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SHORT COMMUNICATION

Plant defensin expression triggered by fungal pathogen invasion depends on EDR1 protein kinase and ORA59 transcription factor in *Arabidopsis thaliana*

Ayumi Kosaka, Haruka Suemoto, Suthitar Singkaravanit-Ogawa, and Yoshitaka Takano [ID]

Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

ABSTRACT

Arabidopsis thaliana exhibits durable 'non-host' resistance against the hemibiotrophic fungal pathogen *Colletotrichum tropicale* that infects mulberry plants. *Arabidopsis* non-host resistance comprises two layers of defense: preinvasive and postinvasive resistance. The EDR1 protein kinase contributes to *Arabidopsis* preinvasive resistance against *C. tropicale* by inducing the expression of plant defensin (*PDF*) genes. Here we report that the expressions of multiple *PDF* genes were strongly induced in *Arabidopsis* upon invasion by *C. tropicale*. Invasion by a necrotrophic pathogen, *Alternaria brassicicola*, also induced *PDF* expression. Importantly, *PDF* expression triggered upon invasion by both pathogens was inhibited in *edr1* mutants, indicating the requirement of EDR1 for *PDF* expression in postinvasive resistance by *Arabidopsis*. Analysis of *ora59* mutants also revealed that this gene is critical for induced *PDF* expression following pathogen invasion. Furthermore, inoculation assays of *A. brassicicola* indicated that *ORA59* is involved in postinvasive resistance against the pathogen, suggesting invasion-triggered *PDF* expression contributes to postinvasive resistance in *Arabidopsis*.

Non-host resistance can be defined as the immunity of an entire plant species against all tested isolates of a particular pathogen. It confers a durable plant defense mechanism against the majority of potential pathogens.[1] Durability of non-host resistance depends on its multi-layered defense system, i.e., non-host resistance consists of both preinvasive resistance and postinvasive resistance. *Colletotrichum tropicale* isolate S9275 (hereafter called *Ctro*) is known to cause anthracnose disease in mulberry plants but is not adapted to *Arabidopsis thaliana*.[2,3] In contrast to the adapted pathogen *Colletotrichum higginsianum*, which causes necrotic lesions on inoculated *Arabidopsis* leaves, nonadapted *Ctro* fails to induce lesions, indicating that *Arabidopsis* exhibits non-host resistance to this pathogen.[3,4] *PENETRATION2* (*PEN2)* and *PEN3* are involved in preinvasive resistance (the control of pathogen entry) against *Ctro* when it uses a hyphal-tip entry mode in the presence of 0.1% glucose.[3,5–7] *PEN2* encodes an atypical myrosinase that hydrolyzes 4-methoxyindol-3-ylmethylglucosinolates as in planta substrate for antifungal responses.[8,9] *PEN3* encodes an ATP binding cassette (ABC) transporter. Genetic interaction analysis of *pen2* and *pen3* mutants suggested that PEN3 is likely involved in exporting toxic compounds including PEN2-catalyzed metabolites.[10] However, whereas *Ctro* invades *pen2* mutants successfully, these are still not fully susceptible to *Ctro*, because postinvasive resistance is activated and this terminates further pathogen growth.[11] This observation highlights the importance of postinvasive resistance for plant survival against the attack of numerous pathogenic fungi.

Non-host preinvasive resistance toward *Ctro* also involves *ENHANCED DISEASE RESISTANCE 1* (*EDR1*).[12] *EDR1* encodes a protein kinase homologous to the mitogen-activated protein kinase belonging to the Raf family.[13] It was reported that the EDR1 protein kinase positively regulates the expression of antimicrobial plant defensin (*PDF*) genes in response to the entry of *Ctro*.[12] Currently, it remains to be elucidated whether EDR1 regulates *PDF* expression during postinvasive defense.

Here we investigated the expression of *PDF1.2a* in *Arabidopsis* invaded by the nonadapted *Ctro*. Because *Ctro* starts to invade *pen2* mutants at around 14 hours post inoculation (hpi) of *Ctro* with 0.1% glucose, whereas *Ctro* cannot invade WT (Col-0) plants,[3,4] we measured the expression of *PDF1.2a* at 6 hpi as the preinvasive stage and 17 hpi as the postinvasive stage. At 17 hpi, when *Ctro* invaded *pen2-1* but not WT plants, we found that *PDF1.2a* expression was highly induced in the *pen2-1* mutant but not in the WT plants (Figure 1a). Compared with the mock-treated plants (plants treated with 0.1% glucose), *PDF1.2a* expression was induced by *Ctro* at 6 hpi in WT plants (Figure 1b), consistent with our previous finding.[12] At 6 hpi, the *PDF1.2a* expression level in the *pen2-1* mutant showed no clear difference difference from that in WT plants (Figure 1b). However, the induced level of *PDF1.2a* at 17 hpi in the *pen2-1* mutant was much greater than at 6 hpi in the *pen2-1* mutant (Figure 1a). *PDF1.2a* was also highly expressed in the *pen2-2* mutant,[5] the other allele of *pen2* mutants, at 17 hpi by *Ctro* (Supplementary Figure 1A). We also investigated the expression of three additional *PDF* genes, *PDF1.2b*, *PDF1.2c* and *PDF1.3* at 17 hpi with *Ctro*. We found that all

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a

b
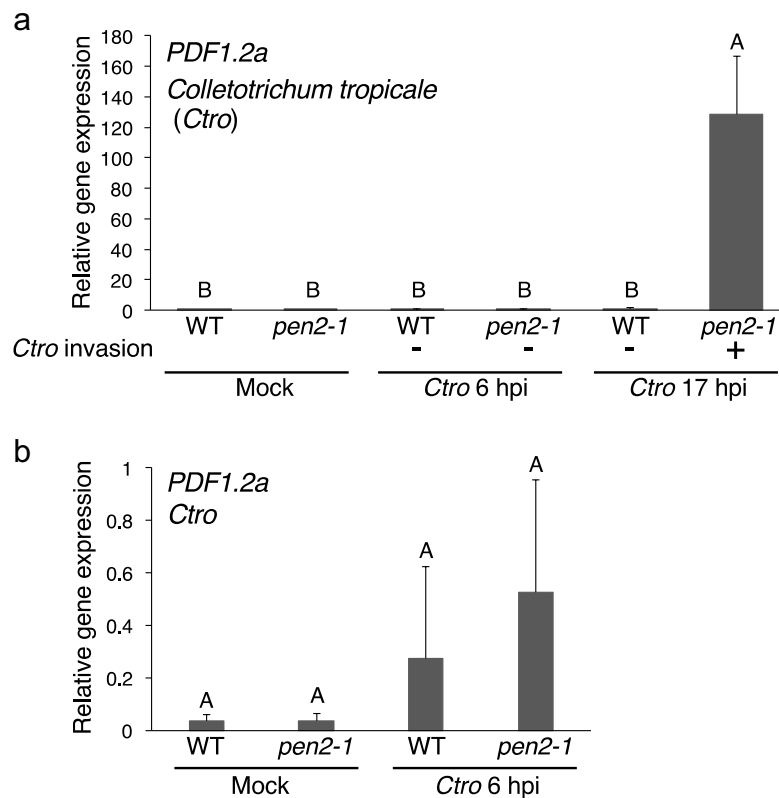
Figure 1. The expression of *PDF1.2a* was highly induced upon *Ctro* invasion. a. RT–qPCR analysis of *PDF1.2a* in *Arabidopsis* plants inoculated with *Ctro*. A conidial suspension from *Ctro* (5×10^5/mL with 0.1% glucose) was spray-inoculated onto 4- to 5-week-old *Arabidopsis* plants. The leaf samples were collected at 6- and 17-hours post-inoculation (hpi). As a control, 0.1% glucose without *Ctro* was sprayed onto the plants and the leaf samples were collected at 17 h after this 'mock' treatment. Total RNA was extracted using PureLink RNA Mini kits (Thermo Fisher Scientific, Waltham, MA, USA) and treated with DNase. Takara Prime Script™ RT kits (Takara Bio Inc., Shiga, Japan) was used for the cDNA synthesis. Takara TB Green™ Premix Ex Taq™ I was used for RT–qPCR with the primers listed in Supplementary Table 1. *Arabidopsis UBC21* (At5g25760) was used as an internal control for normalizing the level of cDNA.[14] RT–qPCR analysis was performed using a Thermal Cycler Dice Real Time System TP810 (Takara Bio Inc.). Means and standard deviations (SDs) were derived from three independent samples. b. The expression of *PDF1.2a* was induced at 6 hpi compared with the mock treatment. The RT–qPCR data on mock and *Ctro* 6 hpi are shown with a different scale on the Y-axis in Figure 1a. The statistical significance of differences in gene expression level was determined by Tukey's honestly significant difference (HSD) test ($P < .01$). The experiment was repeated twice, with similar results.

tested *PDF* genes were highly expressed in *pen2-1* at 17 hpi with *Ctro* (Supplementary Figure 1B).

We then tested whether EDR1 could regulate *PDF* expression not only in preinvasive but also in postinvasive defense. Notably, the expression of *PDF1.2a* was not induced in the *edr1* mutant at 17 hpi with *Ctro* (Figure 2) even though the mutant is defective in preinvasive resistance against *Ctro*.[12] This indicated the requirement of EDR1 for the induced expression of *PDF1.2a* triggered by *Ctro* invasion. We found previously that the expression of *PDF1.2a* in preinvasive defense was recovered in the *edr1 myc2* mutant, in contrast to the *edr1* mutant.[12] Interestingly, we found that *PDF1.2a* expression was not restored in the *edr1 myc2* mutant plant at 17 hpi with *Ctro* (Figure 2), suggesting that EDR1 positively regulates *PDF* expression upon *Ctro* invasion, uncoupled from the MYC2-dependent repression of *PDF* expression that was observed at the preinvasive stage.

OCTA-DECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59 (ORA59) is a transcription factor that belongs to the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily and binds to the GCC-box motif in the promoter region of *PDF1.2a*.[15] It was reported that EIN3 physically interacts with ORA59, resulting in destabilization of ORA59.[16] It was also shown that *Arabidopsis* plants treated with interfering RNA
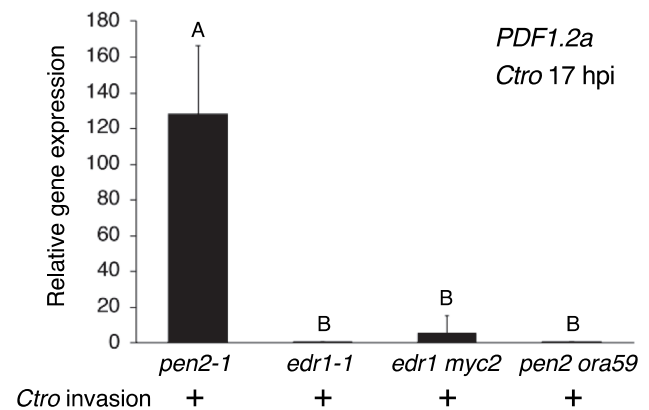
Figure 2. The induced expression of *PDF1.2a* upon *Ctro* invasion depends on both EDR1 and ORA59. *Ctro* inoculation and subsequent RT–qPCR analyses were performed as described in Figure 1. The genotyping primers used for generation of the *pen2 ora59* mutants are listed in Supplementary Table 1. Means and SDs were derived from three independent experiments. The statistical significance of differences in gene expression level was determined by Tukey's honestly significant difference (HSD) test ($P < .01$). The experiment was repeated twice, with similar results.

(RNAi) for *ORA59* showed reduced *PDF1.2a* expression, whereas *Arabidopsis* plants overexpressing *ORA59* displayed increased *PDF1.2a* expression in response to the necrotrophic

pathogens *Botrytis cinerea* and *Alternaria brassicicola*.[17] To assess the involvement of ORA59 in *PDF1.2a* expression upon *Ctro* invasion, we newly generated a *pen2 ora59* double mutant by crossing *pen2-1* and *ora59-1* plants.[18,19] Then, the generated double mutant was inoculated with *Ctro*. Reverse transcription-quantitative polymerase chain reaction (RT–qPCR) analysis indicated that *PDF1.2a* expression was clearly reduced in the *pen2 ora59* mutant at 17 hpi of *Ctro*, in contrast to the *pen2* mutant (Figure 2). These results indicate that the induced expression of *PDF1.2a* triggered by *Ctro* invasion depends on both EDR1 and ORA59.

We next tested whether EDR1 and ORA59 might be required for *PDF* expression triggered upon invasion by a necrotrophic fungus, *Alternaria brassicicola* strain Ryo-1 (hereafter called *Ab*), in addition to *Ctro*. Because our recent study revealed that *PEN2* is dispensable for preinvasive resistance against *Ab*, [20] we used the *ora59* mutant instead of the *pen2 ora59* mutant for *Ab* inoculation. *Ab* was inoculated onto WT (Col-0), *edr1* and *ora59* mutant plants, and leaf samples were collected at multiple time points (4, 12, 24, 48, 72 and 96 hpi). Subsequently, the expression levels of two *PDF* genes (*PDF1.2a* and *PDF1.3*) were investigated using RT–qPCR. We recently revealed that conidia of *Ab* started to germinate at 4 hpi but did not invade *Arabidopsis* plants at this time point. At 12 hpi, germinated conidia of *Ab* started to invade, and from 12 to 24 hpi, the fungal entry rate increased dramatically.[20] We found that the expressions of the two *PDF* genes tested were not induced at 4 hpi but were significantly induced in WT plants at 12 hpi and further elevated at 24 hpi (Figure 3a and Supplementary Figure 2A).

These results suggest that the expressions of *PDF* genes were triggered by *Ab* invasion. Interestingly, the expressions of both *PDF1.2a* and *PDF1.3* in WT plants were reduced at 48 hpi but increased again at 72 hpi (Figure 3a and Supplementary Figure 2A). Importantly, this induced expression was not observed in the *edr1* or *ora59* plants, indicating the involvement of EDR1 and ORA59 for induced *PDF* expression upon *Ab* invasion (Figure 3a and Supplementary Figure 2). To investigate the relationship between *EDR1* and *ORA59* in transcriptional regulation, we investigated the expression of *EDR1* in *ora59* plants and the expression of *ORA59* in *edr1* plants in *Ab* inoculation. The result suggested that *EDR1* is dispensable for the expression of *ORA59*, and also *ORA59* is dispensable for the expression of *EDR1* (Supplementary Figure 2B). It will be important to study the relationship between EDR1 and ORA59 from the aspect of *PDF* expression in the future.

PAD3 is essential for the biosynthesis of camalexin (a phytoalexin of *Arabidopsis*) and is required for *Arabidopsis* immunity to *Ab*.[21,22] We recently reported that the expression of *PAD3* was induced upon *Ab* invasion and was involved in postinvasive resistance against *Ab*.[20] We investigated whether EDR1 and ORA59 are involved in the induced expression of *PAD3* upon *Ab* invasion. The RT–qPCR analysis revealed that *PAD3* expression was not detectable at 4 hpi, started to be induced at 12 hpi and was elevated at 24 hpi in WT (Figure 3b), which was consistent with our previous report.[20] In contrast to the *PDF* genes, *PAD3* expression was not diminished in *edr1* or *ora59* mutants, suggesting that EDR1 and ORA59 are dispensable for *PAD3* expression upon *Ab* invasion.
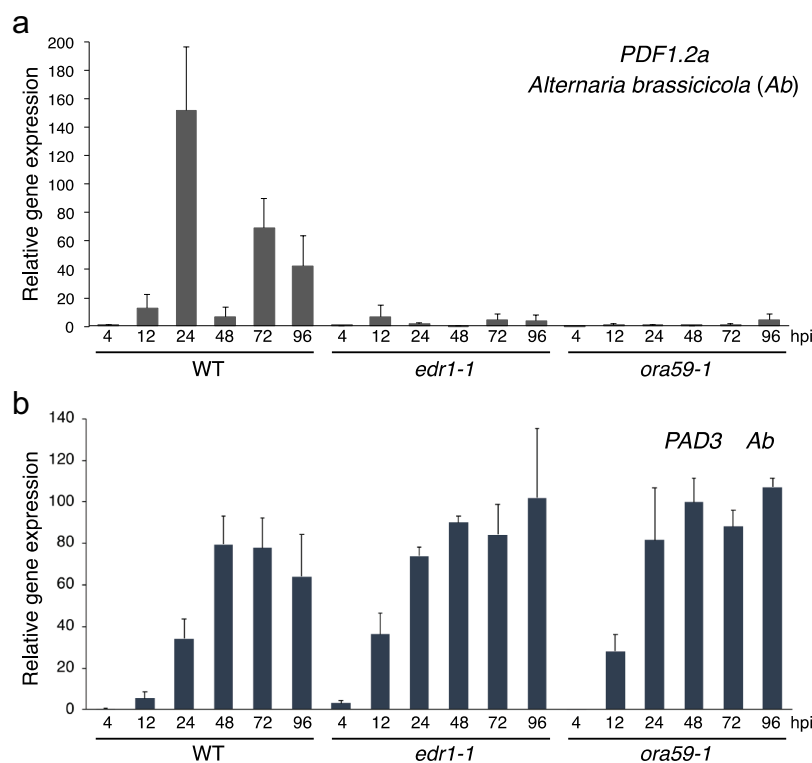
Figure 3. Both EDR1 and ORA59 are required for *Ab*-invasion triggered expression of *PDF1.2a* but not of *PAD3*. **a.** The invasion of *Ab* strongly induced the expression of *PDF1.2a*, which depended on EDR1 and ORA59. A suspension of *Ab* conidia (5×10^5/mL) was spray-inoculated onto 4- to 5-week-old *Arabidopsis* plants, and inoculated leaves were collected at 0, 4, 12, 24, 48, 72 and 96 hpi. Subsequent RT–qPCR analysis was performed as described in Figure 1. The means and SDs were derived from three independent experiments. **b**. EDR1 and ORA59 are dispensable for *Ab* invasion-triggered expression of *PAD3*. Primers used for the RT–qPCR of *PAD3* transcripts are listed in Supplementary Table 1. The means and SDs were derived from three independent samples. The experiment was repeated twice, with similar results.

a

b
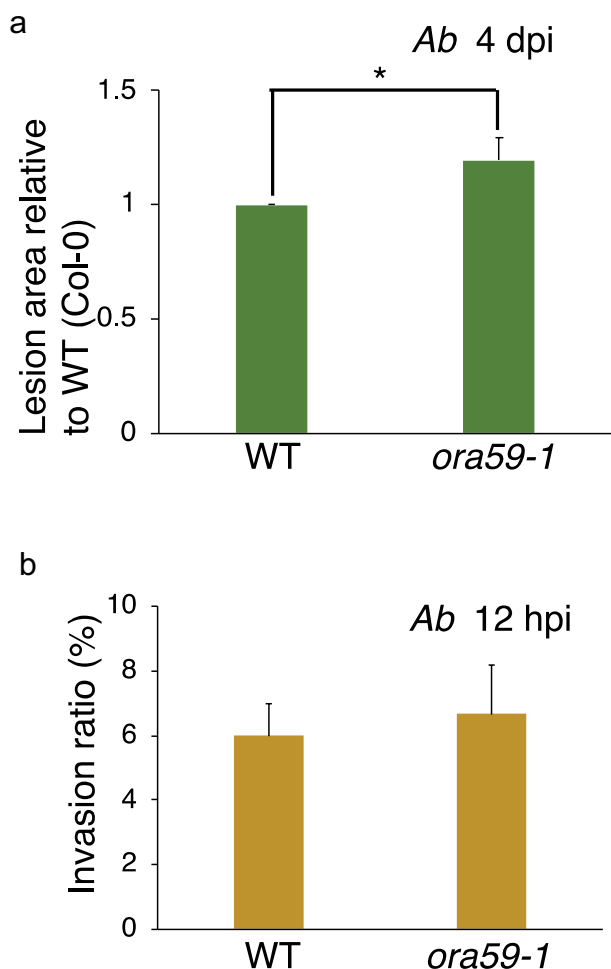
Figure 4. ORA59 contributes to postinvasive resistance against *Ab*. **a**. Quantitative analysis of lesion development caused by *Ab*. A suspension of *Ab* conidia (5×10^5/mL) was drop-inoculated onto mature leaves of 4–5-week-old *Arabidopsis* plants. At 4 dpi, lesion development was measured. The means and SDs were calculated from three independent experiments. For the statistical analysis, lesion development in *ora59* was compared with WT Col-0 using two-tailed Student's *t* tests (*$P <$ .05). **b**. Quantitative analysis of invasion ratio by *Ab*. A conidial suspension (1×10^5/mL) of *Ab* was drop-inoculated onto 4 to 5-week-old plants and kept at 100% humidity. The inoculated leaves were collected at 12 hpi, and then subjected to trypan blue staining assays. The presence or absence of invasive hyphae from at least 50 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments. Statistical comparisons of the *Ab* invasion ratios on WT Col-0 and tested mutants were conducted using two-tailed Student's *t* tests. The invasion ratio of the *ora59* mutants was not significantly different from that of WT (Col-0).

Interestingly, the subsequent expression patterns of *PAD3* were not consistent with those of the *PDF* genes (Figure 3b). In contrast to the latter, a drastic reduction in expression at 48 hpi was not observed in the case of *PAD3*. This finding suggests that the regulation of expression of the *PDF* genes is distinct from that for *PAD3* although their expression is commonly activated upon pathogen invasion. We hypothesize that the expression of the *PDF* genes is induced by *Ab* invasion but then is quickly repressed, whereas the *PAD3* expression is induced by *Ab* invasion and is sustained in contrast to the *PDF* genes. The reason why the expression of the *PDF* genes is recovered at 72 hpi is probably that *Ab* starts to invade neighboring cells around this time point, which triggers the transient expression of the *PDF* genes in these new invaded cells.

We reported previously that the *edr1* mutant displayed enhanced lesion development following inoculation with *Ab*,[12] indicating the involvement of EDR1 for *Arabidopsis*' immunity against *Ab*. Here we tested whether ORA59 might also be involved in the immunity of *Arabidopsis* against *Ab*. Inoculation assays for *Ab* revealed that the *ora59* mutant displayed enhanced lesion development compared with WT plants (Figure 4a). We also found that the *PAD3* expression was slightly enhanced in both *edr1* and *ora59* plants in comparison with the WT plants (Figure 3b), which is likely due to enhanced susceptibility of these plants to *Ab*.

To assess any possible involvement of ORA59 in preinvasive resistance against *Ab*, conidia of *Ab* were inoculated on WT and *ora59* plants, and the invasion ratio was determined at 12 hpi. We found that there was no significant difference in the invasion ratio between WT and *ora59*, suggesting that ORA59 is not required for preinvasive resistance (Figure 4b). These findings suggest that ORA59 is involved in postinvasive resistance against *Ab*. Because ORA59 is required for *PDF* expression at the postinvasive stage of *Ab*, this finding suggests that *PDF* genes contribute to postinvasive immunity in *Arabidopsis*.

Currently, it remains unclear how *Arabidopsis* plants recognize pathogen invasion to activate *PDF* expression. We recently reported that the expression of *GLIP1*, encoding a secreted antimicrobial protein, was induced upon *Ab* invasion and that the induced expression of *GLIP1* was largely reduced in the presence of a *bak1-5* mutation, suggesting that the involvement of a pattern recognition receptor in *Ab* invasion-triggered expression of *GLIP1*.[20,23] However, the *Ab*-triggered expressions of *PDF* genes were not reduced in the presence of the *bak1-5* mutation.[20] Thus, the EDR1–ORA59–PDFs pathway unlikely depends on a group of pattern recognition receptors whose function is blocked by *bak1-5*. Further studies on recognition machineries for pathogen invasion are essential for understanding the molecular background of postinvasive resistance in higher plants.

Disclosure of potential conflicts of interest

The author has declared that no competing interests exist.

ORCID

Yoshitaka Takano 🆔 http://orcid.org/0000-0003-1427-1322

References

1. Heath M. Nonhost resistance and nonspecific plant defenses. Curr Opin Plant Biol. 2000;3:315–319. doi:10.1016/s1369-5266(00)00087-x. PMID: 10873843.

2. Cannon PF, Damn U, Johnston PR, Weir BS. *Colletotrichum—current status and future directions*. Stud Mycol. 2012;73:181–213. doi:10.3114/sim0014. PMID: 23136460.

3. Hiruma K, Onozawa-Komori M, Takahashi F, Asakura M, Bednarek P, Okuno T, Schulze-Lefert P, Takano Y. Entry mode-dependent function of an indole glucosinolate pathway in Arabidopsis for nonhost resistance against anthracnose pathogens. Plant Cell. 2010;22:2429–2443. doi:10.1105/tpc.110.074344. PMID: 20605856.

4. Shimada C, Lipka V, O'Connell R, Okuno T, Schulze-Lefert P, Takano Y. Nonhost resistance in *Arabidopsis-Colletotrichum* interactions acts at the cell periphery and requires actin filament function. Mol Plant-Microbe Interact. 2006;19:270–279. doi:10.1094/MPMI-19-0270. PMID: 16570657.

5. Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, et al. Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science. 2005;310:1180–1183. doi:10.1126/science.1119409. PMID: 16293760.

6. Lipka U, Fuchs R, Lipka V. Arabidopsis non-host resistance to powdery mildews. Curr Opin Plant Biol. 2008;11:404–411. doi:10.1016/j.pbi.2008.04.004. PMID:18499508.

7. Kosaka A, Takano Y. Nonhost resistance of *Arabidopsis thaliana* against *Colletotrichum* species. J Gen Plant Pathol. 2018;84:305–311. doi:10.1007/s10327-018-0799-y.

8. Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, et al. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science. 2009;323:101–106.

9. Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM. Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science. 2009;323:95–101.

10. Stein M, Dittgen J, Sánchez-Rodríguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S. *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell. 2006;18:731–746.

11. Hiruma K, Fukunaga S, Bednarek P, Pislewska-Bednarek M, Watanabe S, Narusaka Y, Shirasu K, Takano Y. Glutathione and tryptophan metabolism are required for Arabidopsis immunity during the hypersensitive response to hemibiotrophs. Proc Natl Acad Sci USA. 2013;110:9589–9594. doi:10.1073/pnas.1305745110. PMID: 23696664.

12. Hiruma K, Nishiuchi T, Kato T, Bednarek P, Okuno T, Schulze-Lefert P, Takano Y. *Arabidopsis ENHANCED DISEASE RESISTANCE 1* is required for pathogen-induced expression of plant defensins in nonhost resistance, and acts through interference of *MYC2*-mediated repressor function. Plant J. 2011;67:980–992. doi:10.1111/j.1365-313X. PMID: 21605210.

13. Frye CA, Tang D, Innes RW. Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc Natl Acad Sci USA. 2001;98:373–378. doi:10.1073/pnas.011405198. PMID: 11114160.

14. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005;139:5–17. doi:10.1104/pp.105.063743. PMID: 16166256.

15. Zarei A, Körbes AP, Younessi P, Montiel G, Champion A, Memelink J. Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the *PDF1.2* promoter in Arabidopsis. Plant Mol Biol. 2011;75:321–331. doi:10.1007/s11103-010-9728-y. PMID: 21246258.

16. He X, Jiang J, Wang CQ, Dehesh K. ORA59 and EIN3 interaction couples jasmonate-ethylene synergistic action to antagonistic salicylic acid regulation of PDF expression. J Integr Plant Biol. 2017;59:275–287. PMID: 28168848. doi:10.1111/jipb.12524.

17. Pré M, Atallah M, Champion A, De Vos M, Pieterse CM, Memelink J. The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. 2008;147:1347–1357. doi:10.1104/pp.108.117523. PMID:18467450.

18. Kim NY, Jang YJ, Park OK. AP2/ERF family transcription factors ORA59 and RAP2.3 interact in the nucleus and function together in ethylene responses. Front Plant Sci. 2018;9:1675. doi:10.3389/fpls.2018.01675. PMID: 30510560.

19. Zhang GB, Yi HY, Gong JM. The *Arabidopsis* ethylene/jasmonic acid-NRT signaling module coordinates nitrate reallocation and the trade-off between growth and environmental adaptation. Plant Cell. 2014;26:3984–3998. doi:10.1105/tpc.114.129296. PMID: 25326291.

20. Kosaka A, Pastorczyk M, Pislewska-Bednarek M, Nishiuchi T, Suemoto H, Ishikawa A, Frerigmann H, Kaido M, Mise K, Bednarek P, et al. *bak1-5* mutation uncouples tryptophan-dependent and independent postinvasive immune pathways triggered in Arabidopsis by multiple fungal pathogens. bioRxiv. 2020. 04.26.052480. doi:10.1101/2020.04.26.052480.

21. Narusaka Y, Narusaka M, Seki M, Ishida J, Nakashima M, Kamiya A, Enju A, Sakurai T, Satoh M, Kobayashi M, et al. The cDNA microarray analysis using an Arabidopsis pad3 mutant reveals the expression profiles and classification of genes induced by Alternaria brassicicola attack. Plant Cell Physiol. 2003;44:377–387. doi:10.1093/pcp/pcg050.

22. Thomma BP, Nelissen I, Eggermont K, Broekaert WF. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. 1999;19:163–171. doi:10.1046/j.1365-313x.1999. PMID: 10476063.

23. Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, Kang NY, Lee S, Cheong H, Park OK. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. Plant Cell. 2005;17:2832–2847. doi:10.1105/tpc.105.034819. PMID: 16126835.