Naringenin confers defence against *Phytophthora nicotianae* through antimicrobial activity and induction of pathogen resistance in tobacco

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**Abstract**

Tobacco black shank caused by *Phytophthora nicotianae* is a serious disease in tobacco cultivation. We found that naringenin is a key factor that causes different sensitivity to *P. nicotianae* between resistant and susceptible tobacco. The level of basal flavonoids in resistant tobacco was distinct from that in susceptible tobacco. Of all flavonoids with different content, naringenin showed the best antimicrobial activity against mycelial growth and sporangia production of *P. nicotianae* in vitro. However, naringenin showed very low or no antimicrobial activity to other plant pathogens. We found that naringenin induced not only the accumulation of reactive oxygen species, but also the expression of salicylic acid biosynthesis-related genes. Naringenin induced the expression of the basal pathogen resistance gene *PR1* and the *SAR8.2* gene that contributes to plant resistance to *P. nicotianae*. We then interfered with the expression of the chalcone synthase (*NtCHS*) gene, the key gene of the naringenin synthesis pathway, to inhibit naringenin biosynthesis. *NtCHS*-RNAi rendered tobacco highly sensitive to *P. nicotianae*, but there was no change in susceptibility to another plant pathogen, *Ralstonia solanacearum*. Finally, exogenous application of naringenin on susceptible tobacco enhanced resistance to *P. nicotianae* and naringenin was very stable in this environment. Our findings revealed that naringenin plays a core role in the defence against *P. nicotianae* and expanded the possibilities for the application of plant secondary metabolites in the control of *P. nicotianae*.
INTRODUCTION

Plant-pathogenic oomycetes pose a serious threat to agriculture, horticulture, forestry, aquaculture and natural ecosystems (Wang, Tyler, et al., 2019). They encompass over 100 species that cause devastating diseases in both plants and animals. The Phytophthora species are the most serious pathogens. They infect a broad group of hosts and cause diseases such as root rot, late blight, downy blight, and downy mildew (Derevina et al., 2016; Kamoun et al., 2015; Panabieres et al., 2016). Tobacco black shank (TBS) disease, caused by Phytophthora nicotianae, threatens production in tobacco-producing areas across the world (Gallup et al., 2018). Infections mainly occur in the adult stage of tobacco, resulting in significant yield loss and quality reduction. Roots and stems are the main parts infected, leading to progressive decay of the diseased tissues, such as root and stem necrosis, wilting, and chlorosis, and finally death (Chen et al., 2020; Csinos & Minton, 1983; Gallup et al., 2006). Mycelia, oospores and chlamydospores from P. nicotianae are found in soil and plant tissues in compost through the winter and can survive for more than 3 years. Soil is therefore the main source of infection, while manure and irrigation promote the spread of pathogens (Antonopoulos et al., 2010; Gallup et al., 2018).

TBS is a very difficult disease to control due to the diversity and high adaptability of P. nicotianae (Gallup et al., 2018; Ji et al., 2014). Its management relies on the integration of different approaches, including planting resistant varieties, fungicide applications, crop rotation, management of water and fertilizer (Shew & Lucas, 1991). So far, four physiological races (0, 1, 2 and 3) of P. nicotianae have been reported. The undomesticated Nicotiana species are an important source of resistant varieties. Tobacco cultivars resistant to race 0 of P. nicotianae conferred by the genes Php or Phi have been widely used across the world; however, cultivars have become susceptible to P. nicotianae as there has been a gradual shift of pathogen populations from race 0 to race 1, making the strategy of TBS-resistant cultivars ineffective in disease control (Johnson et al., 2002; Li et al., 2006; Sullivan et al., 2005). In addition, P. nicotianae has a large arsenal of secreted proteins, termed effectors, that act as weapons to overcome the host resistance and promote infection (Hou et al., 2019; Lee et al., 2018). These are the main reasons that crop rotation is highly recommended to avoid disease outbreaks and propagation of P. nicotianae in the field (Yong et al., 2010). Pesticide application is another important approach to managing TBS. Several fungicides such as mefenoxam and potassium phosphite have been applied for many years but resistance in Phytophthora has been reported for both compounds (Hao et al., 2019; Ji et al., 2014). Some new fungicides against oomycetes, such as fluopicolide, ethaboxam, oxathiapiprolin and mandipropamid, with different modes of action have been developed and they have shown higher efficiency in treatment of Phytophthora (Hao et al., 2019; Ji et al., 2014; Qu et al., 2016). These new fungicides provide another highly effective approach against pathogens in addition to rotation and proper use of all existing fungicides. However, more attention should be paid to the development of resistance to these new fungicides among the populations of P. nicotianae (Panabieres et al., 2016; Qu et al., 2016).

Biocontrol has been shown to be an alternative way to manage TBS efficiently, mainly using biocontrol strains and new compounds that have been isolated from natural organisms. Among biocontrol strains, Pseudomonas fluorescens and Bacillus spp. have been widely studied and used due to the antimicrobial components they release, such as peptides and enzymes (Choudhary & Johri, 2009; Fira et al., 2018; Rajaofera et al., 2018). These antimicrobial components can improve plant growth, as well as induce plant immune response to abiotic stress (Choudhary & Johri, 2009; Sun et al., 2017). In addition, some biocontrol strains, such as the plant growth-promoting rhizobacterium Bacillus amyloliquefaciens Ba168, secrete proteins and peptides that target pathogens directly, for example, by damaging the cell wall and membrane of P. nicotianae (Guo et al., 2020). In addition, various natural agents produced from plants are another source of antimicrobial components. Some secondary metabolites are not involved in growth, development or reproduction of plants directly. Rather, they perform special functions under a given set of conditions such as pathogen attack, water deficit or extreme temperature (Bartwal et al., 2012). The essential oil eugenol and diallyl disulphides have been confirmed to be effective against TBS by destroying mycelial cell membrane integrity, causing an increase in cell membrane permeability and leading to cell death (Jing et al., 2017; Wang et al., 2019). Phytoalexins with anti-septic, anti-inflammatory, antioxidant or antimicrobial activities accumulate soon after pathogen infection (Chirikova et al., 2016; Kumar et al., 2006).

Over 9000 categories of flavonoids have been found in various plants, making them one of the largest families of secondary metabolites (Wang et al., 2011). They are essential factors that are involved in aspects of plant development and defence, and flower and fruit quality (Treutter, 2005; Wang et al., 2011). Flavonoids often accumulate in specialized tissues and there are only a few studies on their function against pathogens (Kariu et al., 2017; Martínez-Castillo et al., 2018; Paczkowski et al., 2017; Tattini et al., 2004). Naringenin is one of the major flavonoids and is mainly found in citrus fruits, including tangerine, lemon, orange and grapefruit (Manchope et al., 2017). Naringenin accumulates when plants are infected by Rhizobium leguminosarum bv. viciae, Pseudomonas syringae pv. pisi or Plasmopodiophora brassicae (Makarova et al., 2016; Paesold et al., 2010). In addition, naringenin has shown anti-inflammatory, antiviral and antifungal activities, for example against Fusarium spp. including F. poae, F. culmorum and F. graminearum, and the rice pathogen Magnaporthe grisea.

**KEYWORDS**

antimicrobial activity, naringenin, NtCHS, oomycete, Phytophthora nicotianae, plant defence
As shown in Table S1, 166 metabolites were identified and characterized by its distinct retention time and mass-to-charge ratio (m/z). Compared to XHJ, the content of 15 metabolites in BH-1 were significantly different in each type of metabolite (Figure 1d and Table S2). These metabolites could be divided into four categories: flavonoids, flavonols, flavanones and others; the proportion of metabolites with a higher content of flavonoids was different in each type of metabolite (Figure 1e,f). The proportions of flavanones and flavonols were 18.75% and 15.90%, respectively. Through metabolite analysis, 16 flavanones were characterized. The content of nine flavanones, including liquiritigenin and naringenin, was slightly higher and the content of three flavanones, including isosakuranetin and hesperetin, was significantly higher in BH-1 compared to XHJ (Table S3). We also characterized 44 flavonols, of which the content of seven was significantly higher in BH-1 than XHJ, such as kaempferol and quercetin (Table S4). These results indicate that there is a significant difference in the basal level of flavonoids between resistant and susceptible tobacco varieties, and this difference might be related to resistance to P. nicotianae in the plants.

2.2 | Naringenin showed antimicrobial activity on P. nicotianae in vitro

For the 16 flavanones and 44 flavonols described above, we tested their antimicrobial activity against P. nicotianae in vitro. P. nicotianae grew on potato dextrose agar (PDA) equally well in the presence of different concentrations of flavonoids. Only three flavanones inhibited the growth of P. nicotianae, and of these naringenin showed the strongest antimicrobial activity (Figure 2a,b). The half-maximum effective concentration (EC_{50}) of naringenin for inhibition of P. nicotianae mycelial growth was 22.01 mg/L, whereas the EC_{50} values of liquiritigenin and hesperetin were 51.43 and 30.20 mg/L (Table S5). In contrast, none of the flavonols, such as kaempferol, quercetin or rutinum, showed any inhibitory activity on the mycelial growth of even at concentrations up to 200 mg/L (Figure S1a,b and Table S5). Naringenin also inhibited growth of other oomycetes, such as Phytophthora capsici, with an EC_{50} value of 50.11 mg/L, but showed low inhibition activity on Pythium aphanidermatum (Figure S2 and Table S6). However, naringenin showed very low or no inhibitory activity on other plant pathogens such as Sclerotinia sclerotiorum, Botrytis cinerea, and Fusarium graminearum (Figure S3). We next tested whether naringenin affected the reproduction of P. nicotianae by microscopic observation. As shown in Figure 2c,d and Table S6, the EC_{50} and EC_{90} values of naringenin for inhibition of P. nicotianae sporangia production were 2.01 mg/L and 6.62 mg/L, respectively. These values are significantly lower than the EC_{50} for mycelial growth. Furthermore, we used reverse transcription-quantitative PCR (RT-qPCR) to confirm that cell growth and reproduction-related genes were down-regulated when P. nicotianae was treated with naringenin (Figure S4). These results indicate that naringenin inhibits not only the mycelial growth but also the reproduction of P. nicotianae.

2.3 | Naringenin induces accumulation of reactive oxygen species

Normally, plants are resistant to the invasion of most pathogens through a burst of reactive oxygen species (ROS), including hydrogen peroxide (H_{2}O_{2}) and the superoxide anion (O_{2}^{-}) (Bray, 2000). The increased H_{2}O_{2} content promotes the up-regulation of genes that are
associated with the plant defence response (Bray, 2000). Flavonoids have been shown to be antioxidant agents by clearing away ROS (Turek & Stintzing, 2013). Quercetin, a flavonoid and powerful antioxidant, has been reported to activate Arabidopsis defence against Pseudomonas syringae pv. tomato DC3000 via an H$_2$O$_2$ burst (Jia et al., 2010). To determine if ROS accumulate in tobacco with naringenin treatment, we tested the content of H$_2$O$_2$ and O$_2^-$ in Honghuadajinyuan (HD) seedlings (Figure 3a,b). HD is an important flue-cured tobacco variety that is susceptible to TBS. As expected, the accumulation of H$_2$O$_2$ and O$_2^-$ was significantly induced with naringenin treatment, indicating that naringenin induced an ROS burst in tobacco.

2.4 | Naringenin induced expression of salicylic acid biosynthesis-related genes and signalling in tobacco

The hormone salicylic acid (SA) is required for the activation of immune responses to biotrophic pathogens in plants (van Butselaar &
Van den Ackerveken, 2020). Various flavonoids mediate the activation of plant-pathogen resistance by the SA-dependent pathway (Jia et al., 2010; Yang et al., 2016). To test whether SA biosynthesis-related genes could be induced by naringenin, the expression of the representative SA biosynthesis-related gene ICS1 was measured by RT-qPCR. The expression of the ICS1 gene increased approximately 2-fold with naringenin treatment (Figure 3c). This result suggests that naringenin induces an increase in SA biosynthesis by the isochorismate-dependent pathway and prompted us to examine whether naringenin induces SA signalling. We measured the expression of the SA signalling gene EDS1 after treatment with naringenin by RT-qPCR. The expression of EDS1 was increased 1.8-fold by naringenin treatment (Figure 3d). This result indicates that naringenin induces SA biosynthesis and activates SA signalling in tobacco.

2.5 Naringenin enhanced plant resistance to *P. nicotianae*

Flavonoids have been shown to be critical to plant defence on pathogenic bacteria and fungi through the induction of PR genes (Mierziak et al., 2014). Quercetin and its derivatives are capable of inducing pathogen resistance to both bacteria and fungi (Jia et al., 2010; Parvez et al., 2004; Yang et al., 2016). To examine whether naringenin induces basal pathogen resistance, we analysed the expression of PR1 and SAR8.2 genes in tobacco after naringenin treatment using RT-qPCR. The SAR8.2 gene is a gene that controls plant resistance to *P. nicotianae* (Shi et al., 2022). As expected, the expression levels of PR1 and SAR8.2 with naringenin treatment were approximately 1.4-fold and 3.5-fold higher than those of the control, respectively (Figure 3e,f). These results suggest that naringenin induces basal pathogen resistance.

2.6 Interference of naringenin biosynthesis led to more susceptibility to *P. nicotianae* in tobacco

The flavonoid biosynthesis pathway has been well studied and most intermediate enzyme steps have been characterized (see Figure 4a). The pathway starts with the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia-lyase (PAL). Under continuous catalysis by cinnamate 4-hydroxylase (C4H) and 4-coumaroyl coenzyme A (CoA) ligase (4CL), cinnamic acid is converted to p-coumarinyl CoA, a substrate of flavonoids. Decarboxylative condensation of p-coumarinyl CoA with three molecules of malonyl CoA into naringenin chalcones by chalcone synthase (CHS) provides substrates for the final synthesis of naringenin by chalcone isomerase (CHI) (Kreuzaler & Hahlbrock, 1972; Pandey et al., 2015). We first tested the expression of NtCHS, NtPAL, NtC4H and Nt4CL in BH-1 and XHJ without and with inoculation of *P. nicotianae*. We found that, compared to XHJ, NtCHS was more highly expressed in BH-1 even when infected with *P. nicotianae*, indicating that NtCHS might...
be a core regulatory gene in the biosynthesis of naringenin in tobacco (Figure 4b). The other three genes (NtPAL, NtC4H and Nt4CL) involved in the synthesis of naringenin did not show significant differences except in the later period (5 days) after tobacco was inoculated with P. nicotianae (Figure S5).

Next, to interfere with the naringenin synthesis pathway, we generated tobacco transgenic lines with NtCHS silenced via RNA interference (RNAi) in the resistant cultivar BH-1 (Figure 4c). Three independent T₂ transgenic lines comprising the NtCHS RNAi construct showed a significant decrease in the expression level of NtCHS (Figure 4d) and a dramatic decrease in naringenin content compared to the wild type (WT) (Figure 4e). These transgenic lines showed more susceptibility to P. nicotianae compared to the WT, but they kept the same sensitivity to Ralstonia solanacearum, a serious pathogen that causes tobacco bacterial wilt (Figures 4f and S6). Naringenin showed very low antimicrobial activity against...
R. solanacearum even at concentrations up to 400 mg/L (Table S7). Furthermore, the biomass of *P. nicotianae* in *NtCHS*-RNAi mutants was up to six times more than that in WT by DNA quantification of the oomycete (Figure S7). These results show that interference in naringenin synthesis in tobacco induced more susceptibility to *P. nicotianae* and naringenin might be a specific antimicrobial agent to *P. nicotianae* because it showed very low or no activity towards other plant pathogens.

### 2.7 Exogenous application of naringenin enhanced resistance to *P. nicotianae*

To explore the possibility of applying naringenin as a natural antimicrobial agent in the management of TBS in the field, we treated susceptible tobacco HD seedlings with naringenin before they were inoculated with *P. nicotianae*. HD seedlings inoculated with *P. nicotianae* but without naringenin treatment were used as the control. After
10 days, we found that HD seedlings treated with naringenin were significantly more resistant to *P. nicotianae* compared to the control (Figure 5a,b). Accordingly, the disease index in HD treated with naringenin was dramatically lower than in the control (Figure 5c). More importantly, the application of naringenin did not negatively affect the agronomic traits of tobacco, such as plant height, leaf length, leaf width, knot spacing and stem girth (Figure S8). In addition, to test the impact of naringenin stability on infection by pathogens, we checked the susceptibility of HD tobacco to *P. nicotianae* 3, 7, 15 and 30 days after treatment with naringenin. The results showed that HD treated with naringenin for 30 days still had the same susceptibility to *P. nicotianae* as HD treated with naringenin for 3 days, and both were significantly more resistant to *P. nicotianae* compared to HD without naringenin treatment. These results show that exogenous application of naringenin improved resistance to *P. nicotianae* significantly and showed good stability of antimicrobial activity in the environment (Figure S9). Therefore, naringenin was shown to be a potential antimicrobial agent for the management of TBS in the field.

3 | DISCUSSION

Plants have evolved diverse biosynthetic routes to produce a range of small organic molecules referred to as secondary metabolites. Secondary metabolites are restricted to specific taxonomic groups and play important roles in diverse aspects of plant life activities, including as components of signalling cascades and in plant defence against herbivores (Pandey et al., 2015). Among all plant secondary metabolites, flavonoids are widely studied as they have a multitude of biological functions, including a function as defence molecules against biotic and abiotic stresses (Treutter, 2005; Zhang et al., 2020). Flavanones and flavonols are categories of flavonoids that differ by a hydroxyl moiety in the 3’ position in flavonols that is lacking in flavanones (Sun et al., 2019). Flavanones include phloretin, glyceollins and naringenin. Aminoethyl-phloretin is a water-soluble phloretin derivative that has been shown to possess antibacterial activity toward both gram-positive and gram-negative bacteria (Wei et al., 2020). Glyceollins are a group of phytoalexins that are mainly isolated from soybeans. Both aminoethyl-phloretin and glyceollins have numerous functions in human health, especially as anticancer agents (Pham et al., 2019). Naringenin is a typical flavanone that has been found mostly in edible fruits such as citrus species, tomatoes and figs, and studies on naringenin have mainly focused on its biological effects on human health (Salehi et al., 2019). There are a few reports on naringenin and its antimicrobial activity on *Escherichia coli* O157:H7 in apple cider and its improvement of resistance to rice blast (Surendran Nair et al., 2020). However, there are no reports on the mechanism by which naringenin provides defence against plant pathogens.

In this study, we found that there was a significant difference in the basal flavonoid metabolite profile between resistant and susceptible tobacco varieties. We treated *P. nicotianae* in vitro with different concentrations of flavanones and flavonols, and found that only three flavanones showed antimicrobial activity, with naringenin showing the highest activity. However, none of the flavonols showed any antimicrobial activity, even at high concentrations up to 200 mg/L. Compared to its activity against *P. nicotianae*, naringenin had a lower antimicrobial activity against other oomycetes.

**FIGURE 5** Resistance to tobacco black shank was enhanced by exogenous application of naringenin. (a) Disease symptoms of Honghuadajinyuan (HD) supplied with 100 ml of a solution of 0.4 g/L naringenin 10 days postinoculation with *Phytophthora nicotianae*. HD was inoculated with *P. nicotianae* but without treatment of naringenin as a control. (b) Enlarged view of the neck with or without treatment of naringenin after HD was inoculated with *P. nicotianae*. (c) The disease index with or without the treatment of naringenin after HD was inoculated with *P. nicotianae* (*n* = 3, error bars = SD). HD is the main cultivated flue-cured tobacco variety but is susceptible to tobacco black shank. Significant difference compared to control by the Mann–Whitney test (**p < 0.01**).
such as *P. capsici*. Naringenin showed no or very low inhibitory activity towards plant pathogens such as *S. sclerotiorum* and *B. cinerea*. Interestingly, there was dramatic inhibition of sporangia production when *P. nicotianae* was treated with a very low concentration of naringenin. The reason for the inhibition of sporangia production by naringenin might be that genes regulating the reproductive process in *P. nicotianae* are directly suppressed by naringenin. We used RT-qPCR to confirm that genes regulating cell growth and reproduction were down-regulated after *P. nicotianae* was treated with naringenin. Naringenin is thus the first flavonoid to be identified as an antimicrobial agent against *P. nicotianae*; it might affect the expression of genes regulating the growth and reproduction of *P. nicotianae* directly.

Flavonoids are well known as ROS scavengers and powerful antioxidants (Pannala et al., 2001; Rice-Evans, 2001). Flavonoids decrease the ROS level through inhibition of pro-oxidant enzymes, including cyclo-oxygenase and lipooxygenase (Eghbaliferiz & Iranshahi, 2016). Phenolics and carotenoids are chemical groups that prevent oxidative damage as a result of their ability to decrease the ROS level, and they also exhibit pro-oxidant activities in vitro in the presence of metal ions (Eghbaliferiz & Iranshahi, 2016). Like phenolics and carotenoids, flavonoids act as pro-oxidants at physiological pH. In this study, we showed that naringenin induced the accumulation of ROS, which suggests that naringenin might act as a pro-oxidant. An et al. also showed that naringenin induced an ROS burst in plants as a defence against *P. syringae* (An et al., 2021). SA has been shown to induce pathogen resistance through ROS accumulation and increased expression of PR genes. We also found that genes regulating SA biosynthesis, SA signalling and basal pathogen resistance were enhanced at the transcriptional level by treatment with naringenin. Furthermore, SAR8.2, a gene in tobacco that regulates plant resistance to *P. nicotianae* (Shi et al., 2022), was substantially up-regulated by treatment with naringenin. These results indicate that naringenin induces plant-pathogen resistance. Naringenin accumulates soon after plants are infected with biotrophic pathogens such as *Rhizobium leguminosarum* bv. *viceae*, *Pseudomonas syringae* pv. *pisi* and *Plasmopodihora brassicae* (Makarova et al., 2016). However, we did not detect significant accumulation of naringenin in tobacco after infection with *P. nicotianae* by metabolite analysis. Interestingly, naringenin has been shown to induce pathogen resistance against *P. syringae* through the activation of NPR1 in *Arabidopsis* 2021, but did not show any antimicrobial activity against *P. syringae* even at high concentrations in vitro (An et al., 2021). Our results demonstrate that naringenin confers plant defence against *P. nicotianae* through induction of plant-pathogen resistance in addition to its antimicrobial activity.

The key genes in the metabolic pathway of naringenin synthesis include PAL, 4CL, C4H, and CHS (Kreuzaler & Hahlbrock, 1972; Pandey et al., 2015). Our results showed that the expression level of *NtPAL*, *NtC4H* and *Nt4CL* did not show a significant change except in the later period (5 days) after BH-1 had been infected by *P. nicotianae* (Figure S5). Remarkably, only *NtCHS* had a higher expression level in BH-1 than that in XHJ, even after BH-1 was treated with *P. nicotianae*. The accumulation of naringenin is positively correlated with the expression of CHS (Pandith et al., 2019).

In this study, without infection of *P. nicotianae*, the basal expression level of *NtCHS* in resistant tobacco variety BH-1 was higher than that in susceptible tobacco variety XHJ. Correspondingly, the content of naringenin in BH-1 was higher than that in XHJ. CHS is a rate-limiting enzyme that controls the supply of substrates in the flavonoid biosynthesis pathway, thus the amount of products downstream are affected by the efficiency of CHS. Naringenin plays a key role in both plant-pathogen resistance and antimicrobial activity after the plant is infected by *P. nicotianae*; however, the plant is not able to synthesize unlimited amounts of naringenin as there is a lack of substrates in the biosynthesis pathway. The expression level of *NtCHS* was down-regulated to keep the flavonoid biosynthesis pathway in order (Figure 4b). RNA interference of *NtCHS* led to a dramatic decrease in naringenin content and the biomass of *P. nicotianae* in *NtCHS*-RNAi tobacco mutants was up to 6-fold higher than that in WT plants. The level of RNA interference in different RNAi transgenic plant lines might vary. In this study, compared to the other two RNAi transgenic lines, the content of naringenin in the *NtCHS*-RNAi-3 transgenic line was much less and this may be the reason that the *NtCHS*-RNAi-3 transgenic line was more susceptible to *P. nicotianae*. Significantly, TBS severity is a quantitative trait and its phenotype might be affected by multiple factors in the environment. Therefore, genes that regulate the plant resistance to TBS and the plant phenotype do not correspond to each other, I, raising difficulties to study the mechanism of plant resistance to TBS.

CHS orchestrates the general flavonoid biosynthesis pathway in tobacco; thus, its down-regulation influences the biosynthesis of all chalcones, flavones, anthocyanins and other derivatives and is not limited to naringenin. In fact, the accumulation of various flavonoids, including naringenin and rutin, decreases significantly in *NtCHS1*-RNAi transgenic plants (Chen et al., 2019). However, only naringenin showed antimicrobial activity against *P. nicotianae*, and other flavonoids such as rutin showed very low or no antimicrobial activity. By contrast, compared to the WT, we found that the *NtCHS*-RNAi tobacco mutants kept the same susceptibility to *R. solanacearum*, a serious pathogen which causes tobacco bacterial wilt. This indicates that RNAi of *NtCHS* induced a reduction in the content of naringenin, and specific antimicrobial activity against *P. nicotianae* by naringenin might be the main reason that *NtCHS*-RNAi tobacco was more sensitive to *P. nicotianae* compared to other plant pathogens such as *R. solanacearum*. Naringenin showed no antimicrobial activity against *R. solanacearum* in vitro even with concentrations of naringenin up to 400 mg/L. Because naringenin induced resistance against both *P. nicotianae* and *P. syringae*, naringenin could also be expected to induce resistance to *R. solanacearum*. CHS regulates flower colour, fertility and gas substances, and we found that CHS was also involved in resistance to *P. nicotianae*. We also found that TBS-susceptible tobacco variety HD became resistant to *P. nicotianae* after treatment with...
naringenin. Naringenin showed high activity with a good stability in the environment (Figure S9). This leads to the potential application of naringenin as a natural antimicrobial agent in the management of TBS in the field in the future.

In conclusion, for first time we found that naringenin might be an antimicrobial secondary metabolite against *P. nicotianae*. Naringenin inhibited *P. nicotianae* through not only its antimicrobial activity, but also its induction of defences against plant pathogens (Figure S10). Our discoveries will enrich the means of prevention and control for TBS. Future work needs to address the question of whether naringenin targets proteins or genes in *P. nicotianae* directly, and whether naringenin interferes in the interaction between *P. nicotianae* and tobacco. Notably, the development of naringenin as a plant-derived fungicide against *P. nicotianae* is important work for the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and *P. nicotianae* inoculation

BH-1 shows a high level of resistance to TBS, whereas XHJ is extremely susceptible. Both of these were obtained from the Tobacco Research Institute, Chinese Academy of Agricultural Sciences. Plants were cultured using Holland nutrient solution and were grown in a growth chamber at 28°C with 16 h light and 8 h dark photoperiod cycles. Sixteen-week-old tobacco plants were infected with *P. nicotianae* by dipping tobacco in a spore suspension for 3 h, then cultured in water. After 5 days, the phenotype of TBS was evaluated using an empirical scale (YC/T39-1996, China), where 0 represents a highly resistant response and 9 represents a highly susceptible response. Disease index scores based on disease severity were used for assessment and calculated using the following formula: disease index (%) = [Σ(disease evaluation scale score × number of plants with each scale score)/(total number of plants observed × highest disease evaluation scale score)] × 100. Three-month-old tobacco plants were infected with *P. nicotianae* according to a described method (Zhang et al., 2018).

The biomass of *P. nicotianae* in infected tobacco plants was quantified using a slight modification of a previously described method (Park et al., 2012). Quantitative PCR was performed using a Light Cycler 96 Real-Time PCR detection system (Roche, http://technical-support.roche.com). The biomass of *P. nicotianae* was calculated using the threshold cycle value (Ct) of *P. nicotianae* WS21 DNA against the Ct of tobacco genomic actin DNA.

4.2 | Inoculation of tobacco with *R. solanacearum*

After 12-week-old tobacco plants were infected with *R. solanacearum*, a small amount of bacteria was picked up with a toothpick and placed into 1 L of nutrient broth (NB) (Qingdao Hope Bio-Technology Co., Ltd). The bacteria were grown in an incubator at 30°C and 200 rpm until the OD_{600} reached 1.0. Two hundred millilitres of suspension bacteria cells was added to each plate of tobacco seedlings. After 15 days, the phenotype of tobacco bacterial wilt was evaluated using an empirical five-point scale (GB/T 23222-2008), where 0 represents a highly resistant response and 4 represents a highly susceptible response. Disease index scores based on the disease severity were used for assessment and were calculated using the following formula: disease index (%) = [Σ(disease evaluation scale score × number of plants with each scale score)/(total number of plants observed × the highest disease evaluation scale score)] × 100.

4.3 | Pathogen isolation and inoculum preparation

*R. solanacearum* and *P. nicotianae* race 0 were obtained from the Plant Protection Laboratory of the Chinese Academy of Agricultural Sciences. *P. capsici*, *P. aphanidermatum*, *Pythium ultimum*, *S. sclerotiorum*, *Colletotrichum gloeosporioides*, *Fusarium moniliforme*, *B. cinerea*, and *F. graminearum* were obtained from Nanjing Agricultural University. Each pathogen was grown separately in 25 ml of potato dextrose agar (PDA; potato 200 g, glucose 20 g, agar 16 g, in 1 L) at 28°C for 14 days. The inoculum method was used as described (Zhang et al., 2018). Then 0.1% KNO₃ was added to the PDA plate containing *P. nicotianae* and kept at 4°C for 25 min, then kept in the light for 30 min at 25°C. The concentration of the spore suspension was determined by a cellometer Auto T4 (Nexcelom Cellometer) and adjusted to 10⁶ spores/ml.

4.4 | Antimicrobial activity test for flavonoids

All flavonoid standards used in this study were purchased from the Solarbio Company. First, 20 mg of standard flavonoid was dissolved in 1 ml of ethanol to make 20 mg/ml stock solution, which was filtered and sterilized before use. PDA containing different concentrations of different flavonoids was prepared for the antimicrobial tests. A single agar plug (5 mm diameter) was removed from the actively growing edge of the fungal culture and placed in a new PDA culture containing flavonoids. Four different pathogens inoculated with PDA or with only ethanol added served as positive controls. The medium was observed after incubating at 28°C for 7 days. The efficacy of each treatment was evaluated by measuring the diameter of each colony. Each treatment contained three replicates. The inhibition rate was calculated as follows: inhibition rate (%) = ([control hyphae diameter – treatment hyphae diameter]/control hyphae diameter) × 100.

4.5 | Antimicrobial activity of naringenin on *R. solanacearum*

First, 0.1 ml of 10⁸ cfu/ml *R. solanacearum* suspension was pipetted onto a Petri dish (diameter of 9 cm). Then the cooled and melted
PDA medium was mixed thoroughly with the R. solancearum suspension. Filter paper was punched into 6-mm diameter discs with a hole punch, immersed in different concentrations of naringenin, then moved to a flat plate. The plates were placed in an incubator at 28°C for 3 days. The diameter of the bacteriostatic circle was measured by the cross-crossing method, and the average weight of each treatment was calculated. Each treatment contained three replicates. The inhibition rate was calculated as follows: inhibition rate (%) = (treatment bacteriostatic circle − control bacteriostatic circle)/control bacteriostatic circle × 100.

4.6 Metabolite estimation and analysis

Eight-week-old seedlings of BH-1 and XHJ grown in Hoagland hydroponic nutrient solution were used for flavonoid metabolomic analysis. The freeze-dried leaves were crushed using a mixer mill (MM 400; Retsch) with a zirconia bead for 1.5 min at 30 Hz. One hundred milligrams of powder extracted overnight at 4°C with 1 ml of 70% methanol. Followed centrifugation at 10,000 × g for 10 min, the extracts were absorbed and filtrated to the single concentration (SCAA-104, 0.22 μm pore size) before LC-ESI-MS/MS (HPLC, Shim-pack UFLC SHIMADZU CBM30A system; MS, Applied Biosystems 4500 Q TRAP) analysis. The sample extracts were analysed by LC-ESI-MS/MS according to methods described previously (Gong et al., 2013). The effluent was connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP; API 4500 Q TRAP LC/MS/MS System) equipped with an ESI Turbo ion-spray interface operating in a positive ion mode and controlled by Analyst v. 1.6.3 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 550°C; ion spray voltage (IS) 5500V; ion source gas I (GSI), gas II (GSII), curtain gas (CUR) set at 55, 60 and 25.0 psi, respectively; collision gas (CAD) high. Instrument tuning and mass calibration were performed with 10 and 100 μM polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi. DP (declustering potential) and CE (collision energy) for individual MRM transitions was done with further optimization of DP and CE. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period.

Based on the self-built database MWDB (Metware database) and the public database of metabolite information, qualitative analysis was performed on the primary and secondary mass spectrometry data. Metabolite quantification was carried out via the metabolite estimation solution (methanol:chloroform:water = 5:2:2) and treated by ultrasonic vibration for 45 min to extract naringenin. The extracted naringenin was analysed using LC-ESI-MS/MS (HPLC, Shim-pack UFLC Shimadzu CBM-20A system; MS, Applied Biosystems 4000 Q TRAP) according to the methods described previously (Zhao et al., 2020). Three biological replicates of each treatment were performed. The chromatographic conditions were as follows: Waters BEH C18 chromatographic column (150 mm × 2.1 mm, 1.7 μm); mobile phase: A phase water, B phase acetonitrile, 0.1% (vol/vol) formic acid in water.

4.7 RNA extraction of plants, cDNA synthesis and RT-qPCR

Total RNA was extracted with a Plant RNA pure kit (ZOMANBIO, ZP405-2), then the reverse transcription of the cDNA was synthesized using HiScript III RT SuperMix for qPCR (Vazyme). The primers for amplification of all genes were designed by Primer Premier v. 5 software. The qPCR was performed by a LightCycler 96 Real-Time PCR detection system (Roche, http://technical-support.roche.com) using ChamQ SYBR Colour qPCR Master Mix (Vazyme, Q411-02/03). The relative expression levels of genes were calculated by the 2−ΔΔCt method and all reactions were repeated three times. Three independent biological replicates were used. The sequences of all primers are shown in Table S8.

4.8 The production of NtCHS-RNAi lines

The production of NtCHS-RNAi and generation of transgenic tobacco lines have been described previously (Chen et al., 2019). PCR identification was used to amplify the transformed seedling DNA with RNAi-F and RNAi-R primers to determine whether the RNAi vector was successfully introduced. For the transformed lines, the silencing efficiency of NtCHS was identified by RT-qPCR. The sequences for all primers used in this experiment are shown in Table S8.

4.9 Naringenin measurement by LC-ESI-MS/MS

Eight-week-old tobacco plants of WT and NtCHS-RNAi lines were sampled and ground into homogenate using liquid nitrogen. After freeze-drying, the 20-mg samples were suspended in 1 ml of extraction solution (methanol:chloroform:water = 5:2:2) and treated by ultrasonic vibration for 45 min to extract naringenin. The extracted naringenin was analysed using LC-ESI-MS/MS (HPLC, Shim-pack UFLC Shimadzu CBM-20A system; MS, Applied Biosystems 4000 Q TRAP) according to the methods described previously (Zhao et al., 2020). Three biological replicates of each treatment were performed. The chromatographic conditions were as follows: Waters BEH C18 chromatographic column (150 mm × 2.1 mm, 1.7 μm); mobile phase: A phase water, B phase acetonitrile, 0.1% (vol/vol) formic acid in water.
acid and 0.2 mM ammonium acetate were added to both phases. Step-stripping sequence: 0–1.0 min, 10% B; 0–9.0 min, 10% B–90% B; 9.0–11.0 min, 90% B–100% B; 11.0–11.1 min, 100% B–10% B; 11.1–13.0 min, 10% B. Column temperature: 30°C; injection volume: 2 μl; flow rate: 0.25 ml/min. Mass spectrum conditions: retention time 4.94 min⁻¹; ion pair 1: quantitative ion 433.1/271.1, collision energy −22 V; ion pair 2: quantitative ion 433.1/150.9, collision energy −42 V.

4.10 | Inhibition of sporangia production

The effect of different concentrations of naringenin on sporangia production of *P. nicotianae* was assayed. A fresh mycelial block was immersed in V8 liquid medium and cultivated at 28°C for 48h. Mycelium was washed with sterile deionized water, then soaked in sterile deionized water containing 0, 0.78, 1.56, 3.13, 6.25, and 12.50 mg/L of naringenin. Following a 24-h incubation period at 28°C in the light, the sporangia were counted by observation with a microscope using three fields. Each treatment contained three replicates. The inhibition rate was calculated as follows: inhibition rate (%) = (control number of sporangia – treatment number of sporangia)/control number of sporangia × 100.

4.11 | Measurement of H₂O₂ content and production rate of O₂⁻ content

Three days after inoculation with *P. nicotianae*, HD roots treated with naringenin were used to measure ROS levels. The H₂O₂ content was determined by measuring the yellow titanium peroxide, which had specific absorption peak at 415 nm, according to the instruction of H₂O₂ Content Detection Kit (Suzhou Keming Biotechnology Ltd Co.). The production rate of O₂⁻ was determined by measuring the formation of red azo compound, which had a specific absorption peak at 530 nm, according to the O₂⁻ Detection Kit (Suzhou Keming Biotechnology Ltd Co.).

4.12 | Exogenous application of naringenin

Under simulated field conditions, *Nicotiana tabacum* ‘Honghuadajinyuan’ (HD) was cultured for 3 months in pots. Then, the stem base of the HD seedlings was treated with 100 ml of solution including 0.04 g of naringenin 1 week prior their inoculation with *P. nicotianae*. Naringenin was added only once. HD seedlings with inoculation by *P. nicotianae* but without prior treatment of naringenin were used as a negative control. After 10 days, the phenotype of TBS was evaluated using an empirical six-point scale (YC/T39-1996, China), where 0 represents a highly resistant response and 9 represents a highly susceptible response. Disease index scores based on disease severity were used for assessment and calculated using the following formula: disease index (%) = [Σ(disease evaluation scale score × number of plants with each scale score)/(total number of plants observed × the highest disease evaluation scale score)] × 100.

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**CONFLICT OF INTEREST**

No conflict of interest declared.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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