PLANT SIGNALING & BEHAVIOR
2016, VOL. 11, NO. 10, e1211222 (4 pages)
http://dx.doi.org/10.1080/15592324.2016.1211222

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Overexpression of Arabidopsis NIMIN1 results in salicylate intolerance

Rajinikanth Mohan[a], Thomson Tai[a], Andy Chen[a], Taylor Arnoff[a], and Zheng-Qing Fu[b]

[a]Department of Biology, Hamilton College, Clinton, NY, USA; [b]Department of Biological Sciences, University of South Carolina, Columbia, SC, USA

ABSTRACT

The transcriptional regulator NPR1 mediates salicylic acid (SA)-induced plant immunity. NPR1 is also required for tolerance to high concentrations of SA. NPR1-interacting protein, NIMIN1, represses immune response by interacting with and negating NPR1. We tested the salicylic acid tolerance of transgenic plants overexpressing NIMIN1 and found that these plants displayed SA intolerance, similar to the *npr1* mutant, due to sequestration of NPR1 by NIMIN1. Plants overexpressing mutated NIMIN1 that cannot interact with NPR1 showed no SA tolerance defect. Gene expression analysis showed that NPR1 is required for SA-stress induced as well as pathogen-induced NIMIN1 expression. These results indicate that over-accumulation of a negative regulator renders plants hypersensitive to SA by limiting NPR1 function. Furthermore, NPR1 activates negative regulators such as NIMIN1 for feedback inhibition of SA signaling to maintain immune homeostasis.

Abbreviations: SA, salicylic acid; NPR1, Non-expressor of PR1; NIMIN1, NIM1 (NPR1)-interacting 1; SAR, systemic acquired resistance

ARTICLE HISTORY
Received 10 June 2016
Revised 29 June 2016
Accepted 1 July 2016

KEYWORDS
NIMIN1; salicylic acid intolerance; salicylic acid sensitivity; NPR1; negative regulator of immunity; feedback inhibition; transcriptional regulator

Salicylic acid (SA) is a hormone produced in plants in response to biotic and abiotic stresses.[1] Infection of plants with bacterial pathogens triggers accumulation of SA as a stress signal, followed by expression of defense genes such as pathogenesis related protein 1 (PR1).[2] Infection or exogenous application of SA, which mimics infection, can induce defense gene expression, while triggering resistance against subsequent pathogen attack,[3] a process defined as systemic acquired resistance (SAR). NPR1 is a master regulator of SAR and functions as transcriptional coactivator of defense-related genes in the nucleus, in conjunction with the bZIP family of transcription factors, namely TGA.[2]

In Arabidopsis, upon pathogen infection, SA is synthesized via the isochorismate synthase pathway, where chorismate into isochorismate, which is subsequently transformed into SA.[4] The level of SA in Arabidopsis is kept in check through a feedback loop involving NPR1, which suppresses further SA accumulation.[5] The biological importance of NPR1 in tolerating SA is evident in the case of *cpr5* mutant, which displayed increased endogenous levels of SA.[6] Chlorosis in young leaves, indicating that NPR1 is required to alleviate the toxicity caused by elevated SA levels. Similarly, young seedlings of *npr1* mutant exhibited chlorosis, that was absent in wild type seedlings, when germinated on Murashige Skoog (MS) medium containing SA, a phenomenon named SA intolerance or sensitivity.[7] These lines of evidence indicate an important role for NPR1 in SA tolerance. More recently, Zhang et al.,[8] showed that nuclear localization of NPR1 is essential for tolerance to SA.

NIMIN1 (NIM1-Interacting; *nim1* is allelic to *npr1*) belongs to a family of small, structurally similar proteins including NIMIN2 and NIMIN3, that were all first identified as interactors of NPR1 in a yeast 2-hybrid screen.[9] NIMIN1 and NPR1 can also interact *in planta* as shown by co-immunoprecipitation in plant extracts.[10] NIMIN1 associates with the C-terminal region of NPR1 through the NPR1-interaction domain, spanning amino acids 49 to 54.[9] The binding of NIMIN1 to NPR1 is an important step in modulating the level of immune response as the *nimin1* mutant displays enhanced NPR1-dependent defense gene expression.[10] Specifically, NIMIN1 regulates the amplitude, rather than timing, of defense gene induction by interacting with and negating NPR1 protein.[10] Like NPR1, NIMIN genes are induced by SA and the NIMIN proteins carry nuclear localization signals targeting them to the nucleus, consistent with their role as transcriptional regulators.[9]

Arabidopsis transgenics highly expressing NIMIN1 mimic the *npr1* mutant by displaying reduced SA-induced PR gene expression and impairment of systemic acquired resistance.[10] In contrast, plants expressing a mutant version of NIMIN1 that cannot interact with NPR1 are similar to wild type plants. These results suggest that the physical sequestration of functional NPR1 by overexpressed NIMIN1 results in an *npr1*-like phenotype. In order to reaffirm the functional importance of NPR1 in SA tolerance, we tested the SA tolerance phenotype of transgenic plants overexpressing myc and histidine tag-fused functional NIMIN1 (35S:NIMIN1:his:myc) and mutant *nimin1-2* protein (35S:*nimin1-2*:his:myc); the *nimin1-2* mutant protein is defective in interaction with NPR1 due to mutation of the amino acids Phenylalanine-49 (Phe-49) and Phe-50 residues in the NPR1-interaction domain to serine residues.[10] As expected, 35S:NIMIN1, but not 35S:*nimin1-2*, replicated the SA intolerance phenotype of *npr1-2* mutant (Fig. 1A), indicating

CONTACT Rajinikanth Mohan rmohan@hamilton.edu Department of Biology, Hamilton College, Clinton, NY 13323, USA

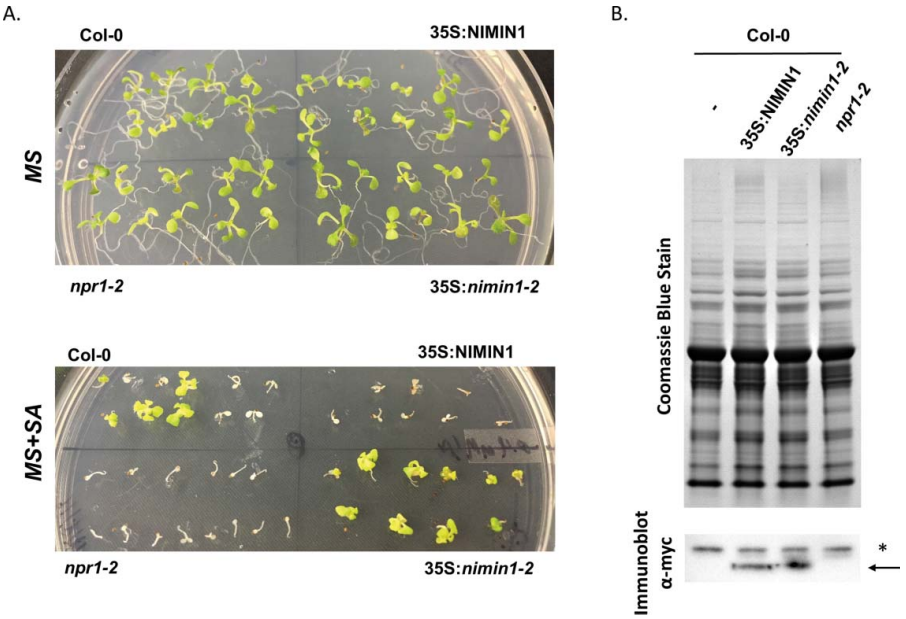

Figure 1. SA intolerance of NIMIN1 transgenics. (A) Wild type, 35S:NIMIN1-his-myc, 35S:*nimin1-2*-his-myc and npr1-2 were grown on Murashige Skoog (MS) plates with (bottom left panel) or without (top left panel) supplementation with 0.4mM SA for about 2 weeks. This result was observed 4 times. (B) Immunoblot analysis of the 44 g genotypes (lower panel); transgenic NIMIN1 protein was detected using an antibody against c-myc (Cell Signaling Technology, 1:2.000). Arrow indicates the band corresponding to NIMIN1 (~20 kDa). Asterisk represents a non-specific band, which along with the Coomassie stain (upper panel) acts a loading control. Confirmed twice.

that sequestration of NPR1 protein renders plants sensitive to SA-mediated stress. Immunoblot analysis was used to ascertain that these plants indeed expressed NIMIN1 fusion proteins. As seen in Fig. 1B, the myc tags associated with NIMIN1 and *nimin1-2* are only detected in the transgenic plants, but not in wild type or *npr1-2* mutant.

Since NIMIN1 overexpression caused SA intolerance, we tested whether the induction of endogenous NIMIN1 contributed to SA intolerance in *npr1* mutant. For this, we assayed the gene expression of NIMIN1 using quantitative PCR in seedlings grown on MS media with and without SA supplementation. NIMIN1 was induced in wild type plants by SA stress and this activation was completely dependent on NPR1, as the *npr1-2* mutant lacked NIMIN1 expression (Fig. 2A). This clarifies that the SA intolerance of *npr1* mutants is not due to NIMIN1 hyperinduction and that the SA intolerance of 35S:NIMIN1 is a result of physical interaction between NIMIN1 and NPR1, demonstrated before.[10] To determine if the NPR1-mediated transcription of NIMIN1 was limited to seedlings displaying SA tolerance, we tested the expression of NIMIN1 in mature plants treated with SA. As observed in Fig. 2B, NPR1 was indispensible for SA-mediated expression of NIMIN1 in soil grown mature plants. Since SA mimics pathogen infection, we tested the pathogen-induced expression of NIMIN1. Indeed, not only was NIMIN1 expressed upon infection by virulent and avirulent bacterial pathogens, its induction was strikingly dependent on NPR1 (Fig. 2C). Together, these results demonstrate that while NIMIN1 inhibits NPR1 functions, including SA tolerance, through physical interaction, the native expression of NIMIN1 in wild type plants in response to SA and during immune response requires NPR1.

Plants lacking NPR1, the bZIP transcription factors TGA2,5,6 and the GRAS-family transcription factor, SCL14, all exhibit intolerance to salicylic acid or its analogs.[11,12] High concentrations of SA (>100 uM) can promote accumulation of reactive oxygen species, leading to oxidative stress.[13] Thus, SA tolerance may be a reflection of the importance of NPR1 and the other proteins in coping with the oxidative stress caused by supraphysiological concentrations of SA. The introduction of SA intolerance by binding of NIMIN1 to NPR1 is reminiscent of a similar phenotype observed in plants expressing a truncated form of TGA2 (35S:TGA2-CT, C-terminal domain alone) that had a dominant-negative sequestration effect on NPR1 protein, resulting in toxicity on SA containing media.[14] The SA intolerance of NIMIN1 overexpressing plants underscores the importance of NPR1 in SA tolerance.

The expression of NIMIN1 gene is inducible by SA[10] in an NPR1-dependent manner[15] and this appears to require a TGA2-binding motif, TGACG, in the promoter.[16] Furthermore, NIMIN1:GUS reporter expression was abolished in plants depleted of SA, indicating a requirement of SA in NIMIN1 induction.[17] From this study, it is evident that NPR1 is additionally required for the transcriptional activation of NIMIN1 during stress caused by elevated concentrations of SA as well as during pathogen infection. NIMIN1 is a *bona fide* negative regulator of plant immunity as NIMIN1 overexpressing plants are more susceptible and *nimin1* mutant is more resistant to bacterial pathogens.[10] The absolute requirement of NPR1 in the activation of negative regulators of immunity has also been observed previously, as the expression of the negative regulators, WRKY38 and WRKY62,[18] and WRKY58 and TGA2 are all dependent on NPR1.[14,19,20] The NPR1-dependent activation of repressors illustrates the need for immune homeostasis during defense to prevent hyperinduction of the immune response. It is striking to note, from a previous report, that NIMIN1

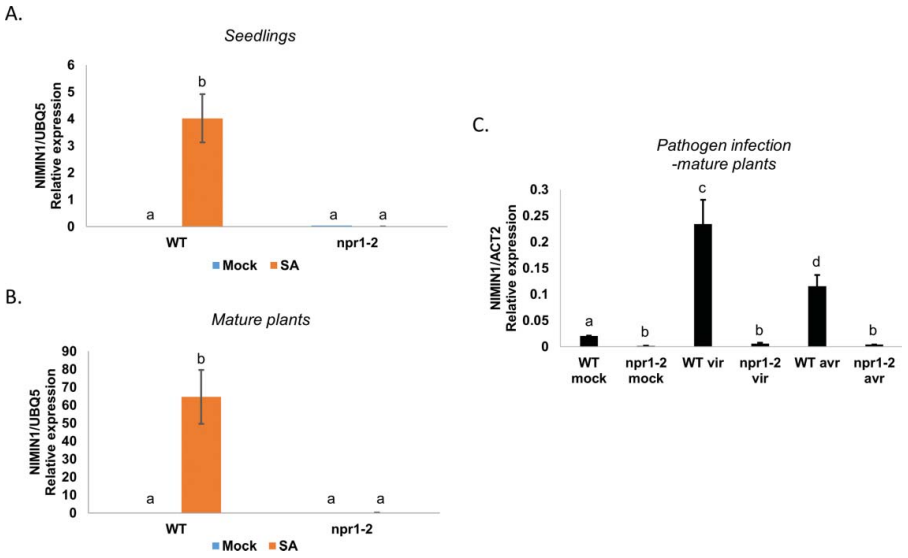

Figure 2. SA- induced expression of NIMIN1 in npr1 mutant. (A) MS grown seedlings (2 wk old). Mock indicates MS plates and SA indicates supplementation of MS with 0.4 millimolar (mM) SA. (B) Mature leaves collected from 4-week old plants, 24 hours after treatment with 5 mM SA or water (mock) by root drenching for 20 minutes. (C) Leaves from 4-week old plants were collected 24 hours after infiltration with 10mM magnesium sulfate ($MgSO_4$) (mock), virulent bacterial pathogen, *Pseudomonas syringae* DC3000 (Pst) (vir) or avirulent Pst with the effector avrRpt2 (avr) suspension in $MgSO_4$ at a dose of 5×10^6 colony forming units per milliliter. RNA was extracted using Trizol (Life Technologies), treated with DNAse I (Ambion), cDNA was prepared using Superscript III reverse transcriptase (Life Technologies) and quantitative RealTime PCR was performed using SYBR Green mastermix (Life Technologies). Compared to the housekeeping gene Ubiquitin 5 (UBQ5) and actin2 (ACT2) were calculated. Data was analyzed using the ΔCt method[21] and represent the average of 3 technical replicates/2 biological replicates with standard deviation. The results were reproduced 2-4 times. For statistical analysis, One way ANOVA was performed, followed by Tukey's multiple comparison test ($p<0.05$). Means with the same letter are not significantly different from each other.

inhibits its own gene expression through interaction with NPR1.[10] In this study, Weigl et al.,[10] reported that the expression of endogenous NIMIN1 was reduced in NIMIN1 overexpressing plants, but not the *nimin1-2* lines, indicating that overexpressing NIMIN1 interacts with NPR1 in the former to prevent its endogenous gene expression.

Overall, the control of negative regulator expression by NPR1 and the physical interaction of NPR1 with the same protein appears to be part of an elegant design to modulate immune response as illustrated by the NPR1 and NIMIN1 mutants and transgenics. Since the *npr1* mutant fails to mount a functional defense response, the activation of negative regulators is precluded, so there is no transcriptional activation of NIMIN1. When SA and NPR1-mediated defense is activated, NPR1 turns on NIMIN1 gene and the synthesized NIMIN1 protein (possibly after reaching a threshold) sequesters NPR1 to keep the amplitude of immune response in check; this is observed as elevated defense gene expression in *nimin1* mutant. When there is excessive accumulation of NIMIN1 protein (as in NIMIN1 overexpression lines), NIMIN1 binds to NPR1 and inhibits NPR1-mediated transcriptional activation of its own gene to maintain immune homeostasis. Thus, NPR1 not only mediates feedback inhibition of SA synthesis,[5] but also promotes feedback inhibition of SA signaling by activation of negative regulators of the SA pathway during plant immunity. Furthermore, the SA tolerance function of NPR1 also implies a possible protective role from collateral damage during immune response.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank Drs. Daniel Klessig and Murli Manohar at BTI, Cornell University, for kindly providing seeds and bacterial strains; Dr. Mark Sasaki at Hamilton College for kindly providing the c-myc antibody.

Funding

This work was supported by the Hamilton College Dean of Faculty and the Class of 1979 award at Hamilton College to A.C.

References

1. Rivas-San Vicente M, Plasencia J. Salicylic acid beyond defence: its role in plant growth and development. J Exp Botany 2011; 62 (10):3321-38; PMID:21357767; http://dx.doi.org/10.1093/jxb/err031
2. Fu ZQ, Dong X. Systemic acquired resistance: turning local infection into global defense. Annu Rev Plant Biol 2013; 64:839-63; PMID:23373699; http://dx.doi.org/10.1146/annurev-arplant-042811-105606
3. White RF. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology 1979; 99(2):410-2; PMID:18631626; http://dx.doi.org/10.1016/0042-6822(79)90019-9
4. Wildermuth MC, Dewdney J, Wu G, Ausubel FM. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 2001; 414(6863):562-5; PMID:11734859; http://dx.doi.org/10.1038/35107108
5. Lu H. Dissection of salicylic acid-mediated defense signaling networks. Plant Signal Behav 2009; 4(8):713-7; PMID:19820324; http://dx.doi.org/10.4161/psb.4.8.9173
6. Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 1997; 9(9):1573-84; PMID:9338960; http://dx.doi.org/10.1105/tpc.9.9.1573
7. Cao H, Glazebrook J, Clarke JD, Volko S, Dong X. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel

protein containing ankyrin repeats. Cell 1997; 88(1):57-63; PMID:9019406; http://dx.doi.org/10.1016/S0092-8674(00)81858-9
8. Zhang X, Chen S, Mou Z. Nuclear localization of NPR1 is required for regulation of salicylate tolerance, isochorismate synthase 1 expression and salicylate accumulation in Arabidopsis. J Plant Physiol 2010; 167(2):144-8; PMID:19716624; http://dx.doi.org/10.1016/j.jplph.2009.08.002
9. Weigel RR, Bauscher C, Pfitzner AJ, Pfitzner UM. NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. Plant Mol Biol 2001; 46(2):143-60; PMID:11442055; http://dx.doi.org/10.1023/A:1010652620115
10. Weigel RR, Pfitzner UM, Gatz C. Interaction of NIMIN1 with NPR1 modulates PR gene expression in Arabidopsis. Plant Cell 2005; 17(4):1279-91; PMID:15749762; http://dx.doi.org/10.1105/tpc.104.027441
11. Zhang Y, Tessaro MJ, Lassner M, Li X. Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell 2003; 15(11):2647-53; PMID:14576289; http://dx.doi.org/10.1105/tpc.014894
12. Fode B, Siemsen T, Thurow C, Weigel R, Gatz C. The Arabidopsis GRAS protein SCL14 interacts with class II TGA transcription factors and is essential for the activation of stress-inducible promoters. Plant Cell 2008; 20(11):3122-35; PMID:18984675; http://dx.doi.org/10.1105/tpc.108.058974
13. Herrera-Vasquez A, Salinas P, Holuigue L. Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. Frontiers Plant Sci 2015; 6:171; PMID:25852720; http://dx.doi.org/10.3389/fpls.2015.00171
14. Fan W, Dong X. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell 2002; 14(6):1377-89; PMID:12084833; http://dx.doi.org/10.1105/tpc.001628
15. Hermann M, Maier F, Masroor A, Hirth S, Pfitzner AJ, Pfitzner UM. The Arabidopsis NIMIN proteins affect NPR1 differentially. Frontiers Plant Sci 2013; 4:88; PMID:23630533; http://dx.doi.org/10.3389/fpls.2013.00088
16. Fonseca JP, Menossi M, Thibaud-Nissen F, Town CD. Functional analysis of a TGA factor-binding site located in the promoter region controlling salicylic acid-induced NIMIN-1 expression in Arabidopsis. Genetics Mol Res 2010; 9(1):167-75; PMID:20198573; http://dx.doi.org/10.4238/vol9-1gmr704
17. Glocova I, Thor K, Roth B, Babbick M, Pfitzner AJ, Pfitzner UM. Salicylic acid (SA)-dependent gene activation can be uncoupled from cell death-mediated gene activation: the SA-inducible NIMIN-1 and NIMIN-2 promoters, unlike the PR-1a promoter, do not respond to cell death signals in tobacco. Mol Plant Pathol 2005; 6(3):299-314; PMID:20565658; http://dx.doi.org/10.1111/j.1364-3703.2005.00288.x
18. Kim KC, Lai Z, Fan B, Chen Z. Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell 2008; 20(9):2357-71; PMID:18776063; http://dx.doi.org/10.1105/tpc.107.055566
19. Spoel SH, Mou Z, Tada Y, Spivey NW, Genschik P, Dong X. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell 2009; 137(5):860-72; PMID:19490895; http://dx.doi.org/10.1016/j.cell.2009.03.038
20. Wang D, Amornsiripanitch N, Dong X. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog 2006; 2(11):e123; PMID:17096590; http://dx.doi.org/10.1371/journal.ppat.0020123
21. Yuan JS, Reed A, Chen F, Stewart CN, Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006; 7:85; PMID:16504059; http://dx.doi.org/10.1186/1471-2105-7-85