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Overexpression of a harpin-encoding gene *popW* in tobacco enhances resistance against *Ralstonia solanacearum*

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Abstract

PopW, a harpin protein identified from *Ralstonia solanacearum*, has multiple beneficial effects in plants, promoting plant growth and development, increasing crop yield, and inducing resistance to pathogens. Tobacco plants transformed with *popW*, the PopW-encoding gene, exhibited a promoted growth rate and enhanced resistance to *Tobacco mosaic virus* (TMV). Here, it is documented that the transgenic tobacco plants overexpressing *popW* exhibited a higher resistance to *R. solanacearum* YN10 infection compared with that of the wild-type plants. In the *popW*-expressing tobacco lines, an enhanced H_2O_2 accumulation and hypersensitive reaction (HR) were activated in the inoculated site. In addition, the resistance was accompanied with increased transcripts in numbers of genes related to defense (including HR), reactive oxygen species (ROS) scavenging, and salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) production. These results suggest that *popW* acted as positive regulator in tobacco resistance against *R. solanacearum via* modulation of SA-, JA-, and ET-mediated signaling pathways. We report for the first time that the expression of a harpin-encoding gene *in vivo* improved plant resistance to *R. solanacearum*.

Additional key words: ethylene, jasmonic acid, hypersensitive reaction, *Nicotiana tabacum*, salicylic acid, transgenic plant.

Introduction

Harpins are acidic, heat-stable, glycine- and leucine-rich, and water-soluble proteins coding by *hrp* gene clusters in many gram-negative phytopathogens (Wei *et al.* 1992, He 1996, Dong *et al.* 1999, Choi *et al.* 2013). The harpins can induce a systemic acquired resistance pathway in diverse plant species acting as kind of an "immune system booster" (Liang *et al.* 2009) and contribute to disease resistance in plants by reducing bacterial growth (Dong *et al.* 1999). PopW is harpin secreted by *Ralstonia solanacearum*, one of devastating gram-negative plant pathogenic bacteria (Li *et al.* 2010). Like many other harpins, external application of PopW induces resistances to a number of plant diseases, including leaf mold caused by *Fulvia fulva* in tomato, false smut caused by *Ustilaginoidea virens* in rice (Wang *et al.* 2014a), downy mildew caused in cucumber by *Pseudoperonospora cubensis* (Zheng *et al.* 2013) as well as to *Tobacco mosaic virus* (TMV) in tobacco (Li *et al.* 2011), and also enhances growth and improves qualities of tobacco, pepper, and cucumber (Zheng *et al.* 2013, Wang *et al.* 2014a).

Except for exogenous application, transformation with harpin-encoding genes improves disease resistance to various bacterial, fungal, and viral pathogens in several plant species (Peng *et al.* 2004, Sohn *et al.* 2007, Pavli *et al.* 2011, Choi *et al.* 2012, Wang *et al.* 2014c). However, there is no research documented that an overexpression of harpin-encoding genes enhances plant resistance to *R. solanacearum*. As devastating bacterial pathogen, *R. solanacearum* infects the cortex and

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Abbreviations: DAB - diaminobenzidine; dpi - days post inoculation; E - empty vector; ET - ethylene; ETI - effector-triggered immunity; HR - hypersensitive reaction; JA - jasmonic acid; PR - pathogenesis-related; PTI - pathogen/microbe-associated molecular patterns triggered immunity; ROS - reactive oxygen species; SA - salicylic acid; TMV - *Tobacco mosaic virus*; WT - wild-type.
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colonizes the xylem in plant roots and secretes cell wall-degrading enzymes and exopolysaccharides, eventually causes bacterial wilt in plants, especially in species of *Solanaceae* and *Musaceae* families (Schell 2000, Yao and Allen 2006, Li *et al.* 2014). Bacterial wilt is one of the most common soil-borne plant diseases in both tropical and subtropical tobacco regions (Jenkins and Averrre 1983, Li *et al.* 2014). There are several symptoms of the tobacco bacterial wilt diseases which frequently causes wilting and yellowing the foliage and discoloration of vascular tissue (Genin and Boucher 2002, Li *et al.* 2014). Compared with many conventional strategies, *e.g.*, chemical and biological control and reinforcement of resistance against pathogen attack using genetic engineering, offers an alternative strategy to control bacterial tobacco wilt (Dang *et al.* 2014). For transgenic tobacco expressing *popW* (coding PopW), except for its enhanced resistance to TMV (Wang *et al.* 2014b), there is a need to testify its resistance to *R. solanacearum.*

To combat pathogens, except for preformed pre-existing barriers, plants have developed two complicated inducible layers of immunity: pathogen/microbe-associated molecular patterns triggered pathogen immunity (PTI), and effector-triggered immunity (ETI) (Jones and Dangl 2006, Dodds and Rathjen 2010). The pathogen triggered immunity commonly activates the generation of reactive oxygen species (ROS), callose deposition, induction of defense-related genes and, in some cases, cell death (Zipfel 2009). In contrast, resistance genes-mediated ETI is faster and quantitatively stronger and is typically accompanied by a hypersensitive reaction (HR) and programmed cell death to restrict pathogens to the infection site (Dodds and Rathjen 2010, Bernoux *et al.* 2011). In addition to local resistance elicited by PTI and ETI, plants can also provoke systemic resistance responses in noninoculated portions. These responses mainly consist of two categories: systemic acquired resistance, which involves salicylic acid (SA) accumulation and induction of a set of pathogenesis-related (PR) proteins together with expression of other defense genes, and induced systemic resistance, conferred by jasmonic acid (JA) and ethylene (ET) signalling (Ton *et al.* 2002, Shah 2009, Fu and Dong 2013).

Until now, several transgenic plant species (*e.g.*, tobacco, rice, sugar beet, cotton, and *Arabidopsis*) transformed with *hrp* genes with enhanced resistance to various bacterial and fungal pathogens are documented (Peng *et al.* 2004, Sohn *et al.* 2007, Shao *et al.* 2008, Miao *et al.* 2010, Pavli *et al.* 2011). Several pathogenicity-related genes, such as *PR-1a*, *PR-1b*, *PR-2*, *PR-3*, and *Chia5*, and genes related to production of ET (*e.g.*, *NT-EFE26*, *NT-1A1C*, *DS321*, *NTACS1*, and *NT-ACS2*), are up-regulated in transgenic plants expressing harpins (Peng *et al.* 2004, Sohn *et al.* 2007, Shao *et al.* 2008, Pavli *et al.* 2011). The *popW* is expressed in stable transformants of tobacco plants to investigate its endogenous effects (Wang *et al.* 2014b). Here, the aim was to show that overexpression of *popW* confers resistance to *R. solanacearum* infection in transgenic tobacco through SA and JA/ET signal pathways.

Materials and methods

Plants, pathogen strain, and growth conditions: Transgenic tobacco plants expressing *popW* constitutively (*Nicotiana tobacum* cv. Xanthi nc) were raised earlier in our lab (Wang *et al.* 2014b). They were grown for four generations and homozygous lines for *popW* were used in this study. Seeds of *popW*-overexpressing lines and wild-type (WT) and empty vector (E) control tobacco plants were sown in *Vermiculite* moistened with water. Two-week-old seedlings were transferred into 300-cm^3 pots containing sterilized (121 °C for 1 h, repeated three times) soil rich of humus and *Vermiculite* in a ratio of 3:1 (v/v) and incubated in a greenhouse at a temperature of 25 to 28 °C, a relative humidity of 60 %, a 10-h photoperiod, and an irradiance of 600 μmol(photons) m^{-2} s^{-1}. Uniform-sized tobacco plants in a 7 to 8-leaf stage were used in this study.

The challenging pathogen *R. solanacearum* YN10 was grown at 28 °C overnight in a liquid medium consisting of yeast extract 5 g, bacto-peptone 5 g, and glucose 10 g in 1 dm^3 containing further 100 mg dm^{-3} rifampicin. The cultured *R. solanacearum* YN10 cells were pelleted by centrifugation, washed once with and resuspended in 10 mM $MgCl_2$, and adjusted to 1×10^7 CFU cm^{-3} by serial dilutions.

Evaluation of resistance against *R. solanacearum* YN10 in tobacco plants: To evaluate resistance of tobacco plants against *R. solanacearum*, the *popW* transgenic lines and control plants (the WT and empty vector controls) were drenched with the *R. solanacearum* suspension (25 cm^3 for each pot). Three independent biological experiments were conducted with three replicates (each containing 12 plants) for each line. Symptom development was monitored daily, and the plants were scored for the disease index (DI) on a scale of 0 to 4 as follows: DI 0 - no visible symptoms; DI 1 - up to 25 % of leaves wilted; DI 2 - 25 to 50 % of leaves wilted; DI 3 - 50 to 75 % of leaves wilted; DI 4 - 75 to 100 % of leaves wilted (Kempe and Sequeira 1983). Disease severity was calculated by summation of all products of the number of diseased plants of the same index and the corresponding index, divided by the product of the total number of all diseased plants and the highest disease index.

Diaminobenzidine and trypan blue staining: The *R. solanacearum* suspension was syringe-infiltrated abaxially into interveinal areas of the third fully expanded tobacco leaves using 0.4 cm^3 of the inoculum at a density of 1×10^7 CFU cm^{-3}. To detect accumulation of H_2O_2, tobacco leaves were stained with 1 g dm^{-3} diaminobenzidine (DAB, pH 3.8) for 10 h at room temperature and subsequently cleared in 96 % (v/v) boiling ethanol for 20 min. Staining with DAB visualizes H_2O_2 as red-brown precipitate under a light microscope. For detection of dead cells in tobacco leaves, trypan blue staining was performed as described previously (Alvarez *et al.* 1998) with slight modifications. Detached leaves were boiled in a trypan blue staining buffer [12.5 % (m/v) phenol, 12.5 % (v/v) glycerol, 12.5 % (m/v) lactic acid, 48 % (v/v) ethanol, and 0.025 % (m/v) trypan blue] for 1 min and incubated overnight at room temperature, followed by destaining (five times) in 70 % (m/v) chloral hydrate, and then observed under a microscope.

Analysis of gene expression: Leaf samples collected at 0, 6, 12, 24, 36, and 48 h post inoculation (hpi) were frozen in liquid nitrogen and stored at -80 °C until use. Then they were ground with liquid nitrogen to a fine powder and the total RNA was extracted from 100 mg of the powder using a *TRIZOL* reagent (*Invitrogen*, Carlsbad, CA, USA). The total RNA was treated with RNase-free DNase (*Takara*, Dalian, China) to remove the genomic DNA, and the concentration of the total RNA was determined using a *Nanodrop1000* (*Thermo Scientific*, Wilmington, DE, USA). Reverse transcription of the RNA was performed with 1 mg of the total RNA using *M-MLV* reverse transcriptase (*Invitrogen*) according to the manufacturer's protocol. Primers used are listed in Table 1 Suppl. Real-time quantitative RT-PCR was performed on a *7500* real-time PCR system (*Applied Biosystems*, Foster City, CA, USA) using *SYBR Premix Ex Taq*™ (*TaKaRa*). According to the manufacturer's protocol, 1.5 mm^3 of the cDNA, 0.4 mm^3 of the PCR forward/reverse primer (10 mM), 10 mm^3 of 2× *SYBR Premix Ex Taq*™, and 0.4 mm^3 of *ROX Reference Dye II* (50×) were suspended in a final volume of 20 mm^3 with double distilled H_2O. A thermal cycling program consisted of an initial polymerase activation step at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and at 60 °C for 35 s. The relative expressions of target genes were normalized to the expression of *NtEF1α*, a housekeeping gene coding elongation factor 1-α in tobacco usually used in RT-PCR analysis (Dang *et al.* 2014).The transcript expression of the selected related genes was quantified based on C_T values using the $\Delta\Delta_{CT}$ method.

Statistical analysis: Data were analyzed by analysis of variance (*ANOVA*), and the differences in these data among treatments were analyzed for significance using the statistical software *Data Processing System* (*DPS v. 7.05*).

Results

To determine the resistance of tobacco plants to *R. solanacearum*, the roots of the T3 transgenic lines and control plants at the 7- to 8-leaf stage were inoculated by drenching with the strain *R. solanacearum* YN10. Wilt symptoms appeared on the WT plants but not on the transgenic ones at 8 d post inoculation (dpi). For the transgenic plants, there was an obvious delay in symptom appearance up to 6 d (Fig. 1). Disease severity data counted at 16 dpi showed that 18 *popW* transgenic lines had a greater disease resistance compared with the control plants. Moreover, especially lines 1-1-2 and 34-1-4 exhibited a significantly lower disease severity than the control plants. Surprisingly, the increased resistance of 1-1-2 reached 81.32 % in relation to empty vector controls (Fig. 2). The wild type and empty vector controls showed whole-plant wilting symptoms (Fig. 3) with a disease severity of 0.96 and 0.98, respectively (Fig. 1) at 20 dpi, whereas slight wilting symptoms were observed in some *popW* transgenic lines (Fig. 3). Among these transgenic lines, the disease severity of the 1-1-2 and 34-1-4 plants were almost a half (0.56 and 0.62) of those in the WT plants (0.96) at 20 dpi. Considering the good resistance to *R. solanacearum* in the transgenic lines 1-1-2, 16-1-1, and 34-1-4, these lines were chosen for further detailed study.

To determine a relationship between *popW* overexpression, H_2O_2 accumulation and HR in tobacco leaves infected with *R. solanacearum*, we tested H_2O_2 production by staining with DAB, and HR-like cell death response by staining with trypan blue at 24 hpi. There was almost no H_2O_2 accumulation detected in the leaves of the control plants, whereas that in the leaves of the *popW* transgenic plants was obviously observed (Fig. 4). Moreover, lesions were apparent upon trypan blue staining in the leaves of the *popW* transgenic plants in contrast to the control ones (Fig. 4). These results indicate that *popW* overexpression increased H_2O_2 accumulation and HR cell death.

To elucidate the molecular mechanism of disease resistance, the quantitative RT-PCR experiments were conducted to compare transcription of 12 known disease resistance-related genes between the control and transgenic lines using specific primers (Table 1 Suppl.). We compared genes encoding proteins that are involved in plant defense. *NtHSR515, NtHIN1*, and *NtHSR203* are genes relative with HR in tobacco (Sohn *et al.* 2007, Pontier *et al.* 1998); *NtPRQ* is pathogenesis-related protein Q (Sonnewald *et al.* 2012); *NtPR2*, *NtPR3*, and

NtNPR1 are the SA-responsive genes (Ward *et al.* 1991, Zhang *et al.* 2011b); *NtPR1b* and *NtPR4* are JA-responsive genes (Shi *et al.* 2014, Sohn *et al.* 2007); *NtACS6* is ET production-associated gene (Chen *et al.* 2003); and *NtGST1* and *NtCAT1* are included in ROS detoxification (Takahashi and Nagata 1992, Takahashi *et al.* 1997) Each of the tested tobacco genes were shown previously to be transcriptionally modified in response to pathogen infection (Pontier *et al.* 1998, Zhang *et al.* 2011, Sonnewald *et al.* 2012, Dang *et al.* 2014).

Before *R. solanacearum* infection, the transcripts of all the 12 disease resistance-related genes except *NtGST1* accumulated more in the transgenic plants compared with the WT plants indicating that these genes were clearly (directly or indirectly) promoted by the overexpression of *popW*.

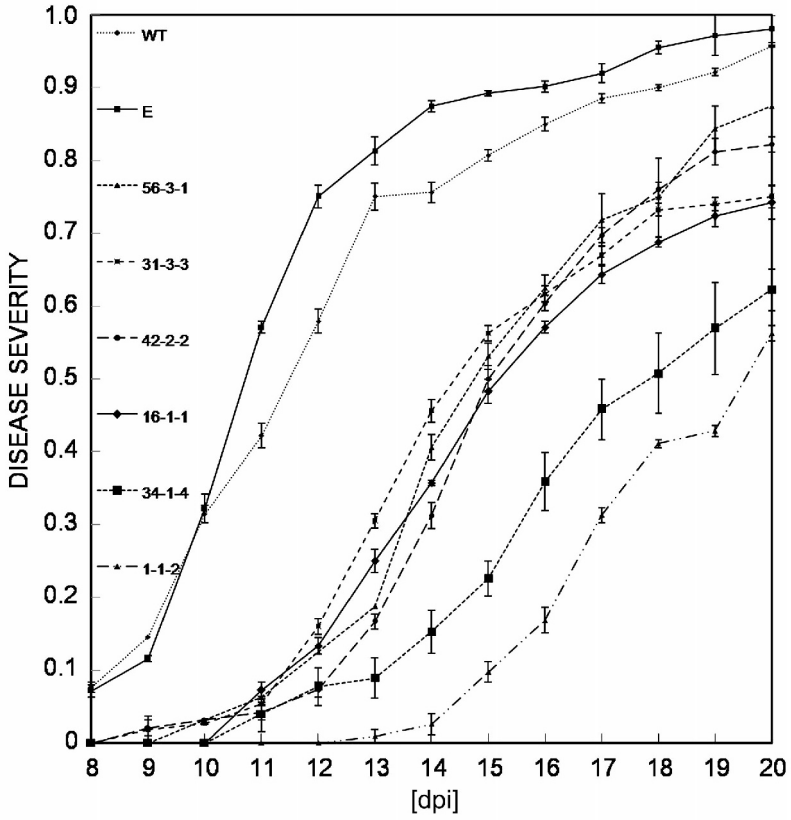

Fig. 1. Disease severity trends in transgenic tobacco plants after inoculation with *Ralstonia solanacearum* YN10. WT - wild-type plants; E - empty vector plants; others are *popW* transgenic lines; dpi - days post inoculation. Means ± SD, $n = 3$.

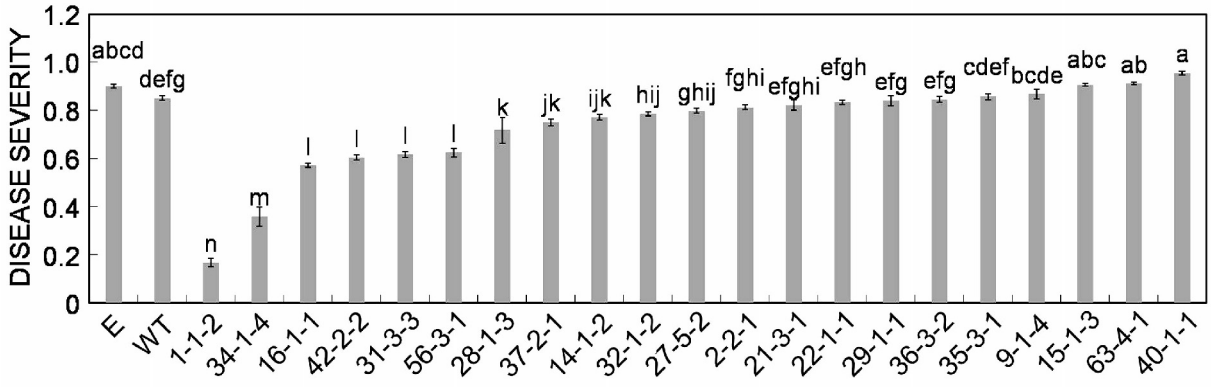

Fig. 2. Disease severity of transgenic tobacco plants at 16 dpi with *Ralstonia solanacearum*. WT - wild-type plants; E - empty vector plants; others are *popW* transgenic lines. Means ± SD, $n = 3$; different letters indicate significant differences ($P < 0.05$) between individual columns.

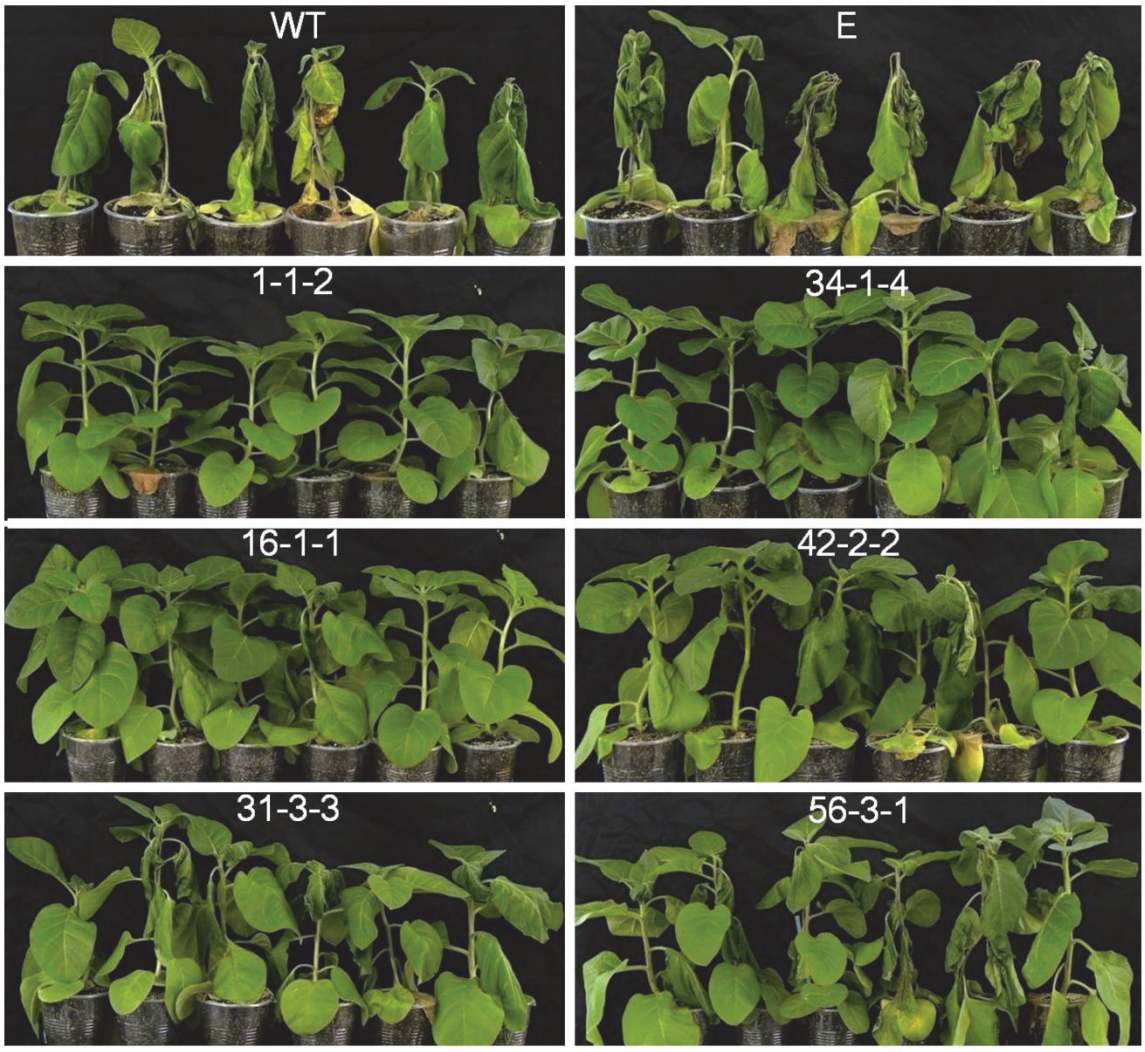

Fig. 3. Phenotypes of tobacco transgenic lines infected with *Ralstonia solanacearum* (photographed at 20 dpi). WT - wild-type plants; E - empty vector plants; others are *popW* transgenic lines.

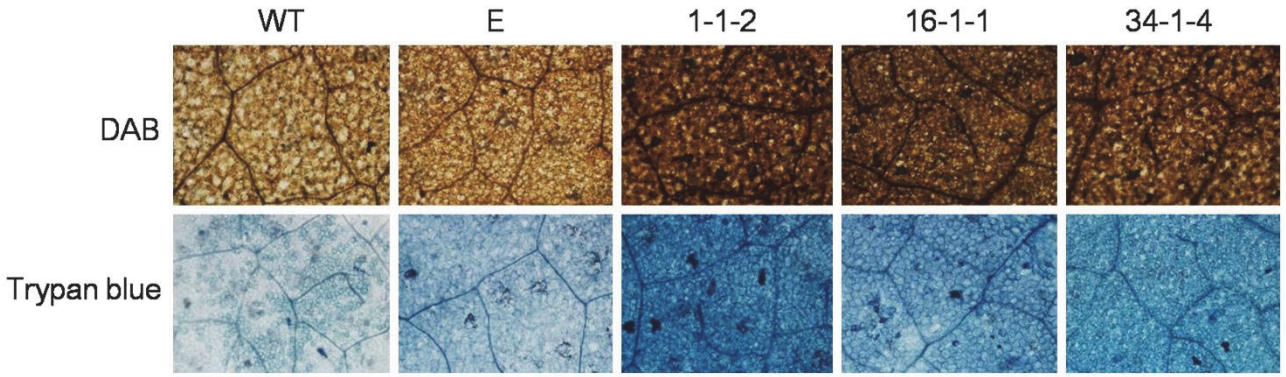

Fig. 4. Detection of H_2O_2 production (stained with diaminobenzidine) and cell death (stained with trypan blue) in transgenic and non-transgenic tobacco leaves 24 h after inoculation with *Ralstonia solanacearum*. WT - wild-type plants; E - empty vector plants; others are *popW* transgenic lines. Each treatment was replicated three times independently.

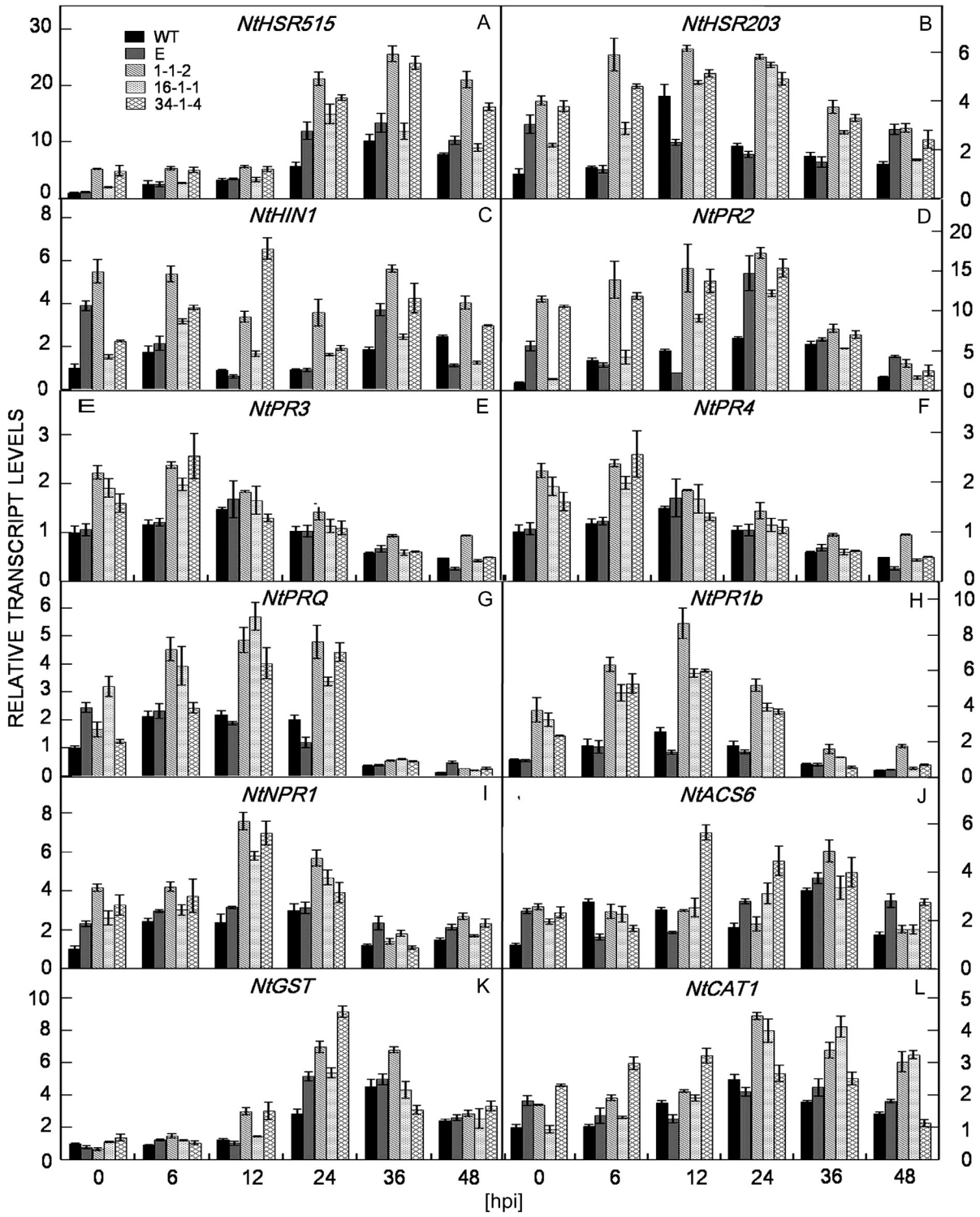

Fig. 5. Relative transcription of tobacco defense-related genes in transgenic and non-transgenic tobacco plants 0, 6, 12, 24, 36, and 48 h post inoculation (hpi) with *Ralstonia solanacearum*. The relative transcriptions were normalized using the transcription of *NtEF1α.* WT - wild-type plants; E - empty vector plants; others are *popW* transgenic lines. Means ± SD, $n = 3$.

The transcription of most of these 12 genes increased in the *popW* transgenic lines in response to *R. solanacearum* infection. Only *NtHIN1* did not exhibit a different transcription before and after *R. solanacearum*

infection, but in the control, its transcription remained lower over the entire time course (Fig. 5*C*). The others exhibited an increased transcription with peaks at different time points following *R. solanacearum* infection, and then their transcription decreased. Besides, their transcripts accumulated to higher levels in the transgenic plants compared with the WT plants. Therefore, *popW* overexpression in the transgenic tobacco plants affected the transcriptional response of most of the tested tobacco genes in response to *R. solanacearum* infection.

Discussion

The harpin PopW has been identified in *R. solanacearum* strain ZJ3721 and demonstrated with multiple beneficial effects when exogenously employed on plants (Li *et al.* 2010, 2011, Zheng *et al.* 2013, Wang *et al.* 2014a). Furthermore, we have previously transferred the PopW-coding gene *popW* into tobacco, and the transgenic plants exhibit an enhanced resistance to the TMV (Wang *et al.* 2014b). The results of the present study show for the first time that the transgenic expression of *popW* elicited a general defense response which resulted in protection against bacterial wilt, a serious disease of tobacco caused by *R. solanacearum*.

Phytopathogen defense responses involve multiple mechanisms that include a rapid production of the ROS and induction of the so-called HR (Lumbreras *et al.* 2010). The oxidative burst which consists of superoxide, H_2O_2, and hydroxyl radicals always triggers plant defense signal transduction pathways, and may have directly antimicrobial effects (Flores-Cruz and Allen 2009). Here, in the leaves of *R. solanacearum*-resistant transgenic lines 1-1-2, 16-1-1, and 34-1-4, we observed a remarkable H_2O_2 accumulation compared with the control (Fig. 4). Otherwise, localized cell-death induced by HR at the infection site could prevent further spreading bacterial pathogens proliferated within living tissues (Heath 2000, Greenberg and Yao 2004). As expected, trypan blue staining also indicated a more serious HR cell death in the inoculated leaves of the transgenic plants (Fig. 4). We speculate that a remarkable H_2O_2 accumulation and HR cell death elicited by the transgenic expression of *popW* restricted the proliferation of *R. solanacearum*, and this restriction contributed to the resistance of the transgenic plants to *R. solanacearum* at least to some extent. A similar phenomenon has been documented in *CaWRKY27* transgenic tobacco plants associated with resistance to *R. solanacearum* infection (Dang *et al.* 2014).

Many secondary signal molecules, including SA, ET, and JA, produced during plant-pathogen interactions initiate various defense responses in plants (Dong 1998, Feys and Parker 2000, Xiao and Chye 2011). Here, compared with the wild-type tobacco plants, the *popW* transgenic lines significantly increased their resistance to *R. solanacearum* attack and induced the expression of defense pathway-related genes (*e.g.*, *NtPR2*, *NtPR3*, *NtPR4*, *NtPRQ*, *NtPR1b*, *NtNPR1*, and *NtACS6*). Besides, the expression of stress-related genes (*e.g.*, *NtHSR515*, *NtHIN1*, *NtHSR203*, *NtGST1*, and *NtCAT1*) was activated (Fig. 5). An increased expression of HR-associated genes in the *popW* transgenic plants was confirmed by trypan blue staining in which the transgenic leaves had more dark blue zones at 24 hpi compared with those in the leaves of the wild-type plants (Fig. 4). Cell death caused by the HR was previously found to be coupled with the accumulation of H_2O_2 (Torres *et al.* 2006). Consistently, DAB staining indicated a correlation between HR and H_2O_2 accumulation in the *popW* transgenic lines inoculated with *R. solanacearum* (Fig. 4). These data suggest that *popW* may act as positive regulator in tobacco plants in response to *R. solanacearum* infection directly or indirectly activating SA-, JA-, and ET-signalling pathways, and the positive regulation was correlated with the H_2O_2 burst and HR.

Plant defense responses against biotrophic pathogens are generally regulated by SA, whereas the responses to necrotrophs are mediated by JA and ET (Farmer *et al.* 2003, Vlot *et al.* 2009). The SA, JA, and ET signals were included in the resistance to necrotrophic *R. solanacearum* in the *popW* transgenic plants, as shown by the increased transcripts of defense-associated marker genes (Fig. 5). SA, JA, and ET have been shown to activate different sets of plant *PR* genes and to act either synergistically or antagonistically during defense signalling (Koornneef and Pieterse 2008, Leon-Reyes *et al.* 2010), which may account for that intriguing finding in the *popW* transgenic tobacco. It has been postulated that NPR1 act as key transcriptional regulator in plant defense responses involving multiple signalling pathways (Peng *et al.* 2004); we also found an up-regulation of *NtNPR1* in the *popW* transgenic tobacco. The *popW* transgenic tobacco has also been shown resistant to the TMV (Wang *et al.* 2014b), a biotrophic virus. Thus, we speculate that the *popW* transgenic tobacco would show an enhanced resistance to both biotrophic and necro-trophic pathogens.

Herein, we present the first set of data showing a remarkably enhanced resistance to *R. solanacearum* in the harpin-encoding gene overexpressing tobacco plants. The defense response and transcriptional expression of multi-defense genes were remarkably altered in the *popW* transgenic tobacco plants, and our results are consistent with a role for the harpin-encoding gene as advantageous elicitor in response to biotic and abiotic stresses (Peng *et al.* 2004, Miao *et al.* 2010, Zhang *et al.* 2011a, Fu *et al.*

2014). Though the *popW* gene was demonstrated as successfully expressed and showed different levels in the transgenic tobacco lines (Fig. 1 Suppl.), the action site of PopW in tobacco need to be elucidated in future studies.

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