RNA-seq, RNA-sequencing. **(B)** Comparative analysis of *C. fructicola*-secreted proteins with other *Colletotrichum spp*: *C. fructicola* (red), *C. higginsianum* (blue), *C. sublineola* (orange), and *C. fioriniae* (green). We chose the 642 predicted secreted proteins to draw a Circos plot. The Circos plot shows secreted proteins as ideograms. A stringent *E*-value (1e-50) was used to identify potential homologs and only the top BLAST hit was included in the Circos plot. Ribbon links convey the linked region between two ideograms with over 90% amino acid residue identities. **(C)** Heat map of the 52 predicted small secreted protein (SSP)-encoding genes differentially expressed during infection at different time points (24, 72, and 96 hpi). Hierarchical clustering shows gene expression clusters. The expression of each gene is based on regularized logarithmic transformation of the average of the best three biological replicates using DESeq2. The color bars represent the values of $\log_2$fold change, ranging from green (−2) to red (12). Candidate effector proteins predicted to be N/O-glycosylated are indicated with black dots. **(D)** Suppression of Bax-induced cell death by *C. fructicola* candidate effectors in *Nicotiana benthamiana* (*Nb*) leaves. *Agrobacterium tumefaciens* (*Ag*) strains expressing individual *C. fructicola* SSPs and Bax were infiltrated into *Nb* leaves. *Ag* carrying GFP was used as a negative control, and Bax was used as a positive control. Bax-induced cell death was scored at 3–5 days after infiltration.

been observed in *Arabidopsis* CESA3-deficient mutants (Caño-Delgado et al., 2003). Reduced cellulose synthesis has been suggested to invoke a wide range of cellular responses, including activating lignin synthesis and defense responses mediated via JA and ethylene (ET) signaling pathways (Caño-Delgado et al., 2003; Hamann, 2012).

Pathogen Perception: Activation of PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI)

Through evolution, plants have developed a two-step innate immunity system to prevent pathogen invasion. The first step involves the recognition of pathogen-associated molecular patterns (PAMPs) through plant pattern recognition receptors (PRRs); this subsequently triggers the PTI (Nicaise et al., 2009). Plant PRRs are either surface-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs), which usually contain ligand-recognition domains, such asleucine rich repeats (LRRs) and lysine motifs (LysMs). Chitin is the major constituent of fungal cell walls and serves as a classic fungal PAMP that can be recognized by several typical plantPRRs (e.g., chitin elicitor receptor kinase 1 [CERK1], chitin elicitor-binding protein [CEBiP]) (Kaku et al., 2006; Miya et al., 2007). We did not observe significant up-regulation of genes encoding CERK1 or CEBiP in *C. fructicola*–infected plants. However, a set of strawberry RLKs were differentially expressed during infection. 53% (23/43) and 60% (26/43) of RLKs were significantly up-regulated at 72 and 96 hpi, while 23% (10/43) of RLKs were down-regulated at both 72 and 96 hpi. (Table S16).

ETI involves the direct or indirect recognition of effectors by plant resistance (R) proteins (Chisholm et al., 2006). The execution of ETI is mediated by intracellular immune receptors (e.g., NBS-LRR proteins) in order to perceive secreted virulence effectors (Zipfel, 2014). In our study, five CC-NBS-LRRs, two TIR-NBS-LRRs, and five NB-ARC domain-containing proteins were differentially expressed (Table S17). Among them, two NB-ARC genes were down-regulated while six were up-regulated during all three stages of infection. However, the successful infection of *C. fructicola* indicated that although some R genes were up-regulated at transcriptional level, they might not fully exert their function and block fungal infection.

Core Immune Phytohormones: SA Is Effectively Triggered

In this study, 50, 88, and 100% of SA-signaling genes were significantly up-regulated at 24, 72, and 96 hpi, respectively. The nonexpressor of pathogenesis-related genes 1 (NPR1) is the key

gene that mediates defense gene expression induced by SA (Mou et al., 2003). We observed an up-regulation of *NPR1* at 72 and 96 hpi. The SA pathway activates many downstream defense related genes, including *PR* genes (van Loon et al., 2006). The PR4 family encodes chitinase and can be induced by *F. culmorum* infection, SA treatment, or JA treatment in wheat (Bertini et al., 2003). In our study, two homologous PR4 genes, PR4a and PR4b, were up-regulated (by 2.3- and 2.4-fold, respectively) at 24 hpi. By 72 hpi, they were up-regulated by 29- and 38-fold, respectively; this increased further to 49- and 60-fold by 96 hpi (Table S18). In addition, one gene coding the cysteine-rich secretory protein (CRISP) was highly expressed at 96 hpi. The homolog of the CRISP in plants is the PR-1 protein; this protein plays an important role in anti-fungal activities (Niderman et al., 1995). These highly up-regulated PR genes seem to indicate their potential role in *C. fructicola* resistance. However, in our previous reports, several relevant immune-related genes were chosen for qRT-PCR assay to evaluate their gene expression level between susceptible (JiuXiang, JX) and less-susceptible plant (Sweet Charlie, SW) (Zhang et al., 2016). Specifically, several SA-related genes, including PR1a and PR5 were up-regulated in both JX and SW. Notably, the gene expression level in less-susceptible plant were significantly higher than that in susceptible plant. For example, at 96 hpi, PR1a was up-regulated by $\sim$30- and 400-folds in susceptible and resistant plant, respectively. Similarly, PR5 was up-regulated by $\sim$10- and 600-folds in susceptible and resistant plant, respectively. These results suggest that although several SA-related genes seem to be up-regulated in successful infection, the extent of the up-regulation is significantly less than that in resistant infection. The relative low level of these genes may not be able to inhibit fungal infection in susceptible infection.

JA is an important plant hormone which plays a role in regulating plant development and stress responses. The largely antagonistic functions of JA and SA have been suggested for plant response to pathogen infection (Verma et al., 2016). There also exists cooperative interplay between JA and SA during early ETI. In parallel to SA accumulation in Arabidopsis, the endogenous JA level also increases during ETI induction, which may reduce plant susceptibility to necrotrophic pathogens in the neighboring tissue; this elevation of JA is dependent on the degradation of JAZ mediated by direct interaction with NPR3-NPR4 (Liu et al., 2016). Lipoxygenases (LOX), especially LOX2, are required for wound-induced JA accumulation in *Arabidopsis* (Bell et al., 1995). In this study, the gene encoding LOX1 was reduced by 10.5-, 3.6-, and 4.3-fold at 24, 72, and 96 hpi, respectively. In addition, the gene encoding LOX2 was significantly down-regulated at 96 hpi. JA biosynthesis is possibly regulated by post-translational modifications of pre-existing enzymes, such as allene oxide cyclase (AOC) (Schaller and Stintzi, 2009). Similar to LOX, the gene encoding AOC4 was reduced by 3.1-, 3.0-, and 4.2-fold at 24, 72, and 96 hpi, respectively (Table S19). These results indicate that JA signaling maymainly inhibited by *C. fructicola* during the entire infection process.

JA and ET operate synergistically in regulating defense responses after pathogen infection (Verma et al., 2016). ET and the related signaling pathway can positively or negatively regulate plant immunity; additionally, this pathway exhibits extensive cross-talk with the SA and JA pathways (Sato et al., 2010; Shakeel et al., 2013). The gene encoding ethylenereceptor2 was down-regulated at 24 hpi. Ethylene response factors (ERFs) act downstream of the intersection between the ET and JA pathways. These transcription factors, such as ERF1, are key elements regulating defense responsive genes (Lorenzo et al., 2003). In our study, the genes encoding the ethylene responsive element–binding factor 2 (EREBF2) and the ethylene-forming enzyme (EFE) were mildly up-regulated after *C. fructicola* infection. Meanwhile, the other eight genes encoding the ethylene-responsive transcription factor were significantly down-regulated at three time points after infection (Table S20).

The endogenous hormone contents of strawberry leaves during *C. fructicola* infection were analyzed to further confirm the role of plant hormone signaling in fungal infections. The results showed significant up-regulation of SA content at 72 and 96 hpi. Meanwhile, JA content was mildly up-regulated at 24 hpi, but then down-regulated at 72 and 96 hpi (**Figure 6**). Our results regarding SA and JA signaling were consistent with previous reports, which demonstrated that the primary mode of interaction between SA and JA pathways was mutual antagonism (Doares et al., 1995; Clarke et al., 2000; Gupta et al., 2000). SA is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens. By contrast, JA and ET are required for resistance against necrotrophic pathogens (Glazebrook, 2005; Bari and Jones, 2009). In our study, the inhibition of ET and JA signaling may be triggered by *C. fructicola* infection. Alternatively, *C. fructicola* induced the activation of SA signaling, which may indirectly inhibit ET and JA signaling.

Other Hormone Signaling

ABA prominently contributes to plant defense response against both abiotic and biotic stress conditions (Fitzpatrick et al., 2011). ABA shows negative interaction with JA-signaling in the modulation of Arabidopsis resistance to the necrotrophic fungi but it can also positively regulate the resistance to some biotrophic pathogens (Denance et al., 2013). Glycosyl hydrolases (GH) belong to a large family of proteins implicated in carbohydrate metabolism andthe remobilization of sugars. ABA significantly increased the activity of α-L-Arabinofuranosidase, one member of glycosyl hydrolase (GH) family, suggesting that some GH might be associated with ABA mediated processes (Alayon-Luaces et al., 2008). Two glycosyl hydrolase superfamily genes and one glycosyl hydrolase family 32 gene were up-regulated at 72 hpi. Meanwhile, five other GH genes were down-regulated at 72 and 96 hpi (Table S21).

Emerging evidence indicates that auxin-signaling genes regulate resistance to different plant pathogens (Denance et al., 2013); additionally, it is possible that components of the auxin synthesis, signaling, and transport pathways could be utilized by pathogens to increase colonization efficiency (Denance et al., 2013). In our study, the temporary up-regulation of auxin related genes, such as auxin-responsive GH3 family protein and indole-3-acetic acid 7 (IAA7), was observed at 24 hpi. However, these auxin-related genes were significantly down-regulated during the middle and late stages of infection. For example, the expression of the early auxin-responsive gene (IAA7) was increased by 7.1-fold

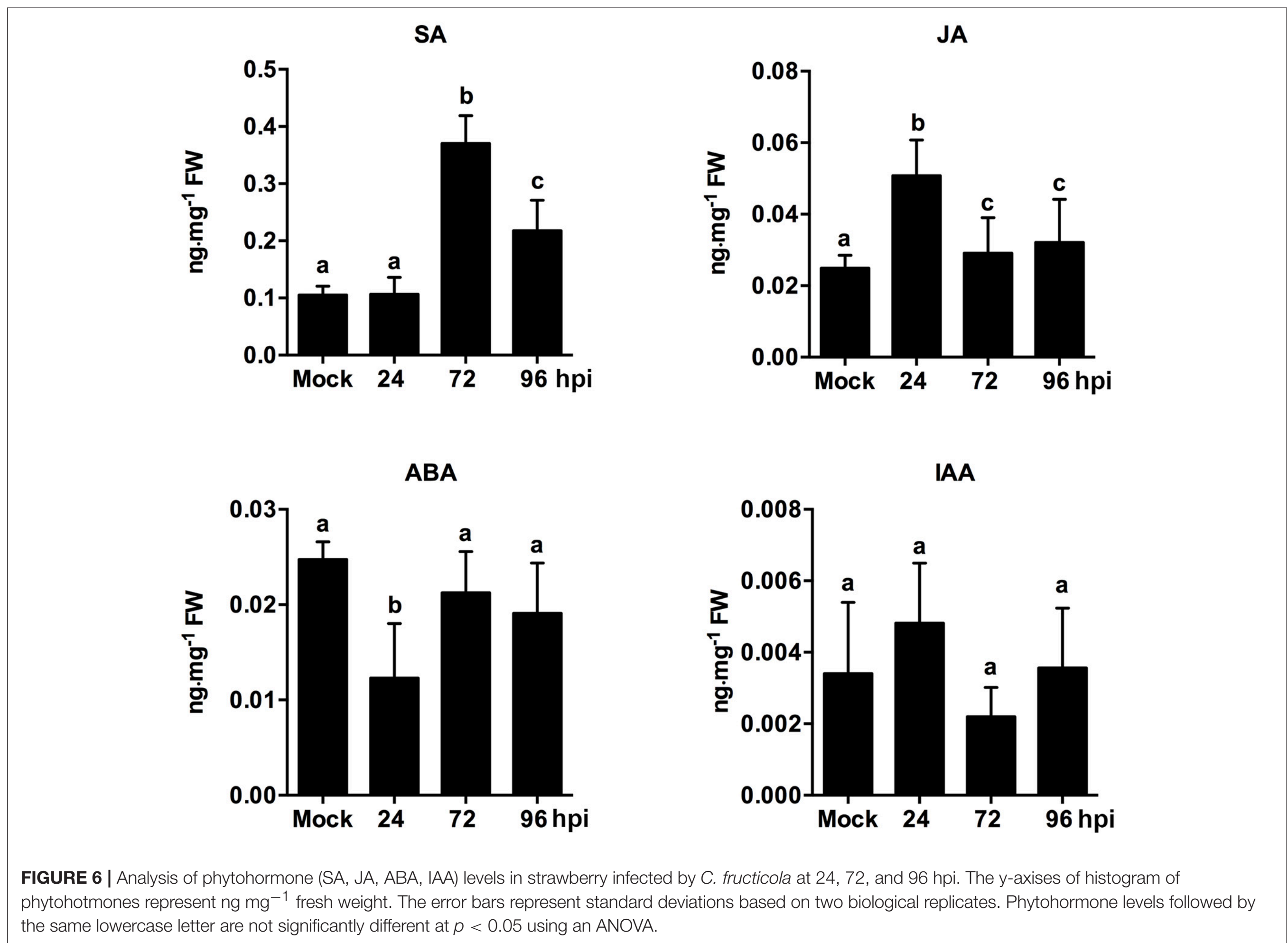

FIGURE 6 | Analysis of phytohormone (SA, JA, ABA, IAA) levels in strawberry infected by *C. fructicola* at 24, 72, and 96 hpi. The y-axises of histogram of phytohotmones represent ng mg^{-1} fresh weight. The error bars represent standard deviations based on two biological replicates. Phytohormone levels followed by the same lowercase letter are not significantly different at $p < 0.05$ using an ANOVA.

at 24 hpi, but was then decreased by 1.6- and 1.8-fold at 72 and 96 hpi, respectively (Table S22). An analysis of phytohormones levels revealed that ABA content was only mildly inhibited at 24 hpi. Other than this, ABA and IAA content were not significantly different at any point during infection (**Figure 6**). ABA play a negatively role in plant resistance to certain necrotrophic fungi (Audenaert et al., 2002; AbuQamar et al., 2006). A negative effect of auxin signaling on plant resistance to Necrotrophic Fungi was recently described (Llorente et al., 2008). In our present study, since ABA and auxin pathways were not significantly triggered during infection, these two pathways may not play an equally important role as SA, JA, and ET pathways. However, the precise function of these two hormone pathways needs to be further analyzed.

Reactive Oxygen Species Production Increases Accompanied by the Obvious Pathological Changes During the Late Stage of *C. fructicola* Infection

Reactive oxygen species (ROS) in plants are normally generated as by-products of oxidative phosphorylation and other diverse biosynthetic pathways (Almeida et al., 2015). The acute accumulation of ROS leads to an oxidative burst that induces cell death and restricts further pathogen infection (Apel and Hirt, 2004). Therefore, generation of ROS is usually accompanied by visual symptoms (i.e., hypersensitive responses or necrosis) in plants. In our study, there were obvious pathological changes at 96 hpi. Seven peroxidases (PODs) were significantly up-regulated at 96 hpi. Of the seven, two were up-regulated by 52- and 131-fold at 96 hpi. Meanwhile, another was up-regulated by 30- and 52-fold at 72 and 96 hpi, respectively (Table S23). The significant up-regulation of POD-encoding genes at 96 hpi may indicate the activation of plant defense mechanisms.

Various Transcription Factors Were Involved in the Regulation of Immune Responses During *C. fructicola* Infection

During PTI and ETI, plants trigger a diverse array of immune responses, such as hormone signaling, ROS generation, and MAP kinase (MAPK). The signal transduction of these immune responses relies on a regulatory network of transcription factors (TF) (Moore et al., 2011). A subset of TF families, such as WRKY, NAC, bHLH, ERF/AP2, MYB, and bZIP, play a role

in the regulation of immune responses. WRKY transcription factors belong to one of the largest families of transcriptional regulators in plants, and are key regulators of PTI and ETI (Eulgem and Somssich, 2007). In this study, we identified a total of 12 differentially expressed WRKY factors (Table S24). Nine genes encoding WRKY factors, including WRKY75, WRKY50, and WRKY23, were significantly up-regulated during the middle-to-late stages of *C. fructicola* infection. Genes encoding three members of the WRKY family, including WRKY33, were down-regulated at all three stages during infection. *Arabidopsis* WRKY33 is a key regulator of host immunity and also regulates the expression of some other WRKY factors as well as defense-associated genes (Birkenbihl et al., 2012). Interestingly, a strawberry homolog of the upstream regulatory gene, mitogen-activated protein kinase 3 (MPK3), was also down-regulated at all stages during infection.

In addition to the WRKY family, the NAC family of proteins is another group of transcriptional regulators that have been identified as important immune components (Jensen et al., 2008). Our study identified six NAC domain-containing proteinsthat were mainly induced at the middle-to-late stages of infection (Table S25). Like WRKY and NAC, other TFs, such as bHLH and ERF, were also differentially expressed after *C. fructicola* infection (Tables S20, S26). The differential expressions of these TFs as observed in the present study suggest that they may play a potential role in the interaction between *C. fructicola* and strawberry.

Fungal–Plant Interactions

The transcriptomic data give a picture of how adapted *C. fructicola* invades host cells and completes the infection process as strawberry works to mitigate immune suppression and slow the infection rate, albeit at a serious price. It should be noted that *C. fructicola* ultimately, successfully infects the plant and causes symptoms; this means that *C. fructicola* wins the "battle" against host resistance. Our transcriptomic data demonstrate that *C. fructicola* utilizes a series of strategies to antagonize or escape host resistance mechanisms at every stage of infection (**Figure 7**).

The establishment of a successful infection is dependent on *C. fructicola* invasion, which is the first step of infection and includes the degradation of the plant cell wall and subsequent penetration into the cell. The top 100 up-regulated genes at 24 hpi provide a glimpse into *C. fructicola*–strawberry interactions (Tables S27, S28). Specifically, at this stage, we found that 15 of the top 100 up-regulated *C. fructicola* genes were identified to encode putative effectors, one of which was a chitin–binding protein (CGGC5_3464). The literature shows that the pathogen is likely to initiate its adhesion to the host cells by expressing accessory molecules, such as chitinases and/or chitin-binding proteins (Chaudhuri et al., 2010). Four homologs of HsbA were within the top 100 up-regulated genes at 24 hpi. As previously mentioned HsbA binds to PBSA hydrophobic surfaces and recruits a polyesterase for the degradation of PBSA (Ohtaki et al., 2006). It prompts us to speculate that four homologs of HsbA are involved in attachment to the strawberry surface and the degradation of constituents from the plant epidermis; however, this requires further elucidation. Meanwhile, the host expresses a series of proteins to stabilize the cell wall to cope with *C. fructicola* invasion. Among the top 100 up-regulated strawberry genes, the most prevalent genes ($n = 5$) encode proteins involved in cell wall reinforcement and catabolism. This includes the glycine-rich cell wall structural protein, pectin acetylesterase family protein, wound-induced protein WI12, chitinase and AMP-dependent glycosyl hydrolase. WI12 preferentially accumulates in the cell wall, which suggests that it plays a role in cell wall reinforcement after wounding and during plant development (Yen et al., 2001). Glycine-rich proteins (GRPs) form a major part of the highly extensible and specialized cell walls. However, there has been no genetic evidence indicating a specific function for GRPs (Ringli et al., 2001). Although we did not thoroughly examine the function of these proteins, their high level of expression in our data indicates that the plant cell wall seems to be reinforced to prevent fungal invasion. However, most of the genes encoding cellulose synthase-like proteins, which participate in cellulose biosynthesis and cell wall modification, were down-regulated (Table S15 and **Figure 7**, upper panel). We conclude that this down-regulation might betargeted by *C. fructicola*, which served to help the fungus break down the cell wall and invade the host cells.

After *C. fructicola* invasion, fungus PAMPs are sensed by host PRRs. A number of strawberry genes encoding RLKs were up-regulated (**Figure 7**, left panel). In addition, several plant chitinase-encoding genes ($n = 7$) were significantly up-regulated, especially during the late stage of *C. fructicola* infection (Table S29). Chitinases were identified to be induced in various plants after fungal infection (Punja and Zhang, 1993). Furthermore, the antifungal activity of chitinases was shown by large number of transgenic plants raised for over expression of chitinase genes (de las Mercedes Dana et al., 2006). To evade recognition by plant PRRs and subsequent elimination, the fungus utilizes numerous effectors to suppress PTI. Typically, secreted LysM Protein1 (SLP1) can bind to chitin and suppress chitin-induced plant immune responses (Mentlak et al., 2012). Our results show that the SLP1-encoding gene was up-regulated at 72 and 96 hpi. The highly-expressed *SLP1* may play an important role in the suppression of PTI, such as chitin-induced immunity; however, this requires further investigation. A subset of these effectors might be perceived by R proteins resulting in a second layer of host defense called ETI. A total of 10 R genes were significantly up-regulated during *C. fructicola* infection; this suggests that they may play a role in antagonizing effectors, although their functions still need to be characterized (**Figure 7** right panel, blue shape).

After recognition of the fungus by PRR and R proteins, the plant initiates downstream anti-fungal innate immunity. In our study, hormone signaling, especially the SA, JA, auxin, ABA, and ET signaling pathways, displayed obvious changes in transcriptional activity (**Figure 7**, middle panel). The early activation of ABA and auxin signaling, the delayed activation of the SA pathway, and the continuous suppression of the JA and ET pathways might contributed to the deployment of basal defenses and the insufficient induction of the defense system in strawberries. The literature showed that the secreted

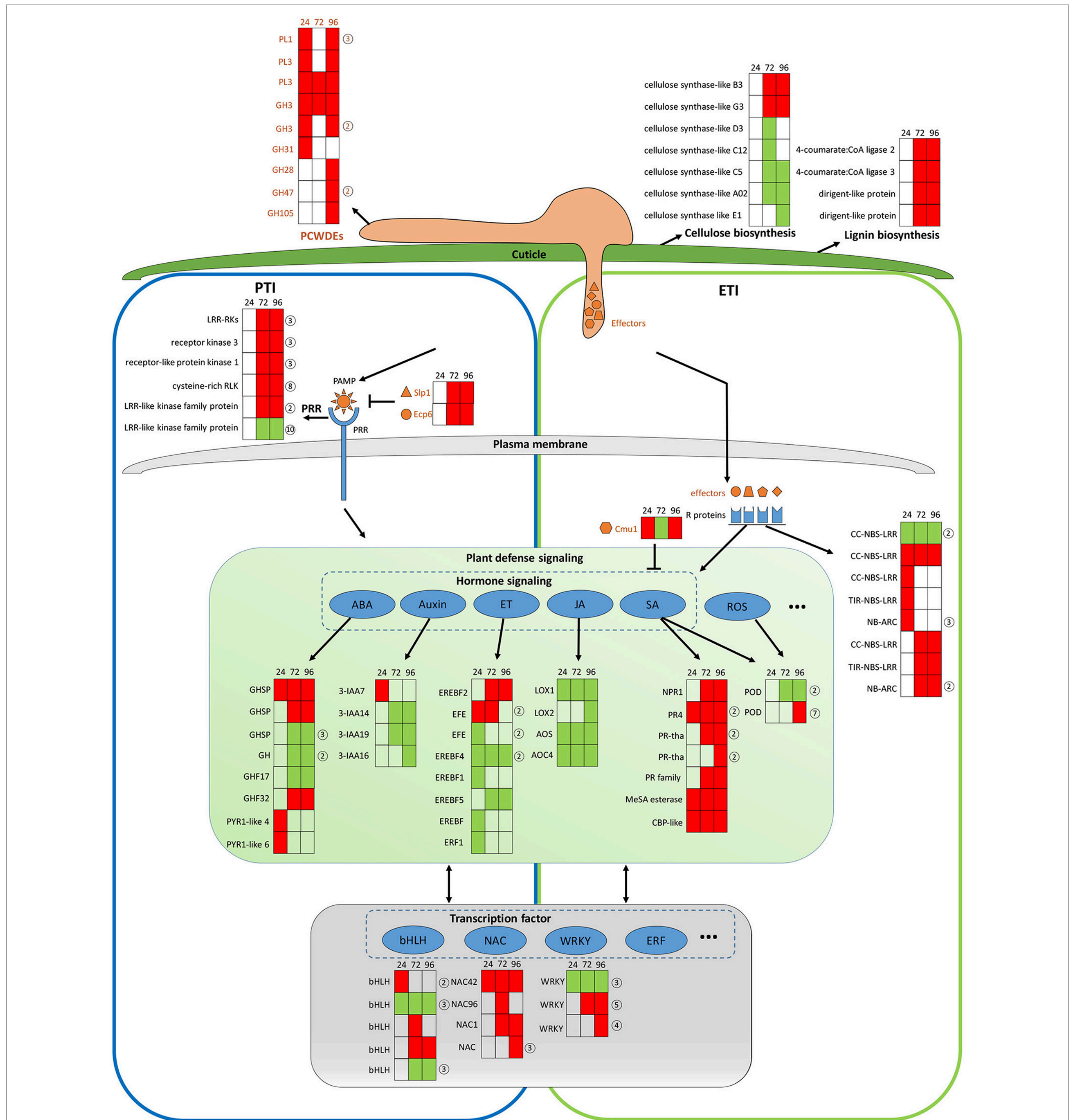

FIGURE 7 | General model of *C. fructicola*–strawberry interaction. The first step of infection includes the fortification of cell wall by strawberry vs. the degradation of the plant cell wall by *C. fructicola* (upper panel). After *C. fructicola* invasion, fungus PAMPs are sensed by host PRRs (PTI panel, left). Fungus secret numerous effectors into and around host cells by invasive hyphae to inhibit plant defense responses (such as PTI) or facilitate colonization. As a response, R proteins recognize effectors and initiates ETI (ETI panel, right). Plant subsequently initiates downstream anti-fungal innate immunity such as SA, ROS signaling (green panel). Various TFs, such as WRKY and NAC, are involved during the whole process of *C. fructicola* infection (gray panel). Red coloring indicates up-regulation, green coloring indicates down-regulation, and no shading represents no significant change (infected leaves at 24, 72, and 96 hpi vs. mock treatment with water containing Tween −20) (adjusted $p < 0.05$). The orange shapes represent the effectors elicited by the fungus. The blue shapes represent R proteins. The full name and gene accession numbers of differentially regulate genes are listed in Tables S4, S12, S15–S26. The numbers in the circles behind the heat maps represent the numbers of the differentially regulated genes in the indicated family. Specifically, PLs and GHs are listed in Table S4. LRR-RLKs, receptor kinase 3, receptor-like protein kinase 1, and cysteine-rich RLK-encoding genes are listed in Table S16. CC-NBS-LRR-and NB-ARC-encoding genes are listed in Table S17. GHSP-and GH-encoding genes are listed in Table S21. EFE-and EREBF5-encoding genes are listed in Table S20. PR4-and PR- encoding genes are listed in Table S18. The POD-encoding gene is listed in Table S23. The bHLH-encoding gene is listed in Table S26. The WRKY-encoding gene is listed in Table S24.

chorismate mutase protein (Cmu1) of fungus *Ustilago maydis* is proposed to suppress SA-dependent plant defense response (Djamei et al., 2011). Therefore, fungi could formulate efficient strategies against strawberry hormone-related defense systems.

A recent report on the strawberry leaf transcriptomes of "Yanli" and "Benihoppe" cultivars infected with *C. gloeosporioides* consistently showed that PR1 expression was up-regulated after infection, thus supporting the SA pathway response (Wang et al., 2017). Similarly, various defense-related genes, such as those encoding PR proteins, peroxidases, and lipoxygenases, were up-regulated in bean leaf following inoculation with *C. lindemuthianum*, which is in accordance with our transcriptomic data (Padder et al., 2016). A dual RNA-seq analysis on *C. graminicola*-maize leaf pathosystem during appressorial maturation, penetration, and colonization revealed that genes encoding secreted proteins, secondary metabolism enzymes, and receptors were over-represented among the differentially expressed genes. Several genes encoding homologs of BAS2, SLP1, and GAS1 were significantly up-regulated following *C. graminicola* infection. The up-regulation of these genes werealsoobserved in the current study, which suggests that these effectors may play important conserved roles in *Colletotrichum* infection (Torres et al., 2016).

In conclusion, the RNA-Seq technique was used to obtain a comprehensive characterization of infection-responsive DEGs from both *C. fructicola* and strawberry. The data revealed the metabolic, host/pathogen-responsive genes, and transcriptional networks associated with *C. fructicola*–strawberry interactions. This study resulted in several novel findings regarding *C. fructicola*–strawberry interactions: (1) an overview of strawberry defense and *C. fructicola* evasion mechanisms during the infection process; (2) a large number of up-regulated fungal genes encode candidate effector proteins during plant invasion; (3) the pivotal role of SA signaling during *C. fructicola* infection; and (4) other plant defense-related genes involved in cell wall fortification and ROS production were differentially regulated in response to *C. fructicola* infection. Future functional studies are needed to confirm the role of these candidate genes in *C. fructicola*–strawberry interactions; in particular, these studies will focus on the *C. fructicola*-specific effector genes and the putative susceptibility-related genes.

MATERIALS AND METHODS

Preparation of Control and Infected Samples for Host Plants and Pathogens

The *C. fructicola* isolate (CGMCC3.17371) was obtained from the Shanghai Academy of Agricultural Sciences, China. A susceptible strawberry (*Fragaria* × *ananassa* Duch.) cultivar, "JiuXiang" was used in this study. The plant growth and inoculation protocols have been previously described (Zhang et al., 2016). Briefly, the plants were selected for their uniform size and color and absence of visual defects. Stolon-derived healthy plants with more than 10 fully expanded compound leaves were inoculated in a growth chamber using a spore density of 1×10^6 spores per mL with 0.01% (v/v) Tween 20 in sterile water. Mock inoculations of plants were made using just the Tween 20 water solution. The mature 5th−6th tri-foliate leaves were harvested at 24, 72, and 96 hpi respectively, for RNA-seq analysis, real-time qRT-PCR and qualification of SA, JA, auxin (indole-3-acetic acid [IAA]), and ABA. The strawberry control samples, mock inoculation with water containing Tween-20 were conducted. Five leaves collected from five independent plants at any time point were polled as a biological replicate. Three independent biological replicates were sequenced for each treatment. For the pathogen control samples, the isolate was cultured on potato dextrose agar (PDA) covered with a layer of cellophane. Mycelium (0.2 g) was harvested from each plate, placed in sterile 2 mL centrifuge tubes, and frozen in liquid nitrogen. Three independent mycelial samples were collected for RNA-sequencing.

Microscopic Observation

Leaf samples were collected at 0, 12, 24, 72, and 96 hpi. Aniline blue staining was performed as described previously (Ge and Guest, 2011). Briefly, leaves were decolorized, rinsed, and stained with 0.025% (w/v) aniline blue in lactophenol. The leaves were then rinsed, mounted on glass slides in 50% fresh glycerol, and examined under a Nikon E200 microscope. Images were captured with a Nikon DS-U3 digital camera.

RNA Extraction, Construction of Illumina Library, and Sequencing

Total RNA was extracted using RNAiso Plus (Total RNA extraction reagent) (TaKaRa, Otsu, Japan). The samples included mycelium grown in medium, mock inoculation and inoculation leaves at three different time points. RNA purity was verified using a nano spectrophotometer (Implen, Westlake Village, CA, USA). RNA concentrations were measured using a Qubit RNA Assay Kit in a Qubit 2.0 fluorometer (Life Technologies, GrandIsland, NY, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit and the Bioanalyzer 2100 system (Agilent Technologies, Wilmington, DE, USA).

Construction of the Illumina library was carried out as previously described (Sun et al., 2016). The cDNA libraries were sequenced on the Illumina HiSeq2500 platform (125 bp paired-end reads) at Shanghai Hanyu Bio-Teche (Shanghai, China). Raw reads were submitted to the NCBI SRA database under accession numbers SRP097590 and SRP099166.

Data Analysis and Real-Time qRT-PCR

Low quality reads (more than 30% bases with $Q < 20$) were filtered from the raw reads to obtain high-quality reads. Genomes of the strawberry (*F. vesca*, http://www.rosaceae.org/species/fragaria/fragaria_vesca/genome_v1.0) and *C. fructicola* Nara gc5 isolate (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/fungi/Colletotrichum_gloeosporioides/) were used as reference sequences. After trimming low-quality bases ($Q < 20$) from the 5′ and 3′ ends of the remaining reads, the resulting high-quality reads were mapped tothe proper reference sequence using Top Hat v1.3.0 (Trapnell et al., 2009), which allows for one mismatch. Gene expression levels in the RNA-seq analysis were measured as reads per kilobase of exon model per million mapped reads (RPKM). Differentially expressed

genes were called via DEGeq package and Cuffdiff which was developed with special attention to cope with biological variance (Anders and Huber, 2010). The MARS model (MA-plot-based method with the random sampling model) in the DEGseq package was used to calculate the expression abundance of each gene via pairwise comparisons (Mycelium vs. fungus *in planta* and mock inoculation vs. infected). The FDR (false discovery rate) method was used to determine the threshold *p*-value for multiple testing. Genes whose expression differences were significant at FDR < 0.001, and |Normalized Fold change|>2 were identified as differentially expressed (Anders and Huber, 2010). The analysis mapped all DEGs to GO terms in the database (http://www.geneontology.org/) to investigate gene ontology enrichment. The PFAM server (http://pfam.sanger.ac.uk/) was used to verify the function of DEGs in *C. fructicola*. PCWDEs were classified using the dbCAN HMMer-based classification system (Yin et al., 2012), applying an *E*-value cut-off of 10E-5. Potential secondary metabolite clusters were identified using SMURF (Khaldi et al., 2010).

Quantitativereal-time PCR (qRT-PCR) was carried out as previously described (Rudd et al., 2015). All primers are listed in the supporting information (Table S1). In brief, total RNA (1 μg) from mycelium, mock inoculated strawberry leaves and *C. fructicola*-infected strawberry leaves (24, 72, and 96 hpi) was reverse transcribed to cDNA with an oligo(dT) primer using SuperScript III (Invitrogen, Carlsbad, CA 92008, USA) according to the manufacturer's instructions. A standard PCR assay for each primer was conducted. The cDNA samples for which one specific band at the desired size was observed were used. The Premix Ex Taq (Perfect Real Time) kit (Takara, Dalian, China, DRR041A) was used for qRT-PCR on an ABI 7500 Real-Time Cycler (Applied Biosystems, Waltham, MA, USA). qRT-PCR was performed with 20 ng of cDNA, 10 μL of enzyme mixture from the kit, 10 pmol of sense primers and 10 pmol of antisense primers in a final volume of 20 μL. The PCR cycles are as follows: 95°C for 15 s, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s and 72°C for 15 s. At the end of the reaction, melt curves were run for all primer pairs in order to check for dimerization. PCR efficiency was >1.85 and amplifications generated single expected amplicons with single, sharp fusion curves. The expression levels of target mRNA were normalized to that of reference genes and calculated by the comparative CT method as described (Schmittgen and Livak, 2008). Each plate was repeated thrice in independent runs for all reference and selected genes. Data were analyzed using the Applied Biosystems 7500 software version 2.0.1.

C. fructicola Secretome Prediction and Analysis Pipeline

We developed a pipeline to predict and classify effectors of *C. fructicola* as previously described (Haddadi et al., 2016). Pfam (Finn et al., 2014) was used to predict biological functions enriched in the secretome of *C. fructicola*. The candidate effectors were then further evaluated with the EffectorP prediction tool (effectorp.csiro.au) (Sperschneider et al., 2016). N- and O-linked glycosylation sites were predicted using the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) and NetOGlyc 2.0 (http://www.cbs.dtu.dk/services/NetOGlyc) servers, respectively.

Agrobacterium tumefaciens Infiltration Assays

Genes for effector candidates were amplified from the *C. fructicola*-strawberry cDNA library using the corresponding primers (Table S1). The PCR products were subcloned between the cauliflower mosaic virus (CaMV) 35S promoter and the green fluorescent protein (S65T mutant, sGFP) reporter gene in pBluscript. The constructs were confirmed by sequencing at Invitrogen. *Agrobacterium tumefaciens* infiltration assays were performed according to previously described methods (Wang et al., 2011)with minor modifications. Briefly, *Agrobacterium* were grown in Luria-Bertani media plus 50 μg/mL kanamycin for 48 h, harvested, and washed with 10 mM $MgCl_2$ three times, re-suspended in 10 mM $MgCl_2$ to a final OD_{600} of 0.8–1.5, and then incubated at room temperature for 3 h prior to infiltration. Leaves of 4–6 weeks old *N. benthamiana* were infiltrated with a needleless syringe and photographed 3–5 days after infiltration. For the suppression of Bax-mediated cell death, the *A. tumefaciens-containing* Bax gene and *A. tumefaciens* strain carrying the individual effector genes were infiltrated in the same spot. *A. tumefaciens* strains carrying Bax, GFP or both Bax and GFP genes, were infiltrated in parallel as controls. The experiment was repeated three times with each assay consisting of three plants each with three leaves inoculation.

Determination of Four Endogenous Phyto-Hormones

The strawberry leaf samples were sent to the Analysis and Testing Center at Beijing Forestry University (Beijing) for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. Free SA, JA, auxin (indole-3-acetic acid [IAA]) and ABA were quantified referring to Pan et al. (2010). In brief, free hormones were extracted from ∼100 mg of leaf tissues first by 90% methanol. The crude plant extracts were then re-dissolved by methanol-water (1:1 in volume) and filtered through 0.24 μm infiltration head before analysis. The UPLC/MS system consisted of an Agilent 1260 detector and ABQ trap 5500 system. The Agilent C18 main column (4.6 × 50 mm, 1.8 μm) was used for separation of different hormonal components. The internal standards were purchased from Sigma Aldrich Company.

Statistical Analysis

The differentially expressed genes (DEGs) were subjected to GO enrichment analysis. DEGs were first mapped to GO terms using a standard database (http://www.geneontology.org/); gene numbers for each term were calculated, and GO terms significantly enriched in DEGs compared to the background genome were determine with a hypergeometric test. All calculated *p*-values were then subjected to Bonferroni correction, using a corrected $p \leq 0.05$ as the threshold. GO

terms that fulfilled this criterion were defined as significantly enriched in DEGs. For phytohormones determination, the data were analyzed by ANOVA, with $p < 0.05$ considered statistically significant.

AUTHOR CONTRIBUTIONS

The study was conceived by KD, QG, and LZ. LZ prepared the *C. fructicola* isolate and plant materials and performed the experiments. LZ, XH, and XZ performed the bioinformatics analysis. CH performed the experiments of *Agrobacterium tumefaciens* infiltration. Q-YZ measured plant hormones in all samples. LZ, KD, and QG prepared the manuscript. All authors contributed to revising the manuscript. All authors had read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00434/full#supplementary-material

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Conflict of Interest Statement: 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.

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