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Serratia marcescens-S3 inhibits *Potato virus* Y by activating ubiquitination of molecular chaperone proteins NbHsc70-2 in *Nicotiana benthamiana*

Ming Ge,[1,†] Mingyue Gong,[2,†] Yubing Jiao,[1] Ying Li,[1] Lili Shen,[1] Bin Li,[3] Yujie Wang,[4] Fenglong Wang,[1] Songbai Zhang[2] and Jinguang Yang[1]

[1]*Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, 266101, China.*

[2]*Hubei Engineering Research Center for Pest Forewarning and Management, Jingzhou, 434025, China.*

[3]*Sichuan Province Company of China Tobacco Corporation, Chengdu, 610017, China.*

[4]*Luoyang City Company of Henan Tobacco Company, Luoyang, 471000, China.*

Summary

The *potato virus* Y (PVY) is a plant virus that causes massive crop losses globally, especially in *Solanaceae* crops. A strain of the plant growth-promoting rhizobacterium (PGPR), *Serratia marcescens*-S3 *was found to inhibit PVY replication in Nicotiana benthamiana*. However, there have been no in-depth studies demonstrating the underlying mechanism. In the current study, we found that ubiquitination of NbHsc70-2 is an important way for *Serratia marcescens*-S3 *to trigger induced systemic resistance (ISR). After the treatment with S. marcescens*-S3, *the protein level of NbHsc70-2 reduced significantly. Inhibiting of ubiquitination increased the accumulation of NbHsc70-2 in plants and reduced S. marcescens*-S3-mediated *resistance to PVY. Furthermore, transgenic engineered Nicotiana benthamiana NbHsc70-2^{KO}* and *NbHsc70-2^{USM}* were constructed using CRISPR-Cas9-mediated *NbHsc70-2* knock-out and ubiquitination respectively. *S. marcescens*-S3 *significantly reduced the inhibition of NbHsc70-2 protein accumulation in NbHsc70-2^{KO}* and *NbHsc70-2^{USM}*. The virulence of PVY was stronger in *NbHsc70-2^{USM}* than the wild-type plants. These results showed that *S. marcescens*-S3 *increases the ubiquitination of NbHsc70-2 to inhibit the recruitment of molecular chaperone NbHsc70-2 to reduce its replication and infection of PVY.*

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For correspondence. *E-mail yangjinguang@caas.cn. **E-mail yangtze2008@126.com.

[†]These authors have contributed equally to this work and share first authorship.

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Introduction

There are different kinds of nonpathogenic bacteria on the surface of plant roots, some of which can promote plant growth and enhance disease resistance. These bacteria are called plant growth-promoting rhizobacteria (PGPR) (Cheol *et al*., 2016). Induced systemic resistance (ISR) refers to the colonization of plant rhizosphere by microorganisms, which induce persistent and systematic broad-spectrum resistance in plants to different pathogens, including viruses. Jasmonic acid (JA) and ethylene (ET) are critical signalling molecules that mediate these reactions (Naylor *et al*., 1998; Glick *et al*., 2007). A few studies have shown that seed treatment with PGPR led to resistance to several cucumber pathogens in greenhouse and field experiments, and evidence suggests that ISR may be involved in the biological control afforded by certain PGPR (Liu, 1996). Although several studies have been conducted to determine the signal transduction pathways of ISR, few studies have focused on the molecular mechanism underlying the post-translational regulation in plants.

Serratia marcescens is a Gram-negative bacterium that is found in a variety of ecological environments. It has been reported that *S. marcescens* produces a variety of extracellular enzymes, including chitinases, metalloproteinases, lipases and nucleases. Extracellular *S. marcescens* nuclease is an extremely active enzyme that nonspecifically degrades RNA and DNA. Its antiviral activity has been shown in honeybees (Panfilova and Salganik, 1983; Alikin *et al*., 2000). It has been reported

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that *S. marcescens* nuclease is important for plant resistance to the *Tobacco mosaic virus* (TMV) (Trifonova *et al*., 2015). On the other hand, prodigiosin, a secondary metabolite produced by *S. marcescens*, inhibited RNA synthesis in the *Zika virus* and activated the expression of Caspase-3, -7 and -8 in the plant cells. The EC50 values of prodigiosin to *Chikungunya virus* and *Semlik forest virus* were 0.03 μM and 0.11 μM, respectively (Varghese *et al*., 2017), indicating that they were very effective. In addition, prodigiosin also inhibited Mcl-1 protein activity and regulated the signal transducer and activator of the transcription 3 (STAT3) signalling pathway, which is activated during the treatment of *Influenza A virus* infection (Denisova *et al*., 2012).

As the definitive species of the *Potyviridae*, *Potato virus* Y (PVY) is a positive-stranded RNA virus. PVY disease is a systemic infectious disease caused by PVY and has caused huge economic losses to agriculture globally. PVY is a member of the largest plant virus genus in the world with the most severe economic impact. There are more than 200 definitive and tentative species in this genus, accounting for approximately 30% of the known plant viruses (Ward and Shukla, 1991; Dogimont *et al*., 1996). There is a lack of effective treatments for the control of PVY.

Replication of positive-stranded RNA viruses is a complex but well-organized process that involves the coordinated functioning of viral proteins and various host factors (Huang *et al*., 2012; Nagy and Pogany, 2012). Heat shock 70 protein (Hsp70) is a highly conserved molecular chaperone, which folds the newly synthesized proteins correctly, refolds the aggregated proteins, assembles and disassembles the large macromolecular protein complexes, translocates organellar and secretory proteins and protects the proteome from stress (Mayer and Bukau, 2005; Richter *et al*., 2010; Young, 2010; Hartl *et al*., 2013). Heat shock cognate 70 kDa protein (Hsc70), is a constitutively expressed cognate of Hsp70 (Sun *et al*., 2016). Previous studies have proved that Hsp70 and Hsc70 may complement each other to maintain cellular integrity during metabolic challenges (Chong *et al*., 2013). Despite their fundamental role in protein quality control, Hsp70 is strongly associated with multiple aspects of the viral infection cycle, such as virion assembly and disassembly, cell entry, genome replication and viral gene expression (Guerrero *et al*., 2002; Chromy *et al*., 2003; Ivanovic *et al*., 2007). It is observed that cytoplasmic Hsp70 plays a variety of roles in *Tomato bushy stunt virus* (TBSV) replication, for instance the insertion of the viral replication proteins into the peroxisomal membranes and the activation of TBSV replication by interacting with viral replication (Pogany *et al*., 2008; Pogany and Nagy, 2015). Recent studies have also indicated that an increase in Hsp70 expression promotes the proliferation of viruses. In addition to specific functions of Hsp70 family proteins in certain aspects of viral infection, these proteins can serve as a mediator for the orchestration of viral infection by interacting with different viral components. For example Hsc70-2 interacts directly with both p23 and coat protein of *Beet black scorch virus* (BBSV) in *N. benthamiana* (Wang *et al*., 2018).

In this study, *S. marcescens*-S3 *showed a notable inhibitory effect on PVY. Since the NbHsc70-2 protein content decreased significantly but the mRNA level of NbHsc70-2* increased, the induction of ubiquitination of NbHsc70-2 protein and resistance to PVY invasion by *S. marcescens*-S3 *were assessed. Our results showed that S. marcescens*-S3 *reduced the content of NbHsc70-2 in N. benthamiana* by increasing the ubiquitination level of NbHsc70-2 to inhibit the proliferation of PVY. These data provided new insight regarding the mechanism through which *S. marcescens*-S3 *induces ISR in N. benthamiana*.

Results

S. marcescens-*S3 inhibited the replication of PVY in tobacco*

Based on previous studies of *S. marcescens*-S3 against TMV (Bi *et al*., 2014), in terms of assessing the inhibitory effect of *S. marcescens*-S3 *on PVY, we first inoculated PVY-GFP and sprayed the S. marcescens*-S3 *solution of* 1×10^8 *CFU* ml^{-1} *or PBS at 3 dpi respectively. After observing the viral infection foci in systemic leaves at 7 d postmechanical inoculation (dpi), we found that compared to S. marcescens*-S3-treated *plants, PBS-treated plants developed more PVY-GFP foci and, subsequently, developed obvious necrotic lesions at 7 dpi in inoculated leaves (Fig. 1A). The transcriptional and translational levels of PVY coat protein* (*CP)* were evaluated using quantitative real-time PCR (qRT-PCR) and western blotting respectively. qRT-PCR results showed that the antiviral efficacy of *S. marcescens*-S3 *against PVY was about 80% (Fig. 1B), and western blotting assays of the CP protein of PVY revealed that the viral accumulation was lower in S. marcescens*-S3-treated *plants than in the PBS-treated plants (Fig. 1C). These results suggested that S. marcescens*-S3 *shows significant antiviral activity against PVY using PGPR ISR*.

The expression of NbHsc70-2 decreased after treatment with S. marcescens-*S3*

To examine the association between ISR and *NbHsc70-2* expression in terms of transcription and post-translational modification, the relative expression of *NbHsc70-2* in the leaves at 7 dpi was measured (Fig. 2A). The results suggested that, regardless of *S.*

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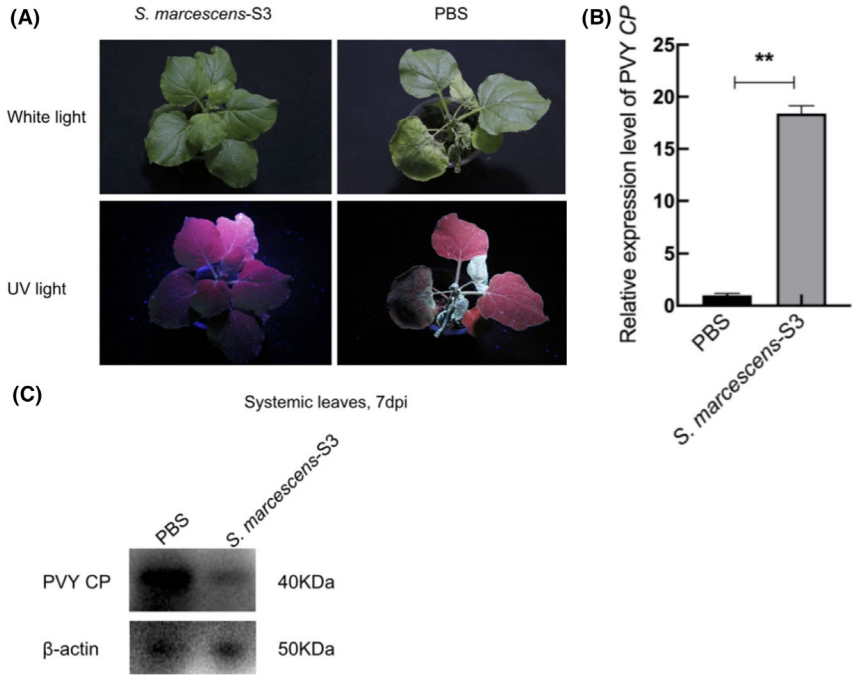

Fig. 1. The effect of *S. marcescens*-S3 *treatment on PVY infection.*
A. Systemic leaves of the PBS-treated plant (right) and *S. marcescens*-S3-treated *plant (left) after PVY inoculation under white light and UV light. Photos were taken 7 days post-PVY-GFP infection (dpi).*
B. The expression of PVY in the PBS- and *S. marcescens*-S3-treated *groups. Error bars indicate the standard deviations of three sets of biological replicates; data were analysed using Student's t*-test; *and* ** *indicates that the difference is significant (*$P < 0.05$).
C. Content of PVY CP protein after treatment with *S. marcescens*-S3.

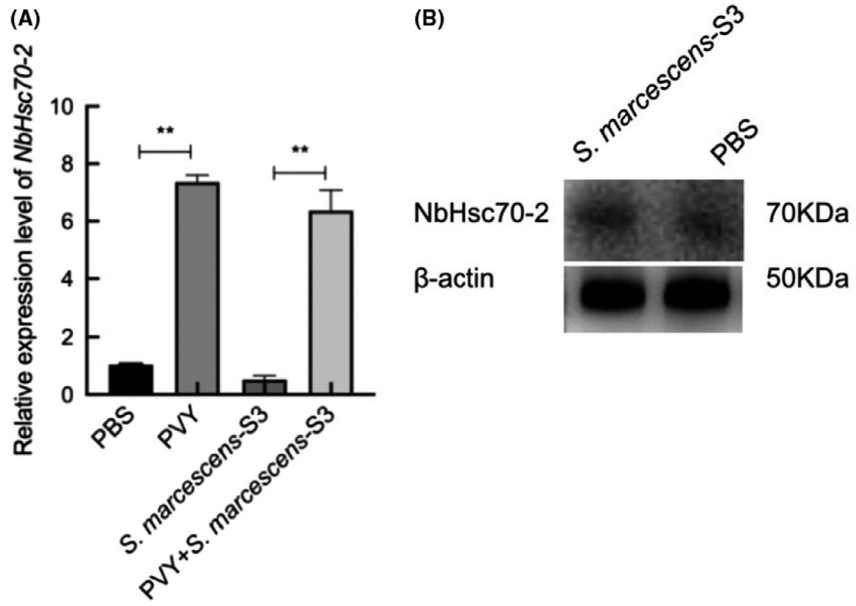

Fig. 2. Effects of treatment with *S. marcescens*-S3 *on transcription and translation of NbHsc70-2.*
A. The transcript level of NbHsc70-2 with treatments of PBS, PVY, *S. marcescens*-S3 *and PVY* + *S. marcescens*-S3. *Error bars indicate the standard deviations of three sets of biological replicates; data were analysed using Kruskal–Wallis t*-test; *and* ** *indicates that the difference is significant (*$P < 0.05$).
B. The protein content of NbHsc70-2 after *S. marcescens*-S3 *treatment.*

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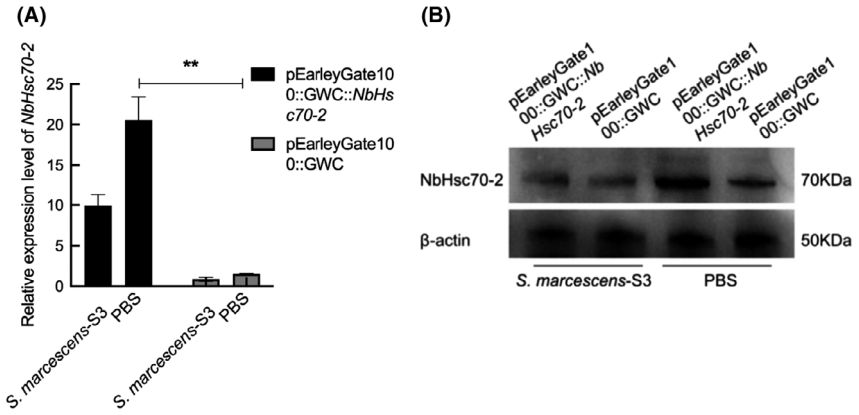

Fig. 3. The effects of transient expression of *NbHsc70-2* on the synthesis of the NbHsc70-2 protein in *S. marcescens*-S3.
A. The expression level of *NbHsc70-2* in the transient expression and control groups treated with *S. marcescens*-S3 *and PBS. Error bars indicate the standard deviations of three sets of biological replicates; data were analysed using Student's t*-test; *and ** indicates that the difference is significant (P* < 0.05).
B. The protein content of NbHsc70-2 in the transient expression and control groups treated with *S. marcescens*-S3 *and PBS.*

marcescens-S3 treatment, the transcription level of *NbHsc70-2* increased significantly after PVY inoculation. The protein level of NbHsc70-2 was evaluated using western blotting using Hsp70/Hsc70-specific antibodies (Fig. 2B). The quantitative value of *S. marcescens*-S3 group is 212.35, and that of PBS group is 131.68. The results indicated that, from day 7 onwards of the *S. marcescens*-S3 treatment, the content of NbHsc70-2 in leaves decreased noticeably compared with those in the PBS-treated group. Overall, these data suggested that *S. marcescens*-S3 did not strongly inhibit the transcription of *NbHsc70-2* during the PVY replication phase. It was proven that *S. marcescens*-S3 reduces the content of NbHsc70-2 protein in *N. benthamiana*.

S. marcescens-*S3 triggered ISR by reducing the protein content of NbHsc70-2*

Based on the above results, we hypothesized that *S. marcescens*-S3 may play a major role in the host ISR during PVY infection. To test this hypothesis, *NbHsc70-2* was transiently expressed using the transient expressing vector. After the treatment with *S. marcescens*-S3, qRT-PCR was used to verify that the expression of *NbHsc70-2* in the transient expression group increased significantly (Fig. 3A). Western blotting was performed to determine the protein concentration in the transient expression and empty vector groups after treatment with *S. marcescens*-S3 and PBS (Fig. 3B). The results showed that the quantitative value of transient expressing group was 83.65 after *S. marcescens*-S3 treatment, and that of empty vector group was 78.29; protein quantitative value of transient expressing group was 135.75 after PBS treatment, and that of empty vector group was 105.96. Compared to the transcription level of *NbHsc70-2*, the protein content of NbHsc70-2 in the transient expression group was not greater than that in the empty vector group after the treatment with *S. marcescens*-S3. *Taken together, S. marcescens*-S3 *had no significant effect on ISR at the transcription level in the plant cells. However, within the transient expression group, NbHsc70-2 was decreased nearly 50% in S. marcescens*-S3 treated plants compared with PBS treated plants at the transcriptional level. These observations suggested that the antiviral activity of *N. benthamiana* treated with *S. marcescens*-S3 *was due to a decrease in the NbHsc70-2 content in the plants, and we speculated a role of ubiquitination of NbHsc70-2 in this process.*

Ubiquitination of NbHsc70-2 was triggered by S. marcescens-*S3*

To verify whether ubiquitination played a crucial role in ISR after the treatment with *S. marcescens*-S3, *we used a proteasome inhibitor MG-132 to inhibit the ubiquitination in the host plants. Western blotting analysis showed changes in the NbHsc70-2 content in the leaves of the plants from different treatment groups. As shown in Fig. 4, the quantitative value of PVY and S. marcescens*-S3 *treatment group was 87.79, and that of PVY treatment group was 144.19, protein quantitative value of PVY and MG-132 treatment group was 108.40 and that of S. marcescens*-S3 *treatment group was 86.00, and the quantitative value of PVY, MG-132 and S. marcescens*-S3 *was 104.96. These data showed that treatment with S. marcescens*-S3 *decreased the accumulation of NbHsc70-2 significantly, and the treatment of leaves with MG-132 and S. marcescens*-S3 *decreased the accumulation of NbHsc70-2. This result indicated that the proteasome inhibitor MG-132 inhibited*

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ubiquitination of NbHsc70-2, and ubiquitination plays a key role in ISR caused by S. marcescens-S3. *The inhibition of ubiquitination led to the failure of NbHsc70-2 protein accumulation inhibited by S. marcescens*-S3 *to inhibit the replication of PVY.*

We also examined the fluorescence signal strength of RFP-tagged NbHsp70-2 (RFP-NbHsp70-2). We found a strong RFP fluorescence signal only in the PVY + MG-132 and PVY groups. A small amount of RFP fluorescence signal was detected in *S. marcescens*-S3 + PVY and *S. marcescens*-S3 groups (Fig. 5A). To investigate whether *S. marcescens*-S3 promote the ubiquitination of NbHsc70-2 in the plant specifically, the recombinant protein RFP-NbHsc70-2 was subjected to a pull-down assay to purify NbHsc70-2, and the difference in ubiquitination of NbHsc70-2 was demonstrated using Western blotting using an antibody against ubiquitin. The results showed that binding of ubiquitin to NbHsc70-2 increased significantly in the PVY-infected group treated with *S. marcescens*-S3 but decreased in the PVY-infected group treated with *S. marcescens*-S3 after treatment with MG-132. However, PVY inoculation had no significant effect on the amount of ubiquitin bound to NbHsc70-2, which may be due to the degradation of NbHsc70-2 (Fig. 5B). Thus, *S. marcescens*-S3 *inhibited the replication of PVY by inducing ubiquitination of the NbHsc70-2 protein.*

The inhibition of NbHsc70-2 protein accumulation by S. marcescens*-S3* failed in NbHsc70-2 knock-out/ ubiquitination site-mutated plants

To further assess the role of ubiquitination of NbHsc70-2 in the process of inhibition of PVY by *S. marcescens*-S3 *in tobacco, firstly, transgenic N. benthamiana* were generated using CRISPR-Cas9 system-mediated knock-out of *NbHsc70-2* (*NbHsc70-2^{KO}*). Then, we identified the potential ubiquitination sites (K560 and K563) in NbHsc70-2 using ubiquitomics data and bioinformatics analysis. Using *NbHsc70-2^{KO} N. benthamiana*, transgenic *N. benthamiana* with the lysine of NbHsc70-2 at K560 and K563 mutated to arginine were generated (*NbHsc70-2^{USM}*). The accumulation of NbHsc70-2 protein was evaluated using western blotting analysis using Hsp70/Hsc70-specific antibodies (Fig. 6A). When the ubiquitination sites of *NbHsc70-2* were mutated, the quantitative value of NbHsc70-2 in the *S. marcescens*-S3 *treatment group was 151.70, which was nearly 50% higher than those in the wild-type (WT) and NbHsc70-2^{KO}* groups. The *NbHsc70-2^{KO}* group showed bands, probably because the antibody also bonds to another protein homologous to *NbHsc70-2* in *N. benthamiana*. However, the expression level of NbHsc70-2 in the *S. marcescens*-S3 *treatment group did not increase significantly compared to that in the WT group after PVY inoculation. The NbHsc70-2 protein content in the NbHsc70-2^{KO}* plants was not significantly affected by *S. marcescens*-S3 *treatment or PVY inoculation. The expression of the PVY CP* was detected using qRT-PCR (Fig. 6B). The results showed that in the *NbHsc70-2^{KO}* and the *NbHsc70-2^{USM}* groups, *S. marcescens*-S3 *treatment had no significant effect on PVY replication, while PVY replication significantly decreased in the WT group. These results together proved that the ubiquitination of NbHsc70-2 protein is crucial for the inhibition of PVY by S. marcescens*-S3.

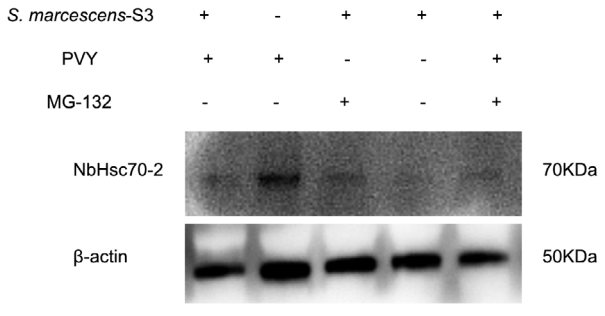

Fig. 4. The effects of the inhibition of ubiquitination on *S. marcescens*-S3-induced *reduction in NbHsc70-2 protein accumulation.*

Discussion

In the current study, we investigated a new pattern of ISR elicited by *S. marcescens*-S3, *a resident flora in the soil, against a virus. We found that S. marcescens*-S3 *significantly inhibited PVY. As a key component of different cellular stresses responses, NbHc70-2 is reported to be induced in viral infection (Gong et al*., 2020) and acts as a regulatory factor in the viral life cycle (Iordanskiy *et al*., 2004). Several lines of evidence from the current study that *S. marcescens*-S3 *acts against PVY by reducing the accumulation of NbHsc70-2 at the post-translational level. We found that the inhibition of ubiquitination led to the failure of S. marcescens*-S3-induced *degradation of NbHsc70-2. Moreover, we demonstrated that NbHsc70-2 knockout or ubiquitination-site mutation significantly reduced the antiviral effect of S. marcescens*-S3. *Notably, NbHsc70-2* knock-out mutant plants exhibited a visible effect of PVY resistance, and this phenotype was similar to that caused by the treatment with *S. marcescens*-S3.

PVY disease control commonly adopted by farmers involves the use of synthetic antiviral agents. Although their excessive application has been attributed to the incidence of resistance, environmental pollution and adverse side effects to human health have also been observed. Hence, biological control of the disease is the

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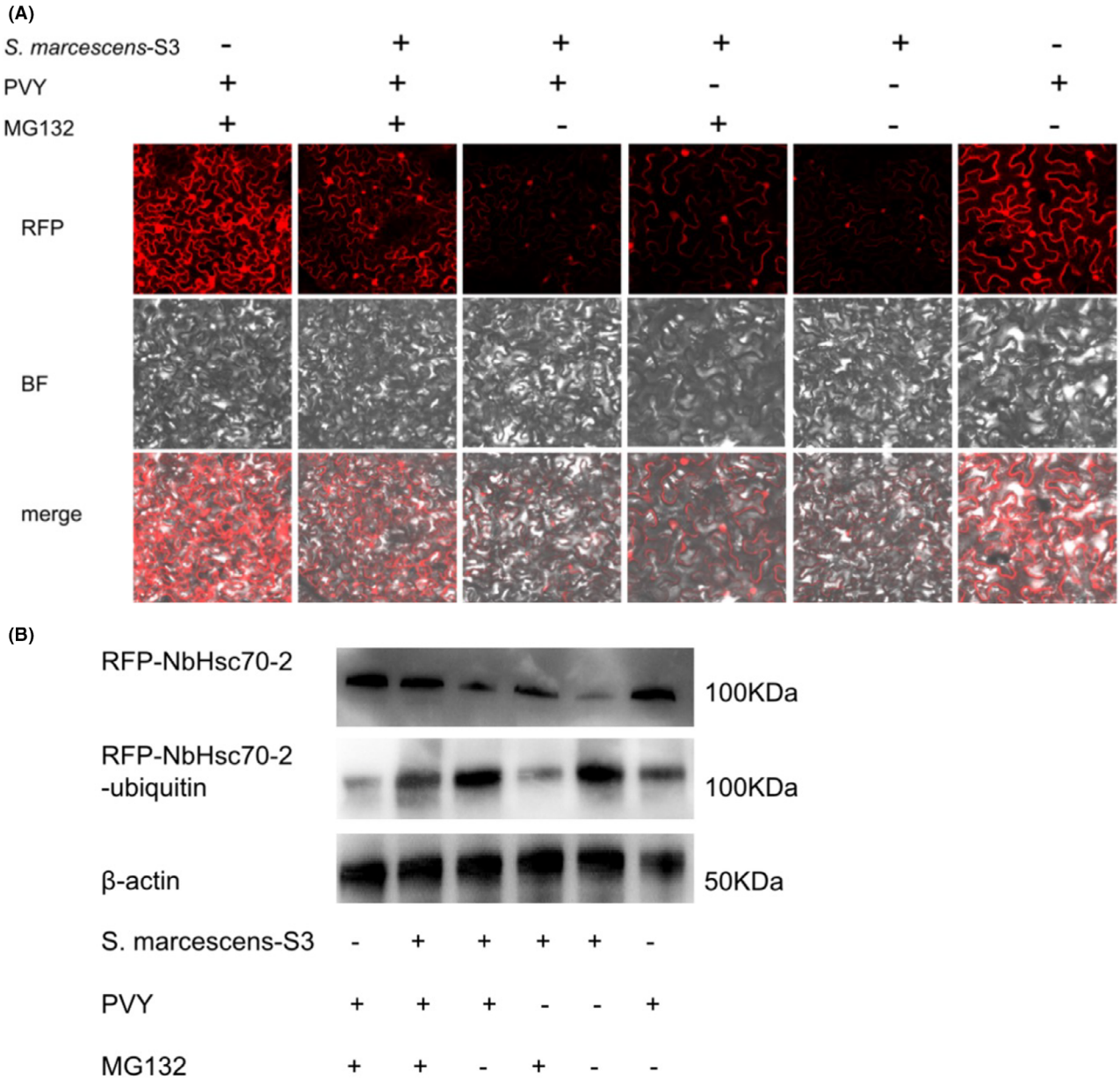

Fig. 5. The fluorescence and pull-down analyses of NbHsc70-2.
A. The fluorescence images of RFP-NbHsc70-2 inoculated *N. benthamiana* leaves. The leaves were treated with *Agrobacterium tumefaciens* carrying RFP-NbHsc70-2. The fluorescence signal was observed 48 h post-treatment.
B. The western blotting assay was performed to measure the RFP-NbHsc70-2 protein content obtained using pull-down purification.

focus of research worldwide (Killani *et al*., 2011). This method is considered a viable alternative for synthetic chemical-based approaches (Kumar *et al*., 2018). Lots of bacteria present in very different environments and also associated with other organisms in pathogenic, commensal or beneficial relationships, some of them, such as *Pseudomonas protegens* can even be found in several insect and myriapod classes (Vesga *et al*., 2021). In recent years, studies have shown that certain PGPR strains trigger broad-spectrum disease resistance, including viral diseases. Among those that have evaluated PGPR-induced ISR to viral infection, only a few have addressed the underlying mechanism (Murphy *et al*., 1999, 2000). The majority of studies on PGPR-induced ISR have focused on pathogenesis-related proteins like JA signal transduction and metabolic pathways and the superposition effects of ISR and systemic acquired resistance (SAR) (Wees *et al*., 2019). To date, several studies have shown that induced resistance by PGPR against a virus occurs downstream of SA or in an

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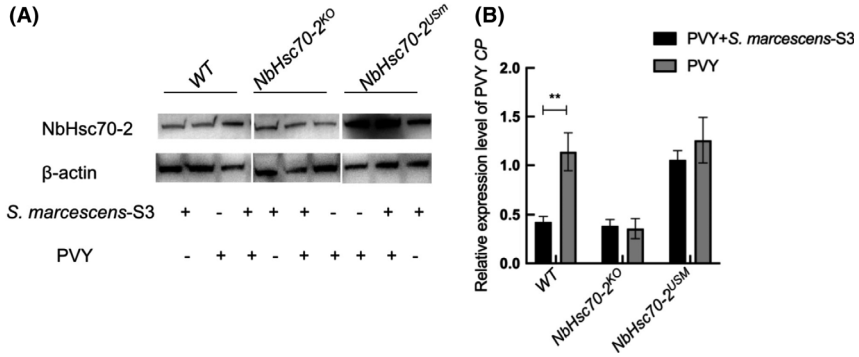

Fig. 6. The expression of NbHsc70-2 and PVY in different genotypes of *N. benthamiana* after treatment with *S. marcescens*-S3.
A. The differential accumulation of NbHsc70-2 in the different genotypes of *N. benthamiana* after the treatment with *S. marcescens*-S3.
B. Relative expression of PVY with different treatments in the *NbHsc70-2*^{KO} and *NbHsc70-2*^{USM} plants. Error bars indicate the standard deviations of three sets of biological replicates; data were analysed using Student's *t*-test; and ** indicates that the difference is significant ($P < 0.05$).

SA-dependent signalling pathway. In a few cases, ISR is SA-independent and develops without the accumulation of PR proteins but is dependent on JA, for example, the *S. marcescens* 90-166 strain resists infection of a variety of plant pathogens, including the *Cucumber mosaic virus* (Ryu *et al*., 2004). However, the mechanism underlying PGPR-induced stress during infection is still unclear.

Hsc70 is a structural Hsp70 that is expressed in all cells. It is an ATP-binding protein (Perez-Vargas *et al*., 2006). Hsp70 regulates the replication of several viruses (Xu *et al*., 2018), and there was no significant difference in the expression of Hsc70 under abiotic stress, such as heat stimulation, compared to that during normal conditions (Jörg and Stefan, 2014). Under biological stress, such as during viral infection, Hsc70 was considered to be involved in the replication, accumulation, movement and folding of viral proteins. In our previous study, we proved that PVY infection increases the expression of NbHsc70-2; that is the expression level of NbHsc70-2 was positively correlated with PVY replication, and NbHsc70-2 protein promoted PVY infection (Gong *et al*., 2020). These results are consistent with another previous report that Hsp70 was required for positive-strand RNA viral replication, and Hsp70 was also a part of the viral replicase complex (Mine *et al*., 2012). These observations suggested that Hsc70, too, may regulat viral replication.

Our previous results proved that *S. marcescens*-S3 *also has a significant control on PVY in N. benthamiana*. Also, we proved that *S. marcescens*-S3 *inhibited the accumulation of NbHsc70-2 induced by PVY at the post-translational level. Ubiquitination is a biological process in which the misfolded proteins in the endoplasmic reticulum are labelled and transported to the 26S proteasome for specific recognition and degradation (Voges et al*., 1999). The function of the ubiquitin-proteasome system (UPS) in different organisms, as well as its central role in mediating different cellular processes (Tian and Xie, 2013). In plants, the UPS functions to modulate pattern-triggered immunity by targeting a wide range of proteins for degradation or refolding (Spoel *et al*., 2009; Ning *et al*., 2016; Chen *et al*., 2018; Furniss *et al*., 2018). MG-132 is an effective, reversible and cell-mediated proteasome inhibitor with an IC50 value of 100 nM. This proteasome inhibitor, which is a chemical reagent that inhibits ubiquitination, can effectively block the proteolytic activity of the 26S proteasome complex (Dang *et al*., 2014). This reagent has been widely used in ubiquitination functional research. Therefore, we blocked the proteolytic activity of the 26S proteasome complex by using MG-132, and the degradation of NbHsc70-2 by *S. marcescens*-S3 *was greatly reduced. Subsequently, NbHsc70-2 was purified using pull-down assay using RFP-tagged NbHsc70-2, and western blotting using an antibody against ubiquitin proved that ubiquitination indeed had a direct effect on the resistance of the plants induced by S. marcescens*-S3. *To further confirm the key role of ubiquitination in S. marcescens*-S3-induced *NbHsc70-2 degradation, we generated transgenic plants whose NbHsc70-2* ubiquitination site was mutated. The results showed that inhibition of ubiquitination reduced the control effect of *S. marcescens*-S3 *on PVY and knock-out of NbHsc70-2* did not change the accumulation of NbHsc70-2, however, the plants with mutated ubiquitination sites accumulated more NbHsc70-2 upon PVY infection. For PVY, knock-out of *NbHsc70-2* did affect its elevated virulence, which also confirms the findings of the previous study (Gong *et al*., 2020), and *S. marcescens*-S3 *was also much less effective against PVY on NbHsc70-2*^{USM}, for NbHsc70-2, irrespective of ubiquitination, determine whether *S. marcescens*-S3 *controls viral replication. Ubiquitination*

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plays a key role in the ISR of plants treated with S. marcescens-S3. *The inhibition of ubiquitination could led to the failure of NbHsc70-2 protein degradation induced by S. marcescens*-S3 *and did not inhibit the replication of PVY.*

In summary, our results showed that the protection of *N. benthamiana* against PVY by a plant growth-promoting rhizobacterium *S. marcescens*-S3 *is because S. marcescens*-S3 *inhibits the accumulation of host protein NbHsc70-2. Further confirmation was provided since S. marcescens*-S3 *indirectly reduced viral replication by increasing the level of ubiquitination of NbHsc70-2. Taken together, these results provided a new direction to elucidate the mechanism through which ISR is triggered by plant growth-promoting rhizobacteria.*

Experimental procedures

Biological material and growth conditions

The growth-promoting rhizobacterium *S. marcescens*-S3 (GenBank number: MK411566.1) was isolated from tobacco rhizosphere soil samples collected in Shanxi Province (Qin *et al*., 2019). The strain was stored in ultra-cold storage before use. Before the experiment, the strain was streaked onto Luria-Bertani (LB) broth and incubated at 28°C for 24 h to check for purity. After transferring single colonies, the bacteria were incubated for 2 days at 28°C on LB broth and were then scraped off plates and suspended in sterilized distilled water (SDW). The resulting bacterial suspensions were adjusted with SDW to 1×10^8 CFU ml^{-1} at OD_{600}.

The plant material used in the current study was *N. benthamiana*. The construction of transgenic *N. benthamiana* was entrusted to Towin Biotechnology (Wuhan, China). Seeds were sown in soil mixtures (soil:-peat = 1:1) and cultivated in an artificial climate-controlled chamber at 25°C, under a 16 h light/8 h dark cycle with 70% relative humidity. PVY and GFP-tagged PVY (PVY-GFP) were constructed as described previously (Sun *et al*., 2018). The experiment involved the use of tobacco plants at the 5–6-leaf stage. The nutrient substrate used was obtained from the World Nutrient Soil Processing Plant in Shouguang city, China, and no additional fertilizer was applied during plant growth.

Extraction of total RNA

Fresh plant leaves were ground into a light-green powder in liquid nitrogen. The powder was then transferred to a 1.5 ml-RNase-free centrifuge tube, and 1 ml of RNA TRIzol reagent was added to fully lyse it at 4°C for 5 min. Then, the supernatant was removed and transferred to a new 1.5 ml RNase-free centrifuge tube. Two hundred microlitres of chloroform were added, after which the tube was shaken vigorously for 15 s to form an emulsion. Then, the tube was incubated at room temperature for 5 min and then centrifuged at 12 000 *g* for 15 min at 4°C. The upper aqueous phase was removed with a pipette and transferred to a new RNase-free centrifuge tube. An equal volume of precooled isopropanol was added, after which the contents were thoroughly mixed. The tube was then incubated at room temperature for 10 min. The mixture was then centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was discarded, and 1 ml of 75% ethanol was added to the tube. The contents were thoroughly mixed, after which the tube was incubated at room temperature for 5 min. The tube was, then, centrifuged again at 12 000*g* for 5 min at 4°C, after which the supernatant was discarded. RNase-free ddH_2O was then added to dissolve the precipitate.

cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Approximately 1.0 μg of RNA was used to produce cDNA using the HiScript® II Q Select RT SuperMix for qPCR Kit (Vazyme, Nanjing, China). qRT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR system together with ChamQ Universal SYBR qPCR Master Mix (Vazyme). qRT-PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) and condition was as follows: 40 cycles each consisting of 95°C for 30 s, 95°C for 5 s and 60°C for 34 s. The β-*Actin* reference gene in *N. benthamiana* was used as the internal control gene. All results are shown as the means of at least three biological replicates with corresponding SE. Data were analysed and normalized to control gene transcript levels. A relative quantitative method ($2^{-\Delta\Delta Ct}$) and the Student's *t*-test were used to evaluate relative expression differences. A *P*-value of < 0.05 was regarded as statistically significant. The sequences of the primers used in this study are listed in Table S1.

Plasmid construction

The oligonucleotides for plasmid construction and the related primers used in the study are listed in the Table S1. Briefly, the *NbHsc70-2* gene (National Center for Biotechnology Information: KX912913.1) was amplified with cDNA of *N. benthamiana*. To measure the transient expression of *NbHsc70-2* and imaging using confocal microscopy, the sequence was introduced into GWC (Sun *et al*., 2018) and Fu46 (Song *et al*., 2020) entry vectors, respectively, using the In-fusion Cloning Kit (Takara, Kyoto, Japan). Next, the reconstructed

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vectors GWC::*NbHsc70-2* and Fu46::*NbHsc70-2* were transformed into pEarleyGate100 expression vector using LR Clonase™ II enzyme mix (Invitrogen, Carisbad, CA, USA) to construct recombinant vectors pEarleyGate100::GWC::*NbHsc70-2* and pEarleyGate100::Fu46::*NbHsc70-2* respectively. The pEarleyGate100::GWC::*NbHsc70-2* and pEarleyGate100::Fu46::*NbHsc70-2* vectors were then transformed into bacterial *Trans1-T1* competent cells (TransGen Biotech, Beijing, China). The positive colonies were analysed using PCR with universal M13 Forward Reverse Primers. The empty vector pEarleyGate100::GWC was used as a negative control in the *NbHsc70-2* transient expression experiment.

Agroinfltration

Plasmids pEarleyGate100::GWC::*NbHsc70-2,* pEarleyGate100::GWC and pEarleyGate100::Fu46::*NbHsc70-2* were introduced into *Agrobacterium tumefaciens* strain LB4404 (Biomed, Beijing, China) cells using the freeze–thaw method. Selected positive colonies were cultured in LB broth medium supplemented with 100 μg ml^{-1} kanamycin and 50 μg ml^{-1} rifampicin at 28°C for 14–16 h. *A. tumefaciens* cells were harvested and resuspended in infiltration media [200 μM $MgCl_2$, 10 mM acetosyringone and 10 mM MES (pH 5.6)]. to achieve a final OD_{600} of 0.6 following growth in the dark at room temperature for 3 h. A syringe without a needle was used to infiltration 100 μl of *A. tumefaciens* cells suspension in *N. benthamiana* leaves.

Western blotting analysis

Protein samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). Anti-Hsp70/Hsc70 rabbit polyclonal antibody (Agrisera, Vännäs, Sweden) and anti-PVY CP rabbit polyclonal antibody (Agdia, Elkhart, IN, USA) were used as primary antibodies. Horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG antibody (Abcam, Eugene, OR, USA) was used as the secondary antibody. Incubation with the antibody was followed by ECL detection (Agdia), and the wavelengths were used for visualization are 400–450 nm. Signals were detected using a luminescent image analyser (LAS-1000 Plus), and the signal intensities were quantified using the ImageJ program (NIH, UK).

Confocal microscopy observation

The aforementioned recombinant plasmid pEarleyGate100::Fu46::*NbHsc70-2* was transformed into *A. tumefaciens* strain LB4404, and the transformed LB4404 cells were infiltrated into *N. benthamiana* leaves. After infiltrated for 48 h, the fluorescence patterns in infiltrated leaves were analysed using Leica confocal laser-scanning microscope (SP8; Leica, Weztlar, Germany). The excitation wavelengths used for RFP were 552 nm, and the emitted light from RFP was detected using a wavelength between 580 and 630 nm respectively.

Pull-down assays

Dynabeads (50 μl) were added to a tube (1.5 mg) and the tube was placed atop a magnet to separate the beads from the solution. The supernatant was removed. Five micrograms of the antibody (Ab) diluted in 200 μl of PBST was added to the tube. The tube was then incubated in a shaker incubator for 1 h at room temperature. The tube was subsequently placed atop a magnet, and the supernatant was removed. The tube was then removed from the magnet, after which the bead-Ab complex was resuspended in 200 μl PBST. The contents were mixed by gentle pipetting. Then, the tube was placed atop a magnet, and the supernatant was removed. Three hundred microlitres of the sample containing the antigen were added, and the mixture was gently mixed with a pipette to resuspend the Dynabeads®-Ab complex. The tube was incubated in a shaker incubator for 1 h at room temperature to allow the antigen (Ag) to bind to the Dynabeads®-Ab complex. Then, the tube was placed atop a magnet. The Dynabeads®-Ab-Ag complex was washed thrice with 200 μl PBST. The contents of the tube were separated using a magnet after each wash, after which the supernatant was removed and resuspended by gentle pipetting. The Dynabeads®-Ab-Ag complex was resuspended in 100 μl wash buffer, after which it was transferred to a new tube. The tube was placed atop a magnet, and the supernatant was removed. Then, 50 μl of SDS sample buffer (including DTT) was added to the tube. The contents were gently mixed with a pipette to resuspend the Dynabeads-Ab-Ag complex, after which the tube was heated for 10 min at 70°C. The tube was then placed atop a magnet, and the supernatant/sample was removed and subjected to western blotting analysis.

Statistical analysis

All experiments were performed in independent biological triplicates. Student's *t*-test and Kruskal–Wallis *t*-test were performed in SPSS (v.21, IBM, Armonk, NY, USA), and the graphs were prepared using GraphPad Prism 8 (significance indicated by $P \leq 0.05$).

Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in this study.

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