The Involvement of Jasmonic Acid, Ethylene, and Salicylic Acid in the Signaling Pathway of *Clonostachys rosea*-Induced Resistance to Gray Mold Disease in Tomato

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ABSTRACT

Tomato gray mold disease caused by *Botrytis cinerea* is a serious disease that threatens tomato production around the world. *Clonostachys rosea* has been used successfully as a biocontrol agent against divergent plant pathogens, including *B. cinerea*. To understand the signal transduction pathway of *C. rosea*-induced resistance to tomato gray mold disease, the effects of *C. rosea* on gray mold tomato leaves along with changes in the activities of three defense enzymes (phenylalanine ammonia-lyase [PAL], polyphenol oxidase [PPO], and catalase [CAT]), second messengers (nitric oxide [NO], hydrogen peroxide [H₂O₂], and superoxide anion radical [O₂⁻]), and stress-related genes (*mitogen-activated protein kinase* [MAPK], *WRKY*, *Lexl2*, and *atpA*) in four different hormone-deficient (jasmonic acid [JA], ethylene [ET], salicylic acid [SA], and gibberellin) tomato mutants were investigated. The results revealed that *C. rosea* significantly inhibited the growth of mycelia and spore germination of *B. cinerea*. Furthermore, it reduced the incidence of gray mold disease, induced higher levels of PAL and PPO, and induced

lower levels of CAT activities in tomato leaves. Moreover, it also increased NO, H₂O₂, and O₂⁻ levels and the gene expression levels of *WRKY*, *MAPK*, *atpA*, and *Lexl2*. The incidence of gray mold disease in four hormone-deficient mutants was higher than that in the corresponding wild-type tomato plants. Among all of these hormone-deficient tomato mutants, JA had the most significant effect in regulating the different signal molecules. Additional study suggested that JA upregulated the expression of *Lexl2*, *MAPK*, and *WRKY* but downregulated *atpA*. Furthermore, JA also enhanced the activity of PAL, PPO, and CAT and the production of NO and H₂O₂. SA downregulated CAT and PAL, whereas ET upregulated PAL but downregulated CAT. This study is of significance in understanding the regulatory pathways and biocontrol mechanism of *C. rosea* against *B. cinerea*.

Keywords: biological control, disease control and pest management, mycology

Tomato (*Solanum lycopersicum*) is a major herbaceous vegetable worldwide that is susceptible to damage from pests (insects, mites, nematodes, and so on) as well as fungal, bacterial, or viral diseases. Tomato gray mold disease is one of the major diseases that threatens tomato production, and it is caused by the fungus *Botrytis cinerea*. This disease can infect not only tomato fruits but also, tomato leaves, stems, flowers, and other parts, rapidly spreading after the onset of infection (Masmoudi et al. 2017). *B. cinerea* is a necrotrophic plant pathogen that is able to infect >1,400 plant species (Elad et al. 2015), including tomato, carrot, cucumber, lettuce, eggplant, grape, apple, pear, cherry, strawberry, kiwi, and so on (Lemonnier et al. 2014). *B. cinerea* infection also activates the accumulation of reactive oxygen species (ROS) and cell death with hallmarks of programmed cell death (PCD) in plant tissues (Ponce et al. 2012). During the infection process, *B. cinerea* secretes cell wall-degrading enzymes (CWDEs), allowing the fungus to penetrate the surface of healthy plants (García et al. 2017). CWDEs may also cause plant cells to undergo PCD, facilitating the growth

of this necrotrophic fungus in dead plant tissues (Celedonio et al. 2016; Mengiste 2012).

Several fungicides have been used to effectively control this disease, especially at the preharvest stage. However, the excessive use of chemical fungicides has contributed to biodiversity impoverishment and negative environmental impact. Furthermore, this also induces fungicide resistance and problems for public health (Gajera and Vakharia 2010). Biological control (biocontrol), which uses antimicrobial agents to limit pathogen infections and activate the plant's own defense system, provides an attractive alternative for managing plant diseases. It is highly efficient and causes no harm to the environment or human health.

The fungus *Clonostachys rosea* has been an attractive biocontrol agent. It protects plants against *B. cinerea* by suppressing gray mold growth and inhibiting spore production (Mouekouba et al. 2014). The treatment of *B. cinerea* infection with *C. rosea* induces several defense mechanisms in tomato, including the fortification of the plant cell wall through the incorporation of phenolic compounds. Several signaling molecules have been identified in plant defense mechanisms, including phytohormones, such as indole acetic acid, abscisic acid, gibberellins (GAs), zeatin, jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and ROS, which are mainly nitric oxide (NO), hydrogen peroxide (H₂O₂), and superoxide anion radical (O₂⁻), as well as several protective enzymes, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and catalase (CAT). These molecules trigger changes in the expression of defense genes, resulting in metabolic alterations that enhance plant defense responses (Mai et al. 2014; Quazi et al. 2015). Among all phytohormones, changes in JA, ET, SA, and GA were the most obvious in gray mold tomato leaves after *C. rosea* treatment (Mouekouba et al. 2014; Wang 2013). The expression of the *WRKY*

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transcription factor and mitogen-activated protein kinase (MAPK) genes was also induced by different biotic and abiotic stresses in numerous plant species (Berri et al. 2009; Ramamoorthy et al. 2008; Zhang et al. 2007), suggesting that these genes may act as regulators for stress response. In addition, *B. cinerea* plus *C. rosea* treatment in tomato leaves could induce higher expression levels of β -xylanase (*Lxyl2*) and ATP synthase CF1 α -subunit (*atpA*) compared with other treatments (Mouekouba et al. 2014). These signaling molecules drive different plant signal transduction pathways through complicated interactions that function independently. In this study, the effects of *C. rosea* on gray mold tomato leaves were investigated using various tomato mutants involved in plant responses to biotic and abiotic stresses. Different phytohormone-deficient mutant tomato leaves were inoculated with *B. cinerea* and *C. rosea* to identify the mechanism by which changes in the signaling molecule and defense enzyme activity induce resistance. The results of this study can help us elucidate the innate immunity mechanisms of *C. rosea* and understand the regulatory relationship between key signal factors involved in plant defenses.

MATERIALS AND METHODS

Microbial culture and tomato plant growth. *C. rosea* was isolated from turfy soil in the suburbs of Jilin City (the northeast region of China) and confirmed by the 5.8S ribosomal DNA (rDNA)-ITS and 18S rDNA sequences. The strain was deposited into the China General Microbiological Culture Collection Center. *C. rosea* was cultured on potato dextrose agar (PDA) plates at 22°C. *B. cinerea* was isolated from infected tomato plants grown in a greenhouse (Wang 2013) and cultured on PDA at 25°C.

Four hormone-related tomato mutants and their associated wild types were used for this study (Table 1). The plants were grown in a mixture of perlite/vermiculite/plant ash (1:6:2) in an artificial climate chamber (model: FYS-HY-7; Nanjing Hengyu Instrument Manufacturing Co., Ltd.). The day and night temperatures were 28 and 18°C, respectively, under a 16-h light and 8-h dark regime. Humidity was maintained at 80%.

Antagonistic experiment in vitro. To test the antagonistic effect of *C. rosea* against *B. cinerea*, *C. rosea* and *B. cinerea* were first cultured separately on PDA at 28°C for 15 days. A 5-mm sterile hole puncher was used to collect the fresh mycelium of *C. rosea* or *B. cinerea* from the culture. The *C. rosea* and *B. cinerea* cells were inoculated to opposite sides of a 90-mm petri dish filled with PDA. The distance between these two strains was 40 mm. The PDA plates were incubated at 28°C, and the inhibitory effect was observed every day for a total of 15 days. Plates only inoculated with *B. cinerea* were used as a control. The inhibition rate of *C. rosea* to *B. cinerea* was calculated at 15 days after inoculation according to the following formula (Zong et al. 2003):

$$\text{Inhibition rate(\%)} = \left[\frac{\text{radius of the control colony} - \text{radius of } B. \text{ cinerea colony confronted with } C. \text{ rosea}}{\text{radius of the control colony}} \right] \times 100\%$$

The experiment was repeated three times, and at least six plates were used in each experiment (three plates for *B. cinerea* only [control] and three plates for *B. cinerea* plus *C. rosea*).

Effect of *C. rosea* on the spore germination of *B. cinerea*.

The fermentation filtrate of *C. rosea* was prepared as follows. Mycelial scrapes from colonized plates were inoculated into a 250-ml flask filled with potato dextrose broth (PDB; 200 g of peeled potato, 20 g of glucose, and 1,000 ml of distilled water) and cultured at 28°C for 48 h with constant agitation (200 r/min). Then, the fermentation broth was centrifuged for 20 min (10,000 $\times$ g/min at 4°C), and the supernatant was filtrated using a 0.22- μ m filter. The sterile fermentation filtrate was maintained at 4°C before use. The *B. cinerea* spore germination was carried out as previously described (Liu et al. 2013; Shu et al. 2004). Briefly, *B. cinerea* spore suspension was added to the sterile fermentation filtrate to a final concentration of 106 spores per 1 ml. One drop of the mixture was placed on a coverslip precoated with collodion. Then, the coverslip was placed on a moisturized petri dish and cultured for 10 h at 28°C. The fermentation filtrate was replaced with fresh PDB medium as a control. Germination was defined when the length of the spore tube was longer than that of the spore diameter. The inhibition rate of spore germination = (spore germination rate of the control – spore germination rate of the treated group)/spore germination rate of the control $\times$ 100%.

Resistance of tomato plants to *B. cinerea* induced by *C. rosea*.

The inoculation of tomato plants with *B. cinerea* and *C. rosea* was described in Abuqamar et al. (2008). Briefly, the *B. cinerea* or *C. rosea* conidia were suspended in distilled water to a final concentration of 1×10^7 spores per 1 ml. The 4-week-old tomato plants were first inoculated with *B. cinerea* conidia suspension (B group) by spraying these directly onto the leaves. *C. rosea* conidia suspension was inoculated at 12 h after the inoculation of *B. cinerea* (B + C group). The same volume of water (no *B. cinerea* or *C. rosea*) was used as a control (CK group). At 18 days after inoculation with *B. cinerea*, the incidence of gray mold disease was investigated, and the disease index was calculated as previously reported (Fang 2007).

Leaf samples were collected from inoculated plants every 12 h (0 to 96 h) and stored at –80°C for enzyme activity assay (refer to the next section). Leaves from at least 10 individual plants were used in each independent experiment. Each experiment was repeated three times.

Enzyme activity assay of PAL, PPO, and CAT in tomato leaves.

Leaf samples were collected as described above. Then, 0.5 g of leaves from each sample were used for protein extraction. The leaf sample was rinsed in ice cold phosphate buffer (pH 7.4), dried with filter paper, and placed in 2 ml of ice cold homogenate medium provided in the enzyme activity kits (Nanjing Institute of Biological Engineering). Then, the leaf tissue was cut into small pieces, homogenized, and filtered through a cheesecloth. Afterward, the solution was centrifuged at 4,000 rpm for 15 min, and the supernatant was used as the enzyme source. Next, the protein concentrations were measured using a commercial protein assay kit (catalog number CW0014; Beijing Kangwei Century Biotechnology Co., Ltd.). The activities of PAL and PPO were carried out using commercially available kits for PAL and PPO (Nanjing Institute of Biological Engineering) according to the manufacturer's instruction. CAT activity was detected as previously described (Li 2000).

Determination of second messengers: NO, H₂O₂, and O₂^{•−}.

The assays for NO, H₂O₂, and O₂^{•−} were carried out using commercially available NO (A013-1), H₂O₂ (A064), and O₂^{•−} assay

TABLE 1. Hormone-related tomato mutants and the associated wild types used in the study

Mutants	Mutation	Wild type	Cultivar origin
<i>Spr2</i> (jasmonic acid deletion mutant)	Jasmonate biosynthetic pathway blocked	CM (Le. CM indicate the wild-type of <i>Spr2</i>)	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences
<i>Nor</i> (ethylene deletion mutant)	Ethylene biosynthetic pathway blocked	AC (Ailsa Craig indicates the wild-type of <i>nor</i>)	Tomato Genetics Resource Center
<i>NahG</i> (salicylic acid deletion mutant)	Salicylic acid biosynthetic pathway blocked	MM (Moneymaker indicates the wild-type of <i>NahG</i>)	Horticultural Institute of Shenyang Agricultural University
<i>Gib3</i> (gibberellin deletion mutant)	Gibberellin biosynthetic pathway blocked	MM (Moneymaker indicates the wild-type of <i>Gib3</i>)	Tomato Genetics Resource Center

kits (A052; all were obtained from Nanjing Institute of Biological Engineering) according to the manufacturer's protocols. The sample protein concentration was measured using a commercial protein assay kit (catalog number CW0014; Beijing Kangwei Century Biotechnology Co., Ltd.).

Real-time polymerase chain reaction of *MAPK*, *WRKY*, *atpA*, and *Lexy12* genes. The leaf samples obtained from different treatments were collected as described above. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was dissolved in 20 µl of RNase-free H₂O and quantified by spectrophotometry. The total RNA extracted from the triplicate of tomato leaves was reverse transcribed using the Easyscript first-strand complementary DNA synthesis super mix (Beijing Transgen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions and stored at -80°C.

The polymerase chain reaction (PCR) conditions are listed in Tables 2, 3, and 4. The primers were designed as previously described (Mouekouba et al. 2014). These are listed in Table 5. β-actin was used as a control. PCR was carried out using the iCycler real-time quantity PCR system (Bio-Rad). The relative expression was calculated using the 2^{-ΔΔC_t} method as previously described (Livak and Schmittgen 2001). These experiments were repeated independently with three biological replicates.

Scanning electron microscopy observation. At 0 (control), 2, 4, 6, 8, 10, and 12 days after the inoculation of gray mold, leaf samples were collected for scanning electron microscopy (SEM) analysis. A small square of 1 to 2 mm² of the leaf (*Spr2* and CM [Le. CM]) was cut between the veins and the edge. The SEM samples were prepared as previously described (Hao et al. 2017). An S-3400 N scanning electron microscope (Hitachi) was used to observe and image the samples. A total of 30 images were taken from each sample at magnifications of 600× and 1,200×. Stomatal opening rate = (stomatal opening number)/(total stomatal number) × 100%.

Transmission electron microscopy observation. The sample collection for transmission electron microscopy (TEM) was the same as that for SEM. The collected samples were prepared for TEM observation as previously described (Gong 2017). An

H-7650 transmission electron microscope (Hitachi) was used to observe the ultrastructure of mesophyll cells and chloroplasts.

Statistical analysis. All data were presented as mean ± standard deviation. The statistical analysis was performed with one-way analysis of variance (SPSS software, v.20; SPSS Inc.). The separations were performed using Duncan's multiple range tests. Differences at *P* < 0.05 were considered to be statistically significant. Student's *t* test was used to compare the resistance of tomato with *B. cinerea* induced by *C. rosea* between treatments. All of the experiments were repeated three times.

RESULTS

***C. rosea* inhibits the colony growth of *B. cinerea*.** The possible inhibition role of *C. rosea* toward *B. cinerea* was examined. In the absence of *C. rosea*, *B. cinerea* grew well on the PDA plate, reaching a radius of 27.6 ± 0.92 mm after 15 days of culture. The colony appeared to be grayish brown and formed many small granular dark sclerotia (Fig. 1G). In the presence of *C. rosea*, the *B. cinerea* grew much slower, reaching a radius of 1.81 ± 0.31 mm at day 4 (Fig. 1A, C, and E). However, the *C. rosea* colony continued to grow and enlarge, and it completely inhibited the growth of *B. cinerea* after 15 days of culture (Fig. 1B, D, and F). The colony of *B. cinerea* became even smaller, with a radius of 0.3 ± 0.03 mm, suggesting that the *B. cinerea* hyphae were degraded and engulfed by *C. rosea* possibly by substances released by *C. rosea*. Finally, the *B. cinerea* was completely covered by *C. rosea*, causing its degradation and death. At the contact area between *C. rosea* and *B. cinerea*, a bacteriostatic ring was clearly observed (Fig. 1B and C). The calculated inhibition rate at 15 days was 98.91 ± 0.13%.

The germination of *B. cinerea* was also inhibited by *C. rosea*. When cultured in fresh PDB medium, the germination rate of *B. cinerea* reached up to 36.73 ± 0.98%, but it only reached 2.25 ± 0.13% when cultured in the *C. rosea* fermentation filtrate. The inhibition rate of spore germination by the *C. rosea* fermentation filtrate was 93.87 ± 0.44%. Among the germinated spores, the tubes were much longer in the fresh PDB medium than in the *C. rosea* fermentation filtrate (Fig. 2).

***C. rosea* enhances the resistance of tomato plants to *B. cinerea*.** Next, the possible effect of *C. rosea* in enhancing the resistance of tomato plants to *B. cinerea* was examined. Compared with the control group, the *B. cinerea*-treated groups (both wild type and mutants) grew slower, and the gray mold disease indexes were significantly higher (Fig. 3), indicating that *B. cinerea* impairs tomato growth. Treatment with *C. rosea* significantly improved tomato growth and significantly decreased the gray mold disease. In all types of tomato plants, the averages of plant heights and leaf numbers of the B + C group were at least one time higher than those of the B group. This is consistent with a previous report from our laboratory (Wang et al. 2015). Leaf lesions were also significantly

TABLE 2. Polymerase chain reaction mix

Materials	Volume, µl
Bio-Rad Super SYBR Green mix	10
Primer F (10 µM)	0.4
Primer R (10 µM)	0.4
Template complementary DNA (25 ng)	2
ddH ₂ O (double distilled water)	7.2
Total volume	20

TABLE 3. Polymerase chain reaction conditions for the *mitogen-activated protein kinase* and *WRKY* genes

Stage	Cycles	Temperature, °C	Time
Stage 1	1	94	5 min
Stage 2	35	94	30 s
		55	30 s
		72	60 s
Stage 3		72	10 min

TABLE 4. Polymerase chain reaction conditions for the *atpA* and *Lexy12* genes

Stage	Cycles	Temperature, °C	Time
Stage 1	1	94	3 min
Stage 2	35	95	45 s
		52	45 s
		72	60 s
Stage 3		72	10 min

TABLE 5. Primers used for the polymerase chain reaction amplification

Gene and primer	Sequence
<i>Mitogen-activated protein kinase</i>	
Forward primer	GCTGACAGATTGTTGCAGGT
Reverse primer	TCCACCCATAAAGATACATCA
<i>WRKY</i>	
Forward primer	GCATTACTGTCAACCATCGC
Reverse primer	AACTTCGCGGATTCTCACTT
<i>atpA</i>	
Forward primer	AGGCTCATATACGGAACGG
Reverse primer	GAGTGAGGCTTATTTGGGTC
<i>Lexy12</i>	
Forward primer	GTGGTGCTGCGTTGGCTGA
Reverse primer	GTGGTGTTGTATTGGGTGT
<i>β-actin</i>	
Forward primer	TGTCCCTATTTACGAGGGTTATGC
Reverse primer	CAGTTAAATCACGACCAGCAAGAT

different among these three groups. Almost no lesions were observed in the control group. Furthermore, leaves in the B + C group had fewer leaf spots, whereas leaves in the B group were covered with gray brown spots and large lesion sizes. In addition, the incidence of gray mold disease in the four hormone-deficient mutants was significantly higher compared with that in corresponding wild-type tomato plants, suggesting that deficiency in the production of these hormones affects the host resistance to *B. cinerea*.

***C. rosea* improves the ultrastructure of *B. cinerea*-infected tomato leaf cells.** To confirm that *C. rosea* could enhance the resistance of tomato plants to *B. cinerea*, the ultrastructure of tomato leaves was examined under different treatments. After 5 days of *B. cinerea* inoculation, the ultrastructure of the green tissue on the edge of the disease spot of a tomato leaf was severely injured in both the wild type and the JA mutant (*Spr2*) (Supplementary Fig. S1).

In the JA mutant (*Spr2*), chloroplasts became round, and the starch granules became nearly full of whole chloroplasts. Furthermore, the

grana were destroyed (blurred, reduced, and no lamellar structure), some mitochondria were degraded, thylakoid disintegration and partial dissolution occurred, and the separation of chloroplasts from the cell wall was observed. In the wild type, the membranes were degraded and ruptured, the chloroplasts became round, partial chloroplasts were degraded and ruptured, the grana were destroyed (loosely arranged, blurred, and reduced), thylakoids were stacked and partially dissolved, and chloroplast and cell wall separation occurred.

After 5 days of *B. cinerea* plus *C. rosea* inoculation, the ultrastructure of the green tissue at the edge of the spot significantly improved, although minor damage could still be observed. In the JA mutant, the morphology of chloroplasts was close to a spindle shape. Furthermore, the starch grains in the individual chloroplasts were bigger, the majority of granum lamellae were clearly visible, and the mitochondria had no obvious changes.

In the wild type, the chloroplast morphology remained intact. Although the starch grains were somewhat larger, there was no separation between the chloroplast and cell wall, and the chloroplast

Fig. 1. The mycoparasitism of *Clonostachys rosea* to *Botrytis cinerea* in potato dextrose agar is shown. **A, C, and E,** At 4 days after inoculation with *C. rosea* and *B. cinerea*. **B1, B2, D1, D2, F1, and F2,** At 15 days after inoculation with *C. rosea* and *B. cinerea*. **A, B1, and B2,** Single-point, **C, D1, and D2,** double-point, and **E, F1, and F2,** triple-point inoculations of the antagonistic effect of *C. rosea* were evaluated: **B1, D1, and F1** present the back side of the plate, whereas **B2, D2, and F2** present the front side of the plate. **G,** Negative control: 15 days after inoculation only with *B. cinerea* (back side of the plate).

granum lamellae had no obvious loosening degradation. Furthermore, some of the granum lamellae were clearly visible, and there was no obvious change in mitochondria.

Changes in activity of defense enzymes in response to *B. cinerea* and *C. rosea* treatment. Compared with controls,

Fig. 2. Inhibition of the *Clonostachys rosea* fermentation solution against the spore germination of *Botrytis cinerea* (100 $\times$): **A**, control and **B**, treatment with the *C. rosea* fermentation solution.

B. cinerea treatment significantly increased the activities of PAL and PPO but decreased the activity of CAT in both the wild type and the four mutants. This was apparent in both the B group (treated with *B. cinerea* only) and the B + C group (treated with *B. cinerea* and *C. rosea*).

The PAL activities reached their peaks at ~60 to 84 h. The PAL activity of *Spr2* (JA deletion) plants was higher than that of the wild type (CM) plants in the B group, but it was lower than that in the wild type of the B + C group (Fig. 4A). A similar trend was observed in the *nor* (ET deletion) mutant, where PAL activity reached its highest peak in the B + C group at 84 h (Fig. 4B). For the *NahG* (SA deletion) and *Gib3* (GA deletion) mutants, the PAL activities of these mutants were normally higher than those of the wild type (Moneymaker, MM) in both the B group and the B + C group (Fig. 4C and D), suggesting that the *NahG* and *Gib3* mutants have an opposite effect compared with *Spr2* and *nor*.

In both the B and B + C groups, PPO activities gradually increased, reached their peaks at ~60 to 84 h, and subsequently decreased. The PPO activity of CM plants was mostly higher than that of *Spr2* plants in both the B group and the B + C group. At 72 h,

Fig. 3. Analysis of the spectral resistance of tomato to *Botrytis cinerea* primed by *Clonostachys rosea*. The incidence of gray mold disease in the different varieties was calculated at 18 days after inoculation with *B. cinerea*. **A to G** represent *Spr2*, CM (Le. CM), *Nor*, AC (Ailsa Craig), *Gib3*, *NahG*, and MM (Moneymaker), respectively. The CK (control check) row indicates the water treatment group, the B row indicates the *B. cinerea* conidia-treated group, and the B + C row indicates the group first treated with *B. cinerea* conidia suspension and then, treated with *C. rosea* conidia suspension after 12 h. **H to J** show the disease indexes of the **H**, *Spr2*, **I**, *Nor*, and **J**, *Gib3* and *NahG* mutants and their wild types. Each experiment was repeated three times for each treatment, with at least 15 strains of single-type tomato. The different letters show the significant difference at $P \leq 0.05$ by Duncan's multiple range. ns, not significant. ** $P < 0.01$ versus *B. cinerea* plus *C. rosea*.

the maximum value of PPO activity in CM leaves in the B + C group was almost three times higher than that of the B group (Fig. 5A).

The PPO activity of the wild-type (Ailsa Craig, AC) plants was significantly higher than that of the *nor* plants at the 60- to 96-h time

points but lower at the 24- and 48-h time points in the B group. In the B + C group, the PPO activities of both *nor* and the wild type (AC) reached their peaks at 36 and 60 h, respectively, with maximum values of 145.9 and 191.9% higher than that of controls, respectively. The PPO

activity of AC plants was significantly higher than that of *nor* plants at 24 and 60 h but lower at 36 and 48 h (Fig. 5B). Similar to PAL activities, the PPO activities of the *NahG* and *Gib3* mutants were higher than the wild type (MM) in both the B group and the B + C group (Fig. 5C and D).

Different from PAL and PPO activities, CAT activities decreased when treated with *B. cinerea* (in both the B group and the B + C group). In the B group, the CAT activities of the *Spr2* and the wild-type leaves reached their lowest levels at 36 and 24 h, respectively, 65.9 and 30.7% lower than that of controls, respectively. Furthermore, the CAT activity of *Spr2* plants was significantly lower than that of the wild-type (CM) plants at three time points (36 to 60 h). In the B + C group, the CAT activities of the *Spr2* and CM leaves reached their lowest levels at 36 and 60 h, respectively, 57.9 and 42.8% lower than that of controls, respectively. In both treatments, the CAT activity in *Spr2* was lower than that in the wild type (Fig. 6A). In the B group, the CAT activities of the *nor* and the wild-type (AC) leaves reached their lowest levels at 72 and 36 h, respectively, 73.2 and 74.9% lower than that of controls, respectively. Furthermore, the CAT activity of AC was significantly lower than that of *nor* in the B + C group from 12 to 72 h. In the B + C group, the CAT activities of the *nor* and AC leaves reached their lowest levels at 48 and 60 h, respectively, 65.3 and 79.1% lower than that of controls, respectively. The CAT activity of *nor* plants was significantly higher than that of AC plants at 60 h (Fig. 6B). Furthermore, the CAT activity of *NahG* was significantly higher than the wild type (MM) in both the B and the B + C groups (Fig. 6C), but no significant difference in CAT activity was observed between the *Gib3* and the wild-type (MM) plants (Fig. 6D).

Changes in second messenger levels in response to *B. cinerea* and *C. rosea* treatment. In the *Spr2* mutant and its wild type (CM), the H_2O_2 levels were relatively steady, except for the high level that increased at 72 h in the B + C group, which gradually

decreased to normal level (Fig. 7A). *B. cinerea* treatment increased the levels of H_2O_2 in the *nor* mutant in both the B and the B + C groups, whereas the H_2O_2 level was higher in the wild type in the B + C group compared with the mutant (Fig. 7B). Furthermore, H_2O_2 levels increased in the *NahG* mutant in both the B and the B + C groups within 60 to 84 h, whereas H_2O_2 levels were higher in the wild type compared with the mutant (Fig. 7C). A similar trend was also observed in the *Gib3* mutant compared with the *NahG* mutant (Fig. 7D).

A similar change in NO levels was observed in all four mutants, in which *B. cinerea* treatment (both the B and the B + C groups) induced a significant increase in NO within 60 to 84 h (Fig. 8). In the *Spr2* and *nor* mutants, NO levels were higher in the wild type compared with the mutant, whereas in the *Gib3* mutant, NO levels were higher in the mutant than in the wild type. However, in the *NahG* mutant, no significant difference in NO level was observed between the wild type and mutant.

A similar change in O_2^- levels was also observed in all four mutants, in which *B. cinerea* treatment (both the B and the B + C groups) caused a significant increase in O_2^- at 24 h (Fig. 9). In the *Spr2* mutant, the O_2^- level was significantly higher in the mutant than in the wild type (Fig. 9A), whereas in the *nor* mutant, the O_2^- level was higher in the wild type than in the mutant (Fig. 9B). However, in the *NahG* and *Gib3* mutants, no significant difference in O_2^- level was observed between the wild type and the mutant (Fig. 9C and D).

The expression of stress-related genes in response to *B. cinerea* and *C. rosea* treatment. *B. cinerea* treatment (both in the B and the B + C groups) caused a significant increase of *WRKY* expression. However, this only occurred in the wild type (CM), and it did not occur in the *Spr2* mutant (Fig. 10A). The highest *WRKY* expression was observed at 24 h, in which the B + C group exhibited a significantly higher *WRKY* expression compared with the B group.

Different from the *WRKY* expression, the *MAPK* gene expression increased in both the wild type and the mutant (*Spr2*), but the *MAPK* gene expression in *Spr2* seemed to be significantly lower than that in

Fig. 6. Analysis of catalase (CAT) activity. Changes in CAT levels in tomato leaves subjected to different treatments. Each experiment was repeated three times. Data are presented as mean $\pm$ standard deviation. The means marked with different letters (**A**, **B**, **C**, and **D**) are significantly different at $P < 0.05$. 1-*Spr2*, 1-CM (Le. CM), 1-*Nor*, 1-AC (Ailsa Craig), 1-*NahG*, 1-*Gib3*, and 1-MM (Moneymaker) indicate the control group (treated with water); 2-*Spr2*, 2-CM, 2-*Nor*, 2-AC, 2-*NahG*, 2-*Gib3*, and 2-MM indicate the B group (treated with *Botrytis cinerea*); and 3-*Spr2*, 3-CM, 3-*Nor*, 3-AC, 3-*NahG*, 3-*Gib3*, and 3-MM indicate the B + C group (treated with *B. cinerea* plus *Clonostachys rosea*). **A**, *Spr2* mutant and wild-type CM tomato plant. **B**, *nor* mutant and wild-type AC tomato plant. **C**, *NahG* mutant and wild-type MM tomato plant. **D**, *Gib3* mutant and wild-type MM tomato plant.

Fig. 7. Analysis of hydrogen peroxide (H_2O_2) levels. Changes in H_2O_2 levels in tomato leaves subjected to different treatments. Each experiment was repeated three times. Data are presented as mean $\pm$ standard deviation. The means marked with different letters (**A**, **B**, **C**, and **D**) are significantly different at $P < 0.05$. 1-*Spr2*, 1-CM (Le. CM), 1-*Nor*, 1-AC (Ailsa Craig), 1-*NahG*, 1-*Gib3*, and 1-MM (Moneymaker) indicate the control group (treated with water); 2-*Spr2*, 2-CM, 2-*Nor*, 2-AC, 2-*NahG*, 2-*Gib3*, and 2-MM indicate the B group (treated with *Botrytis cinerea*); and 3-*Spr2*, 3-CM, 3-*Nor*, 3-AC, 3-*NahG*, 3-*Gib3*, and 3-MM indicate the B + C group (treated with *B. cinerea* plus *Clonostachys rosea*). **A**, *Spr2* mutant and wild-type CM tomato plant. **B**, *nor* mutant and wild-type AC tomato plant. **C**, *NahG* mutant and wild-type MM tomato plant. **D**, *Gib3* mutant and wild-type MM tomato plant.

Fig. 8. Analysis of nitric oxide (NO) levels. Changes in NO levels in tomato leaves subjected to different treatments. Each experiment was repeated three times. Data are presented as mean $\pm$ standard deviation. The means marked with different letters (**A**, **B**, **C**, and **D**) are significantly different at $P < 0.05$. 1-*Spr2*, 1-CM (Le. CM), 1-*Nor*, 1-AC (Ailsa Craig), 1-*NahG*, 1-*Gib3*, and 1-MM (Moneymaker) indicate the control group (treated with water); 2-*Spr2*, 2-CM, 2-*Nor*, 2-AC, 2-*NahG*, 2-*Gib3*, and 2-MM indicate the B group (treated with *Botrytis cinerea*); and 3-*Spr2*, 3-CM, 3-*Nor*, 3-AC, 3-*NahG*, 3-*Gib3*, and 3-MM indicate the B + C group (treated with *B. cinerea* plus *Clonostachys rosea*). **A**, *Spr2* mutant and wild-type CM tomato plant. **B**, *nor* mutant and wild-type AC tomato plant. **C**, *NahG* mutant and wild-type MM tomato plant. **D**, *Gib3* mutant and wild-type MM tomato plant.

the wild type (Fig. 10B). In general, the *MAPK* gene expression was higher in the wild type in the B + C group, except at 84 h, in which the expression in the B group was significantly higher than that in the B + C group.

No significant change in Lexyl expression was observed in the *Spr2* mutant in the control group or B group. However, a

significant increase in Lexyl expression was observed in the B + C group, reaching its highest expression level at 36 h in the wild type (Fig. 10C). For the *atpA* expression, no significant changes were observed in the wild type in either the B group or the B + C group, except at 48 h, where a significant increase was observed in the B + C group. However, a significant increase was observed

Fig. 9. Analysis of superoxide anion radical (O_2^-) levels. Changes in O_2^- levels in tomato leaves subjected to different treatments. Each experiment was repeated three times. Data are presented as mean $\pm$ standard deviation. The means marked with different letters (A, B, C, and D) are significantly different at $P < 0.05$. 1-*Spr2*, 1-CM (Le. CM), 1-*Nor*, 1-AC (Ailsa Craig), 1-*NahG*, 1-*Gib3*, and 1-MM (Moneymaker) indicate the control group (treated with water); 2-*Spr2*, 2-CM, 2-*Nor*, 2-AC, 2-*NahG*, 2-*Gib3*, and 2-MM indicate the B group (treated with *Botrytis cinerea*); and 3-*Spr2*, 3-CM, 3-*Nor*, 3-AC, 3-*NahG*, 3-*Gib3*, and 3-MM indicate the B + C group (treated with *B. cinerea* plus *Clonostachys rosea*). **A**, *Spr2* mutant and wild-type CM tomato plant. **B**, *nor* mutant and wild-type AC tomato plant. **C**, *NahG* mutant and wild-type MM tomato plant. **D**, *Gib3* mutant and wild-type MM tomato plant.

Fig. 10. Expression analysis of *WRKY*, *mitogen-activated protein kinase* (*MAPK*), *Lexyl2*, and *atpA* in the *Spr2* mutant (indicated by M) and wild-type CM (Moneymaker) (indicated by W). Data are presented as mean $\pm$ standard deviation. At each time point, the different letters indicate the significant differences between treatments ($P < 0.05$; $n = 9$). ns, not significant. **A**, **B**, **C**, and **D** represent the expression of *WRKY*, *MAPK*, *Lexyl2*, and *atpA* genes, respectively.

in the *Spr2* mutant in the B + C group but not in the B group (Fig. 10D).

JA induces stomatal closure in tomato leaves at different treatments. The SEM analysis revealed that the stomatal opening ratio of the JA mutant (*Spr2*) treated with *B. cinerea* was significantly higher compared with that of CM (control, $P < 0.01$) (Fig. 11A and Supplementary Fig. S2). At all time points, the stomatal opening ratio of the *Spr2* mutant in the B + C group was also higher than that of CM (0 to 10 days: $P < 0.01$; 12 days: $P < 0.05$) (Fig. 11B).

At 2, 6, and 12 days, the stomatal opening ratio of CM leaves in the B + C group was significantly lower than that of CM leaves in the B group (2 and 6 days: $P < 0.01$; 12 days: $P < 0.05$). However, at 10 days, the opening ratio of the stomata of CM leaves in the B + C group was significantly higher than that of the CM leaves in the B group ($P < 0.01$) (Fig. 11C).

At the time points of 2, 4, 8, and 10 days, the stomatal opening ratio of the *Spr2* mutant leaves in the B + C group was significantly higher than that in the B group (2 and 8 days: $P < 0.05$; 4 and 10 days: $P < 0.01$). However, at 12 days, the opening ratio of the stomata of JA mutant leaves in the B + C group was significantly lower than that in the B group ($P < 0.01$) (Fig. 11D).

DISCUSSION

C. rosea is a mycoparasitic fungus that can control several important plant diseases. It has the ability to parasitize and kill other fungi, such as *B. cinerea*, and utilize the dead fungal biomass (Karlsson et al. 2015). Compared with *B. cinerea* treatment, visible lesion was significantly reduced in the *B. cinerea* plus *C. rosea* treatment on tomato leaves, indicating the ability of *C. rosea* to resist *B. cinerea* in tomato leaves. In this study, it was demonstrated that *C. rosea* inhibits *B. cinerea* growth both in vivo and in vitro. In vitro, *C. rosea* completely blocked the growth of *B. cinerea* and spore germination. The spore germination experiment strongly suggested that *C. rosea* releases compounds that possess antibiotic properties and that these compounds may play a role in protecting

plants from invading pathogens (Ezra et al. 2004). In vivo, it inhibits gray mold disease in both wild-type and mutant tomato plants.

Compared with the wild type, *C. rosea* treatment significantly changed several signal molecules in the mutants of *Spr2*, *nor*, and *NahG*, suggesting that JA, ET, and SA are likely involved in the development of resistance to *B. cinerea* in tomato leaves.

Changes in defense enzymes in tomato leaves under *C. rosea* treatment. PAL, PPO, and CAT play important roles in protecting plants against various pathogens. PAL is involved in the phenylpropanoid pathway that takes part in the synthesis of phenolic compounds phytoalexin and lignin, which are important in pathogen resistance (Paiva et al. 1991; Pellegrini et al. 1994). PPO participates in the oxidation of many types of phenolic compounds, leading to the production of quinones, which are toxic to several pathogens (Thipyapong et al. 2004, 2007; Wang and Constabel 2004). In this study, it was found that *B. cinerea*-infected leaves triggered an increase in PAL and PPO activity. Furthermore, the treatment with *C. rosea* further enhanced the activity of PAL/PPO enzymes. In addition, the activity of PAL/PPO in the wild type was significantly higher than that in the *Spr2* mutant. However, the activity of PPO in the wild type was significantly lower than that in the *nor* mutant. *Spr2* is involved in the biosynthesis pathway of JA, which is a positive regulator of PAL/PPO in the *C. rosea*-induced resistance to tomato gray mold disease. This is consistent with a previous study, which showed that JA can enhance the insect resistance of cotton seedlings by increasing the activity of PAL and other enzymes in cotton leaves (Yang et al. 2013). Methyl jasmonate has also been shown to significantly increase PPO levels (Boughton et al. 2006). However, PAL activity in *NahG* mutant plants was significantly higher compared with that of wild-type plants at several time points in either the *B. cinerea*-treated group or the *B. cinerea*- and *C. rosea*-treated group, suggesting that SA may play a negative role of PAL in *C. rosea*-induced resistance to tomato gray mold.

CAT is a key antioxidant enzyme that catalyzes the decomposition of cellular H_2O_2 and thereby, protects cells from oxidative stress. The H_2O_2 produced during electron transport in

Fig. 11. Jasmonic acid (JA) induces stomatal closure in tomato leaves in different treatments. **A**, The effect of *Botrytis cinerea* treatment on the ratio of aperture stomata in JA and CM (MoneyMaker) tomato leaves. **B**, The effect of *Clonostachys rosea* plus *B. cinerea* treatment on the ratio of aperture stomata in JA and CM tomato leaves. **C**, The effect of *B. cinerea* and *C. rosea* plus *B. cinerea* treatments on the ratio of aperture stomata in CM tomato leaves. **D**, The effect of *B. cinerea* and *C. rosea* plus *B. cinerea* treatments on the ratio of aperture stomata in JA tomato leaves.

mitochondria and the fatty acid β -oxidation process is mainly removed by CAT (Nan and Fan 2008; Tiwari et al. 2002). In this study, it was found that the activity of CAT enzyme in the *Spr2* mutant was lower than that in the wild type, indicating that the *Spr2* mutant enhances oxidative stress in plants because of the lack of JA, which may place the positive regulation of CAT in the *C. rosea*-induced resistance to tomato gray mold disease. However, ET and SA may have a negative regulation of CAT. It has been reported that SA can increase the content of H_2O_2 and cause cell death (Hao et al. 2014; Mejía-Teniente et al. 2013).

Changes in second messengers in tomato leaves under *C. rosea* treatment. Redox homeostasis in plant cells is a key determinant of cell function. Imbalance of redox status leads to severe injury or cell death (Bykova and Rampitsch 2013; Vranova et al. 2002). Cell redox homeostasis closely correlates to accumulation of ROS (such as H_2O_2 and O_2^-), which are important signaling molecules that participate in the regulation of physiological processes. Particularly, ROS play a significant role in plant resistance to pathogens by contributing to the induction of defense genes (Lehmann et al. 2015).

Rapid release of ROS is one of the early events known as oxidative burst (L'Haridon et al. 2011). It was found in this study that *B. cinerea* infection induced a high level of O_2^- at 24 h among all four mutants and their wild types. The *Spr2* mutant produced a higher level of O_2^- compared with the wild type in both the *B. cinerea*-treated group and the *B. cinerea*- plus *C. rosea*-treated group. However, the *nor* mutant produced a lower level of O_2^- compared with the wild type. These results suggest that JA may inhibit the accumulation of O_2^- , where ET may promote the accumulation of O_2^- . Furthermore, the *Spr2*, *nor*, and *NahG* mutants produced a lower level of H_2O_2 compared with their wild types in both the *B. cinerea*-treated group and the *B. cinerea*- plus *C. rosea*-treated group. These results suggest that JA, ET, and SA may cause

accumulation of H_2O_2 . *B. cinerea* could produce oxalic acid, which can suppress the formation of ROS (Cessna et al. 2000; Prins et al. 2000). Compared with the control group or the *B. cinerea*-treated group, the *B. cinerea*- plus *C. rosea*-treated group produced more ROS, which enhanced the resistance of tomato toward *B. cinerea* infection. A similar phenomenon was reported in which the removal of oxalic acid produced by *B. cinerea* enhanced the protection of *Arabidopsis thaliana* (Schoonbeek et al. 2007).

NO is a regulator of physiological processes and functions as a messenger in immune response. NO also promotes seed germination and root growth, and it prolongs leaf elongation (Palavan-Unsal and Arisan 2009). As a regulator of disease resistance, it can initiate the death of hypersensitive cells and activate the expression of a series of defense genes (Hong et al. 2008).

In this study, it was revealed that the wild type (CM) produced a higher level of NO compared with the *Spr2* mutant, suggesting that JA may promote the accumulation of NO during *B. cinerea* infection. This is consistent with a previous report in which JA treatment induced the production of NO (Casimiro et al. 2001). It was also found that the effect of NO levels was higher in the *B. cinerea* plus *C. rosea* treatment than in other treatments.

Changes in the expression of MAPK and WRKY in the *Spr2* mutant and its wild type. The MAPK gene family and the WRKY transcription factor family play an important role in plant growth and other physiological and biochemical processes (Rushton et al. 2010; Ülker and Somssich 2004). Studies on early events after pathogen recognition have established the importance of MAPK and WRKYs in plant defense response (Mouekouba et al. 2014).

In this study, the JA signaling pathway was selected to investigate the role of these two genes in *C. rosea*-induced resistance to tomato gray mold disease. It was found that the expression of MAPK and WRKY was higher under *B. cinerea* plus *C. rosea* treatment and that

Fig. 12. The potential signaling pathway model of *Clonostachys rosea* primed the innate immunity to tomato gray mold disease in mesophyll cells. *B. cinerea*, *Botrytis cinerea*; CAT, catalase; ET, ethylene; H_2O_2 , hydrogen peroxide; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; NO, nitric oxide; O_2^- , superoxide anion radical; PAL, phenylalanine ammonialyase; PPO, polyphenol oxidase; ROS, reactive oxygen species; SA, salicylic acid.

these two genes were expressed lower in the *Spr2* mutant than in the wild type, suggesting that JA may promote the expression of *WRKY* and *MAPK* via the production of NO. A model for the potential signaling pathway is illustrated in Figures 11 and 12.

Changes in the expression of *LexY2* and *atpA* in the *Spr2* mutant and its wild type. β -xylanase (encoded by *Lexyl2*) is one of the key enzymes of xylan degradation. Xylan degradation could be used to modify the plant cell wall structure in response to infection. However, little is known about the role of β -xylanase in the *C. rosea*-induced resistance to *B. cinerea* infection in tomato. It was found that *B. cinerea* plus *C. rosea* treatment induced *Lexyl2* expression in either the wild-type or *Spr2* leaves. This is in agreement with a previous study (Mouekouba et al. 2014). It was also revealed that JA upregulates *Lexyl2* expression in *B. cinerea* plus *C. rosea* treatment, suggesting that *Lexyl2* may play an important role in *C. rosea*-induced resistance to *B. cinerea*, possibly by modifying the cell wall structure.

The ATP synthase CF1 α -subunit is a photosynthetic-related protein (Shao et al. 2011) that is located on the chloroplast membrane. ATPase plays an important role in promoting plant resistance to abiotic and biotic stress (Chen et al. 2000; Wu et al. 2013). It is encoded by the *atpA* gene on the chloroplast genome, and it is relatively conservative in evolution and low in mutation rate (Singh et al. 1985). It triggers a series of resistance reactions by proton transportation, improving cytoplasmic pH. The ATP synthase CF1 α -subunit also facilitates energy increase to enhance the resistance of plants (Shao et al. 2011). It was found that *B. cinerea* plus *C. rosea* treatment induced a higher level of *atpA* expression compared with the control group or the *B. cinerea*-treated group. It was also revealed that the *atpA* levels of the *Spr2* mutant were significantly higher than those of the wild type in the *B. cinerea*- plus *C. rosea*-treated group, suggesting that JA inhibits *atpA* expression.

JA induces stomatal closure in tomato leaves in different treatments. The stomatal aperture in leaf epidermis is formed by a pair of guard cells, which regulate opening and closing of the aperture. Many factors, such as light, drought, external Ca^{2+} , SA, and MeJA, could affect the opening/closing of apertures, which helps the plant to control transpiration, gas exchange, and innate immunity (Prodhan et al. 2018). Previous studies demonstrated that guard cells can sense microbes and close the pore, a process known as stomatal defense or stomatal immunity.

This study revealed that the stomatal opening ratio of leaves significantly increased when JA was absent in either *B. cinerea* treatment alone or the *B. cinerea* plus *C. rosea* treatment in tomato leaves. This suggests that JA effectively induces stomatal closure. In addition, *C. rosea* treatment caused stomatal closure at the early stage of the disease. The stomatal opening ratio decreased significantly at 2 to 4 days after inoculation, and the number of closed stomata increased. Significant changes of H_2O_2 and NO also occurred at the same time. In the B + C treatment, H_2O_2 increased from 60 h and peaked at 72 h, whereas NO peaked at 60 and 84 h (Figs. 8 and 9). *C. rosea* promotes the production of H_2O_2 and NO, which may induce the contraction of guard cells in leaves, thus accelerating stomatal closure. The effect of H_2O_2 and NO on stomata is consistent with that shown in previous studies (Desikan et al. 2006; Xie et al. 2014). The levels of PAL and PPO also increased at the early stage of disease (2 to 4 days), which correlates with the increased stomata closure. These two enzymes may have a high correlation with stomatal regulation in the model of *C. rosea* primed the innate immunity. Among the genes (*WRKY*, *MAPK*, *Lexyl2*, and *atpA*) that we tested, only *MAPK* showed a positive correlation with stomatal closure rate. In the later period of disease resistance, the expression of CAT could be directly or indirectly increased, and the stability of H_2O_2 was inhibited, whereas the opening of the stomata increased. Additional studies are needed to understand how these factors regulate stomatal opening and closure by regulating the related second messengers, such as Ca^{2+} , H_2O_2 ,

and NO, in *C. rosea*-induced innate immunity against tomato gray mold disease.

Based on these data and previous studies, a model was proposed for the possible roles of JA, ET, and SA in the signaling pathway of *C. rosea*-induced resistance to gray mold disease in tomato (Fig. 12). JA, ET, or SA response enhances the resistance to *B. cinerea* infection. However, GA is likely not involved in the process, because the disease symptoms in GA mutants were indistinguishable from those of wild-type plants.

In conclusion, it was revealed that *C. rosea*-induced resistance to *B. cinerea* is affected by multiple factors, including protective enzymes (PAL, PPO, and CAT), the levels of ROS and NO, and the expression of several stress response genes (*MAPK*, *WRKY*, *Lexyl2*, and *atpA*). All of these are affected by hormone-related genes, especially *Spr2*. This study sheds new light on the molecular mechanism of *C. rosea*-induced resistance to gray mold disease in tomato.

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