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Report

# Salicylic Acid Inhibits Pathogen Growth in Plants through Repression of the Auxin Signaling Pathway

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## Summary

**The phytohormone auxin regulates almost every aspect of plant development. At the molecular level, auxin induces gene expression through direct physical interaction with the TIR1-like F box proteins, which in turn remove the Aux/IAA family of transcriptional repressors [1–4]. A growing body of evidence indicates that many plant pathogens can either produce auxin themselves or manipulate host auxin biosynthesis to interfere with the host's normal developmental processes [5–11]. In response, plants probably evolved mechanisms to repress auxin signaling during infection as a defense strategy. Plants overaccumulating the defense signal molecule salicylic acid (SA) frequently display morphological phenotypes that are reminiscent of auxin-deficient or auxin-insensitive mutants, indicating that SA might interfere with auxin responses. By using the Affymetrix ATH1 GeneChip for *Arabidopsis thaliana*, we performed a comprehensive study of the effects of SA on auxin signaling [12]. We found that SA causes global repression of auxin-related genes, including the *TIR1* receptor gene, resulting in stabilization of the Aux/IAA repressor proteins and inhibition of auxin responses. We demonstrate that this inhibitory effect on auxin signaling is a part of the SA-mediated disease-resistance mechanism.**

## Results and Discussion

To uncover the molecular basis of SA-mediated plant immunity, we began by analyzing transcriptome changes in the model plant *Arabidopsis thaliana* in response to an SA analog, benzothiadiazole S-methylester (BTH). A detailed description of this analysis has been previously published [12]. Interestingly, among the 1133 BTH-repressed genes, a significant number (21 genes) was related to auxin signal transduction (p value $< 2.12 \times 10^{-3}$, Table 1). These repressed genes include *AUX1* and *PIN7*, encoding an auxin importer and exporter, respectively [13, 14]; genes for the auxin receptors TIR1 and AFB1; and the well-characterized auxin-inducible *SAUR* genes and *Aux/IAA*-family genes [15]. Additionally, among genes upregulated by BTH, two of them encode auxin-conjugating enzymes [16], implying that BTH might also affect auxin homeostasis by lowering the levels of free auxin. Together, these data suggest that BTH treatment results in an overall reduction in auxin responses, which might attenuate the effectiveness of pathogen-derived auxin and contribute to disease resistance. In a different microarray experiment, we found that 18 of these 21 auxin-related genes were also repressed in systemic tissue after induction of systemic acquired resistance (SAR) [17]; thus, downregulation of auxin-related genes might be a part of the SAR response. In the microarray for NPR1 direct transcriptional targets [18], many of these genes had low signal levels: Eight were called "absent." Of the rest, only one gene was repressed more than 2-fold in both replicates, suggesting that these auxin-pathway genes are likely to be indirectly regulated by NPR1.

To investigate whether SA treatment affects auxin levels, we measured free auxin (indole-3-acetic acid; IAA) in SA-treated plants under conditions similar to those of the microarray experiment [12]. We found no significant changes in free-auxin levels 24 and 48 hr after SA treatment (Figure S1A in the Supplemental Data available online). This suggests that induction of the auxin-conjugating enzymes has no immediate effect on free-auxin levels and that SA and BTH repress auxin-mediated genes predominantly at the signaling level.

To validate this prediction, we tested the effect of SA on the inducibility of the auxin-signaling reporter *DR5::β-glucuronidase* (*GUS*) [19] by using increasing concentrations of SA. As shown in Figure 1A, SA significantly inhibited auxin-mediated expression of this reporter at 0.5 mM, a concentration that is normally used for inducing *Pathogenesis-related* (*PR*) gene expression and disease resistance [20]. This result was also observed with in situ staining for reporter activity in roots, in which the *DR5* promoter is most active. As shown in Figure 1B, exogenous treatment of seedlings with 1 μM synthetic auxin, 1-naphthalacetic acid (NAA), stimulated strong GUS activity, indicated by a bright-blue color in the root. Addition of 0.5 mM SA abolished this effect. Similarly, SA also blocked *DR5::GUS* induction by both the natural auxin IAA and another synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) (data not shown). This effect was not observed with an inactive SA analog 4-hydroxybenzoic acid (4-HBA) or another plant hormone jasmonic acid (Figures S2A and S2B), confirming its SA specificity.

Several SA overaccumulating mutants, such as *cpr5*, *cpr6*, and *snc1*, exhibit morphologies, namely reduced apical dominance and stunted growth, that are reminiscent of auxin deficiency [21–23]. We first measured free-auxin levels in *cpr6* and *snc1* and found that they

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Table 1. Expression of Annotated Auxin-Related Genes Modulated by BTH

| Array Element | Locus Identifier | Annotation | 8 Hr F.C. | 24 Hr F.C. | p Value |
|---|---|---|---|---|---|
| 264014_at | At2g21210 | Auxin-responsive protein | −4.78 | −11.47 | 1.81E-03 |
| 254746_at | At4g12980 | Auxin-responsive protein, putative | −4.29 | −4 | 2.18E-03 |
| 252965_at | At4g38860 | Auxin responsive SAUR protein | −5.81 | −7.52 | 2.23E-03 |
| 259773_at | At1g29500 | Auxin responsive SAUR protein | −4.61 | −5.78 | 2.27E-03 |
| 259787_at | At1g29460 | Auxin responsive SAUR protein | −2.25 | −2 | 2.39E-03 |
| 265319_at | At2g22670 | Indoleacetic acid-induced protein 8 (IAA8) | −2.62 | −3.53 | 2.55E-03 |
| 259783_at | At1g29510 | Auxin responsive SAUR protein | −4.46 | −5.85 | 2.80E-03 |
| 264900_at | At1g23080 | Auxin efflux carrier PIN7 | −2.67 | −2.96 | 2.89E-03 |
| 267092_at | At2g38120 | Amino acid permease (AUX1) | −2.6 | −2.82 | 2.89E-03 |
| 252972_at | At4g38840 | Auxin-inducible SAUR gene | −3.97 | −4.12 | 3.02E-03 |
| 251199_at | At3g62980 | Auxin receptor TIR1 | −1.7 | −2.04 | 3.23E-03 |
| 265454_at | At2g46530 | Transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related | −2.75 | −2.92 | 3.47E-03 |
| 259790_s_at | At1g29430, At5g27780 | Auxin responsive SAUR protein | −2.27 | −1.66 | 3.83E-03 |
| 267310_at | At2g34680 | Leucine-rich repeat protein | −2.16 | −2.39 | 6.19E-03 |
| 266215_at | At2g06850 | Endo-xyloglucan transferase (EXT) (EXGT-A1) | −10.66 | −13.59 | 8.26E-03 |
| 245076_at | At2g23170 | IAA-amido synthase that conjugates amino acids to auxin | 1.46 | 2.11 | 8.65E-03 |
| 255417_at | At4g03190 | F box family protein (FBL18), AFB1 | −2.02 | −2.03 | 1.14E-02 |
| 248801_at | At5g47370 | Homeobox-leucine zipper protein 2 (HAT2) | −1.5 | −2.07 | 1.32E-02 |
| 252970_at | At4g38850 | Auxin-responsive protein/small auxin up RNA (SAUR-AC1) | −1.82 | −2.16 | 2.14E-02 |
| 250012_x_at | At5g18060 | Auxin-responsive protein, putative | −1.87 | −3.32 | 2.16E-02 |
| 253908_at | At4g27260 | IAA-amido synthase that conjugates amino acids to auxin | 2.41 | 1.87 | 4.00E-02 |

Fold changes (F.C.) and BTH-dependency p values were calculated on the basis of gene-expression levels at 0, 8, and 24 hr after induction.

were significantly lower than those in the wild-type ($p \leq 0.05$; Figure 2A). This indicates that although exogenous treatment of plants with SA had little immediate effect on free-auxin levels, the presence of high endogenous SA did have a long-term effect on auxin homeostasis. To test whether these SA-accumulating mutants display altered responses to auxin, we introduced the *DR5::GUS* reporter into these mutants through genetic crosses. We then quantified the auxin sensitivity of these plants by measuring the activity of the *DR5::GUS* reporter. As shown in Figure 2B, induction of the reporter by NAA was clearly diminished in these mutants, indicating that SA accumulation causes insensitivity to exogenously applied auxin.

To rule out the possibility that SA inhibits the uptake of auxin, we crossed SA-accumulating mutants with an auxin-overproducing mutant, *yucca*. As a result of high endogenous auxin levels, *yucca* plants display long hypocotyls and epinastic cotyledons at the seedling stage, narrow rosette leaves with long petioles during the adult stage, and increased apical dominance after bolting [24]. Interestingly, SA-accumulating mutants suppressed most of the phenotypes associated with *yucca*. For example, the *snc1 yucca* double mutant had small rosette with curly leaves, typical of the *snc1* single mutant (Figure 2C and Figure S3), and the *cpr6 yucca* double mutant exhibited reduced apical dominance as in *cpr6* (Figure 2D). Only at the seedling stage were auxin phenotypes still evident, presumably because SA had not accumulated to high levels early in development. Because the *yucca* mutant was generated through activation tagging, the *YUCCA* gene is no longer regulated by its endogenous promoter or sensitive to SA repression. Not surprisingly, free-IAA levels in the *yucca snc1* and *yucca cpr6* double mutants were not significantly different from that found in the *yucca* single mutant (Figure S1B), indicating that suppression of *yucca* phenotypes by *snc1* and *cpr6* is mainly due to repressed auxin response, not reduced auxin synthesis.

As suggested by our expression-profiling experiment, one mechanism by which SA might inhibit auxin signaling is through transcriptional repression of the auxin receptor genes (Table 1). Reduction in TIR1 and ABF1 levels would lead to reduced degradation of Aux/IAA repressors and thus inhibit auxin responses. To test this hypothesis, we used the reporter *HS::AXR3NT-GUS*, which encodes a fusion between the coding sequences of amino terminus (NT) of one auxin-response repressor, *AXR3*, and GUS-encoding *uidA* gene driven by a heat-shock-inducible promoter (*HS*). After heat shock, the level of the AXR3NT-GUS fusion protein can be measured using MUG assay [4]. To distinguish transcriptional induction from increased protein stability, we also measured the transcript levels of GUS by using semiquantitative RT-PCR. As shown in Figure 3A, transcription of the reporter was induced after heat shock, and this induction was further enhanced in the presence

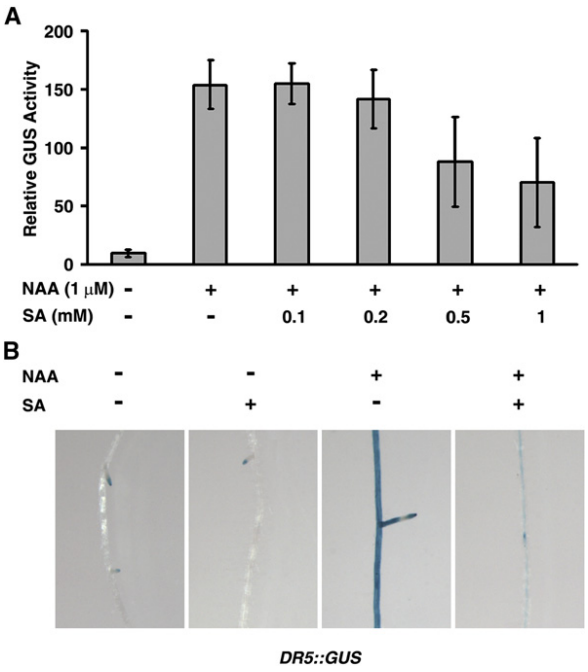


Figure 1. SA Suppresses Auxin Response

(A) Seven-day-old *DR5::GUS* seedlings were incubated overnight with 1 μM NAA and increasing concentrations of SA, as indicated. GUS activity was measured as previously described [20]. Error bars represent SDs.

(B) GUS staining in representative root segments after treatment with 1 μM NAA with or without 0.5 mM SA. This experiment was repeated multiple times, with more than ten seedlings per treatment, with similar results.

of SA. GUS activities correlated with this increase in transcription. However, 40 min after the heat shock, transcript abundance dropped to background levels, and GUS activity could then be used as a measure of protein stability. For the sample treated with 1 μM NAA alone, GUS activity started to decline. In contrast, in the sample treated with both NAA and SA (0.5 mM), GUS activity continued to increase, suggesting that SA has a stabilizing effect on AXR3NT-GUS. To confirm this result, we directly examined endogenous Aux/IAA repressor protein levels after SA treatment. Aux/IAA proteins are normally short lived and difficult to detect on a western blot [4]. However, SA treatment resulted in a drastic increase in the amount of Aux/IAA proteins detected with an antibody against AXR2/IAA7 (Figure 3B). Examination of *AXR2* transcript levels showed that this gene was not affected by SAR induction [12, 18]. As expected, 4-HBA did not delay the degradation of AXR3NT-GUS (Figure S2C). From these results, we conclude that SA significantly increases Aux/IAA repressor protein levels, and such an increase leads to inhibition of auxin responses.

Stabilization of the auxin repressor proteins by SA might be due to transcriptional repression of the auxin receptor genes (Table 1) or posttranscriptional mechanisms. Navarro et al. reported a microRNA-mediated pathway that causes a rapid reduction in auxin receptor levels in response to the defense elicitor flg22 [25]. We found that the auxin induction of *DR5::GUS* was unaffected after a short SA treatment of 2 hr (data not shown). The level of miR393, the microRNA involved in flg22-mediated response, was not altered 24 hr after SA treatment (Figure S4). Apparently, the mechanism by which SA downregulates auxin signaling is independent of miR393.

Besides repressing the transcription of auxin receptor genes, SA might interfere with auxin signaling in a more direct manner, namely by disrupting the interaction between Aux/IAA proteins and auxin receptors. To test this possibility, we examined the effect of SA on the ability of GST-IAA1 to pull down Myc-tagged TIR1 in vitro. *TIR1-myc* expression is driven by a dexamethasone (Dex)-inducible promoter, and this induction is not altered by SA (data not shown). As previously reported, GST-IAA1 could pull down TIR1 in an auxin-dependent manner [1, 4]. However, we found that this interaction was unaffected by SA, whether it was added at the point of TIR1 induction or only in the pulldown reaction (Figure 3C). These in vitro data strongly argue that SA stabilizes Aux/IAA proteins mainly by limiting auxin receptors that are required for Aux/IAA protein degradation. However, we cannot completely exclude the possibility that SA can modify Aux/IAA proteins in vivo to make them more resistant to degradation.

A growing body of evidence indicates that pathogen infection perturbs auxin homeostasis [5–11, 25]. During an infection experiment with the virulent oomycete pathogen *Hyaloperonospora parasitica* Noco2, we noticed that the auxin-responsive reporter *DR5::GUS* was induced. Intriguingly, the induction pattern of *DR5::GUS* coincided with the growth of conidiophores, whereas leaves free of pathogen growth only displayed background levels of staining (Figure 4A). Increases in free auxin and auxin sensitivity were also observed in plants expressing the *Pseudomonas syringae* type III effector AvrRpt2 and in response to *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000) infection (Z. Chen and B. Kunkel, personal communication). Exogenous auxin application has also been shown to promote the development of *Pst*DC3000 disease symptoms ([25], Z. Chen and B. Kunkel, personal communication). We found that coinoculation of *P. syringae* pv. *maculicola* ES4326 (*Psm*4326) with 50 μM of NAA not only enhanced disease symptoms but also moderately promoted pathogen growth (Figure 4B). Although the exact role of auxin in pathogenesis is unknown, we observed that auxin application could inhibit the full induction of SA-mediated *PR-1* expression (Figure 4C). Our results lend support to the hypothesis that auxin might downregulate host defense responses [26].

On the basis of the strong inhibitory effect of SA on auxin responses, as well as the likely role of auxin in promoting pathogenesis, it is logical to hypothesize that repressing the auxin pathway is an important aspect of the defense response. Therefore, we tested the disease-resistance phenotype of *axr2-1* plants, which are insensitive to auxin because of production of a nondegradable form of the AXR2/IAA7 repressor protein [27]. When infected with the bacterial pathogen *Psm*4326 at a high inoculum ($OD_{600}$ = 0.001), wild-type plants were unable to prevent the proliferation of the pathogen. In contrast, the *axr2-1* mutant displayed a 10-fold reduction in bacterial growth (Figure 4D). This result clearly

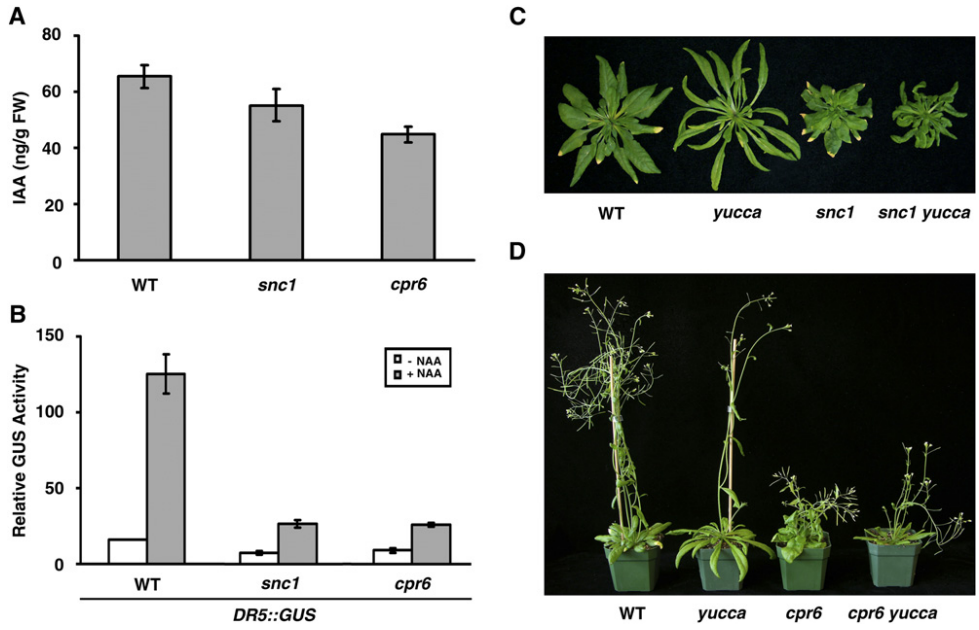


Figure 2. SA-Accumulating Mutants Exhibit Lower Levels of Free IAA and Reduced Sensitivity to Auxin

(A) Free-IAA levels were measured in 4-week-old wild-type, *cpr6*, and *snc1* plants. Error bars represent SDs for three independent samples.

(B) The *DR5::GUS* reporter was introduced into SA-accumulating mutants, and the responsiveness of the reporter to exogenous auxin was determined for seven-day-old seedlings in a MUG assay. Error bars represent the SDs for three independently collected samples.

(C) Among the progeny from a *snc1*-*yucca* cross, seedlings with *yucca*-associated long cotyledons were transplanted. Plants homozygous for *snc1* started to suppress *yucca* phenotypes as the plants aged. Shown here are rosettes of six-week-old plants. Inflorescences were removed for a clearer view of the rosette.

(D) Flowering *cpr6 yucca* plants showed reduced apical dominance typical of the *cpr6* single mutant. Note that the short-silique phenotype of *yucca* was also suppressed.

shows that blocking auxin responses can lead to heightened disease resistance.

The importance of repressing auxin signaling to disease resistance might be more evident when other forms of SA-mediated defenses are absent. In *NahG* transgenic plants, a bacterial salicylate hydroxylase, encoded by the *NahG* gene, degrades SA. As a result, these plants cannot defend against *Psm*4326 even when challenged with a low bacterial inoculum ($OD_{600}$ = 0.0001, Figure 4E). If inhibition of auxin responses is

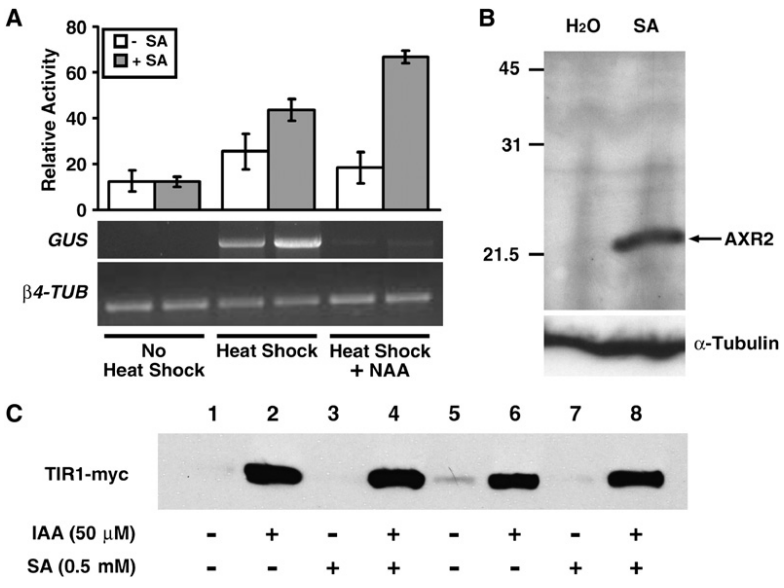


Figure 3. SA Stabilizes Auxin-Response Repressors

(A) Seven-day-old seedlings containing *HS::AXR3NT-GUS* were incubated in either water or 0.5 mM SA for 24 hr. GUS activity was then examined before heat shock, immediately after a 2 hr heat shock, or after seedlings had been incubated for 40 min after heat shock in the presence of 1 μM NAA. Error bars represent the SDs for three independent samples. *AXR3NT-GUS* transcript levels were measured for each sample with RT-PCR. β-Tubulin subunit 4 (*β4-TUB*) transcript levels were used as loading controls.

(B) The level of the AXR2 protein after 24 hr water or SA treatment was examined with an antibody raised against the endogenous AXR2 protein. Because of constitutive degradation, the protein in water-treated samples usually cannot be detected. α-tubulin was probed as a loading control.

(C) Inducible *TIR1-myc* transgenic seedlings were treated with Dex overnight to turn on TIR1-Myc synthesis. Total protein was extracted and TIR1-Myc was pulled down with purified GST-IAA1 in the absence or the presence of 50 μM IAA. SA was added either during Dex induction (lanes 3 and 4) or during the pulldown assay (lanes 7 and 8).

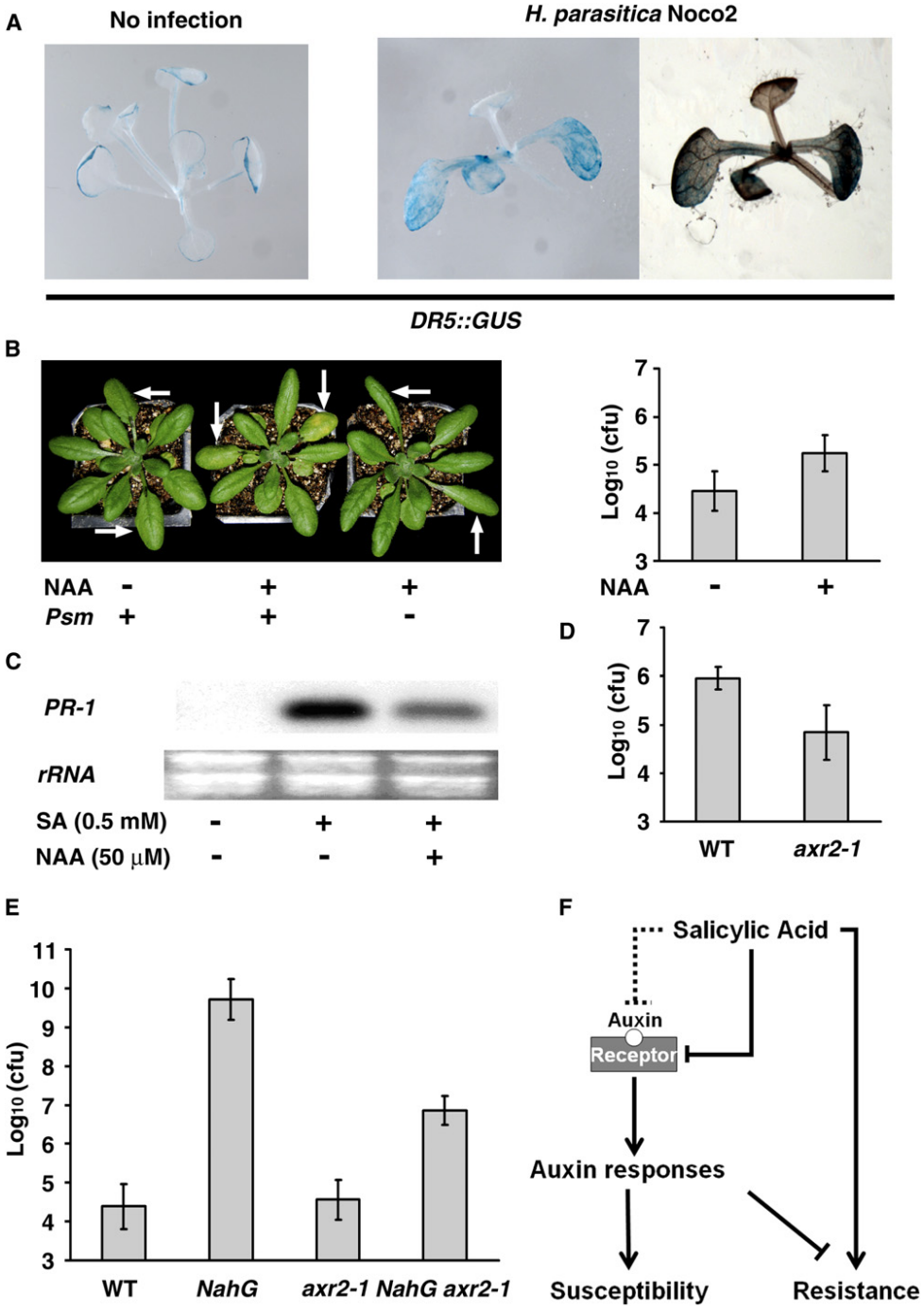


Figure 4. Crosstalk between Auxin and SA Affects Plant Defense Responses

(A) Plants carrying the *DR5::GUS* reporter were either mock treated or infected with *Hyaloperonospora parasitica* Noco2 ($1 \times 10^4$ spores/ml) and stained for GUS activity 7 days postinoculation (dpi). We photographed the same infected plant with different background lighting to reveal conidiophores. Plants shown here are representative of multiple samples.

(B) Four-week-old plants were inoculated with *Psm*4326 (OD600 = 0.0005), 50 μM NAA, or *Psm*4326 together with 50 μM NAA. Disease symptoms were photographed (left panel), and bacterial growth was measured (right panel) 3 dpi. Arrows indicate infiltrated leaves. Eight samples were taken for each treatment. The experiment has been repeated with similar results. Bacterial growth in NAA-treated plants is significantly different from control plants ($p = 0.0048$, Student's t test). Error bars represent 95% confidence intervals. "cfu" stands for colony-forming unit.

(C) Four-week-old plants were treated with water, SA (0.5 mM), or SA plus NAA (50 μM) for 24 hr. SA response was examined by probing *PR-1* transcripts on a Northern blot. Ribosomal RNA (*rRNA*) is shown as a loading control.

(D) Four-week-old plants were infiltrated with a high dose of *Psm*4326 (OD600 = 0.001). Bacterial growth was quantified 3 dpi. The absolute values of bacterial growth in *axr2-1* are significantly different from those of the wild-type ($p = 0.0027$, Student's t test). Error bars represent 95% confidence intervals.

(E) The assay was performed in the same manner as for (D), except with a lower dose of pathogen (OD600 = 0.0001). Error bars represent 95% confidence intervals.

a component of SA-mediated resistance, then blocking auxin signaling should restore some resistance in *NahG* plants. Indeed, as shown in Figure 4E, the auxin-insensitive *axr2-1* mutation drastically reduced *Psm*4326 growth in *NahG* plants. SA is well known for activating multiple types of defense responses (See model in Figure 4F). It is therefore not surprising that *axr2-1* did not fully restore resistance in *NahG* plants. Our results are in agreement with those of a complementary study, in which overaccumulation of an auxin receptor protein compromised resistance [25]. Together, our results establish that inhibiting auxin sensitivity is an integral component of the SA-mediated defense response.

In summary, by using expression profiling, we discovered that SA inhibits the auxin signaling pathway as part of the plant defense mechanism. This inhibition is accomplished through SA-mediated stabilization of the auxin-response repressors. In addition, auxin might not be the only plant growth hormone targeted by SA. In the BTH-treatment microarray experiment [12], several genes known to be involved in the gibberellin pathway were also found to be downregulated, indicating the presence of an elaborate interaction network between developmental and defense pathways in plants.

### Supplemental Data

Experimental Procedures and four figures are available at http://www.current-biology.com/cgi/content/full/17/20/1784/DC1/.

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(F) A model for SA-mediated inhibition of the auxin pathway as part of the defense response. Pathogens manipulate auxin responses to both promote susceptibility (solid arrow) and attenuate defense gene expression in the host (solid blocked arrow). To counter pathogen attack, SA stabilizes auxin-response repressors by downregulating auxin receptors (solid blocked arrow). Long-term SA accumulation might also reduce auxin levels (dashed blocked arrow). This antagonism of auxin signaling, together with other SA-mediated responses, leads to disease resistance.

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