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NtPR1a regulates resistance to *Ralstonia solanacearum* in *Nicotiana tabacum* via activating the defense-related genes

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ABSTRACT

Pathogenesis-related proteins (PRs) are associated with the development of systemic acquired resistance (SAR) against further infection enforced by fungi, bacteria and viruses. PR1a is the first PR-1 member that could be purified and characterized. Previous studies have reported its role in plants' resistance system against oomycete pathogens. However, the role of *PR1a* in Solanaceae plants against the bacterial wilt pathogen *Ralstonia solanacearum* remains unclear. To assess roles of *NtPR1a* in tobacco responding to *R. solanacearum*, we performed overexpression experiments in Yunyan 87 plants (a susceptible tobacco cultivar). The results illuminated that overexpression of *NtPR1a* contributed to improving resistance to *R. solanacearum* in tobacco Yunyan 87. Specifically speaking, *NtPR1a* gene could be induced by exogenous hormones like salicylic acid (SA) and pathogenic bacteria *R. Solanacearum*. Moreover, *NtPR1a*-overexpressing tobacco significantly reduced multiple of *R. solanacearum* and inhibited the development of disease symptoms compared with wild-type plants. Importantly, overexpression of *NtPR1a* activated a series of defense-related genes expression, including the hypersensitive response (HR)-associated genes *NtHSR201* and *NtHIN1*, SA-, JA- and ET-associated genes *NtPR2*, *NtCHN50*, *NtPR1b*, *NtEFE26*, and *Ntacc oxidase*, and detoxification-associated gene *NtGST*1. In summary, our results suggested that *NtPR1a*-enhanced tobacco resistance to *R. solanacearum* may be mainly dependent on activation of the defense-related genes.

1. Introduction

In environment, plants are frequently exposed by a series of abiotic and biotic stresses and have evolved a variety of mechanisms to resist them. Some specific proteins would be produced when plants change their genes rapidly in response to different stresses. Pathogenesis-related proteins (PRs) are coded by the host plant but induced only in pathological or related situations [1]. Not only do PRs accumulate locally in the infected part, but also they could be induced systemically, which develop systemic acquired resistance (SAR).

PRs have been classified into 17 families, contained: four families of chitinases (PR-3, -4, -8 and -11), one β-1,3-glucanases (PR-2), one proteinase inhibitors (PR-6), and one specific peroxidase (PR-9), as well as the PR-1 family with unknown biochemical properties, the thaumatin-like PR-5 family, and the birch allergen Betv1-related PR-10 family [2–4]. PR1a is the first PR-1 member that is purified and characterized [5]. *PR1a*-overexpressing plants exhibited increased tolerance to the oomycete pathogens *Phytophthora parasitica* var. *nicotianae* and *Peronospora tabacina* [6]. However, whether overexpression of *PR-1a* in tobacco enhanced resistance to bacteria pathogens *R. solanacearum* remains unclear.

The bacterial wilt caused by the soil-borne plant pathogenic bacterium *R. solanacearum* is among the most devastating bacterial diseases [7]. There are many managements such as chemical pesticides, biological agents, agricultural practices, and cultivating resistant varieties used to control this bacterial disease. However, the effects are not satisfactory in the field until now. Making use of resistance-related genes to control disease is an environmental-friendly and effective way. In this study, we explored the role of the tobacco (*Nicotiana tabacum*) pathogenesis-related protein 1a gene (*NtPR1a*) responded to *R. solanacearum*, overexpressed *NtPR1a* in susceptible tobacco plants enhancing resistance to the bacterial pathogens *R. solanacearum*. The results provide some evidence for future control measures and resistance breeding.

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2. Materials and methods

2.1. Plant materials

N. tabacum (cultivar: Yunyan 87) was used in this study as wild type (WT). The seeds of the cultivar were surface sterilized with 75% alcohol for 30 s, 10% H_2O_2 for 10 min, washed five times with sterile ddH_2O, then placed on MS medium for 2–3 weeks [8,9]. The sterile plants were prepared for transgenic plants. Regenerated shoots and healthy resistant shoots were grown on selective shooting medium and rooting medium, respectively, which both contained 100 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin. The transgenic plants used for this study were obtained by asexual reproduction. Well-developed rooted plants were transferred to soil and then grown in a growth room at 25 ± 2 °C, with 70% relative humidity, and 16/8 h photoperiod.

2.2. Application of plant hormones

Three plant hormones, salicylic acid (SA), methyl jasmonate (MeJA), and ethephon (ET), were selected for evaluating the response of *NtPR1a*. In detail, tobacco plants with four to five true leaves were sprayed with 2 mM SA, 0.1 mM MeJA, and 7 mM ET (SA was dissolved in 10% ethanol, and the others in sterile ddH_2O). Control plants were sprayed with 10% ethanol and sterile ddH_2O, individually [10]. The leaves of treated plants were harvested at the indicated timepoints for preparation of RNA.

2.3. Pathogens and inoculation procedures

R. solanacearum strain CQPS-1 was used as highly virulent pathogen [11], and was cultured at 180 rmp, 30 ± 1 °C in B medium [12]. On the one hand, for monitoring bacterial growth in leaf tissues and evaluating relative *NtPR1a* expression levels in response to *R. solanacearum* infections, infiltrated inoculation was used according to Tang et al. [10]. On the other hand, soil drenching was used for pathogenicity assays as previously described [11].

2.4. DNA preparation, RNA extraction, and cDNA synthesis

Genomic DNA of tobacco leaves was extracted by Plant Genomic DNA Kit (Tiangen). The leaves from different treatment (appr. 0.1 g) were frozen in liquid nitrogen and ground into powder using mortars. Total RNA was extracted by TRNzol reagent (Tiangen). Then RNA samples were reverse transcribed with iScript™ cDNA Synthesis Kit (BIO-RAD).

2.5. Construction of plant expression vector and Agrobacterium transformation

The full-length cDNA of *NtPR1a* was cloned by the primers with specific restriction sites (*Bgl*II and *Bst*EII). The primers used in this study were listed in Supplementary Table S1. The plant expression vector pVCT2024 was used in this study. The *NtPR1a* cDNA was inserted into the *Bgl*II and *Bst*EII of the vector pVCT2024, under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The positive recombinant plasmid, pVCT2024-*NtPR1a*, was transformed into *Agrobacterium tumefaciens* strain EHA 105. Leaf discs of *N. tabacum* cv. Yunyan 87 were transformed with the pVCT2024-*NtPR1a*-containing *A. tumefaciens* strain EHA 105, as described by Oh *et al* [13]. Transgenic plants were selected with kanamycin (100 mg L^{-1}) and further confirmed by *NtPR1a* specific primer PCR and quantitative real-time PCR (qRT-PCR), respectively.

2.6. Histochemical staining

The leaves of WT and *NtPR1a*-overexpressing (*NtPR1a-OE*) plants before and 1 days after inoculating *R. solanacearum* were stained with 3, 3′- diaminobenzidine (DAB, 1 mg ml^{-1}) or nitro blue tetrazolium (NBT, 0.1 mg ml^{-1}) at 25 °C in the dark for 24 and 6 h, respectively [14]. After destaining, the leaves were soaked and preserved in fresh ethanol at room temperature and imaged.

2.7. Gene expression analysis by qRT-PCR

The relative transcript levels of various genes were evaluated by qRT-PCR with SsoFast™ EvaGreen® Supermix (BIO-RAD) and the CFX96™ real-time system (BIO-RAD). The primers were listed in Supplementary Table S1, and *NtUBI3* was used as the reference gene. The amplifications were performed as previously described [14]. The expression levels were analyzed using the ΔΔCq method.

3. Results

3.1. The expression of NtPR1a responding to R. solanacearum and exogenous plant hormones

To investigate if *NtPR1a* can be affected under *R. solanacearum*, the transcriptional profile of *NtPR1a* was detected by qRT-PCR during the compatible interaction with strain CQPS-1. As shown in Fig. 1A, the expression of *NtPR1a* was enhanced after inoculation of *R. solanacearum*, which reached the maximum at 3 days post inoculation (dpi), almost 79.2-fold compared with 0 dpi.

Since plant hormones play crucial roles in the regulation of the expression of defense genes and the reactions against biotic and abiotic stresses [15], the expressions of *NtPR1a* were examined by using qRT-PCR after treated with exogenous SA, ET, and MeJA. The results showed that the relative expression levels of *NtPR1a* increased after applying exogenous SA, reaching maximal levels at 6 hpt (approximately 191.9-fold relative to control plants, Fig. 1B). In addition, ET application strongly induced the expression levels of *NtPR1a* at 3 hpi (54.5-fold relative to control plants), and then gradually decreased (Fig. 1C). However, the expression level of *NtPR1a* was reduced at 3 hpi, and then only increased approximately 4-fold within 6–12 hpi (3.8-fold and 3.4-fold, respectively, see Fig. 1D), under MeJA treatment.

3.2. Overexpression of NtPR1a enhances tobacco disease resistance to R. solanacearum

To evaluate the effects of *NtPR1a* gene on *R. solanacearum* infection, the tobacco *NtPR1a* cDNA, under control of the 35S promoter, was transformed into tobacco plants (cv. Yunyan 87) (Fig. 2A). From all transgenic lines of overexpression-*NtPR1a*, three lines (OE3, OE5, and OE6), which showed high levels of *NtPR1a* transcripts (Fig. 2B), were selected. The tested lines were verified by PCR amplification using gene-specific primers (*NtPR1a*^{B}) (Supplementary Table S1, Fig. 2C). There were not any morphological differences observed between *NtPR1a-OE* lines and WT plants.

To assess its role in tobacco resistance against *R. solanacearum* infection, a highly virulent strain CQPS-1 was used to inoculate the *NtPR1a-OE* lines and WT plants. From the result of soil drenching, *NtPR1a-OE* lines showed more severely wilting symptoms and had lower disease incidence than WT plants (Fig. 2D and E). For monitoring the dynamic change of bacterial number, the colony-forming units (cfu) of the pathogen at 1, 3, and 5 dpi were measured after inoculating *R. solanacearum*. The results showed that the bacterial growth was significantly reduced in *NtPR1a-OE* lines than in WT plants (Fig. 2F).

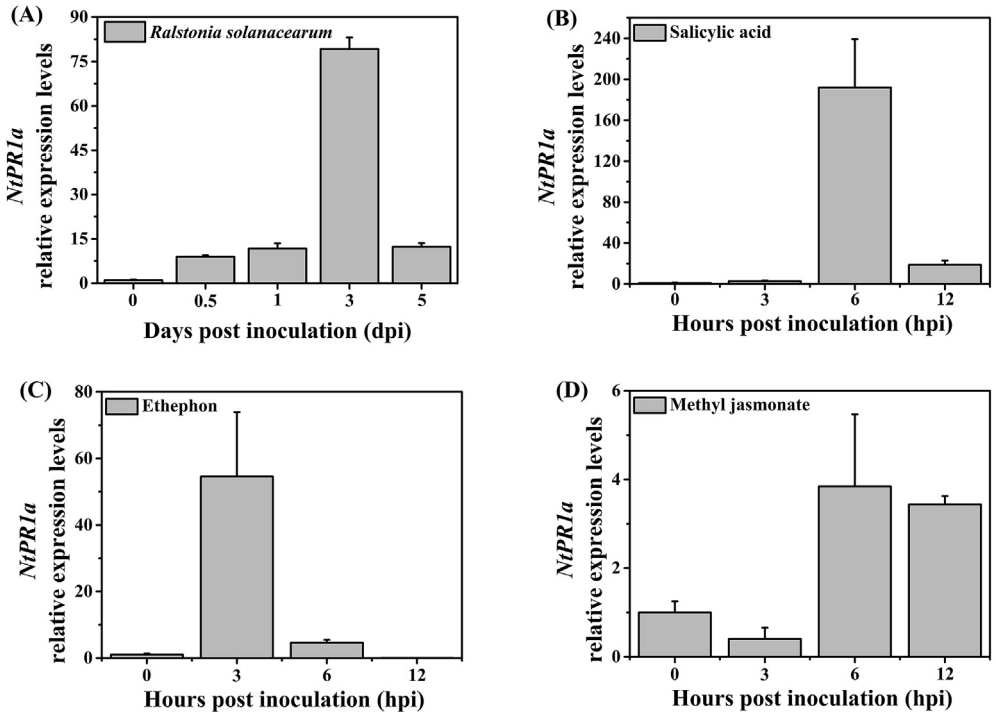

Fig. 1. The expression pattern of *NtPR1a* in tobacco wild-type plants response to *R. solanacearum* (A) and exogenous hormones (B: salicylic acid, C: methyl jasmonate, D: ethephon).

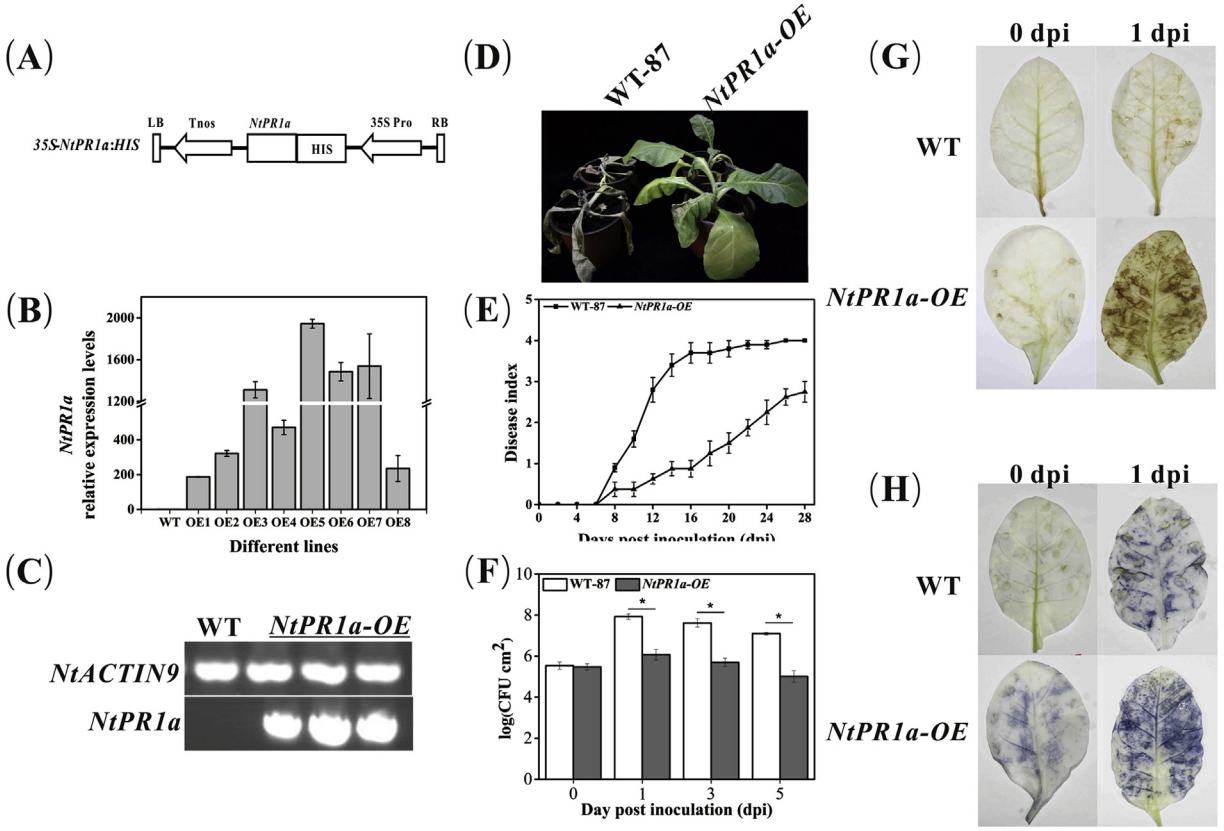

Fig. 2. Overexpression of *NtPR1a* enhances tobacco resistance to *R. solanacearum*. (A) Schematic diagram of the 35S::NtPR1a-HIS fusion protein construct. (B) Relative transcripts of *NtPR1a* in *NtPR1a-OE* lines and wild-type Yunyan87 (WT-87) plants tested by RT-qPCR. (C) PCR analysis of *NtPR1a* gene in *NtPR1a-OE* line and WT-87 plant. (D–E) The symptoms in 20 days and disease index of the *NtPR1a-OE* lines and WT-87 plants after irrigating by *R. solanacearum* strain CQPS-1. (F) *R. solanacearum* growth assay. (G–H) Histochemical staining: DAB and NBT staining, respectively. Error bars indicate the standard error.

3.3. Overexpression of NtPR1a increases the accumulation of H_2O_2 and O_2^- production

H_2O_2 and O_2^- productions, a part of reactive oxygen species (ROS), are associated with the development of hypersensitive response (HR). DAB staining and NBT staining were used to detect the accumulation of H_2O_2 and O_2^- production, individually [16,17]. From the results of histochemical staining by DAB (Fig. 2G) and NBT (Fig. 2H), we know that *NtPR1a*-overexpressing plants had more accumulation of H_2O_2 and O_2^- production after inoculating *R. solanacearum* compared with WT plants.

3.4. Overexpression of NtPR1a activates the expression of defense-related genes

We next investigated transcriptional responses of other known defense-related genes in the *NtPR1a*-overexpression plants by qRT-PCR. The transcript levels of known defense genes were examined, such as the HR-associated genes *NtHSR201* and *NtHIN1*, SA-responsive genes *NtPR2* and *NtCHN50*, JA-responsive *NtPR1b*, ET production-associated genes *NtEFE26* and *Ntacc oxidase*, and ROS detoxification-associated genes *NtGST1* and *NtCAT1* [18–23]. The relative differences of defense-related genes were normalized to the reference gene *NtUBI3* [24].

Before infecting pathogen (Fig. 3), the relatively expression levels of *NtHSR201*, *NtPR2*, *NtPR1b*, *NtEFE26*, and *NtGST1* in *NtPR1a*-overexpression plants were significantly increased compared with WT plants ($p < 0.01$). *NtHIN1*, *NtCHN50*, and *Ntacc oxidase* in the over-expressed plants were also increased relative to those of WT plants ($p < 0.05$). However, there was no significant difference of *NtCAT1* in the over-expressed and WT plants. After one day post of *R. solanacearum* inoculation (Fig. 4), similar to the untreated expression pattern, except for *NtCAT1*, the expression pattern of other defense genes in *NtPR1a-OE* leaves was still higher than in WT plants, in which the transcriptional levels of *NtPR2* and *NtCHN50* exhibited significantly higher. However, *NtHIN1*, *NtPR2*, *NtCHN50*, *NtEFE26*, *Ntacc oxidase*, *NtGST1*, and *NtPR1b* were reduced compared with WT plants at 3 dpi, and no significant difference was found.

4. Discussion

According to previous studies, the members of PR-1 were well known for the of anti-fungal and -oomycetes activities [4,25,26]. The PR-1 family had many members, and PR1a was one of three acidic PR-1 proteins [2,4]. In this study, we explored the function of *NtPR1a* during the interaction of tobacco and *R. solanacearum*. The results showed that overexpression of *NtPR1a* in the *N. tabacum* remarkably induced the resistance of plant against bacterial wilt.

In the 1970s, PR-1 proteins were first described in tobacco infected by tobacco mosaic virus (TMV) [27]. Then, several studies showed that overexpression of PR-1 in plants increased the resistance to fungi, oomycetes, and bacteria [6,26,28,29]. Our data supplemented the effect of the PR-1 member (*NtPR1a*) on bacteria. The functions of PR-1 protein have always been focused. There were researches demonstrated that PR-1 proteins had antifungal activity and could inhibited the growth of the zoospore germination [25,30], which may be attributed to the sterol-binding activity of PR-1 proteins [31]. However, this was not the only explanation for the function of PR-1 proteins [32]. On the one hand, PR-1 proteins embedded a C-terminal peptide, which involved in plant immune signaling [32–34]. On the other hand, PR-1 proteins could interact with some pathogen effectors directly, for example, with the effector ToxA and Tox3 [32,35,36]. As a member of PR-1, the role of NtPR1a in plants responding to *R. solanacearum* is still unclear. From our data, we knew that overexpression of *NtPR1a* would reduce the number of *R. solanacearum* clones in plants, increase the ROS of plants, and induce the expression of defense-related genes.

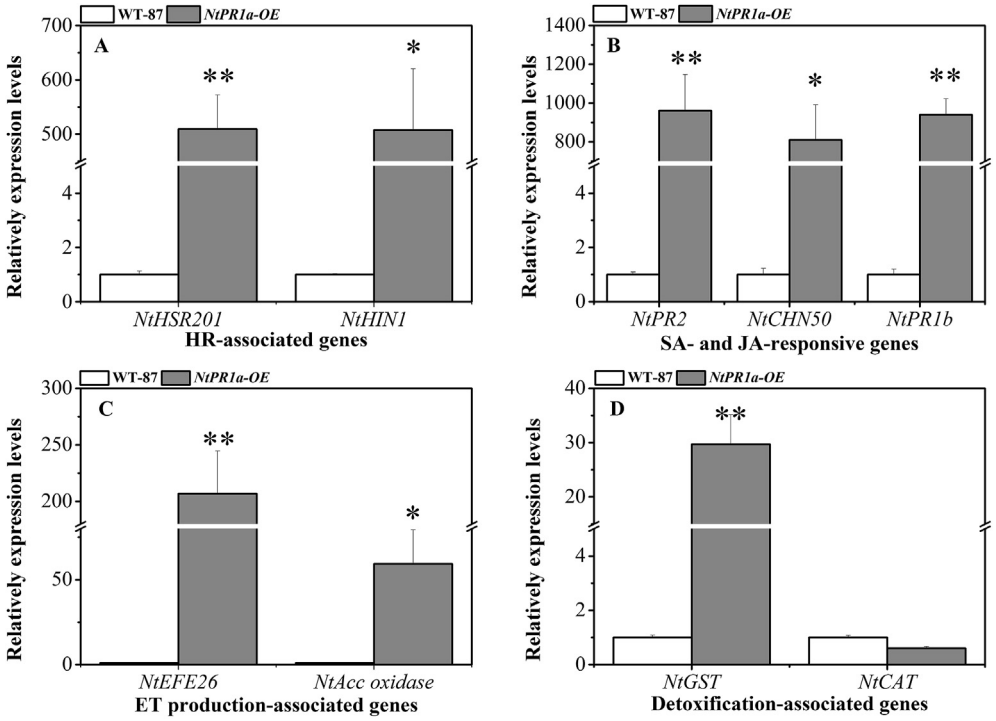

Fig. 3. The transcript levels of defense marker genes in *NtPR1a-OE* plants and WT-Yunyan87 (WT-87) plants. Defense-related gene transcript levels in WT-Yunyan87 were used as controls. Error bars indicate the standard error, values were based on at least three independent replicates. Asterisks indicate a significant difference ($^*p < 0.05$; $^{**}p < 0.01$).

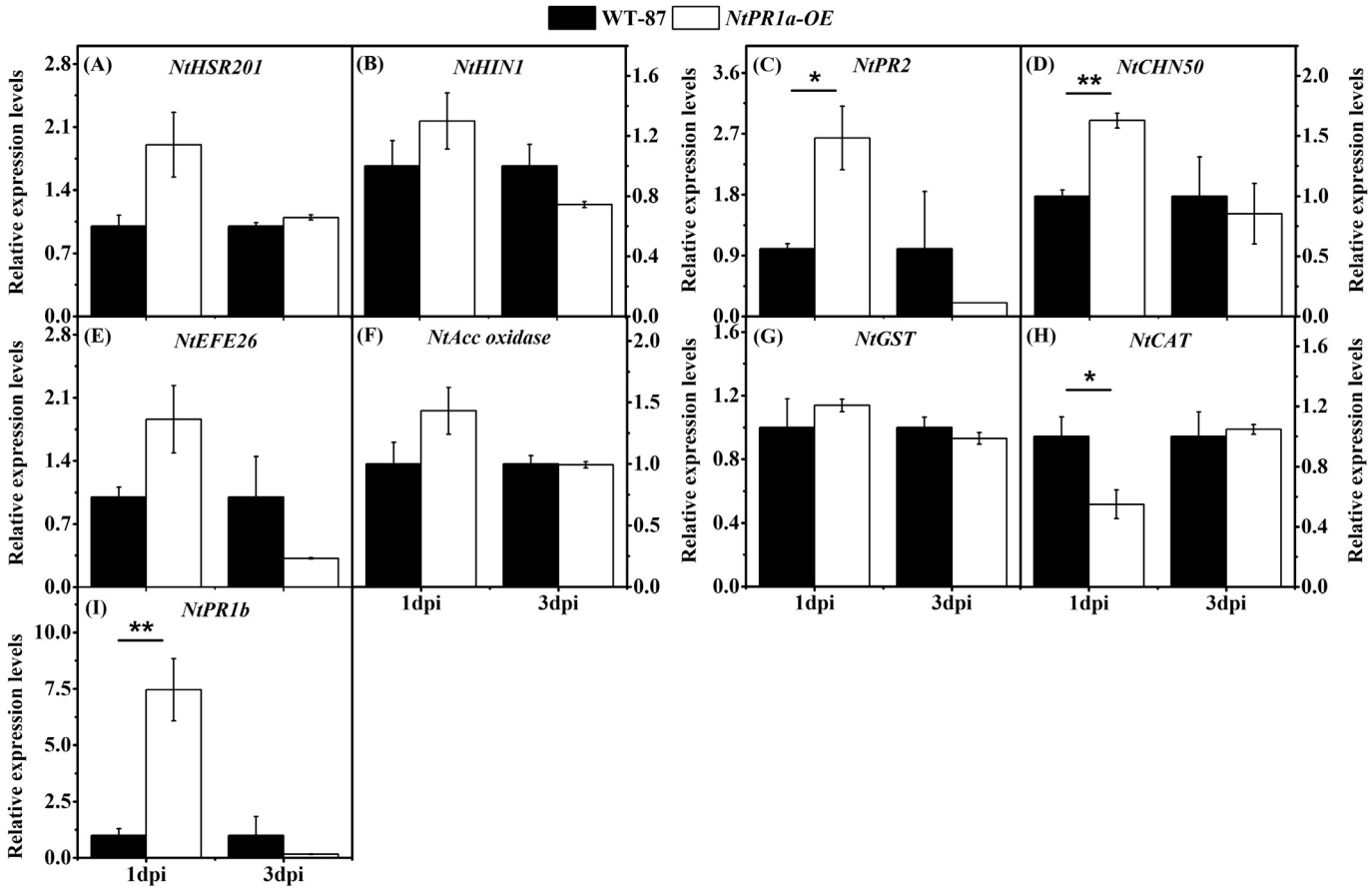

Fig. 4. The transcript levels of defense marker genes in *NtPR1a-OE* plants and WT-Yunyan87 plants after inoculating *R. solanacearum*. Defense-related gene transcript levels in WT-Yunyan87 (WT-87) were used as controls. (A–B) HR-associated genes *NtHSR201* and *NtHIN1*; (C–D) SA-responsive genes *NtPR2* and *NtCHN50*; (E–F) ET production-associated genes *NtEFE26* and *Ntacc oxidase*; (G–H) detoxification-associated genes *NtGST1* and *NtCAT1*; (I) JA-responsive gene *NtPR1b*. Error bars indicate the standard error, values were based on at least three independent replicates. Asterisks indicate a significant difference ($^{*}p < 0.05$; $^{**}p < 0.01$).

ROS, the by-products of normal metabolism produced by plants, contains hydrogen peroxide (H_2O_2), superoxide radical (O_2), hydroxyl radical (•OH) and singlet oxygen ($^1O_2$) etc [37]. The high levels accumulation of ROS in plant cells, such as the production of localized O_2 and H_2O_2 from the oxidative burst, would lead to the programmed cell death, which is known as HR in plant defense mechanisms [38]. Our results demonstrated that the *NtPR1a*-overexpressing plants under *R. solanacearum* attacks had higher levels of O_2 and H_2O_2 than wild-type plants (Fig. 2G and H). As the toxic molecules with strong oxidant power, high levels of ROS would be harmful to plant cells. To keep the steady state, plants have a series of scavenging mechanisms - antioxidants like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) [39–41]. However, our data showed that the relatively expression level of *NtCAT1* in transgenic plants with *R. solanacearum* inoculation was significantly lower than that in the wide-type plants (Fig. 4), which may imply the activator role of *NtPR1a* in ROS production.

SA pathway is one of the most important way in which plants response to phytopathogens [42]. *PR1a/c* was also regarded as one of responsive genes in the SA pathway. From our study, overexpression of *NtPR1a* significantly increased the expressions of other SA-responsive genes like *NtPR2* and *NtCHN50* before and after inoculation (Figs. 3 and 4). PR-1 proteins always serve as part of important molecular markers in SAR, and play crucial role in plant innate immune response when plants suffer from biotic and abiotic stress [6,43–45]. The overexpression of ET production-associated genes, JA-responsive genes, HR-associated genes, and detoxification-associated genes in the transgenic tobacco plants suggested that the transcription of *NtPR1a* would induce overexpression of other resistance-related genes, which verifies the effect of *PR1a* in SAR.

Plants have a variety of mechanisms resisting pathogens attacks. Previous studies showed that there were a series of proteins functioning in tobacco resistance to *R. solanacearum* [10,14], but there was no specific resistance gene found. Nowadays, many researchers commit to finding accurate genetic factors for controlling this resistance, for example, transcriptome analysis and quantitative trait loci (QTL) mapping experiment are designed [46]. Further researches of exploring the interaction between these genes and pathogens should be done, which may provide a new strategy for disease resistance breeding.

Author contributions

The experiment was designed by WD and YL, then YL, QL, and YT accomplished the experiments, YL analyzed the data, YL and WD wrote and revised the paper.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.017.

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