INTRODUCTION

Strawberry (Fragaria × ananassa) is an important economic plant widely cultivated all over the world (Giampieri et al., 2017). Anthracnose causes up to 80% of plant death in nurseries and over 50% of yield loss in strawberry fields (Sreenivasaprasad & Talhinhas, 2005). Three Colletotrichum species have been reported as causal agents of strawberry anthracnose: Colletotrichum gloeosporioides and Colletotrichum fragariae frequently infect strawberry leaves, stems, and roots, while Colletotrichum acutatum often causes strawberry fruit rot (Denoyes-Rothan et al., 2003; Peres et al., 2005). However, many studies have indicated that C. gloeosporioides is the most prevalent agent of strawberry anthracnose in China, whereas C. acutatum and C. fragariae cause anthracnose to a lesser extent (Damm et al., 2012; Han et al., 2015; Xie et al., 2010). C. gloeosporioides threatens the health of the

HSP17.4 mediates salicylic acid and jasmonic acid pathways in the regulation of resistance to Colletotrichum gloeosporioides in strawberry

Xianping Fang 1 | Weiguo Chai 2 | Shuigen Li 1 | Liqing Zhang 1 | Hong Yu 2 | Jiansheng Shen 3 | Wenfei Xiao 2 | Aichun Liu 2 | Boqiang Zhou 1 | Xueying Zhang 1

1 Institute of Forestry and Pomology, Shanghai Academy of Agricultural Sciences, Shanghai, China
2 Institute of Biotechnology, Hangzhou Academy of Agricultural Sciences, Hangzhou, China
3 Jinhua Academy of Agricultural Sciences, Jinhua, China

Correspondence
Xueying Zhang, Institute of Forestry and Pomology, Shanghai Academy of Agricultural Sciences, Shanghai 201403, China.
Email: zhangxueying@saas.sh.cn

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Abstract
In this study, we used virus-mediated gene silencing technology and found that the HSP17.4 gene-silenced cultivar Sweet Charlie plants were more susceptible to Colletotrichum gloeosporioides than the wild-type Sweet Charlie, and the level of infection was even higher than that of the susceptible cultivar Benihopp. The results of differential quantitative proteomics showed that after infection with the pathogen, the expression of the downstream response genes NPR1, TGA, and PR-1 of the salicylic acid (SA) signalling pathway was fully up-regulated in the wild-type Sweet Charlie, and the expression of the core transcription factor MYC2 of the jasmonic acid (JA) pathway was significantly down-regulated. The expression of the proteins encoded by these genes did not change significantly in the HSP17.4-silenced Sweet Charlie, indicating that the expression of HSP17.4 activated the up-regulation of downstream signals of SA and inhibited the JA signal pathway. The experiments that used SA, methyl jasmonate, and their inhibitors to treat plants provide additional evidence that the antagonism between SA and JA regulates the resistance of strawberry plants to C. gloeosporioides.

KEYWORDS
Colletotrichum gloeosporioides, HSP17.4, jasmonic acid, salicylic acid, strawberry
strawberry during the entire plant growth cycle, especially at the seedling and transplanting stages (Wu et al., 2019). Currently, the control of *C. gloeosporioides* is primarily based on the use of biocides, but this is not a long-term solution to the problem because of their high cost and environmental pollution. Strawberry cultivar Benihopp has delicious fruits but is susceptible to anthracnose, while Sweet Charlie has a light flavour but displays good resistance to anthracnose (Vincent et al., 1999; Zhang et al., 2016). The use of excellent resistance gene resources is the most economical and effective way to improve strawberry varieties, and the identification and use of such genes depends on the in-depth analysis of the mechanism of resistance of strawberry (Guidarelli et al., 2011). The interaction between strawberry and *C. gloeosporioides* has been studied in plant physiology and histopathology (Amil-Ruiz et al., 2016; Guidarelli et al., 2011). Because proteins are the direct facilitator of biological activities, they more directly reflect the process of interactions between plants and pathogens (Fang et al., 2015). A new anthracnose resistance response protein, HSP17.4, that causes resistance to anthracnose was identified in the strawberry cultivar Sweet Charlie by comparative proteomics technology in our previous study (Fang et al., 2012). The level of expression of the response protein in *Colletotrichum*-infected leaves of Sweet Charlie increased by more than 2.5 times in each period (24, 48, and 72 hr), which was confirmed by the analysis of mRNA and protein levels. Western blotting results showed that the level of expression of HSP17.4 in the susceptible cultivar Benihopp was far lower than that of the highly resistant cultivar Sweet Charlie, which indicated that HSP17.4 could be an important candidate protein (gene) related to strawberry anthracnose resistance (Fang et al., 2012).

HSP17.4 is a member of the small heat-shock protein (sHSP) family. The sHSP family is a group of proteins with a molecular weight of 12–43 kDa and an α-crystalline domain at the C-terminus (Perez et al., 2009), and it was originally found to be a class of proteins in cells exposed to high temperature stimulation. Most sHSPs are expressed under heat stress (Swindell et al., 2007). Some sHSPs can also respond to stresses such as cold, drought, salt, and even ultraviolet light, toxic substances, peroxides, diseases, and pests. Their antistress functions are diverse (Gorovits et al., 2007) and various organisms, including plants, can also produce these types of response proteins under stress (Chivasa et al., 2006). In tomato (*Solanum lycopersicum*), sHSPs can regulate the changes in soluble sugar content in postharvest fruits and maintain the stable state of reactive oxygen species, thus improving the ability of tomato fruits to respond to stress (Zhang et al., 2018). However, the type of signalling pathways that sHSPs are involved in to facilitate resistance to stress in plants is still unknown. Therefore, further study on the relationship between HSP17.4 and stress in strawberry caused by *C. gloeosporioides* is of substantial significance to further understand the molecular mechanisms of resistance to stress.

With the deepening of research and the continuous improvement of related technologies, as the research hotspot of life sciences, tobacco rattle virus-based virus-induced gene silencing (TRV-VIGS) and proteomics have become powerful tools in biology to decipher gene function and contribute to breeding programmes and have been widely used in the study of plant stress resistance, growth, and developmental mechanisms (Gong et al., 2019; Ismayil et al., 2020; Wang et al., 2020; Zhu et al., 2015). On the one hand, this study further confirmed the biological function of HSP17.4 in the regulation of resistance to anthracnose in strawberry through VIGS. On the other hand, label-free quantitative proteomics based on liquid chromatography–mass spectrometry was used to compare the differences in expression of proteins in the response of empty carrier-transfected or HSP17.4-silenced Sweet Charlie plants to *C. gloeosporioides*, with the goal of analysing the specific protein signalling pathway of HSP17.4 involved in the resistance to *C. gloeosporioides* and exploring its mechanism of disease resistance, which provides an important research basis for disease resistance gene cloning and molecular breeding in the future.

## 2 RESULTS AND DISCUSSION

### 2.1 Silencing efficiency of HSP17.4 and identification of anthracnose resistance

After the TRV2::HSP17.4 vector was injected, the HSP17.4 gene was significantly silenced in the roots, stems, and leaves of Sweet Charlie and Benihopp strawberry (Figure 1a). The results of disease resistance analysis showed that 15 days after inoculation with *C. gloeosporioides*, the disease indices of the Sweet Charlie and Benihopp wild type, not silenced, and silenced plants were wild type (6.75, 19.56) < TRV2::00 (4.93, 20.03) < TRV2::HSP17.4 (39.55, 48.64) (Figure 1b). When the HSP17.4 gene was silenced, the resistance of Sweet Charlie to disease was reduced and the plant disease index increased significantly, although this cultivar normally has a high resistance to anthracnose. Similarly, after the susceptible variety Benihopp was injected with TRV2::HSP17.4 vector, the resistance of the plant to disease was reduced further, and obvious symptoms occurred in the roots, stems, and leaves (Figure 1c).

### 2.2 Overview of protein identification and the sample repeatability test

In this study, a total of 3,132 proteins were identified using label-free quantitative proteomics technology, of which 2,301 proteins contained quantitative information (Table S2). The molecular weight of the identified proteins was primarily distributed up to 100 kDa. The mass error of most peptides was less than 5 ppm, and the peptide length was generally 7 to 20 amino acids (Figure 2a–c). For differentially expressed proteins, a statistical analysis was performed for each Q group (Figure 2d,e; Tables S3 and S4), and the gene ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and protein domain analyses were conducted.
Cluster analysis was performed to find the functional correlation of proteins with different differential expression multiples.

For biologically replicated samples, whether the quantitative results of biological replicates met statistical consistency was tested. In this experiment, the relative standard deviations calculated among the replicates of each sample of A1–A4 were 0.0912, 0.0751, 0.0723, and 0.0704, respectively, showing good repeatability (Figure 2f).

2.3 Functional classification and enrichment of differentially expressed proteins in the response of TRV2::HSP17.4 and TRV2::00 plants to \textit{C. gloeosporioides}

The differentially expressed proteins in response to pathogenic stress were classified by GO function, and we found that based on biological processes, TRV2::HSP17.4 and TRV2::00 Sweet Charlie plants occupied a relatively large proportion in the single-tissue process, stimulation response, and biological regulation. In the analysis of cell composition, both macromolecular complexes and membrane proteins comprised a large proportion. In the analysis of molecular functions, a large number of differentially expressed proteins was related to ligand activity in TRV2::HSP17.4 Sweet Charlie plants. The TRV2::00 plants had a higher proportion of catalytically active proteins in response to pathogen stress (Figure S1).

GO enrichment analysis showed that compared with the TRV2::00 Sweet Charlie control, HSP17.4-silenced plants exhibited a significant decrease in the abundance of proteins related to oxidative stress metabolism, lignin and chitin metabolism, membrane protein, and redox activity after pathogen stress (Figures S2 and S3). The KEGG pathway enrichment analysis showed that the proteins involved in amino acid synthesis, plant disease interaction, carbon metabolism, and the MAPK signal pathway in TRV2::HSP17.4 and TRV2::00 Sweet Charlie plants were up-regulated or down-regulated following the same trend under pathogen stress, while proteins in the TRV2::00 plants were specifically enriched in the plant hormone signal transduction pathway under \textit{C. gloeosporioides} stress (Figure 3).

2.4 HSP17.4 silencing inhibited the activation of salicylic acid downstream signals and the inhibition of jasmonic acid downstream signals induced by the pathogen

The results of mass spectrometry showed that the protein expression levels of NPR1, TGA, and PR-1, which are involved in
FIGURE 2  Statistical analysis of differential proteomics results. The TRV2::HSP17.4 plants treated with sterile water (control) and inoculated were designated A1 and A2, respectively, and the TRV2::00 plants treated with sterile water (control) and inoculated were designated A3 and A4, respectively. (a) The relationship between molecular weight and peptide mass distribution. (b) The relationship between peptide mass error and peptide score distribution. (c) The relationship between peptide length and number. (d-e) Differential multiple grouping (d) and probability map (e) of differentially expressed proteins.
the downstream signalling of salicylic acid (SA), in TRV2::HSP17.4 Sweet Charlie were almost unchanged in plants infected or not infected with *Colletotrichum gloeosporioides* (up-regulated 1.24-, 1.05-, and 1.11-fold, respectively; Figure 4a). However, in TRV2::00 Sweet Charlie, pathogen stress induced the expression of NPR1, TGA, and PR-1 (up-regulated 1.92-, 1.83-, and 1.95-fold, respectively), while the expression level of MYC2, the core transcription factor of the jasmonic acid (JA) pathway, was down-regulated by 1.74-fold (Figure 4b).

To further confirm the quantitative results of mass spectrometry, PR-1 and MYC2, key proteins in the two pathways, were selected for western blot analysis. Under pathogen stress, PR-1 and MYC2 in the TRV2::00 plants were up-regulated 2.56-fold and downregulated 2.21-fold, respectively. No significant difference was observed in the TRV2::HSP17.4 plants (Figure 4c). The quantitative reverse transcription PCR (RT-qPCR) test showed that under pathogen stress, the expression levels of the PR-1 and MYC2 genes in the TRV2::00 plants also changed significantly (Figure 4d). The quantitative results of our western blot and mass spectrometry analyses were highly consistent. Moreover, HSP17.4 silencing itself caused a slight change in protein expression of PR-1 and MYC2 (down-regulated 1.31- and up-regulated 1.37-fold, respectively; Figure 4c) and a significant decrease in protein and mRNA expression of TGA (down-regulated 1.70- and 9.54-fold, respectively) (Figure 4e) in the strawberry plants. In TRV2::00 Sweet Charlie, *C. gloeosporioides* strongly induced mRNA expression of callose synthase 7 (CalS7) (up-regulated 30.2-fold). Compared with TRV2::00 Sweet Charlie, HSP17.4 silencing itself resulted in a noticeable decrease of CalS7 mRNA expression (down-regulated 15.7-fold) in the TRV2::HSP17.4
FIGURE 4 The effect of Colletotrichum gloeosporioides infection on the salicylic acid (SA) and jasmonic acid (JA) pathways in TRV2::HSP17.4 and TRV2::00 strawberry plants. (a) The differential expression of proteins in the SA pathway in TRV2::HSP17.4 plants. (b) The differential expression of proteins in the SA and JA pathways in TRV2::00 plants. (c) Western blot analysis of HSP17.4, MYC2, and PR-1. (d) Changes in differential mRNA expression of HSP17.4, MYC2, and PR-1 in TRV2::HSP17.4 and TRV2::00 plants infected or not infected with pathogen. (e) Changes in differential protein expression of TGA and differential mRNA expression of TGA and CalS7 in TRV2::HSP17.4 and TRV2::00 plants infected or not infected with pathogen. TRV2::00, TRV2 empty carrier plants; TRV2::HSP17.4, HSP17.4 gene-silenced plants; WB, western blot; MS, mass spectrometry.
plants. However, the expression of CalS7 was not activated in the C. gloeosporioides-infected TRV2::HSP17.4 plants (Figure 4e).

These results indicate that in the susceptible HSP17.4-silenced Sweet Charlie plants, C. gloeosporioides stress did not activate the SA pathway and synergistically suppressed the JA signal like the disease-resistant control plants.

2.5 | The activation of SA and synergistic suppression of the downstream signals of JA significantly increased the resistance of strawberry plants to C. gloeosporioides

Owing to the strong resistance of the TRV2::00 Sweet Charlie plant itself, no obvious symptoms appeared 15 days after inoculation with C. gloeosporioides. Interestingly, we found that SA pretreatment did not weaken the resistance of TRV2::00 Sweet Charlie at all, while the spray treatment of methyl jasmonate (MeJA) slightly weakened the resistance of TRV2::00 Sweet Charlie. Surprisingly, when sprayed with 2-aminoindan-2-phosphonic acid (AIP) (SA synthesis inhibitor), the plants showed very strong symptoms of disease (Figure 5a). It could be observed that the SA content in the plants decreased sharply, and the expression of HSP17.4 and PR-1 was also largely down-regulated (Figure 5b). When sprayed with MeJA, the contents of JA and the expression of MYC2 in the plants were both increased, but the resistance to pathogens was weaker than that of the non-sprayed control plants. When SA and sodium diethyldithiocarbamate (DIECA) (MeJA synthesis inhibitor) were used at the same time, the SA content and HSP17.4 and PR-1 expression in the plant reached their highest states, while the JA content and MYC2 expression were strongly suppressed (Figure 5b). The plants had the greatest degree of resistance without any symptoms of disease (Figure 5a).

**FIGURE 5** The effects of different hormone combinations on anthracnose resistance and key protein expression in strawberry Sweet Charlie plants. (a) Phenotypic changes in leaf and root disease resistance. (b) Changes in the content of salicylic acid (SA) and jasmonic acid (JA), and the differential mRNA expression of HSP17.4, MYC2, and PR-1. T1, Pathogen; T2, SA+Pathogen; T3, AIP+Pathogen; T4, MeJA+Pathogen; T5, DIECA+Pathogen; T6, SA+DIECA+Pathogen. AIP, 2-aminoindan-2-phosphonic acid; MeJA, methyl jasmonate; DIECA, sodium diethyldithiocarbamate
Plant hormones, including SA, JA, and ethylene (ET), can effectively participate in various signaling pathways to help plants resist the invasion of different pathogens (Robert-Seilaniantz et al., 2011). Biotrophic or hemibiotrophic pathogens, such as *C. gloeosporioides*, can cause plant hypersensitivity by activating the SA signaling pathway, which eventually leads to systemic acquired resistance (Fu & Dong, 2013). In contrast, necrotic pathogens or insect bites often activate the JA/ET signaling pathway, which triggers the emergence of induced systemic resistance in plants (Yan & Xie, 2015). SA, JA, and ET all induce the production of many downstream antidisease-related molecules, including a large number of transcription factors, disease-related proteins, and small-molecule metabolites. SA is an important signal molecule for the invasion of corresponding pathogens. It primarily accumulates in plants during the process of disease resistance, and the process of accumulation has priority over the expression of pathogenesis-related (PR) genes and the production of disease resistance. In addition, SA is an important signal molecule in the process of signal transduction of acquired resistance. Many studies have shown that treatment with SA can increase the resistance of strawberries to *C. gloeosporioides* and stimulate the expression of downstream related disease resistance genes (Grellet-Bournonville et al., 2012; Shu et al., 2018). Infection with *C. gloeosporioides* obviously activated the SA-mediated disease resistance signal pathway in strawberries. Therefore, regulating the phytohormone-mediated disease resistance signal pathway is of substantial significance in the study of the mechanism of resistance to anthracnose in strawberry (Amil-Ruiz et al., 2016).

In conclusion, sHSPs have rich functions and genetic diversity. They can maintain the stability of natural proteins in response to biotic or abiotic stresses, and they play an important role in protecting membrane structure, protein degradation, and the regulation of multiple signal pathways (Gorovits et al., 2007). This study showed that HSP17.4 participates in the activation of downstream signals of SA and synergistically suppresses downstream signals of JA, contributing to the resistance of strawberry plants to *C. gloeosporioides* (Figures 4 and 5). Therefore, in the strawberry Sweet Charlie defence system induced by *C. gloeosporioides*, the two hormone signal pathways of SA and JA had cross-talk and mutual antagonism, and the response of the dual hormone signal was achieved through the mediation of HSP17.4 (Figure 6).

**FIGURE 6** A proposed model depicting how HSP17.4 mediates salicylic acid (SA) and jasmonic acid (JA) pathways in the regulation of resistance to strawberry anthracnose. HSP17.4 participates in the activation of downstream signals of SA and synergistically suppresses downstream signals of JA, contributing to the resistance of strawberry Sweet Charlie plants to *Colletotrichum gloeosporioides*.
3 | EXPERIMENTAL PROCEDURES

3.1 | Plant materials and reagents

Two-month-old strawberry stolon seedlings were cultured in the artificial climate chamber of the Institute of Forestry & Fruit Trees, Shanghai Academy of Agricultural Sciences, Shanghai, China. Monoclonal cultures of C. gloeosporioides coming from the collection of Zhejiang University, China, and identified by morphological characteristics and rDNA internal transcribed spacer sequence characterization (Xie et al., 2010) were grown on potato dextrose agar (Sigma) in a constant-temperature shaker (150 rpm; Infors) at 27 °C for 7 days and used for artificial inoculation. The VIGS experiment materials were the strawberry varieties Sweet Charlie and Benihopp, and the proteomics experiment materials were the leaves of HSP17.4-silenced (TRV2::HSP17.4) and TRV2 empty carrier (TRV2::00) Sweet Charlie plants 15 days after inoculation with sterile water or C. gloeosporioides. The TRV1 and TRV2 plasmids were provided by Professor Gong Zhenhui’s Laboratory at Northwest Agricultural & Forestry University, Xianyang, China. Agrobacterium tumefaciens GV3101, Escherichia coli DH5α, and C. gloeosporioides were preserved at −80 °C in the laboratory of the Institute of Forestry & Fruit Trees, Shanghai Academy of Agricultural Sciences, Shanghai, China. SA, MeJA, and their inhibitors were purchased from Shanghai Aladdin Reagent Co., Ltd.

3.2 | VIGS target fragment acquisition and vector construction

The HSP17.4 protein fragment sequence (NCBI accession number GI2911276) identified by differential proteomics was used in homologous cloning, based on BLAST searches against the cultivated strawberry protein database (http://strawberry-garden.kazusa.or.jp/) using the homologous gene sequence in the Arabidopsis database TAIR (https://www.arabidopsis.org/). The total RNA of the Sweet Charlie leaves was extracted, and the first strand of cDNA was synthesized. The strawberry HSP17.4 full-length coding region sequence was amplified by PCR. The recovered product was used to transform E. coli, and the plasmid was extracted from E. coli cells with the correctly sequenced HSP17.4 gene to amplify the fragments needed to construct the VIGS vector.

A 274-bp nucleotide sequence with a specific site was selected from the conserved region of the Sweet Charlie HSP17.4 gene to design primers. An XbaI restriction site was introduced in the upstream primer (HSP17.4-F: 5’-GCTCTAGAATTCTTGAGCACCAGGTGG-3’), and a KpnI restriction site was introduced in the downstream primer (HSP17.4-R: 5’-GGGGTACCATTTCTCTGGCAACCGGAAACT-3’). The synthesized cDNA was used as a template for PCR amplification. After the target amplified fragment was sent to Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China) for sequencing, the plasmid was extracted and digested with XbaI and KpnI. The VIGS virus vector TRV2 plasmid was simultaneously digested with the same restriction enzymes. The target fragment after the double restriction digestion was ligated with the vector using T4 DNA ligase, and the recombinant plasmid TRV2::HSP17.4 was obtained after the verification by restriction digestion. The TRV1, TRV2, and TRV2::HSP17.4 plasmids were transformed into A. tumefaciens GV3101 using the freeze-thaw method.

3.3 | Agrobacterium-mediated VIGS method

A total of 50 mg/L kanamycin, 50 mg/L gentamicin, 10 mM MES, and 20 μM acetosyringone was added to 100 ml Luria Bertani (LB) liquid medium, which was inoculated with a 1% solution of TRV1, TRV2, or TRV2::HSP17.4 bacteria and cultured at 28 °C under shaking at 220 rpm to an OD_{600} of 1.2 to 1.5. The bacterial cells were collected by centrifugation and suspended in VIGS resuspension buffer (10 mM MgCl₂, 10 mM 2-(N-morpholino) ethanesulfonic [MES], and 200 μM acetosyringone). The final concentration of the infection solution was adjusted to an OD_{600} of approximately 1.5. TRV1 was mixed with TRV2::HSP17.4 or TRV2::00 bacterial solution at a ratio of 1:1 vol/vol and placed at room temperature for 3 hr. Stolon seedlings of 2-month-old Sweet Charlie and Benihopp were selected, and 2-ml syringes without needles were used to inject the mixed bacterial solution into the upper tender leaves of the plant from the back, so that the bacterial solution filled the entire leaf. Two leaves of each seedling were injected. After infection, the plants were grown under 70% relative humidity, at 16 °C, and under dark conditions for 24 hr before transfer to 25 °C/20 °C, 16 hr light/8 hr dark, and 70% relative humidity in an artificial climate chamber. Twenty plants were included in each treatment with four replicates.

3.4 | Detection of HSP17.4 silencing efficiency, inoculation, and identification of resistance to C. gloeosporioides

The total RNA from the roots, stems, and leaves of the unloaded control and gene-silenced Sweet Charlie and Benihopp strawberries was extracted and analysed using fluorescent RT-qPCR. GAPDH2 was used as the internal reference to detect the level of HSP17.4 mRNA in the TRV2::HSP17.4 and TRV2::00 plants. The detailed detection was conducted as previously described (Fang et al., 2012).

Fifteen days after Sweet Charlie and Benihopp were transfected with TRV2::HSP17.4 or TRV2::00, they were inoculated with C. gloeosporioides using a spore suspension concentration of 10⁷ cfu/ml, and sterile water was used as a control. The inoculation was carried out by spraying the whole plants with a spore suspension or sterile water, and then plants were returned to the culture room. The culture conditions were set to 30 °C/25 °C, 16 hr light/8 hr dark, and 90% relative humidity. Twenty plants were included in each treatment with four repetitions. The infection type (IT) and the disease index were evaluated for each strawberry plant at 15 days after inoculation. ITs were categorized from 0 to 5 (Smith & Black, 1987) as follows: 0, healthy plant with no visible lesions; 1, <3 mm long petiole lesion;
2. 3–10 mm long petiole lesion; 3. 11–20 mm long petiole lesion; 4. >20 mm long petiole lesion; 5. leaf wilted or plant dead. The disease index was calculated as follows: disease index (%) = \([0 \times X_3] + (1 \times X_4) + (2 \times X_5) + (3 \times X_6) + (4 \times X_7) + (5 \times X_9)/[(X_3 + X_4 + X_5 + X_6 + X_9) \times 5]\) × 100, where \(X_9\) to \(X_2\) indicate plants with IT 0 to 5, respectively.

### 3.5 Extraction and quantification of protein

Approximately 3 g of TRV2::HSP17.4 and TRV2::00 Sweet Charlie leaves was collected 15 days after inoculation with sterile water and C. gloeosporioides. The TRV2::HSP17.4 sterile water control and inoculation groups were designated A1 and A2, respectively, and the TRV2::00 sterile water control and inoculation groups were designated A3 and A4, respectively. The leaves were ground into fine powder in liquid nitrogen and then added to 2 ml centrifuge tubes. Approximately 1 ml of protein extraction solution I (containing 10% acetone in trichloroacetic acid and 0.07% β-mercaptoethanol) was added to precipitate the crude protein (−20 °C, 1 hr), and the samples were centrifuged at 4 °C at 13,000 \(\times g\) for 20 min. The precipitate was then collected, and the supernatant was discarded. Protein extract II was used to suspend the crude protein (−20 °C for 1 hr), and the samples were centrifuged at 4 °C at 13,000 \(\times g\) for 20 min.

The precipitate was then collected, and the supernatant was discarded. Protein extract II was used to suspend the precipitate, which was washed three times (−20 °C, 1 hr). The dry powder of the crude protein was obtained by drying the solution under vacuum. A total of 0.5 g of powder was weighed, and 10 ml of lysis solution (containing 8 M urea, 10 mM dithiothreitol [DTT], 2 mM EDTA, and 1 mM benzyl sulfonyl fluoride) was added. After mixing well, the samples were placed at 4 °C for 1 hr and shaken three to five times during this period. The solution was then centrifuged at 13,000 \(\times g\) at 20 °C for 15 min. The precipitated insoluble matter was discarded, and the supernatant was retained. The Coomassie blue staining method (Gurusamy & Christof, 2006) was used for protein quantification and the solution was aliquoted and stored at −80 °C for later use.

### 3.6 Reduction alkylation, trypsin digestion, and C18 column desalting

A 100 μg protein sample was used for the digestion. A volume of 100 mM DTT was added to reach a final concentration of 10 mM, and the reduction reaction lasted for 1 hr at 56 °C. After cooling to room temperature, 500 mM iodoacetamide was added to reach a final concentration of 50 mM, and the reaction was conducted without light for 45 min. A volume of 100% trichloroacetic acid was added to reach a final concentration of 10% and protein was precipitated at 4 °C for 2 hr. Cold acetone was used to wash the precipitated particles three times, and the particles were obtained after centrifugation. The particles were dissolved in 100 mM NH4HCO3 and subjected to ultrasound for 5 min. Trypsin was added to the mixture of protein at a 1:50 ratio, and the solution was incubated at 37 °C for 12 hr. The peptide segments after enzymolysis were vacuum-dried after desalting with a C18 SPE column (Qiagen).

### 3.7 Liquid chromatography–mass spectrometry analysis

The vacuum-dried peptides were dissolved in an aqueous solution of 0.1% formic acid (FA) and then separated using an EASY-nLC 1000 ultra-high-performance liquid system (Thermo Fisher Scientific). Mobile phase A was an aqueous solution that contained 0.1% FA and 2% acetonitrile (ACN). Mobile phase B was an aqueous solution that contained 0.1% FA and 98% ACN. The liquid gradient was set as 5%–8%, 6 min; 8%–30%, 34 min; 30%–60%, 5 min; 60%–80%, 3 min; 80%, 7 min for equilibration; 80%–5%, 3 min; and 5%, 7 min for equilibration. The flow rate was maintained at 200 nl/min. The peptides were separated using an ultrahigh-performance liquid system, injected into the NSI ion source for ionization, and then analysed using a Q Exactive mass spectrometer (Thermo Fisher Scientific). The voltage of the ion source was set to 2 kV. The peptide precursor ions and their secondary fragments were detected and analysed by high-resolution Orbitrap. The three strongest precursor ions were automatically selected in each primary spectrum for secondary scanning.

### 3.8 Qualitative analysis by database search and quantification

The secondary mass spectrum data were retrieved using Proteome Discoverer v. 1.4 software. The search parameter settings were as follows: the database was the cultivated strawberry protein database (http://strawberry-garden.kazusa.or.jp/). An antidatabase was added to calculate the false discovery rate caused by random matching, and a common pollution database was added to the database to eliminate the influence of contaminating proteins in the identification results. The differentially expressed proteins were relatively quantified using Thermo Fisher Sieve v. 2.2 software. The peak area was used as the quantitative value of each peptide. The protein differential quantification standard must meet A2/A1 or A4/A3 > 1.50 or < 0.67, and \(p < .05\). We divided it further into four parts based on its differential expression multiple, which were designated Q1 to Q4, that is, Q1 (0 < ratio ≤ 1/2), Q2 (1/2 < ratio ≤ 1/1.5), Q3 (1.5 < ratio ≤ 2), and Q4 (ratio > 2).

### 3.9 Information annotation and functional classification of the differentially expressed proteins

The UniProt protein database (http://www.ebi.uniprot.org) was used to perform a GO analysis on the differentially expressed proteins identified. GO annotation and functional classification of the differentially expressed proteins were conducted based on the processes they were involved in, including biological processes, cellular components, and molecular functions.
3.10 | Cluster analysis and functional enrichment of the differentially expressed proteins

Based on the identified proteins, Fisher’s exact test was used to test the significance of differential expression. GO enrichment and KEGG pathway enrichment analyses were used to analyse these proteins. Test p values less than .05 were considered significant. Finally, the pathways were classified based on the hierarchical classification method on the KEGG website (https://www.kegg.jp). On this basis, the cluster analysis for each Q group (Q1 to Q4) was conducted based on protein differential expression multiples to find the functional correlation among proteins with different differential expression multiples.

3.11 | Western blotting and transcripional analysis of the key differentially expressed proteins in hormone signalling pathways

The extracted and quantified total protein solution from the leaves of TRV2::HSP17.4 and TRV2::00 Sweet Charlie plants was diluted to 1 mg/ml, and 20 μl was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). Then proteins were transferred to a membrane at a constant current of 15 mA at 4 °C overnight. The membrane was removed from the rotating cell and slowly shaken in blocking solution for 1 hr. The membrane was incubated overnight at 4 °C with primary antibody (Fang et al., 2012): GAPDH2, HSP17.4, PR-1 (AS10687, Agrisera), or MYC2 (AS121869, Agrisera) (1:1,000). Next, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2,500) at room temperature for 1 hr under shaking conditions. A Novex ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific, CA, USA) was used for reconstitution, and the solution was passed through a 0.1-μm filter membrane, placed in a sample vial, and established for testing. Each treatment was repeated three times. Phytohormones were detected using a liquid chromatography–mass spectrometer (Agilent 1290-AB Sciex QTRAP 6500, Agilent Technologies). The column was an Agilent ZORBAX SB-C18 (2.1 × 150 mm, 3.5 μm), and the column temperature was 30 °C. Under the positive ion mode, the mobile phase A was 0.1% FA solution; B was methanol; the injection volume was 2 μl, and the gradient elution and multiple reaction monitoring mode were used for detection. SigmaPlot software (SYSTAT Software) was used to analyse the data obtained from plant hormone detection, and MS Excel was used to analyse the significance of the difference (α = .05).

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Protein sequence data are available in the protein database at www.ncbi.nlm.nih.gov with the accession number GI2911276. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Xiangping Fang https://orcid.org/0000-0001-7461-9170
Liqing Zhang https://orcid.org/0000-0002-3936-4376

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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