Control of *Tobacco mosaic virus* by PopW as a Result of Induced Resistance in Tobacco Under Greenhouse and Field Conditions

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ABSTRACT

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In a previous study, we isolated a new harpin protein, PopW, from the bacterium *Ralstonia solanacearum* ZJ3721 that can induce a hypersensitive response in tobacco, *Nicotiana tabacum*, leaves. In the current study, we demonstrate that, in a greenhouse experiment, PopW induced tobacco-acquired resistance against the *Tobacco mosaic virus* (TMV) with a biocontrol efficacy of 80.9 to 97.4% at a concentration as low as 25 µg/ml in both PopW-treated and neighboring leaves. The resistance induced by PopW is systemic acquired resistance mediated by salicylic acid, which was certified by the development of resistance being

accompanied by the expression of the pathogenesis-related-1 gene (*PR1*) 8 h after PopW was sprayed onto the tobacco leaves. In addition, hydrogen peroxide began to accumulate 10 h after PopW spraying, peaking at 24 h with a maximum concentration of 1.97 µM/g fresh weight. The activities of phenylalanine ammonia lyase (EC4.3.1.5), polyphenol-oxidase (EC1.14.18.1), and peroxidase (EC1.11.1.7) also increased, peaking at different times in the PopW-treated tobacco leaves. PopW also reduced the level of TMV disease in field trials with a biocontrol efficacy of 45.2%. Furthermore, PopW both increased tobacco yield (by 30.4 more than in control plants) and improved tobacco foliar quality, with an increase of 50.2% in the number of first-class tobacco leaves from treated compared with untreated plants. All of these results indicate that the new harpin protein PopW has the potential to be an effective biocontrol agent against TMV in tobacco.

Incompatible interactions between plants and pathogens are characterized by the induction of various defense mechanisms (12). One common feature of many incompatible interactions is the development of a hypersensitive response (HR) in higher plants, which is characterized by rapid, local death of plant cells at the sites of pathogen infection (4). The rapid host cell death in infected tissues might serve directly to contain the invading pathogen. In addition, a local HR is often associated with activation of plant defense responses, including expression of a large number of defense-related genes, in the surrounding and even distal, uninfected parts of the plants, leading to the development of systemic acquired resistance (SAR) (51).

SAR is associated with local and systemic increases in endogenously synthesized salicylic acid (SA) and a coordinated expression of genes encoding pathogenesis-related (PR) proteins (19,42). SA is a necessary intermediate in the SAR signal transduction pathway because SA-non-accumulating *NahG* plants, expressing the bacterial SA hydroxylase gene *NahG*, are impaired in SAR (42,43).

Plant defense responses are initiated by the direct or indirect recognition of elicitors, microorganism-derived molecules that function as signal molecules in the plant (29–31,40). As application of a purified elicitor to plants usually mimics an attack by an avirulent pathogen, elicitors are useful in studying the molecular mechanisms of both HR and SAR (40). Harpin proteins, produced

by gram-negative plant-pathogenic bacteria, generally affect virulence in host plants and induce HR in nonhost plants; in addition, they are acidic, glycine rich, protease sensitive and heat stable (1,35,49). As well as inducing HR, several beneficial effects also are induced following application of harpin proteins to plants. For example, HrpN_{Ea} from *Erwinia amylovora* (9,11,33,47) and HrpZ_{Pss} from *Pseudomonas syringae* pv. *syringae* induce a range of resistance to various plant pathogens (38). Insect repellency occurs in HrpN_{Ea}-treated cucumber, *Cucumis sativus*, in that striped cucumber beetles, *Acalymma vittatum*, prefer to colonize control plants rather than plants treated with HrpN_{Ea} (53). Harpin-induced defenses often accompany enhanced plant growth (7,8,48,53). Moreover, treatment with HrpN_{Ea} increases the drought tolerance of *Arabidopsis thaliana* growing under conditions of water deficit (8,52). Overall, harpins have been recognized as multifunctional elicitors in plants. However, until now, a harpin with a similar function in *Ralstonia solanacearum* had not been reported.

In a previous study, we isolated a cell-wall associated, *hrpB*-dependent, 39.79 kDa new harpin, PopW, from *R. solanacearum* that is widely distributed in different *R. solanacearum* strains and can induce an HR in tobacco (*Nicotiana tabacum*) leaves (21). In the present study, we investigated the role of purified PopW in inducing plant resistance and characterized the defense-related cellular and molecular responses of plants treated with PopW and the signal pathways involved in this process.

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* The e-Xtra logo stands for “electronic extra” and indicates that Figures 1, 3, and 5 appear in color online.

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MATERIALS AND METHODS

Plant material and growth conditions. *N. tabacum* ‘Xanthi nc’ harboring the *N* gene, which confers resistance against *Tobacco mosaic virus* (TMV) (50) was cultivated at 25°C in a growth

chamber under a 12-h light/12-h dark cycle. Plants were watered on alternate days and were supplied with half-strength Hoagland's nutrient solution once a week as described elsewhere (44).

Detection of the biocontrol efficacy of PopW against TMV in tobacco. The extraction and purification of PopW was performed as described previously (21). PopW protein was sprayed onto tobacco leaves with a concentration of 25 µg/ml once the seedlings had been growing for 10 weeks. TMV was rubbed onto the tobacco leaves 2 days after the PopW treatment. The TMV-inoculated leaves included the PopW-treated leaf and the first and second leaves above and below the treated leaf. The TMV concentration was adjusted in order to induce about 200 lesions per leaf of control untreated plants. A quantified assay for PopW-induced tobacco SAR against TMV was performed according to the method of Wang et al. (46): biocontrol efficacy = [(spot number of control – spot number of treated group)/spot number of control] × 100%.

Determination of transcript levels of *PR* genes in tobacco. A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for *PR1* in tobacco was performed as described by Wang et al. (45). The *PR1* primers were designed based on published sequences (34) and deposited in the National Center for Biotechnology Information database with the accession number D90196 (<http://www.ncbi.nlm.nih.gov/>). Primer sequences are as follows: forward primer, 5'-ATGCCCATACAGCTCG-3'; and reverse primer, 5'-GAGGATCATAGTTGCAAGAG-3'. Amplification of a constitutively expressed gene, the translation elongation factor gene *EF1a*, served as an internal control in RT assays, using specific primers (5'-AGACCACCAAGTACTACTGCAC-3'; 5'-CCACCAATCTTGACACATCC-3') synthesized based on a highly conserved region of the *EF1a* cDNA sequence (GenBank accession no. AF120093) to produce a 495-bp sequence.

Survey of hydrogen peroxide accumulation in tobacco leaves. The oxidative burst, during which large quantities of reactive oxygen species (ROS) like H₂O₂, has been reported as one of the earliest and most effective defense reactions of plants. Qualitative and quantitative methods were used to examine H₂O₂ accumulation in PopW-treated tobacco leaves. 3,3'-Diaminobenzidine 4 HCl (DAB) staining was used to visualize the accumulation of H₂O₂ according to an adapted procedure from Orozco-Cárdenas and Ryan (32) and Thordal-Christensen et al. (41). Briefly, the leaves were harvested and immediately vacuum infiltrated for 20 min with a phosphate-buffered saline buffer, pH 7.4, containing 0.5% (wt/vol) DAB. The leaves were subsequently placed under light for 10 h and then boiled for 20 min in 80% ethanol. The intensity and patterns of DAB staining were assessed visually. The quantification of H₂O₂ content was performed as described by Jiang et al. (15).

Activity measurement of defense-related enzymes. Phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO) are the SAR-related enzymes in plant, and their activities are related to plant resistance, while peroxidase (POD) participates in oxidative burst. The activities of them were assayed after the tobacco leaves were sprayed with PopW. Extraction of PAL and PPO followed the methods described by Rivero et al. (36). In total, 0.5 g of leaf tissue was homogenized in 5 ml of boric acid extracting buffer (0.05 M boric acid, 5 mM mercaptoethanol, 1 mM EDTA, and 0.05 g of polyvinylpyrrolidone) in ice bath. The supernatant was collected after centrifugation at 12,000 rpm for 20 min. The method of extracting POD was similar as above, except the buffer was 0.05 M phosphate with a pH of 7.0 (25). The activity of the enzymes was assayed as described by Li et al. (23).

Biocontrol efficacy of PopW against TMV in field. The biocontrol efficacy of PopW against TMV in tobacco was conducted in a field with serious TMV disease in the recent 3 years in Fuquan (107°30'E, and 26°40'N), Guizhou Province, China

in 2009. Each plot was 120 m² (10 m × 12 m) containing 120 tobacco seedlings. There were two treatments (PopW at 25 µg/ml and the water control) with four replicates. The 2-month-old tobacco seedlings were transplanted to each plot and 4 days after transplantation, they were sprayed with either PopW (25 µg/ml) or the water control until they were soaking wet.

In all experimental plots, normal agronomic practices were applied to culture the tobacco plants without the use of any other pesticides. On the 100th day after spraying, disease index, quality, and yield were recorded. The quality of tobacco leaves was evaluated as described by Liang et al. (24). The disease severity was evaluated by visual observation and a rating scale of 0 to 4, in which 0 = no symptoms observed; 1 = light mottling and a few thin yellow veins; 2 = mottling and vein clearing unevenly distributed on the leaf; 3 = mottling, leaf distortion, and stunting; and 4 = severe mottling, leaf curling, and stunting. Disease severity and biocontrol efficacy were calculated as follows.

Disease severity = [Σ(the number of diseased plants in this index × disease index)/(total number of plants investigated × the highest disease index)] × 100%.

Biocontrol efficacy = [(disease severity of control × disease incidence of antagonist-treated group)/disease incidence of control] × 100%.

Data analysis. Analysis of variance (ANOVA) for biocontrol efficacy, TMV spot number, and tobacco yield was performed using the SAS general linear model (GLM) procedure (SAS Institute, version 6, Cary, NC). Mean comparisons were conducted using the ANOVA test and a least significant difference (LSD) test (*P* = 0.05). Standard deviations, ANOVA results, and LSD results were recorded.

RESULTS

PopW induced tobacco resistance against TMV. PopW induced a strong resistance against TMV infection in tobacco plants under greenhouse conditions (Fig. 1A and B). The average disease spot number in blank control leaves was 162.0, which was higher than in PopW-treated leaves with the average spot number of 4.3. The average disease spot number in SA-non-accumulating NahG plants was 165.3, while the number of spots on PopW-treated and on the above and under PopW-treated leaves ranged from 4.3 to 31.0 (Fig. 1A and B). Biocontrol efficacy reached 97.3% in the PopW-treated leaves, whereas it ranged from 80.9% to 94.9% in the leaves above and under the PopW-treated leaves. These results indicate that PopW was able to trigger SAR in leaves that were next to PopW-treated leaves.

PopW induced expression of *PR* genes in tobacco. *PR* genes are useful molecular markers of SAR induced by harpin treatment or infection with incompatible pathogens (6,37). Expression of *PR1* in PopW-treated tobacco leaves was assayed by semiquantitative RT-PCR, confirming the signal pathway triggered by PopW in plants. The PCR products were detected by electrophoresis and stained with ethidium bromide; the staining intensity of the *EF1a* products was used to confirm the uniform loading of RNA in the different samples, and helped to evaluate relative levels of gene expression. Transcription of *PR1* was rapidly and strongly induced in the tissue of tobacco leaves treated with PopW; transcription was visualized at 8 h after spraying and reached a peak 12 h after treatment. Finally, *PR1* expression disappeared 48 h after treatment and was not induced in the control plants (Fig. 2).

H₂O₂ bursts in tobacco leaves coordinated with SAR induced by PopW. The accumulation of ROS is characteristic of plant tissues undergoing HR (18). To examine whether there was an ROS burst in leaves treated with PopW during SAR, levels of H₂O₂ were monitored at various times after spraying with PopW. The elicitor provoked rapid H₂O₂ accumulation in the treated leaves (Fig. 3A). The first transient peak (0.99 µM/g FW)

appeared approximately 10 h after treatment, and a higher peak (1.97 $\mu\text{M/g}$ FW) appeared 24 h after treatment. HR was also visible at the infiltration sites 24 h after PopW treatment (21). By contrast, the level of H_2O_2 was consistently lower in the leaves of control plants. The accumulation of H_2O_2 was also detected in the form of a brown precipitate using the DAB staining method (Fig. 3B). Numerous irregular brown spots were visible 10 h after spraying with PopW (Fig. 3B-b) and reached a maximum 24 h after treatment (Fig. 3B-c). No brown precipitate was visible in the control plants (Fig. 3B-a). All of these results indicate that PopW can trigger the accumulation of H_2O_2 in PopW-treated tobacco leaves.

An increase in the activity of defense-related enzymes as a result of PopW treatment. PopW also influenced the activities of PAL, PPO, and POD. The activity of PPO significantly increased 2.9 folds in leaves treated with PopW compared with control leaves, reaching a peak in the former of 863.67 U/g 10 h after PopW treatment (Fig. 4A); The activity of POD in PopW-treated leaves also increased rapidly, reaching a peak of 1,110.50 U/g 24 h after PopW treatment, and keep higher level from 10 to 36 h (Fig. 4B). The activity of PAL in PopW-treated leaves was higher than in the control leaves, with maximum activity recorded 12 h after treatment (1,215.33 U/g). It declined to bottom only 106 h after treatment (Fig. 4C).

PopW can effectively control TMV and increase tobacco yield in field. Although PopW is able to induce resistance in tobacco in greenhouse experiments, it does not mean that it would be able to do so in the field. Therefore, experiments to investigate

the biocontrol efficacy of PopW against TMV disease were conducted in the field to further confirm the effect of PopW on disease control. The results showed that the application of PopW to tobacco plants effectively suppressed TMV infection in the study field up to 100 days after treatment, with the biocontrol efficacy reaching 45.2% (Table 1). Additionally, PopW also increased the yield of tobacco leaves, with a weight of 2,782.5 kg/ha which was 30.4% more than the yield of control plants. Furthermore, the color of tobacco leaves in control plants was more yellow than PopW-treated ones, and the shape of tobacco leaves in control plants was mottling, distorting, and stunting (Fig. 5). PopW also improved the quality of tobacco leaves, with an increase in the number of first-class tobacco leaves of 50.2% compared with control plants (Table 1).

Fig. 2. Kinetics of *PR* gene expression in tobacco plants treated with PopW as assessed by semiquantitative reverse transcription-polymerase chain reaction. Expression of *EF-1a* was used as an internal control. The molecular weight of *PR-1* and *EF-1a* are 360 and 495 bp, respectively. The same results were obtained from three replicates.

Fig. 1. Induction of tobacco resistance to *Tobacco mosaic virus* (TMV) by PopW. **A**, The disease spot number and **B**, symptoms of different leaves pretreated with PopW (25 $\mu\text{g/ml}$) by spraying 2 days before TMV inoculation on treated (photo 5 and bar g) and the first and second upper (photo 3, bars c and d) of lower leaves (photo 4, bars e and f) of the elicitor-treated leaves on the same plants; photo 1 and bar a are the NahG plants treated with PopW (25 $\mu\text{g/ml}$), and photo 2 and bar b are controls treated with water. The photographs were taken 4 days postinoculation. Data presented are the means of three replicates and error bars represent the standard deviation ($n = 9$).

DISCUSSION

The rapid and effective activation of disease resistance responses is essential for plant defenses against pathogen attack. These responses are initiated when pathogen-derived molecules (elicitors) are recognized by the host (26). Harpin is a group of

glycine-rich, heat-stable and protease K-sensitive proteins produced by gram-negative bacterial pathogens, including *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* spp. (22,49). In the current study, we showed that the purified harpin, PopW, previously isolated from *R. solanacearum* ZJ3721, can elicit disease resistance against TMV under greenhouse and field conditions, as

Fig. 3. H₂O₂ burst in PopW-treated leaves. **A**, H₂O₂ accumulation in the intercellular washing fluid of PopW-treated leaves. Data presented are the means of three replicates and error bars represent the standard deviation ($n = 3$). **B**, Histochemical identification of H₂O₂ by DAB staining in leaves at 10 h (b) and 24 h (c) after spraying with PopW. The stained spots are indicative of H₂O₂ accumulation in treated and control leaves (a) of tobacco.

Fig. 4. Activity of **A**, polyphenoloxidase (PPO), **B**, peroxidase (POD), and **C**, phenylalanine ammonia lyase (PAL) in tobacco leaves treated with PopW. Data presented are the means of three replicates and error bars represent the standard deviation ($n = 3$).

well as increasing the yield and improving plant quality at the same time.

The first harpin, encoded by the gene *hrpN*, was isolated from *E. amylovora*, and named harpin_{Ea} (49). It is able to induce resistance against three diseases: southern bacterial wilt of tomato, TMV, and Gliocladium leaf spot of cucumber (47). In the present study, we showed that PopW can control TMV both under greenhouse and field conditions. Li et al. (22) showed that purified *X. oryzae* pv. *oryzae* harpin, harpin_{Xoo}, could suppress the infection of TMV in tobacco with a control efficacy of 78.8%, which is more effective than harpin_{Ea} in inducing TMV resistance (64.4%). The present study demonstrated that an HR elicitor, PopW, can also activate tobacco SAR against TMV, with a control efficacy ranging from 80.9 to 97.4% in greenhouse experiments. Additionally, it also can control TMV in field trials, with a biocontrol efficacy of 45.2% (Table 1).

As well as its disease control function, we also found that PopW can increase the yield of tobacco and improve the quality of tobacco leaves, with a 50.2% increase in the number of the first-class leaves compared with control plants under field conditions (Table 1). To our knowledge, this is the first report of a harpin from *R. solanacearum* controlling plant diseases and improving plant quality. These characteristics suggest that PopW could potentially be used as a biological control agent in agricultural management.

As in many other plant species, pathogen-induced SAR is associated with local and systemic increases in endogenously

synthesized SA and a coordinated expression of genes encoding PR proteins, which are considered good molecular markers of SAR (3,10,19,37,42). SA is a necessary intermediate in the SAR signal transduction pathway, and it has various roles in different plant–pathogen interactions. Harpin was shown previously to induce the expression of *PR* genes (9). However, in other systems, SA has been shown not to be involved in hypersensitive cell death, and acquired resistance mechanisms have been demonstrated to operate independently of SA (13,16). These findings are supported by the discovery that several defense responses can be activated without increases in the levels of SA or the expression of *PR1a*, a marker gene for SA. Although PopW exhibited characteristics of inducing SAR against TMV tobacco in greenhouse experiments, we investigated the expression of *PR1* using semi-quantitative RT-PCR to determine definitively whether *PR* genes accumulated in PopW-treated tobacco. The results showed that *PR1* was detected 8 h following treatment, reaching a maximum expression level at 12 h (Fig. 3). Furthermore, we also investigated the control efficacy of PopW against TMV in NahG tobacco, and the results showed that PopW could not induce resistance to suppress TMV disease. Therefore, SAR induced by PopW in tobacco is mediated by SA and accompanied by *PR1* expression, given that SA-non-accumulating NahG plants, expressing the bacterial SA hydroxylase gene *NahG*, are impaired in SAR.

We also assessed the potential involvement of H₂O₂ in the PopW-induced defense response. ROS can serve as a second mes-

TABLE 1. Effect of PopW on tobacco disease severity, yield, and quality in the field^z

Treatments	Disease severity (%)	Biocontrol efficacy (%)	Yield (kg/ha)	Yield increase (%)	Increase of first-class tobacco leaves (%)
PopW	11.2 a	45.2 ± 2.1	2,782.5 ± 112.5 a	30.4 ± 2.4	50.2 ± 7.3
control	20.5 b	...	2,134.2 ± 71.6 b	...	...

^z Plants were sprayed with PopW (25 µg/ml) to control *Tobacco mosaic virus* (TMV) in tobacco, and control plants were treated with the same volume of sterile water. Means from 30 plants followed by different letters within a column are significantly different between treatments as determined by the least significant difference test ($P = 0.05$). Data were expressed as the average mean ± standard deviation of four replicates.

Fig. 5. Symptoms of *Tobacco mosaic virus* disease on tobacco leaves treated with **A**, water control and **B**, PopW (25 µg/ml) in a field experiment in Fuquan (107°30'E, and 26°40'N), Guizhou Province, China.

senger in defense signaling pathways (5). Exogenous application or overproduction of H₂O₂ and several prooxidants in plants leads to the induction of *PR1* expression and greater resistance to pathogens (2,17). H₂O₂ can activate pathways aimed at avoiding cellular apoptosis (27). However, in other systems, H₂O₂ has been shown not to be involved in the production of phytoalexins or as a signal regulator for cell death (14,28,39). In summary, the role of H₂O₂ during interactions between plants and pathogens or elicitors needs to be further elucidation. Our results show that PopW induced a strong H₂O₂ burst in treated leaves compared with untreated leaves: phase I peaked 10 h, and phase II peaked 24 h, after treatment. The biphasic nature of the oxidative burst is reminiscent of the oxidative burst in incompatible plant–pathogen interactions (20). DAB staining also revealed that PopW-induced H₂O₂ was not homogeneously distributed over the leaf surface, but instead was concentrated in discrete areas.

In conclusion, PopW can induce different forms of plant resistance against TMV in tobacco under greenhouse and field conditions through SA-mediated pathway. However, more work is needed to explore the functions and correlated mechanisms of PopW further to make it a suitable biological control agent for use in an agricultural setting.

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