

Research article

## Two pathways act in an additive rather than obligatorily synergistic fashion to induce systemic acquired resistance and *PR* gene expression

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### Abstract

**Background:** Local infection with necrotizing pathogens induces whole plant immunity to secondary challenge. Pathogenesis-related genes are induced in parallel with this systemic acquired resistance response and thought to be co-regulated. The hypothesis of co-regulation has been challenged by induction of *Arabidopsis PR-1* but not systemic acquired resistance in *npr1* mutant plants responding to *Pseudomonas syringae* carrying the avirulence gene *avrRpt2*. However, experiments with *ndrl* mutant plants have revealed major differences between avirulence genes. The *ndrl-1* mutation prevents hypersensitive cell death, systemic acquired resistance and *PR-1* induction elicited by bacteria carrying *avrRpt2*. This mutation does not prevent these responses to bacteria carrying *avrB*.

**Results:** Systemic acquired resistance, *PR-1* induction and *PR-5* induction were assessed in comparisons of *npr1-2* and *ndrl-1* mutant plants, double mutant plants, and wild-type plants. Systemic acquired resistance was displayed by all four plant lines in response to *Pseudomonas syringae* bacteria carrying *avrB*. *PR-1* induction was partially impaired by either single mutation in response to either bacterial strain, but only fully impaired in the double mutant in response to *avrRpt2*. *PR-5* induction was not fully impaired in any of the mutants in response to either avirulence gene.

**Conclusion:** Two pathways act additively, rather than in an obligatorily synergistic fashion, to induce systemic acquired resistance, *PR-1* and *PR-5*. One of these pathways is *NPR1*-independent and depends on signals associated with hypersensitive cell death. The other pathway is dependent on salicylic acid accumulation and acts through *NPR1*. At least two other pathways also contribute additively to *PR-5* induction.

### Background

In response to local infection with necrotizing pathogen, plants display a whole plant enhanced immunity to secondary challenge (systemic acquired resistance or SAR)

[1]. A characteristic set of genes termed pathogenesis-related (*PR*) is induced both locally and in distal tissues in parallel with SAR induction [2]. Accumulation of salicylic

acid (SA) has been shown to be necessary for induction of SAR [3].

The Arabidopsis *ndr1-1* mutant showed drastically impaired induction of SAR and *PR-1* gene expression in response to infection with *Pseudomonas syringae* bacteria carrying the avirulence (*avr*) gene *avrRpt2* [4]. This mutant is also incapable of showing a hypersensitive response (HR) to these bacteria [5,6] except at very high levels of inoculum [7]. The HR is a macroscopic leaf collapse. The HR occurs in response to infection with avirulent bacteria at levels of inoculum that cause a large percentage of leaf cells to undergo programmed cell death (PCD) [8]. In contrast to results with *avrRpt2*, *P. syringae* bacteria carrying the avirulence genes *avrB*, *avrRpm1*, *avrPphB* or *avrRps4* elicit an exaggerated HR relative to that on the wild-type Columbia parent [5,7]. In a study in which bacteria carrying *avrB* were chosen as a representative member of this class, in further contrast to results with *avrRpt2*, these bacteria were capable of eliciting SAR and *PR-1* induction on *ndr1-1* mutant plants [4].

However, induction of SA accumulation by *ndr1-1* mutant plants was impaired at early time points to a similar extent in response to bacteria carrying either *avr* gene [4]. These results suggested that at least two pathways control SAR and *PR-1* gene expression. One pathway is directly dependent on SA accumulation. The second pathway is not directly dependent on SA levels. The correlation between ability to elicit the HR and to elicit SAR and *PR-1* induction led to the model that the second pathway was caused by signals associated with hypersensitive cell death. Despite the severe impairment in SA accumulation within the first 12 hours post-inoculation of *ndr1* plants, bacteria carrying *avrB* could nonetheless induce *PR* gene expression and SAR because of the HR-associated pathway. Bacteria carrying *avrRpt2* could not because both pathways were impaired by the *ndr1* mutation. This study could not address the question as to whether the HR-associated pathway alone was sufficient to induce SAR and *PR-1* because SA accumulation did occur late in the response.

Induction of SAR has been reported to be dependent upon the *NPR1* gene [9–11]. In these studies, neither SA/SA analogs nor avirulent bacteria were able to induce SAR on *npr1* mutant plants. The biological inducer of SAR tested in these experiments was *P. syringae* bacteria carrying *avrRpt2*. In contrast to *ndr1* plants, *npr1* plants do show the HR in response to *P. syringae* carrying *avrRpt2* (data not shown). These results could be taken to suggest that the HR-associated pathway is not sufficient to induce SAR in the absence of the *NPR1*-dependent, SA-associated pathway (but see below for why this conclusion is not correct).

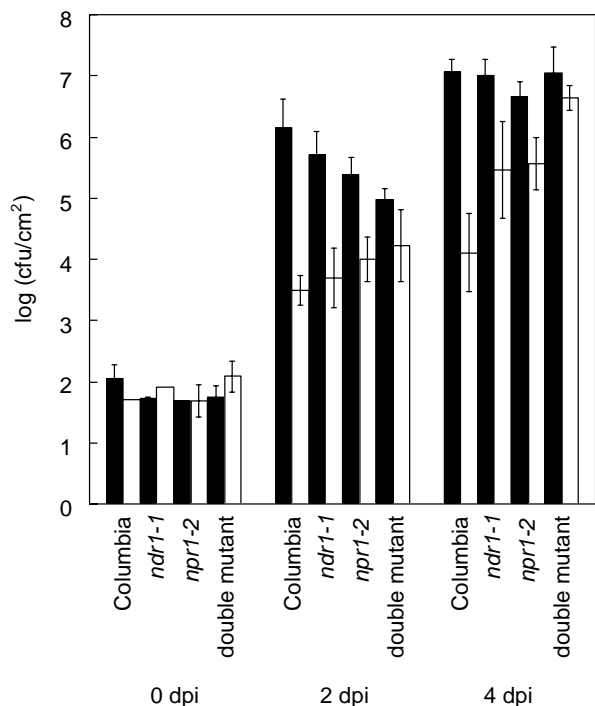
In contrast to SAR induction, induction of *PR-1* by avirulent bacteria has been shown to occur in *npr1* mutants [11]. It is, however, reduced in extent and delayed. If *npr1* mutants are indeed fully blocked in SAR induction, then the pathways leading to *PR-1* induction can not be the same as those leading to SAR. However, we have argued on the basis of results with *ndr1* mutant plants that HR "triggering" in response to the *avrRpt2/RPS2* combination is weaker than that in response to *avrB/RPM1* [4]. Weaker "triggering" results in a lower probability of individual cells undergoing PCD early in the HR, prior to the onset of potentiation caused by increased SA levels [4,12]. Signaling elicited by *avrRpt2* clearly differs in many respects from that directed by the other *avr* genes. Use of bacteria carrying *avrB* to elicit SAR would allow a more rigorous test of whether the *NPR1*-dependent pathway is essential for SAR induction.

To clarify the signaling circuitry governing SAR and *PR* gene induction, we have constructed a *ndr1/npr1* double mutant. This line was compared with wild-type Columbia Arabidopsis and both single mutant lines for the ability to display biological induction of SAR and *PR* gene expression. We conclude that SAR, *PR-1* induction and *PR-5* induction are regulated in parallel by the two pathways we have previously delineated [4], but the effects of these pathways are additive rather than obligatorily synergistic. Moreover, additional pathways also contribute additively to *PR-5* induction.

## Results

### Systemic acquired resistance elicited by DC3000•*avrB*

If *NPR1* is truly essential for SAR [9], *npr1* mutant plants should not be able to undergo SAR in response to *P. syringae* pv. tomato DC3000 (hereafter DC3000) carrying *avrB*. Alternatively, if HR-associated signals leading to SAR act independently of *NPR1*, DC3000•*avrB* might be able to elicit SAR on *npr1* plants. The results of this experiment are presented in Figure 1. Columbia, wild type plants showed a three order of magnitude reduction in growth of virulent bacteria in plants that had received a prior inoculation with DC3000•*avrB*. SAR was also displayed by *ndr1-1* mutant plants. These results are comparable to those published previously [4]. Clear SAR was also displayed by the *npr1-2* mutant. The SAR response was statistically significant (Student's *t* test,  $P < 0.05$ ) for all three lines in this experiment and in a replicate experiment. This assay is not sufficiently sensitive to quantitate small differences in SAR between the lines. However, it is clear that all three lines did display SAR. As it is likely that both mutant lines are null alleles (previously established for *ndr1-1* [6] and argued below for *npr1-2*), the hypothesis that *NPR1* is essential for SAR is thus rejected.



**Figure 1**  
**DC3000•*avrB* elicits systemic acquired resistance on the *ndr1-1/npr1-2* double mutant and both single mutants** Plants were either inoculated with  $2 \times 10^7$  bacteria  $\text{mL}^{-1}$  DC3000•*avrB* (white bars) or not inoculated (black bars). Two days later, inoculated leaves were excised. Remaining leaves were pressure infiltrated using a syringe with  $5 \times 10^4$  bacteria  $\text{mL}^{-1}$  DC3000•empty vector. Data points represent means of triplicate (Day 0) or quintuplicate (Day 2 or 4) determinations of *in planta* bacterial growth.

To test epistasis relationships between these mutants, a double mutant was constructed (see Methods). The double mutant was also tested for SAR in response to DC3000•*avrB* (Figure 1). Statistically significant SAR was seen at the two day time point but not at the four day time point (Student's *t* test,  $P < 0.05$ ). In a replicate experiment, SAR was statistically significant only at the four day time point. The combination of the two mutations was thus sufficient to abolish most of the SAR response. Residual SAR may have been due to HR-associated signals in that the double mutant did display a HR to DC3000•*avrB*. The macroscopic HR phenotype was exaggerated, like that of the *ndr1-1* single mutant (data not shown).

#### Induction of *PR-1* gene expression in response to DC3000•*avrB* and DC3000•*avrRpt2*

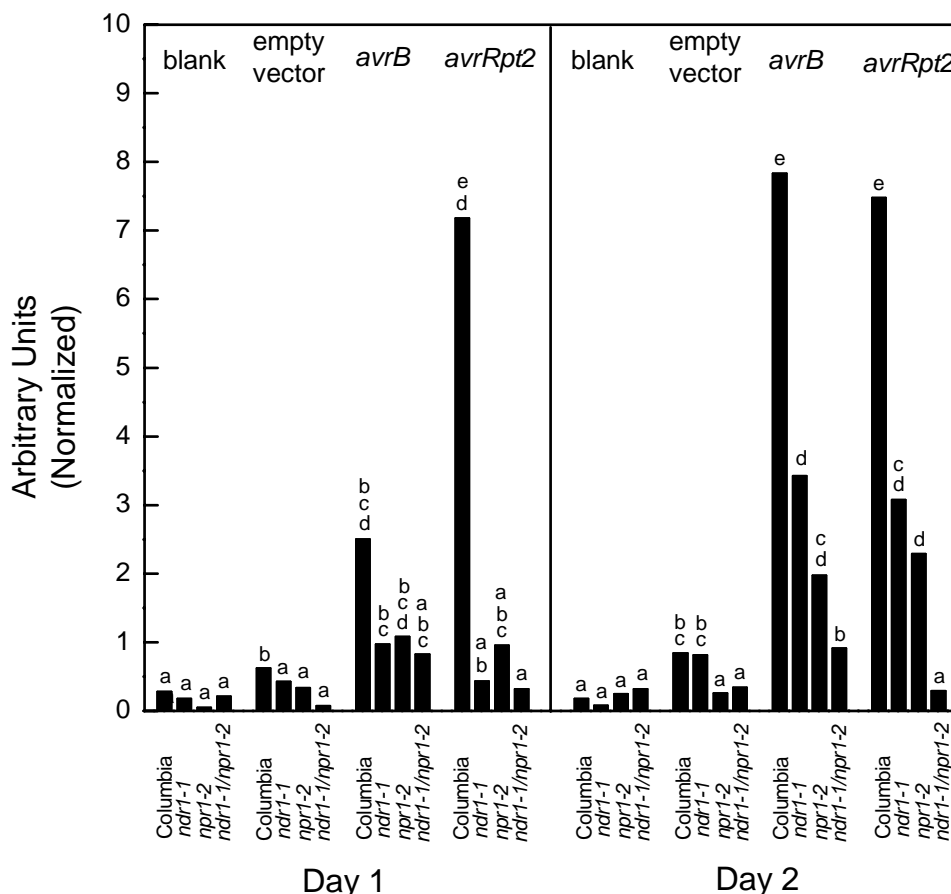
We have previously argued that *PR-1* gene expression is controlled by two separate pathways [4]. One pathway re-

sults from SA accumulation and is dependent upon the NPR1 gene product. The second pathway results from signals correlated with the HR and is *NPR1*-independent. The *ndr1-1/npr1-2* double mutant was used to further test this hypothesis.

The double mutant, each single mutant and Columbia wild-type plants were infected with  $1 \times 10^6$  bacteria  $\text{mL}^{-1}$ . At this level of inoculum, most leaf cells did not undergo PCD (data not shown). Macroscopic tissue collapse was therefore not seen, allowing *PR-1* gene expression to be quantitated using Northern blots. The data is presented in Figure 2. Columbia wild-type Arabidopsis showed high levels of *PR-1* induction in response to either DC3000•*avrB* or DC3000•*avrRpt2*. The level of *PR-1* induction shown by either single mutant line in response to either bacterial strain was greatly reduced relative to that shown by Columbia. However, both single mutants still showed *PR-1* induction at the two day time point in response to either bacterial strain. The level of *PR-1* induction shown by the double mutant in response to DC3000•*avrRpt2* was not significantly different from that shown in response to either DC3000•empty vector or the  $\text{MgCl}_2$  blank (Student's *t* test,  $P > 0.05$ ). In this experiment, when there was no PCD and NPR1 activity was blocked by mutation, no induction of *PR-1* resulted. By contrast, DC3000•*avrB* did elicit highly significant *PR-1* induction (Student's *t* test,  $P < 0.01$  for comparison to DC3000•empty vector or blank). As NPR1 activity was blocked by mutation, PCD-associated signals likely explain the residual *PR-1* induction. These results confirm the original model that the two pathways for *PR-1* induction are additive.

#### Induction of *PR-5* gene expression in response to DC3000•*avrB* and DC3000•*avrRpt2*

The two postulated pathways acting in an additive fashion sufficed to explain bacterial induction of SAR and *PR-1* gene expression. Experiments were next undertaken to extend the model to the control of *PR-5* induction. DC3000•*avrRpt2* or DC3000•*avrB* were inoculated into *ndr1-1/npr1-2* double mutant plants, the single mutants, or the Columbia wild type plants. *PR-5* gene expression was quantitated by Northern blots. The data is presented in Figure 3. At the one day time point, the double mutant showed significant impairment in *PR-5* induction relative to wild type in response to either avirulent bacterial strain (Student's *t* test,  $P < 0.05$ ). However, at the two day time point, only the impairment in response to DC3000•*avrRpt2* was statistically significant at this level. If the criteria for statistically significant differences was relaxed to  $P < 0.1$ , then the same qualitative picture was shown as with *PR-1* in comparison of single mutants to wild type Columbia. Columbia plants showed differences at this level of significance from *ndr1-1* or *npr1-2* plants in

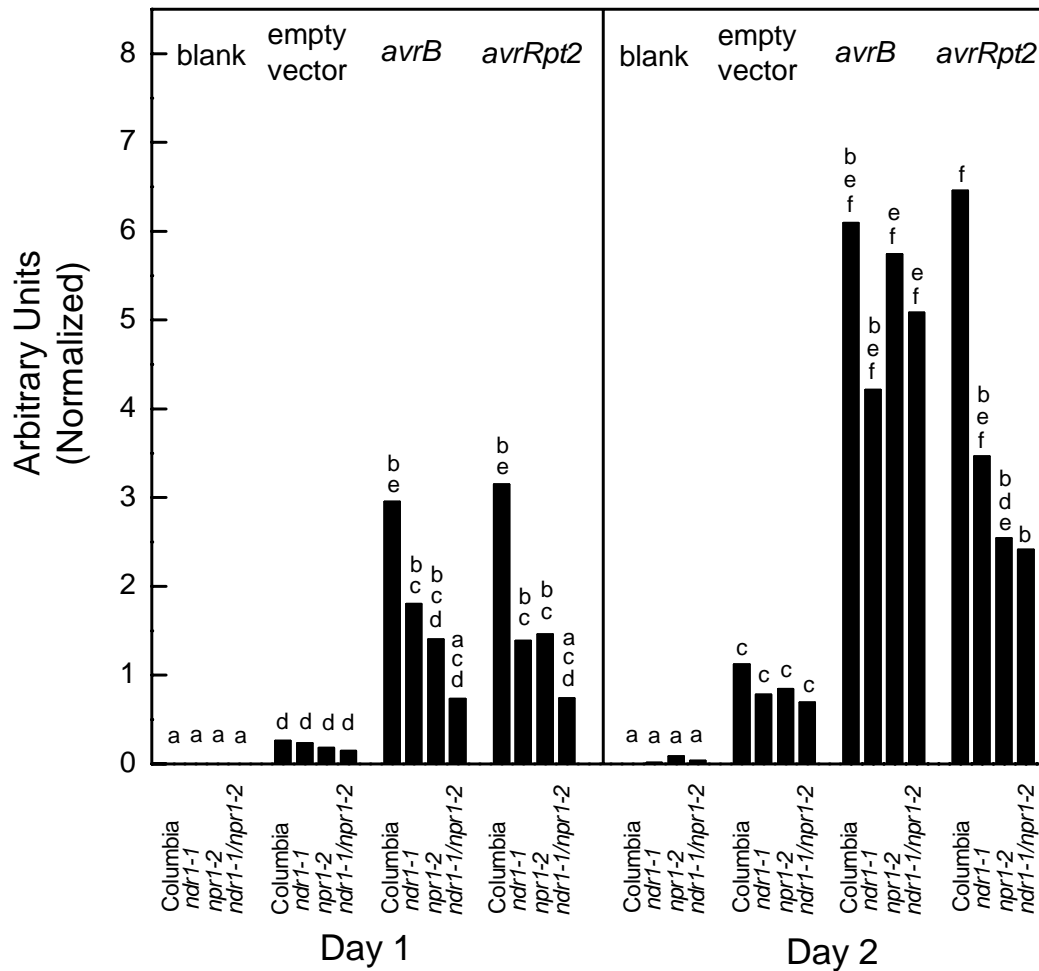


**Figure 2**  
**Induction of PR-1 gene expression** Plants of the indicated genotype were inoculated with  $1 \times 10^6$  bacteria  $\text{mL}^{-1}$  DC3000 carrying the specified avirulence gene or the empty vector or a 10 mM  $\text{MgCl}_2$  blank. At the indicated time points, leaf samples were taken for total RNA preparation. Probes derived from the PR-1 cDNA were used for Northern blots. Quantitation was with a phosphorimager. All blots were stripped and probed again with a radiolabeled probe made from the ROC1 cDNA as a control for RNA loading. These values were used for data normalization. Each bar represents a mean of data from 3 separate experiments. Differences between means were assessed for statistical significance using Student's *t* tests. Lowercase letters indicate statistically significant differences between means ( $P < 0.05$  or in many cases greater significance). Comparisons of means were made separately for the two time points.

induction of PR-5 in response to DC3000•*avrRpt2* at both time points. These results are consistent with contributions to PR-5 induction coming from both the HR-associated pathway and the SA/NPR1-dependent pathway. However, PR-5 gene expression occurred in spite of blockade of both pathways in experiments with DC3000•*avrRpt2* and the double mutant. This result was very different from what was seen with PR-1 induction or SAR. Clearly, the two pathways we have delineated do not constitute a complete picture of signaling leading to PR-5 gene expression.

### Discussion

PR genes are thought to be tightly associated with SAR because they are co-regulated with SAR [13,14] and because many PR gene products have been shown to have antimicrobial activity [2]. The complete impairment of *npr1* plants in SAR induction [9] as contrasted with a partial impairment in PR-1 induction [11] in response to *P. syringae* carrying *avrRpt2* challenged the hypothesis of co-regulation. However, *avrRpt2* was shown to differ markedly from the other characterized *P. syringae* *avr* genes in the responses it elicited on *ndr1* mutant plants [4,5,7]. The apparent challenge to the hypothesis of co-regulation could have been because of the choice of an atypical *avr* gene. Therefore, SAR and PR gene expression of *npr1* mutant



**Figure 3**  
**Induction of PR-5 gene expression** Experimental procedures and data analysis were done the same way as with PR-1 gene expression experiments (Figure 2).

plants were assessed in response to a second *avr* gene (*avrB*). Markedly different results were obtained. Mutant *npr1* plants were not fully impaired in induction of SAR, PR-1 gene expression or PR-5 gene expression in response to *P. syringae* carrying *avrB*. These results are consistent with the hypothesis of co-regulation. The apparent NPR1-dependence of SAR induction in response to bacteria carrying *avrRpt2* is likely quantitative rather than qualitative.

A potential alternative explanation for these results might be residual function of mutant gene products in the plant lines used. The *ndr1-1* mutant is an unambiguous null mutant in that a deletion removes most of the coding region and the proximal promoter region [6]. However, none of the characterized *npr1* mutants have a large deletion [15,16]. As such, attempts to define allelic series have

relied upon the level of residual function associated with these mutants in assays of gene expression or fungal disease resistance [15,16]. However, the complexity of signaling leading to these outputs complicates analyses of these phenotypes. A "null" mutant might still show gene expression or disease resistance caused by pathways that do not require the corresponding gene product.

What is required is an assay where the output is due solely to an NPR1-dependent pathway. The experiment presented in Figure 2 provided such data. The combination of *npr1-2* with *ndr1-1* in a double mutant prevented the *avrRpt2*-elicited HR under the conditions used. Under these conditions, DC3000•*avrRpt2* failed to elicit any PR-1 gene expression (the level was statistically indistinguishable from that seen with inoculation of a MgCl<sub>2</sub> blank).

Impairment of SA accumulation in the double mutant (Zhang, Gutsche and Shapiro, manuscript in preparation) resembled that in the *ndr1* single mutant [4] at early time points prior to the onset of *NPR1*-dependent negative feedback on SA accumulation [10,11]. Nonetheless, SA accumulation likely occurred by the time points taken in SAR and PR gene expression experiments reported herein.

This assay depends solely on *NPR1* function. The complete lack of *PR-1* induction supports our contention that *npr1-2* is indeed a null mutant or perhaps a severe hypomorph. This contention is further supported by published data showing that the *npr1-2* allele failed to interact with putative target TGA transcription factor genes in a two-hybrid assay [17] or in an *in vivo*, reporter gene-based assay [18]. The treatment of *npr1-2* seedlings with exogenous SA failed to induce increases in *PR-1* gene expression under some experimental conditions [19] albeit not under all conditions [15]. The potential alternative explanation that results presented herein were caused by residual gene product function in the mutant lines used is thus not correct.

The idea that SAR and PR gene expression are co-regulated has also been challenged by experiments with the *eds5* and *sid2* mutants [20]. These mutants have been implicated in SA biosynthesis. They showed impairments in *PR-1* induction in response to bacteria that were very similar to those displayed by *ndr1* plants but marginal if any impairment in *PR-2* or *PR-5* induction. Direct comparison with results presented here required addressing an issue with the potential to affect interpretation of that study. In that study, PR gene expression was quantitated from uninfected parts of leaves that had been infected with  $1 \times 10^8$  mL<sup>-1</sup> DC3000•*avrRpt2*. This level of inoculum was sufficiently high to cause "false positive" spurious HR-like responses to DC3000•empty vector [5]. Indeed, when *sid2* mutant plants were inoculated with  $2 \times 10^7$  mL<sup>-1</sup> DC3000•*avrRpt2*, no HR was seen (data not shown).

The high levels of inoculum used in the cited study can only be used without causing "false positive" HRs if a bacterial strain that is not a pathogen of Arabidopsis is used to deliver the *avr* gene product [5,7]. When *sid2* plants were inoculated with  $1 \times 10^8$  mL<sup>-1</sup> of a nonpathogen strain (*P. syringae* pv. *glycinea* Race 5) carrying *avrRpt2*, HRs were seen (data not shown). The identical results with the two bacterial strain backgrounds imply that the cited results with *sid2* mutant plants were obtained under conditions in which high levels of bacterial inoculum overcame some effects of the mutation. The *ndr1-1* mutation can also be overcome at high levels of inoculum. However, the level at which *ndr1-1* phenotypes are overcome is higher than that for *sid2* phenotypes in that  $1 \times$

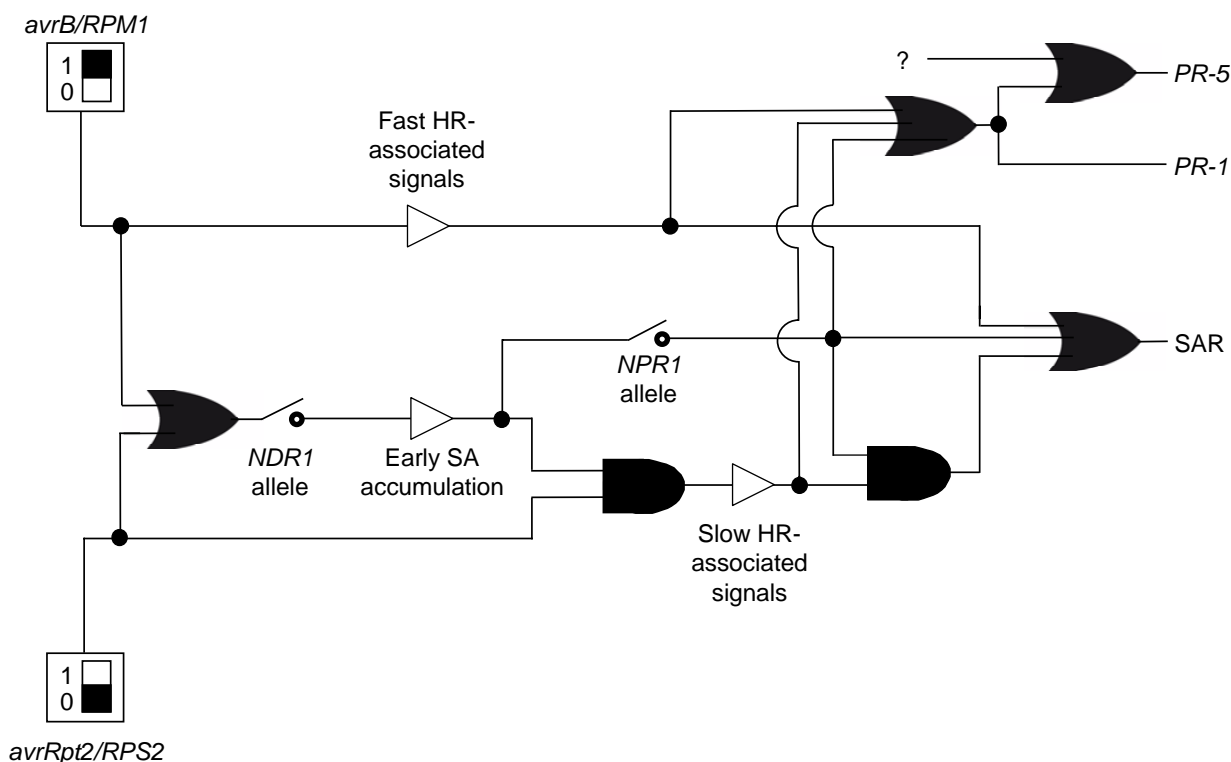
$10^9$  mL<sup>-1</sup> of a nonpathogen strain carrying *avrRpt2* is required to yield reproducible HRs on *ndr1-1* plants [7].

Clearly, the interpretation of results with *PR-5* induction depends critically on the experimental design. The relatively unimpaired *PR-2* and *PR-5* induction in *sid2* plants might imply that the HR-associated signals were quantitatively more important than the SA-associated signals. The same two pathways invoked to explain SAR and *PR-1* induction could potentially account for *PR-2* and *PR-5* induction if this assumption were correct.

The results presented herein, however, argue strongly that *PR-5* induction is indeed regulated differently than SAR or *PR-1* induction. The two pathways identified as making the major contributions to *PR-1* and SAR induction were shown to contribute to *PR-5* induction. However, significant *PR-5* induction occurred in the double mutant in response to DC3000•*avrRpt2* (Figure 3). DC3000•*avrRpt2* elicited HRs on Columbia or *npr1-2* plants but not on *ndr1-1* plants or double mutant plants (data not shown). As such, these results can not be explained by HR-associated signals or SA-associated, *NPR1*-dependent signals. There must be additional pathways contributing to *PR-5* induction. This conclusion is consistent with the original interpretation of PR gene expression data in *eds5* and *sid2* mutants that *PR-1* and *PR-5* are regulated differently [20].

The data presented in Figures 2 and 3 suggest that there must be at least two additional pathways contributing to *PR-5* induction. DC3000•empty vector did not elicit significant *PR-1* induction on the double mutant at either time point (Student's *t* test,  $P > 0.05$ ). However, the analogous experiment with *PR-5* induction gave the opposite results. Moreover, at both time points, the *PR-5* induction elicited by infection with DC3000•empty vector was to a similar extent regardless of allele status at the *NDR1* or *NPR1* loci (no statistically significant differences, Student's *t* test,  $P > 0.05$ ). These bacteria elicited *PR-5* gene expression in spite of the absence of a functional *avr* gene, the absence of PCD, and mutations in both the *NPR1* and *NDR1* genes. As even *E. coli* has been shown to induce plant defense-related gene expression [21], it should not be surprising that bacteria can elicit changes in plant gene expression via many different signaling pathways. Indeed, conserved features of bacteria can act as elicitors of gene expression through pathways distinct from those associated with gene-for-gene disease resistance [22].

However, an additional pathway leading to *PR-5* gene expression that stems from *avrRpt2* function must also exist. DC3000•*avrRpt2* elicited no statistically significant induction of *PR-1* by the double mutant at either time point (Student's *t* test,  $P > 0.05$ ). However, clear induction of *PR-5* was seen in the analogous experiment. The level of



**Figure 4**  
**Boolean representation of signaling network** The binary switches used to represent allele status at the *NPR1* and *NDR1* loci were rendered according to standard depictions in engineering texts. All other symbols are according to Genoud, Trevino Santa Cruz and Métraux [24]. Signal generators were rendered as rectangles with black boxes inside them set at one or zero. "Or" gates, indicating that either input is sufficient to give the specified output, were rendered as bullet shapes with concave left sides. "And" gates, indicating that both inputs are required to give the specified output, were rendered as bullet shapes with flat left sides. Signaling outputs that also serve as inputs to downstream events were rendered as open triangles. Branches in the pathway were indicated with filled circles to suggest the resemblance to contact points in electrical circuit diagrams.

*PR-5* induction was higher than in response to DC3000•empty vector (Student's *t* test, *P* < 0.05). As the double mutant was susceptible to disease caused by this bacterial strain (data not shown), perhaps this residual gene induction was due to the virulence function of *avrRpt2*[23]. Mutant *npr1* plants have previously been shown to be impaired in induction of *PR-1* but not *PR-5* in response to virulent bacterial pathogen [19].

These details are presented in a model of the signaling network for induction of *SAR*, *PR-1* gene expression and *PR-5* gene expression by avirulent *P. syringae* bacteria (Figure 4). We have used a representation scheme proposed recently for plant signal transduction networks that is based on Boolean formalisms used in engineering and computer

science [24]. Either of two "generators" shown can elicit signaling. One generator is infection of plants carrying a functional *RPS2* allele with bacteria carrying *avrRpt2*. The second generator is infection of plants carrying a functional *RPM1* allele with bacteria carrying *avrB*. An "or" gate is used to show that either way of eliciting signaling resulted in accumulation of SA. The *NDR1* allele status was represented with a "switch". The *ndr1-1* allele was pictured as analogous to an electrical switch where the contact is in an open position, impairing early SA accumulation.

The HR progresses more slowly in response to *avrRpt2* than in response to *avrB*[4,25,26]. Evidence has been presented, however, that differences between HRs are not merely differences in kinetics [27]. As such, the *avrRpt2*-

elicited HR and the *avrB*-elicited HR were pictured as two separate components. When assayed under standard conditions, *ndr1-1* mutant plants did not show the *avrRpt2*-elicited HR. An "and" gate was used to show that both infection with bacteria carrying *avrRpt2* and early accumulation of SA were needed to give the characteristic, "slow HR". By contrast, *ndr1-1* mutant plants did show the HR to bacteria carrying *avrB*. A straight line that is not connected via a gate to SA accumulation thus leads to "Fast HR-associated signals".

SAR is gated by an "or" gate with three possible inputs. As DC3000•*avrB* elicited SAR on the *ndr1-1/npr1-2* double mutant, neither of these gene products was essential for SAR induction. The early SA accumulation was not likely to have been essential either, because that was markedly impaired by the *ndr1-1* mutation. However, the characteristic *avrB*-elicited, "fast HR" did occur in the double mutant. As such, a straight line deriving from "Fast HR-associated signals" is one of the inputs to this "or" gate. Another input is an "and" gate. The evidence requiring this gate is that SAR elicited by DC3000•*avrRpt2* was prevented by either a *npr1* mutation [9] or a *ndr1* mutation [4]. We conclude that the characteristic *avrRpt2*-elicited "slow HR" can only contribute to SAR in combination with *NPR1*-dependent, early SA-elicited signaling, necessitating the "and" gate. The final input is SA-elicited, *NPR1*-dependent signaling acting alone [10].

*PR-1* induction is also gated by an "or" gate with three alternative inputs. The "Fast HR" alone (this study), the "slow HR" alone [11], or SA-elicited, *NPR1*-dependent signaling [9–11] were each sufficient by themselves to give *PR-1* induction. Induction of *PR-5* gene expression can also be via any of these three inputs acting independently. However, we (this study) and others [19,20] have presented evidence that other pathways can also contribute to *PR-5* induction. As such, *PR-5* is gated by an "or" gate. One input is a branch point from the "or" gate leading to *PR-1* induction. The second input is pictured as a question mark. Evidence has been presented herein that this second input represents at least two pathways. However, the details of these pathways are not known and are thus not pictured.

No attempt has been made to represent the kinetics of signaling events in Figure 4. Boolean formalism can incorporate kinetic information through use of gating delays. Differential equations-based kinetic modeling is an alternative means of capturing the rich dynamics of signaling networks [28,29]. As time-resolved data sets become available and feedback loops are delineated, it may be necessary to use these methods to understand system dynamics and control [30]. However, the authors agree with Genoud et. al. (2001) that the scheme used in that work

and in Figure 4 of this paper has advantages for representing the topology of signaling networks. This formalism allows clear differentiation of additive from obligatorily synergistic signaling interactions through use of "or" and "and" gates, respectively.

## Conclusions

In conclusion, the additive effects of two pathways explain most if not all induction of SAR and *PR-1* gene expression by the avirulent bacteria examined. One of these pathways stems from signals associated with hypersensitive cell death and is *NPR1*-independent. The other pathway stems from SA accumulation and signals through *NPR1*. By contrast, *PR-5* induction by avirulent bacteria involves at least two additional pathways that also act in an additive fashion.

## Methods

### Bacteria and plant growth

*Pseudomonas syringae* was cultured according to published methods [31]. Arabidopsis was grown in a HTPC walk-in chamber (Convicon, Winnipeg, MN) or a MTR-30 reach-in chamber (Convicon, Winnipeg, MN) as previously described [4]. Columbia, *ndr1-1* and *ndr1-1/gl-1* seed used were previously described [4,5]. Seeds of *npr1-2* and *sid2-1* mutants were generously provided by Dr. Jane Glazebrook (Torrey Mesa Research Institute) and Dr. Christiane Nawrath (University of Fribourg), respectively.

### Isolation and validation of the *ndr1-1/npr1-2* double mutant line

The *ndr1-1/npr1-2* double mutant was generated using pollen from a *npr1-2* plant to fertilize flowers of a *ndr1-1/gl-1* plant. The success of the cross was validated by the hairy (non-glabrous) phenotype of leaves of the F<sub>1</sub> plant. DNA was prepared from leaves of 5-week old F<sub>2</sub> progeny from this cross for *npr1-2* detection or cultured roots grown from F<sub>3</sub> seeds for *ndr1-1* detection.

The *ndr1-1* deletion was detected by Southern blotting done essentially as previously described [6]. To make a radiolabeled probe, part of the *NDR1* locus was amplified using *ndr1-1* genomic DNA as template. The primers used in the amplification were as follows: 5'-TCCTAAGGTTTCGTTTTGGGTC-3' and 5'-GGGACGGTTTCAATTCTGTGATAG-3'. The resulting PCR product was gel purified. This PCR product was subsequently used as template DNA in a labeling reaction using the Radprime DNA labeling system (Invitrogen, Carlsbad, CA).

The *npr1-2* point mutation [15] was detected using a CAPS (cleaved co-dominant amplified polymorphic sequence) marker [32]. The primers used in PCR were as follows: 5'-CCTGATGTATCTGCTCT-3' and 5'-GCTTAATGCAGATGGTG-3'. The amplified products were cut with *FspI* to



yield a 330 bp fragment and a 134 bp fragment if wild type (*NPR1*) DNA was used as the template. *FspI* does not cut DNA amplified from *npr1-2* mutant plants.

#### Systemic acquired resistance experiments

SAR experiments were performed as previously described [4] except that hand inoculation using a syringe was used instead of vacuum infiltration to introduce the challenge pathogen. Triplicate samples of four leaf disks each were used on Day 0, and quintuplicate samples were used on Days 2 and 4. Error bars represent standard deviations of data means. Significance of differences between means at a given time point were determined using Student's *t* tests. Values of *P* were read from a *t* table. A replicate experiment gave comparable results. In accordance with established practice in the field [33], data was not combined between the two replicate experiments.

#### Northern analysis

Plant growth and inoculations were as described for SAR experiments. Leaf tissue samples were collected at indicated time points. Samples were flash frozen in liquid nitrogen and total RNA was isolated according to Nawrath and Métraux [20]. RNA concentration was determined spectrophotometrically. 25 µg of total RNA was used per lane. Gene specific probes were made from gel-purified DNA fragments using the Radprime DNA labeling system (Invitrogen, Carlsbad, CA). The template DNA used for making probes to detect expression of *PR-1*, *PR-5* and *ROC1* (cyclophilin, used to normalize for RNA loading) were *PR-1* and *PR-5* cDNAs [14] and an *EcoRI* fragment of pCG22 [34], respectively. AGI numbers corresponding to the genes used as probes are AT2G19990, AT1G75040 and AT4G38740, respectively. Northern Blots were performed using standard procedures [35]. Quantitation used a Storm PhosphorImager (Amersham Biosciences, Sunnyvale, CA).

#### Authors' contributions

CZ isolated the *ndr1-1/npr1-2* line used, established homogeneity of both mutations, carried out the *PR* gene expression studies, and contributed to preparation of the manuscript. AS conceived of the study, performed the genetic cross to make the *ndr1-1/npr1-2* line, carried out the SAR experiments, developed the Boolean model of the signaling pathway, and contributed to preparation of the manuscript. Both authors read and approved the final manuscript.

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