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Research Paper

Arabidopsis GH3.5 regulates salicylic acid-dependent and both NPR1-dependent and independent defense responses

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole acetic acid; KB, king's B; PR, pathogenesis-related; R, resistance; SA, salicylic acid; SAR, systematic acquired resistance

Key words: GH3.5, salicylic acid, defense response, double mutants, NPR1, NahG, *Pseudomonas syringae*

The cross-talk between plant disease resistance and development is fundamental to understanding systemic physiological processes during pathogen attack. Our previous study showed that the Arabidopsis *GH3.5* gene acts as a bifunctional modulator of the salicylic acid (SA)-mediated resistance and the auxin-mediated susceptibility during the Arabidopsis-*Pseudomonas syringae* interaction as well as development. Here, we further study the role and mechanism of *GH3.5* involved in the SA-dependent defense pathway. Transcript and histochemical analysis of the *GH3.5* promoter::*GUS* reporter expression indicate that *GH3.5* is expressed with a strong temporal and spatial manner with predominant expression in the divisional tissues. Upon bacterial challenge, GUS activity is induced in the junction tissue around the infiltrated zone with higher levels in the vasculature with a pattern different between the incompatible and compatible interactions. Exogenous SA application enhances disease resistance in the activation-tagged mutant *gh3.5-1D*, while the *GH3.5*-mediated defense enhancement is depleted in the SA deficient *gh3.5-1D/NahG* double mutant, indicating that *GH3.5* modulates defense response through the SA-dependent pathway. Furthermore, bacterial growth in the *gh3.5-1D/npr1* double mutant treated with SA indicates that *GH3.5* enhances the SA-mediated defense response through both *NPR1*-dependent and independent pathways.

Introduction

It has been widely recognized that SA plays a central role in plant defense against pathogens and is required for the induction of a set of *PR* genes and synthesis of defense compounds.[1] Exogenous SA application can induce a set of *PR* genes and establish the systematic acquired resistance (SAR), resulting in broad-spectrum disease resistance.[2] Transgenic plants expressing the *NahG* gene, encoding a salicylate hydroxylase that catalyzes the conversion of SA to catechol, fail to accumulate SA after pathogen infection and are compromised in SAR, basal resistance and some *R* gene-mediated resistance.[3,4] Furthermore, the Arabidopsis *sid1/eds5* and *sid2/eds16* mutants deficient in pathogen-induced SA accumulation are also impaired in SAR, certain *R* gene-mediated resistance and basal resistance.[5,6] Extensive studies have also shown that the *NPR1* gene (also known as *NIM1* and *SAI1*) functions as the key regulator of the SA-mediated SAR. The mutants of the gene, *npr1/nim1/sai1*, lost the expression of SA-induced *PR* genes and SAR.[7-10]

The *GH3* gene family, an early auxin-responsive gene group, was first isolated by differential screening from etiolated seedling hypocotyls of soybean (*Glycine Max*) after treatment with 2,4-dichlorophenoxyacetic acid (2,4-D).[11,12] One of Group II GH3 proteins of Arabidopsis, GH3.5 (At4g27260), adenylated both indole acetic acid (IAA) and salicylic acid (SA) in vitro,[13,14] suggesting that GH3.5 could potentially function in modulating and integrating both the auxin and SA signaling pathways. We conducted extensive genetic, molecular and biochemistry analysis, and demonstrated that GH3.5 acts as a bifunctional modulator of SA and auxin signaling during the Arabidopsis-*Pseudomonas syringae* (*P. syringae*) interaction.[15] The activation-tagged mutant *gh3.5-1D* that overexpresses *GH3.5* accumulated high SA levels and increased expression of *PR-1* in local and systemic tissues in response to avirulent *P. syringae*. By contrast, two T-DNA insertion mutants of *GH3.5* partially compromises the systemic acquired resistance associated with diminished *PR-1* expression in systemic tissues. The *gh3.5-1D* mutant also accumulated high levels of free IAA after pathogen infection and impairs different *R* genes-mediated resistance, revealing another dimension to the complex and dynamic plant-pathogen interaction.[15] A similar activation-tagged mutant of the *GH3.5* gene, *wes1-D*, was also recently reported to accumulate SA and exhibit enhanced disease resistance to *Pst* DC3000 at the flowering stage with spray-inoculation under the long day conditions, which also exhibited the altered light response.[16,17]

Recently, GH3.12/PBS3/GDG1/WIN3, a group III GH3 member, was shown to positively regulate SA-dependent disease resistance.[18-20] Taken together, these studies indicate that some members of the GH3 family play a critical role in SA signaling and

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induced defense responses. However, how these GH3s regulate the SA-mediated defense response is still largely unknown. For example, we have shown that although SA accumulation and the expression of *PR-1* were elevated in local and systemic tissues in response to avirulent pathogens, the *R* gene-mediated local resistance was compromised in *gh3.5-1D* due to counteracting by the auxin-mediated susceptibility,[15] as the elevated auxin levels within host tissue promote *P. syringae* virulence and the type III effector AvrRpt2 may be one of the virulence factors of *P. syringae* that modulate host auxin physiology to promote disease.[21] It is also intriguing that the rice *GH3.8* gene plays a role in the SA- and jasmonate-independent basal immunity through suppressing expansin expression, indicating that the cell wall is actively involved in the plant immunity.[22]

In this work, we investigate the roles of *GH3.5* in SA-dependent defense pathway with double mutants (transgenes) *gh3.5-1D/Nahg* and *gh3.5-1D/npr1,* and demonstrate that *GH3.5* modulates defense response through SA-dependent and both NPR1-dependent and independent pathways.

Results and Discussion

To assess developmental regulation of the *GH3.5* gene, we detected the *GH3.5* transcript in young seedlings and different organs of the adult wild-type plant by northern blot analysis. As shown in Figure 1A, except low level in the rosette leaves, high expression levels of *GH3.5* were detected in one-week-old whole seedling, root, stem, buds and blooming flowers of the adult plant. This pattern is slightly different from the previous observation with RT-PCR Southern blot which showed that *GH3.5* was expressed at a relatively high level in rosette leaves.[16] In order to further confirm the *GH3.5* expression pattern, we developed transgenic plants (GH3.5-GUS) carrying a *GH3.5* promoter-*GUS* reporter fusion gene, and detected GUS activity in different tissues. In one-week-old seedlings, GUS activity was mainly centralized at the meristems, newly-born true leaves, root tips, lateral root primordia and developing lateral roots (Fig. 1B–E). GUS activity was also detected at the vascular bundles of cotyledons. In the buds, GUS was mainly stained at sepals and developing stamens (Fig. 1F). In the blooming flowers, GUS activity was mostly distributed at stigma, filaments and anthers (Fig. 1G). Taken together, these results demonstrate that *GH3.5* is expressed with a strong temporal and spatial manner with predominant expression in the divisional tissues where auxin is produced and functions.

To examine roles of *GH3.5* in the SA-dependent defense pathway, we first examined the exogenous SA-induced disease resistance (SAR) in *gh3.5-1D*. Wildtype and *gh3.5-1D* plants were first treated with 1 mM SA or buffer (mock) and inoculated with virulent *Pst* DC3000 two days later. We observed that SA treatment alleviated disease symptom in *gh3.5-1D* plants compared with wildtype plants (Fig. 2A). Bacterial growth titer decreased 0.73 Log in the SA-treated Col-0 plants compared to the mock-treated plants at day 3 post inoculation (dpi), confirming that SA could trigger a disease resistance response in wildtype plants as expected (Fig. 2B). Interestingly, the SA-induced resistance was indeed stronger ($p < 0.05$) in *gh3.5-1D* compared with the wildtype, which exhibied a 1.5 Log decrease of bacterial growth titer compared to the mock-treated plants at 3 dpi. The result demonstrates that *GH3.5* positively modulates SA signaling, leading to a slight but reliable increased resistance to virulent pathogen in *gh3.5-1D* after exogenous SA treatment.

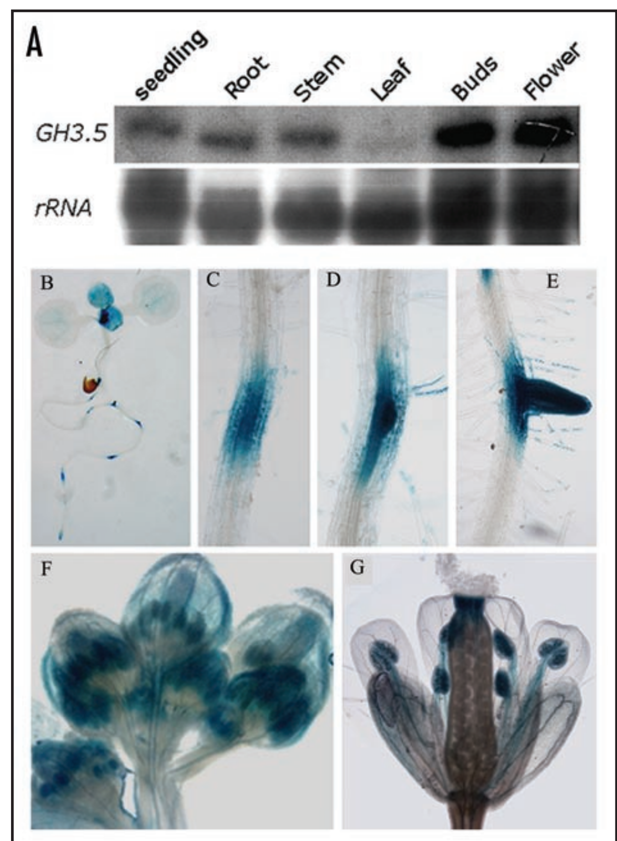

Figure 1. Expression pattern of *GH3.5*. (A) Northern blot analysis of *GH3.5* in different tissues of Col-0. (B) GUS activity in 7-day-old seedling of the *GH3.5-GUS* transgenic plant. (C–E) GUS activity during lateral root development in the *GH3.5-GUS* plant. (F) GUS activity in buds of the *GH3.5-GUS* plant. (G) GUS activity in the blooming flower of the *GH3.5-GUS* plant.

Therefore, this result further supported the previous observation that the pathogen-induced SAR was indeed stronger induced in *gh3.5-1D* than the wildtype but was counteracted by the simultaneously augmented auxin-mediated susceptibility, leading to the normal resistance outcome in term of bacterial growth.[15]

GH3.5 is inducible by *P. syringae* with different induction patterns by avirulent and virulent strains in wildtype plants.[15] To further examine the induction pattern of *GH3.5*, GUS activity was examined histochemically in the GH3.5-GUS transgenic plants. We observed that *GH3.5-GUS* was expressed at a low basal level in $MgCl_2$-treated leaves (Fig. 3A and B). When challenged with bacteria, GUS activity was induced in the junction tissue around the infiltrated zone with higher levels in the vasculature in response to both virulent and avirulent pathogens. Interestingly, GUS activity was mainly centralized around the chlorotic areas, with limited extension into the vasculature in the compatible interaction. Taken together, the different induction patterns of *GH3.5* by the virulent and avirulent pathogens further support the hypothesis that *GH3.5* plays different roles in the compatible and incompatible interactions.[15]

Since the activation of the SA pathway is associated with the expression of certain *PR* genes such as *PR-1*,[2] we further analyzed the expression of *PR-1* induced by exogenous SA in both *gh3.5-1D* and wildtype plants. As shown in Figure 4A, the kinetics of *PR-1* induction was altered in *gh3.5-1D* compared with the wildtype after SA treatment. In contrast to a prolonged elevation of the *PR-1* transcript

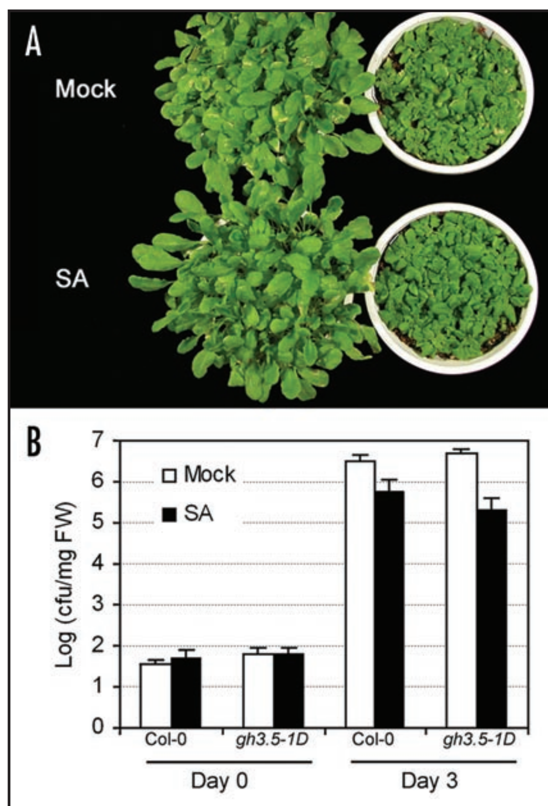

Figure 2. SA-induced disease resistance in Col-0 and *gh3.5-1D*. (A) Disease symptom of *Pst* DC3000 in mock and SA-treated Col-0 (left) and *gh3.5-1D* (+/-) (right). Five-week-old plants of the wildtype and mutants were sprayed with 1 mM SA and further inoculated with *Pst* DC3000 at a dose of 10^5 cfu ml^{-1} (OD600 = 0.0002). All controls were infiltrated with 10 mM $MgCl_2$ for mock inoculation. Photos were taken at 3 dpi. Similar results were observed in two independent experiments. (B) Growth of *Pst* DC3000 in SA-treated leaves of Col-0 and *gh3.5-1D*. Bacterial growth assay was performed at 0 and 3 days after inoculation in Col-0 and *gh3.5-1D* (+/-). All values are means ± SE (n = 6). The SA-induced resistance was significantly stronger ($p < 0.05$) in *gh3.5-1D* than the wldtype. cfu, colony-forming units. Similar results were observed in two independent experiments.

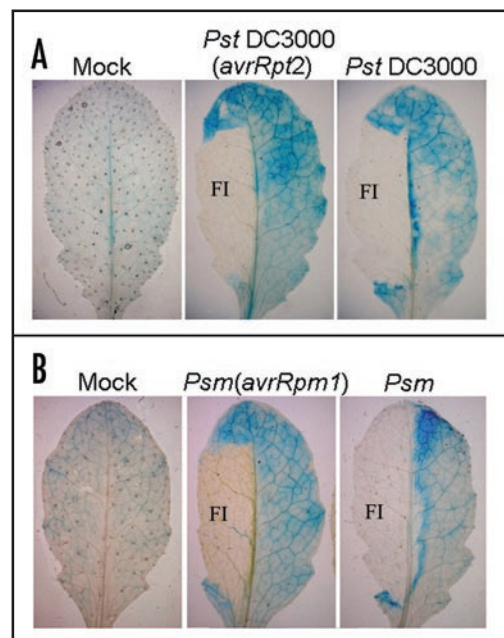

Figure 3. Histochemical assay of the expression pattern of the *GH3.5-GUS* reporter in responses to pathogen. (A) Induction of the *GH3.5-GUS* fusion reporter by *Pst* DC3000(*avrRpt2*) and *Pst* DC3000 at 10^7 cfu ml^{-1} at 3 dpi. FI, filter-infected areas. (B) Induction of the *GH3.5-GUS* fusion reporter by *Psm(avrRpm1)* and *Psm* at 10^7 cfu ml^{-1} at 3 dpi. Leaves were infiltrated with 10 mM $MgCl_2$ for mock inoculation (A and B).

from 6 to 48 h in the wildtype control, the SA-mediated induction of the *PR-1* gene exhibited a sharper pattern with a peak at 12 h and no *PR-1* expression at 48 h in both heterozygous and homozygous *gh3.5-1D* plants. These results indicated that *GH3.5* regulates *PR-1* in response to SA treatment, with a significant effect on the kinetics of *PR-1* expression. This kind of defense gene induction was previously observed during incompatible interactions in bean and parsley cells,[25,26] suggesting that this kind of expression pattern may be related to the establishment of an efficient resistance.

To determine whether the *GH3.5*-enhanced defense depends on SA accumulation, we generated a double mutant between the *gh3.5-1D* mutant and the *NahG transgenic* plants known to be defective in SA accumulation.[3,4] The expression of *PR-1* was first analyzed in these plants after inoculation with *Psm*(*avrRpm1*). As shown in Figure 4B, *PR-1* was only slightly induced in both *NahG* and *gh3.5-1D/NahG* plants, which is in sharp contrast to a strong *PR-1* induction in *gh3.5-1D*, demonstrating that the increased expression of *PR-1* in *gh3.5-1D* is dependent on SA accumulation. In addition to the reduced *PR-1* induction, bacterial growth assays revealed that both the SA-induced SAR and basal resistance were abolished in *gh3.5-1D/NahG* plants like in *NahG* plants at 3 dpi (Fig. 4C; reviewed in ref. 15). These results confirmed that the enhancement of defense responses by over-expressing *GH3.5* indeed depends on SA accumulation.

We have shown that *GH3.5* is inducible by pathogen and SA.[15] However, the induction of *GH3.5* by *Psm*(*avrRpm1*) in *NahG* and *sid2-2*[27] plants, both accumulating low levels of SA in response to pathogen, was quite same to that in the equivalent wildtype plants (Fig. 5A). Similar induction of *GH3.5* was also observed in the *npr1* mutant. These results further support the postulation that the pathogen-induced expression of the *GH3.5* gene is mediated most likely by an SA-independent pathway, and plays an important role in strengthening the SA pathway.[15] To further address the *GH3.5*-mediated SA pathway enhancement in SAR, we developed another double mutant *gh3.5-1D/npr1* since NPR1 is a major regulator of SAR,[28] and conducted the SA-mediated SAR assay. As shown in Figure 5B, there was a 0.51 Log ($p < 0.05$) decrease of bacterial growth in *gh3.5-1D/npr1* plants than in *npr1* plants after SA treatment, a growth value higher than that in *gh3.5-1D* treated with SA (Fig. 2), suggesting that SA treatment induced resistance through both the *NPR1*-dependent and independent pathways in *gh3.5-1D*.

Although extensively studies have been done on SA signaling, little is known about how SA acts. For instance, it is well established that the NPR1-regulated expression of *PR* genes is required for the induction of SAR,[9,28] but understanding of the molecular mechanism underlying SAR against a broad spectrum of pathogens is limited. The recent finding that methyl salicylate is a mobile signal for SAR opens a door to these questions.[29] Our data has revealed that *GH3.5* plays an important role in the regulation of SA signaling in plant defense, since *gh3.5-1D* exhibited a stronger SA-induced disease resistance accompanied by a greatly altered kinetics of

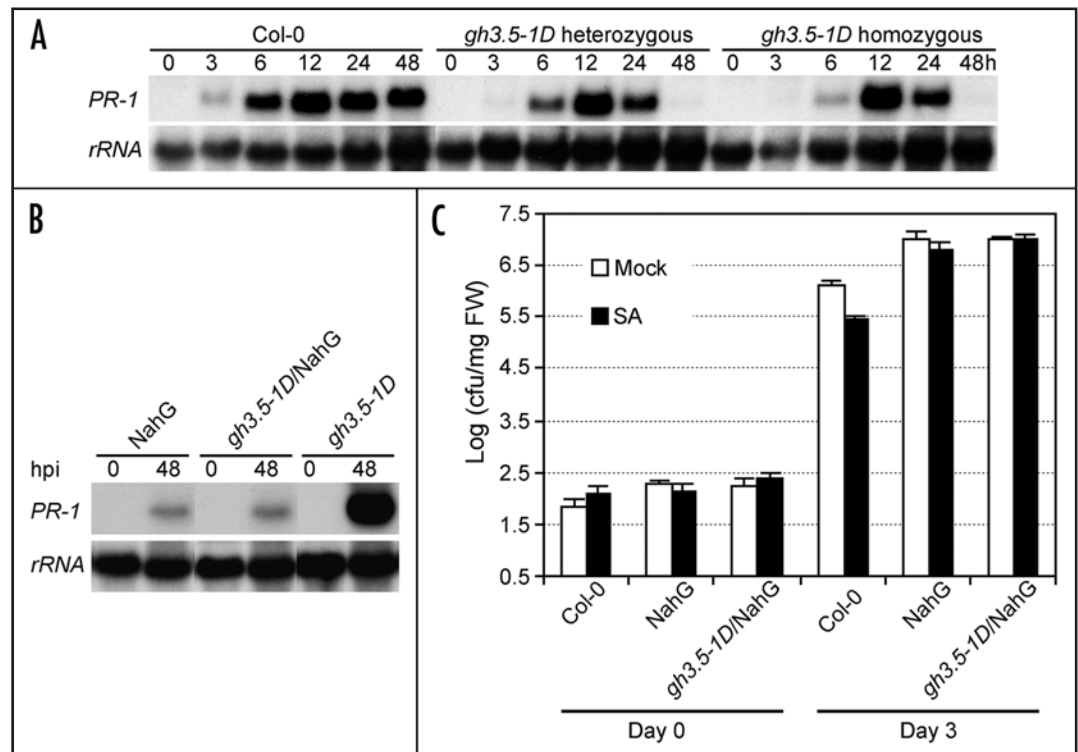

Figure 4. *GH3.5* modulates SA-dependent defense response. (A) Northern blot analysis of *PR-1* induction by exogenous application of SA (0.5 mM) over a time course of 0 to 48 h after treatment in *gh3.5-1D* heterozygous (*gh3.5-1D*$^{+/-}$) and homozygous (*gh3.5-1D*$^{-/-}$) plants, in comparison with Col-0 plants. The experiment was biologically repeated once. (B) Expression of *PR-1* in *NahG*, *gh3.5-1D/NahG* double mutant and *gh3.5-1D* plants after infection with *Psm(avrRpm1)* at 10^7 cfu ml^{-1}. Leaves were collected at 0 and 48 hpi. The experiments were repeated once with similar results. (C) Growth of *Pst* DC3000 in Col-0, *NahG* and *gh3.5-1D/NahG* plants after pre-treatment with SA (1 mM) or buffer (mock). Bacterial titers were repeated twice with similar results. All values are the mean ± SE (n = 6).

PR-1 expression. Furthermore, the *PR-1* induction was no longer strengthened in the *gh3.5-1D/NahG* double mutant that exhibited no SAR. These results indicate that *GH3.5* enhances the SA-mediated disease resistance, providing the further evidence that GH3.5 act as a positive regulator of the SA pathway in response to pathogen. Interestingly, the *GH3.5*-enhanced SA pathway is both *NPR1*-dependent and independent since the *gh3.5-1D/npr1* double mutant still exhibited partial SAR. Consistent with this, our previous microarray analysis revealed some *NPR1*-dependnet *WRKY* and *TGA* transcription factors, and *NPR1*-independent *α-DOX1*.[15] Our current study also adds evidence that the SA-dependent defense responses are either NPR1-depdendent or independent.[30-34] How these signaling pathways are integrated to defense pathogens is still a big challenge to plant biologists.

Materials and Methods

Plant materials and growth conditions. The activation-tagged mutant *gh3.5-1D* was previously described.[15] The *sid2* mutant was provided by Prof. Fred Ausubel, the *npr1* mutant was provided by Prof. Xinnian Dong. Seeds were surface sterilized and germinated on 1/2 MS agar medium. Plants were grown in a growth room under 22–23°C, 60% relative humidity, 85 μmol s^{-1}m^{-2} fluorescent illumination, with 9/15 h day/night for pathogen inoculation, and 16/8 h day/night for physiological analysis.

Construction of double mutant. For construction of the *gh3.5-1D/NahG* double mutant, *gh3.5-1D* was crossed with NahG (obtained from Novartis), a transgenic line expressing a bacterial salicylate hydroxylase that is unable to accumulate SA.[4] The double mutant plants with *gh3.5-1D* and *NahG* were selected in F_3 progenies. Homozygous *NahG* plants were confirmed by PCR. These double mutant plants are morphologically similar to *gh3.5-1D*. For construction of the double mutant *gh3.5-1D/npr1*, the *gh3.5-1D* was

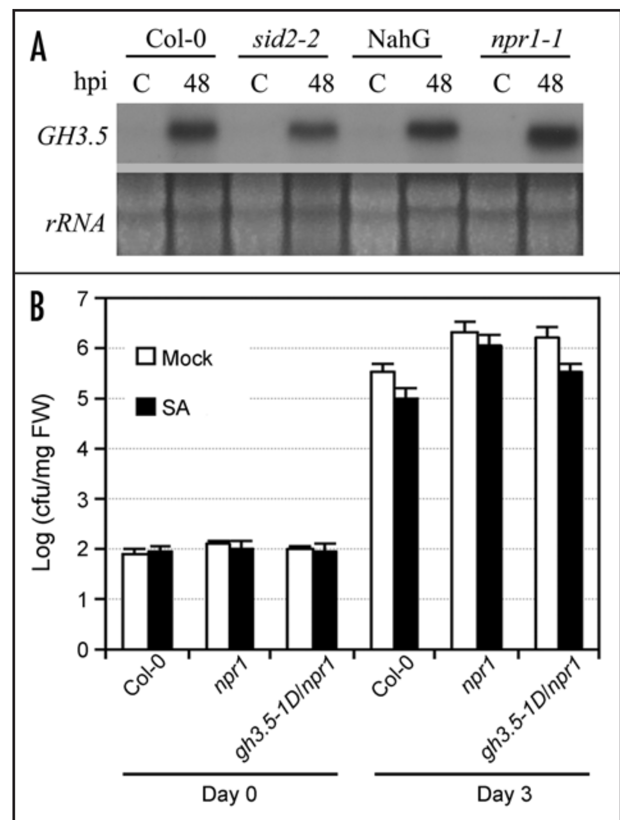

Figure 5. SA-independent induction of *GH3.5* and NPR1-dependent and independent SA-induced defense. (A) The induction of *GH3.5* by *Psm*(*avrRpm1*) at 10^7 cfu ml^{-1} in Col-0, *NahG*, *sid2-2* and *npr1-1* plants, indicating that the *GH3.5* induction was not affected by SA deficiency in these mutants. (B) Growth of *Pst* DC3000 in Col-0, *npr1* and *gh3.5-1D/npr1* plants after pretreatment with SA (1 mM) or buffer. Bacterial titers were repeated twice with similar results. All values are the mean ± SE (n = 6).

crossed with the *npr1-1* mutant.[9] The *gh3.5-1D*/*npr1* plants were selected in F_3 progenies and the mutation of *npr1* was confirmed with cleaved-amplified polymorphic sequence marker using restriction endonuclease *Nla*III. The *gh3.5-1D*/*npr1* double mutant plants are morphologically similar to *gh3.5-1D*.

Promoter activity. For the *GH3.5* promoter-*GUS* fusion reporter, the PCR primers 5'-TTAGTAAGTTTCAGTCGACGTTCTAGA-3' and 5'-TTGGATCCTCAGGCGTGGTTTAAGAG-3', were used to amplify the 1.6-kb upstream region of the *GH3.5* from genomic DNA. The PCR product was inserted into the pBI101 vector. The construct was then introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into Arabidopsis (Col-0) by floral dip to produce more than 50 independent *GH3.5-GUS* transgenic plants. Homozygous transgenic plants were selected in the progenies. Histochemical assay for GUS activity in *GH3.5-GUS* plants was performed as described.[23]

Bacterial strains, inoculation and disease assessment. *Pseudomonas syringae* strains and inoculation were previously described.[15] In brief, bacterial pathogens grown in King's B (KB) medium were collected and re-suspended in 10 mM $MgCl_2$. Leaves of 5-week-old plants were infiltrated with a bacterial suspension. All procedural controls were infiltrated with 10 mM $MgCl_2$ for mock inoculation. Bacterial growth assay was performed as described.[24] Except where otherwise noted, heterozygous *gh3.5-1D(+/-)* plants were used for all of bacterial growth experiments.

SA treatment. Salicylic acid (Sigma-Aldrich, MO) was dissolve in distilled water. Five-week-old plants of the wild-type and mutants were sprayed with 1 mM SA containing 0.01% Silwett L-77 or buffer alone. Two days later, plants in each treatment were further inoculated with *Pst* DC3000 at a dose of 10^5 cfu ml^{-1} (OD600 = 0.0002). Bacterial growth titers were counted at 0 and 3 days after inoculation.

Northern blot analysis. Total RNA was isolated from leaf tissues using TRIzol reagent according to the manufacturer's protocol (GIBCO BRL). Each 10 μg RNA of samples were separated on a formaldehyde-agarose gel, then blotted to Hybond-N^+ membranes (Amersham). A 353-bp fragment of the *GH3.5* 3' non-coding region was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara) for hybridization and autoradiograph as previously described.[15] Northern blot was also performed to determine the transcript levels of the pathogenesis-related gene *PR-1*. The filters were reprobed with a 2.5-kb fragment of Arabidopsis 18S rRNA for loading normalization.

Acknowledgements

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