RESEARCH LETTER

Trichokonins from Trichoderma pseudokoningii SMF2 induce resistance against Gram-negative Pectobacterium carotovorum subsp. carotovorum in Chinese cabbage

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Abstract

Peptaibols, mainly produced by Trichoderma, play a pivotal role in controlling plant disease caused by fungi, virus, and Gram-positive bacteria. In the current study, we evaluated the control effect of Trichokonins, antimicrobial peptaibols from Trichoderma pseudokoningii SMF2, on soft rot disease of Chinese cabbage caused by a Gram-negative bacterium Pectobacterium carotovorum subsp. carotovorum and analyzed the mechanism involved. Trichokonins treatment (0.3 mg L⁻¹) enhanced the resistance of Chinese cabbage against Pcc infection. However, Trichokonins could hardly inhibit the growth of Pcc in vitro, even at high concentration (500 mg L⁻¹). Therefore, the direct effect of Trichokonins on Pcc may not the main reason why Trichokonins could control soft rot of Chinese cabbage. Trichokonin treatment led to an obvious increase in the production of reactive oxygen species hydrogen peroxide and superoxide radical, a significant enhance of the activities of pathogenesis-related enzymes catalase, polyphenoloxidase and peroxidase, and upregulation of the expression of salicylic acid-responsive pathogenesis-related protein gene acidic PR-1a in Chinese cabbage. These results indicate that Trichokonins induce resistance in Chinese cabbage against Pcc infection through the activation of salicylic acid signaling pathway, which imply the potential of Trichoderma and peptaibols in controlling plant disease caused by Gram-negative bacteria.

Introduction

Pectobacterium carotovorum subsp. carotovorum (Pcc, formerly known as Erwinia carotovorum subsp. carotovorum), a Gram-negative bacterium, is the casual agent of bacterial soft rot, a severe disease of field crops (corn, rice, and sugar beet), ornamental plants (begonia, dieffenbachia, dahlia, and philodendron) and vegetables (potato, tomato, onion, crucifers) (Aysan et al., 2003; Fiori & Schiaffino, 2004; Zhang et al., 2007; Baz et al., 2012). Chinese cabbage (Brassica rapa L. ssp. pekinensis), a member of crucifers, is one of the most popular vegetables among Chinese people, and it is widely cultivated in the northern area of China. Soft rot has been one of the most destructive diseases in Chinese cabbage (Zhang et al., 2007). Because of the Pcc invading the inner parts of the plants, the conventional chemical products such as copper may not provide adequate control for the soft rot disease (Aysan et al., 2003). Moreover, chemical methods are not economical in the long run because of their potential for damaging the environment, polluting the atmosphere, and leading to the development of resistant strains among the target organisms with repeated use (Naseby et al., 2000). Therefore, much attention has been focused on the biologic control agents (BCAs), because they are efficient, reliable, and safe for the environment (Lyon & Newton, 1997). Several BCAs have been used for controlling soft rot pathogens, such as Pseudomonas and Bacillus (Geels & Schippers, 1983; Kloeper, 1983; Colyer & Mount, 1984; Raju et al., 2006; Zhao et al., 2013).
**Trichoderma** spp. are well known as important BCAs of plant diseases. Previous reports have mainly focused on the antimicrobial activity of *Trichoderma* spp. against plant fungal pathogens and the related mechanisms (Howell, 2003; Harman et al., 2004). Antimicrobial metabolites produced by *Trichoderma* spp. play essential roles in their antagonistic activities (Reino et al., 2008). Peptaibols are among these important metabolites from *Trichoderma* spp. against phytopathogens (Daniel & Filho, 2007). Peptaibols have been reported to be able to inhibit the growth of fungal pathogens, to trigger a defense response, or to reduce the susceptibility of plant to pathogens (Yun et al., 2000; Viterbo et al., 2007; Luo et al., 2010). Trichokonins are a kind of peptaibols which are composed of 20 amino acid residues. Three Trichokonins, TK VI, TK VII, and TK VIII, have been isolated and purified from *Trichoderma pseudokoningii* SMF2 (Song et al., 2006). These Trichokonins had antimicrobial activity against a range of Gram-positive bacteria and fungal phytopathogens. They also could induce morphological alteration in *Bacillus subtilis* (Su et al., 2012), programmed cell death in plant pathogens (Shi et al., 2012) and resistance in tobacco against Tobacco Mosaic Virus (Luo et al., 2010). However, these Trichokonins were not active against Gram-negative bacteria in vitro (Song et al., 2006), and their activities against Gram-negative bacteria in vivo have not been tested.

In previous study, we found that *T. pseudokoningii* SMF2 could control bacterial soft rot infection in Chinese cabbage in the field (Hu et al., 2009), but the controlling mechanisms underlying this process are still unknown. The present study described the control effect of the Trichokonins from *T. pseudokoningii* SMF2 on *Pcc* infection to Chinese cabbage and the mechanism involved.

### Materials and methods

#### Plant materials and growth conditions

Chinese cabbage seeds were purchased from Shandong Denghai Seed Industry Co., Ltd., China. Plants were grown in a growth chamber with a photoperiod of 16/8 h (light/dark) (1.87 W m\(^{-2}\)) and 75–80% relative humidity, at 25 ± 1 °C. The culture substrate was purchased from Luqing Seed Co., Ltd., China. Experiments were carried out when the plants reached the eight true-leaf stage.

#### Preparation of Trichokonins

Trichokonins stock solution with a concentration of 10 mg mL\(^{-1}\) was prepared as previously described (Luo et al., 2010). In different experiments, sterile water (with 1% v/v Tween-80) was used for further dilution of Trichokonins stock solution.

### Culture condition

*Pcc* was grown at 37 °C in Mueller Hinton Broth (MHB; Hangzhou Microbial Reagent Co., Ltd.; China) overnight and was diluted with fresh MHB to a concentration of approximately 6 × 10\(^8\) colony-forming units (CFU) mL\(^{-1}\).

#### Induction of resistance in Chinese cabbage against *Pcc* by Trichokonins

Each Chinese cabbage plant was sprayed with 2 mL Trichokonins (0.15, 0.2, 0.3, or 0.6 mg L\(^{-1}\)) or 2 mL distilled water containing 1% (v/v) Tween-80 (control solution). After 1 day, the petioles of the third to fifth leaves were injured using a bundle of 10 sterile needles, and 10 μL of the test bacterial suspension was dropped onto the injury (Kyeremeh et al., 2000). Then, the seedlings were kept for 48 h in a humidified chamber (RH, 95%) at 25 °C (Han, 2006). The area macerated by each treatment was measured using a ruler. Inhibition rate = (lesion length of control Chinese cabbage - lesion length of treated Chinese cabbage)/ lesion length of control Chinese cabbage × 100%. Each treatment consisted of three replicates (each replicate containing six individual plants).

#### Susceptibility test

The minimal inhibitory concentration (MIC) was determined for Trichokonins according to a modified microtiter-broth dilution method (Wiegand et al., 2008). The MIC was defined as the lowest concentration that inhibited the visible growth of bacteria compared with the control sample (Thomas et al., 2006).

The antibacterial activity was determined using turbidimetry. *Pcc* suspension (1.5 mL, 5.5 × 10\(^8\) CFU mL\(^{-1}\)) was mixed with 1.5 mL of Trichokonins solution (0.1, 1, 10, 100, or 1000 mg L\(^{-1}\)) in a test tube, which resulted in a final Trichokonins concentration of 0.05, 0.5, 5, 50, or 500 mg L\(^{-1}\). A tube with 1.5-mL *Pcc* suspension and 1.5-mL sterile water was used as controls. The tubes were incubated at 28 °C for 24 h with shaking, and the multiplication of bacteria was measured by recording their OD\(_{600}\) nm on a UV/VIS-550 spectrophotometer (Jasco; Japan). Each treatment consisted of four replicates.

#### Elicitor activity tests

The production of superoxide anion radical (O\(_2^−\)) and hydrogen peroxide (H\(_2\)O\(_2\)) in the leaves of Chinese cabbage plants treated with Trichokonins was examined using the procedure of Fitzgerald et al. (2004). Seedlings were cultured in MS medium containing 0.3 mg g\(^{-1}\) Trichokonins for 24 h, and then, the top leaf was harvested.

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Seedlings cultured in an MS medium without Trichokonins were taken as a control. The leaves were vacuum-infiltrated with nitrotetrazolium blue chloride (NBT) or 3, 3-diaminobenzidine (DAB), incubated overnight at 28 °C, fixed and cleared in alcoholic lacto-phenol solution, and examined for the formation of precipitates.

**Enzyme extraction and assays**

Induction of defense enzymes was assessed in the leaves of the treated and control Chinese cabbage plants. Chinese cabbage plants were sprayed with 2 mL of 0.3 mg L⁻¹ Trichokonins or 2 mL water as control solution. After 0, 1, 2, 3, or 4 days treatment, the fourth leaves were used for protein extraction. One gram of plant material was ground in a mortar and pestle with 1 g quartz sand and 3 mL of 0.5 mM Tris-HCl buffer (pH 6.8) at 4 °C, then filtered through four layers of cheesecloth, and centrifuged for 30 min at 12 000 × g at 4 °C. The protein concentration of the supernatant was determined according to the Bradford method using bovine serum albumin as standard (Bradford, 1976). Peroxidases (POD, E.C.1.11.1.7) activity was determined as described by Rathmell and Sequeira (1974). Polyphenol oxidases (PPO, E.C. 1.14.18.1) activity was assayed using Flurkey’s method (1985). Catalase (CAT, E.C. 1.11.1.6) activity was measured as described by Aebi (1984). Each treatment consisted of three replicates, and each replicate contained three Chinese cabbage plants.

**Real-time quantitative RT-PCR analysis**

To determine the effect of Trichokonins on expression of **PR-1a** gene, Chinese cabbage seedlings were sprayed with 2 mL of 0.3 mg L⁻¹ Trichokonins or 2 mL water as control solution. Each treatment consisted of three replicates (each replicate containing three Chinese cabbage plants). The fourth to fifth leaves were collected at 0, 3, 6, or 9 h after treatment, and total RNA was extracted using a plant RNA purification reagent (TaKaRa, Japan) according to the manufacturer’s instructions. The quality of the extracted RNA was tested by 1.0% agarose electrophoresis. The expression level of **PR-1a** gene was determined using reverse transcriptase (TaKaRa, Japan) in a 25-µL reaction volume. For qRT-PCR, a fragment of the **PR-1a** gene (181 bp; AF528177) was amplified with the **PR-1a**-specific primers (Luo et al., 2010). The actin gene was used as a reference gene. Real-time PCRs were carried out with 1 µL of cDNA template, 250 nM of each primers, 1 × SYBR Green PCR Master Mix (Shanghai Shinegene Molecular Biotechnology Co., Ltd, Shanghai City, China), and RNase freewater in a final volume of 25 µL. Reactions were performed on an ABI PRISM_7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR cycling parameters were set at 94 °C for 4 min, followed by 35 cycles of 94 °C for 20 s, 63 °C for 30 s, and 72 °C for 30 s. After completion of the cycling parameters, dissociation melt curve analysis (60–90 °C every 0.5 °C for 1 s) was conducted to discount the effects of primer-dimer formation and contamination.

The relative fold change was calculated according to the 2⁻DDCT method (Livak & Schmittgen, 2001). Each sample repeated three times, and a representative result was displayed for individual assays.

**Statistical analysis**

The data were analyzed by analysis of variance (ANOVA) using SPSS (version 17.0). The significance of differences was determined according to Duncan’s multiple range test (DMRT). P values < 0.05 were considered to be significant. All data were expressed as means ± standard deviation calculated from at least three replicates for one representative experiment. All experiments were repeated three times. And asterisks indicate values significantly different from controls.

**Results**

**Biocontrol effect of Trichokonins on soft rot of Chinese cabbage**

The inhibitory activity of different concentrations of Trichokonins against Chinese cabbage soft rot was analyzed. After 48 h treatment with Trichokonins, the lengths of lesions in the leaves of Chinese cabbage were significantly shorter than those of the control (Table 1). The lesion inhibition rates in Chinese cabbage dose dependently increased with Trichokonins concentration, reaching the maximum of 48.77% at 0.3 mg L⁻¹ Trichokonins. This result indicated that Trichokonins had control effect on soft rot disease in Chinese cabbage, consistent with our field experiment result (Hu et al., 2009).

**Susceptibility of Pcc to Trichokonins**

To analyze whether the disease controlling effect in vivo was due to the growth inhibition of Trichokonins against
the phytopathogen, the MIC of Trichokonins against Pcc was measured. The results indicated that the bacterium grew in all the wells on the microtiter plates with Trichokonins (0.05–500 mg L$^{-1}$). Even under the highest concentration (500 mg L$^{-1}$) of Trichokonins, the absorbance of Pcc suspension only decreased by 3.07% (Fig. 1).

**Elicitor activity of Trichokonins in Chinese cabbage**

Because of Trichokonins having little inhibition against Pcc in vitro, we suspect that Trichokonins may act as an elicitor of plant defense reactions in Chinese cabbage for protection against Pcc. The production of reactive oxygen species (e.g. O$_2^-$ and H$_2$O$_2$) in plants treated by Trichokonins was analyzed. Compared with the control plants, higher levels of H$_2$O$_2$ and O$_2^-$ were produced in the leaves of Chinese cabbage plants cultured in 0.3 mg L$^{-1}$ Trichokonins solution for 1 day (Fig. 2a and b).

**Effect of Trichokonins treatment on the activities of some pathogenesis-related enzymes in Chinese cabbage**

To gain further insight into the plant response to Trichokonins treatment, the activities of three pathogenesis-related enzymes, CAT, POD, and PPO, in Chinese cabbage were measured. While the activities of CAT, POD, and PPO in the control plant leaves treated with water remained constant, Trichokonins treatment caused a rapid and significant increase in the activities of the CAT, POD, and PPO in the treated plant leaves, which all reached the maximum levels after 1 day treatment and then fell to the levels similar to the control after 2 days treatment (Fig. 3a–c). The maximum activities of CAT, POD, and PPO in the treated leaves were, respectively, 2.4 (CAT)-, 4.5 (POD)- and 9.1 (PPO)-fold of those in the control leaves.

**Expression of PR1 gene in Chinese cabbage treated with Trichokonins**

To determine whether salicylic acid (SA) signaling pathway was involved in the Trichokonins-induced resistance against Pcc in Chinese cabbage, the expression level of PR-1a was analyzed. As shown in Fig. 4, the expression level of PR-1a gene significantly increased in Chinese cabbage after treatment with Trichokonins for 6–9 h. In contrast, there was little change in the expression level of PR-1a gene in the control plants treated with water.

**Discussion**

Recently, we found that T. pseudokoningii SMF2 can prevent the soft rot development caused by Gram-negative Pcc in Chinese cabbage under field conditions (Hu et al., 2009). In this study, we tried to study the involved mechanism and found that Trichokonins, a kind of peptaibols secreted by SMF2, induce resistance in Chinese cabbage against Pcc infection. Our results showed that, although Trichokonins had inhibitory activity against Pcc infection to Chinese cabbage in vivo, they could hardly inhibit Pcc in vitro, even at the high concentration of 500 mg L$^{-1}$. Therefore, the direct antagonistic effect of Trichokonins on Pcc may not

**Table 1. Inhibition effect of Trichokonins on Pcc infection to Chinese cabbage plants**

<table>
<thead>
<tr>
<th>Trichokonins concentrations (mg L$^{-1}$)</th>
<th>Lesion length (cm)</th>
<th>Inhibition rate (%)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>2.85 ± 0.21 a</td>
<td>–</td>
</tr>
<tr>
<td>0.15</td>
<td>2.51 ± 0.08 ab</td>
<td>11.93 ± 1.36 c</td>
</tr>
<tr>
<td>0.2</td>
<td>2.06 ± 0.15 b</td>
<td>27.72 ± 3.06 b</td>
</tr>
<tr>
<td>0.3</td>
<td>1.46 ± 0.07 c</td>
<td>48.77 ± 2.23 a</td>
</tr>
<tr>
<td>0.6</td>
<td>1.58 ± 0.14 c</td>
<td>44.56 ± 3.62 a</td>
</tr>
</tbody>
</table>

$^*$Inhibition rate = (lesion length of control Chinese cabbage - lesion length of treatment Chinese cabbage)/ lesion length of control Chinese cabbage × 100%. Values are mean of nine plants ± standard deviation of three replicates. For each row, values followed by a different lower case letter are significantly different at $P < 0.05$, according to Duncan’s multiple range test.
the main reason why Trichokonins could control soft rot of Chinese cabbage. As Trichokonins have been found to be able to induce resistance in tobacco plants against TMV previously (Luo et al., 2010), resistance induction of Trichokonins in Chinese cabbage was investigated. The results showed that Trichokonins induced significant production of reactive oxygen species. The production of reactive oxygen species has been assumed to be one of the earliest defense responses (Nanda et al., 2010; Singh et al., 2010). Therefore, Trichokonins could induce early defense responses in Chinese cabbage plants against soft rot caused by Pcc. The results also showed that the defense-related enzymes (POD, PPO, and CAT) in Chinese cabbage seedlings could be induced by Trichokonins, allowing it to be concluded that the protection of Chinese cabbage plants from Pcc is primarily caused by the activation of plant defense mechanisms. These defense-related enzymes induced in other plants by various elicitors have also been reported (Luo et al., 2010; Sun et al., 2013; Zhang et al., 2013). It seems that these enzymes respond to elicitors in a similar way, increasing to a peak and then decreasing. However, the increasing or decreasing rates of these enzymes and their highest levels in different plants are different, probably due to the difference in the responses of different plants to various elicitors.

Trichoderma might trigger biochemical and molecular changes characteristic of systemic acquired resistance, mainly associated with the expression of pathogenesis-related proteins (PRs) (Hermosa et al., 2012; Mathys et al., 2012). In the present study, we found that Trichokonins could upregulated the expression of PR-1a gene, a SA-responsive PR gene, in Chinese cabbage. This result suggests that the SA-mediated defense pathway may be involved in Trichokonins-induced resistance against
This systemic acquired resistance mechanism is similar to that of the antivirus mechanism of Trichokonins (Luo et al., 2010).

In summary, although the Trichokonins from T. pseudokoningii SMF2 could not inhibit the Gram-negative bacterium \( Pcc \) in vitro, they could induce indirect systemic acquired resistance in Chinese cabbage to control \( Pcc \) soft rot via activation of SA signaling pathway. This represents the first report that peptaibols have control effect on plant disease caused by Gram-negative bacteria. Our results imply the potential of \textit{Trichoderma} and peptaibols in controlling plant disease caused by Gram-negative bacteria.

**Acknowledgements**

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